Physiology of Neuronal Subtypes in the Respiratory–Vocal Integration Nucleus Retroamigualis of the Male Zebra Finch

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INTRODUCTION

Vocalizations in vertebrates range from innate and acoustically simple contact and alarm calls to more complex learned sounds used for courtship and, in humans, as a medium for language (Doupe and Kuhl 1999). Many songbirds, in particular, have rich vocal repertoires characterized by complex spectral and temporal features. Notably, bird song and human speech, unlike most other animal vocalizations, are learned through auditory experience during a juvenile critical period. A further parallel to human speech is that the maintenance of adult song in some songbird species, including the zebra finch, continues to depend on auditory feedback. These behavioral parallels make birdsong an especially useful system to address the neural mechanisms important to learned vocal control.

Song is produced by passing air through the syrinx, where sounds are modulated by the activation of up to seven pairs of muscles that are innervated by the tracheosyringeal portion of the hypoglossal nucleus (XIIIs) (Suthers et al. 1999; Vicario and Nottebohm 1988). Thus two major output routes play a role in song production: one controls the syringle muscles and the other controls spinal motor neurons (MN) innervating ventilatory muscles. These output pathways must be precisely coordinated to produce appropriate vocalizations. While the majority of songbird studies have focused on the telencephalic structures responsible for high level patterning of song, less attention has been paid to the neuronal structures in the lower brainstem that control the motor output involved in song production and where respiratory–vocal integration might be expected to occur (Sturdy et al. 2003; Wild 1993b). In particular, the physiological properties of the brain stem respiratory–vocal network in songbirds remain largely undescribed (but see Sturdy et al. 2003).

Anatomical studies suggest that a complex formed by the nucleus retroamigualis (RAm) and paramarginal (ParM) in the ventrolateral medulla of birds is an essential site on which to focus such a physiological analysis. The avian RAm is the equivalent of the mammalian nucleus retroambiguus (NRA), which serves as the final common pathway for vocalization (Holstege 1989). The NRA is under the control of the parieto-oculal gray (PAG) and the pontine call site, both of which, when stimulated, are able to elicit vocalizations by virtue of their connections to phonatory motor neurons, through NRA. NRA is involved in respiratory control by virtue of its projections to respiratory spinal MNs, as well as in respiratory–laryngeal coordination during active vocalizations (Chen and Aston-Jones 1996; Gerrits and Holstege 1996; Holstege 1989; Luthe et al. 2000). In the squirrel monkey, NRA shows a high density of vocalization-related cells (Luthe et al. 2000), and in the cat, NRA neurons exhibit increased firing rate during vocalization (Katada et al. 1996). Kainic acid injection into NRA abolishes the PAG-induced vocalizations, showing that NRA is necessary for their production (Shiba et al. 1997). However, direct stimulation of NRA in mammals produces vocalizations that do not have a normal structure. This has been attributed to the failure of NRA to produce a normal motor pattern for the orofacial modulation of sound (Davis et al. 1996). Instead, it has been proposed that NRA may be involved in the control of the expiratory phase of vocalization by balancing expiratory force through its spinal projections and...
vocal fold adduction through its connections to vocal motor neurons (Luthe et al. 2000).

Similar to NRA in mammals, RAm in birds receives descending projections from the midbrain, specifically from the dorsomedial nucleus of the intercollicular complex (DM) (Wild et al. 1997), a region implicated in the generation of innate vocalizations. In turn, RAm and PAm project to spinal MNs innervating expiratory and inspiratory muscles, respectively, as well as to the avian vocal motor nucleus (XIIts; Reineke and Wild 1997, 1998). In songbirds, RAm also receives paraventricular-positive projections from the telencephalic nucleus robustus archopallialis (RA), the output from the telencephalic vocal control circuitry that is essential to learned vocal control (Nottebohm and Arnold 1976; Vicario 1993; Vu et al. 1994; Wild 1993a,b, 1994, 2004; Wild et al. 2000, 2001; Yu and Margoliash 1996). These various connections suggest that RAm plays an essential role in respiratory patterning of vocal motor neuron activity during quiet respiration and during phonation. Indeed, XIIts, RAm, and PAm exhibit ventilatory-related activity (Manogue and Paton 1982; Reineke and Wild 1997, 1998; Sturdy et al. 2003; Wild 1993b). In addition, RAm is known to provide both excitatory and inhibitory inputs to XIIts that, together, are thought to modulate the pattern of vocal MN discharge during both quiet respiration and during singing (Sturdy et al. 2003). One idea is that RAm drives the rhythmical pattern of expiratory-related discharge characteristic of XIIts neurons during quiet respiration and that the song premotor nucleus RA acts through RAm and XIIts to impose an activity pattern on both respiratory and syringal muscles that underlies the species-typical song.

Despite this general model, the cellular organization of RAm remains poorly understood. Double retrograde labeling studies show that RAm neurons that target vocal MNs in XIIts are an anatomically distinct pool from those that innervate respiratory MNs in the thoracic spinal cord (Wild 2004; unpublished data), pointing to distinct subtypes of RAm neurons. However, the intrinsic electrophysiological properties of different RAm neuron subtypes remain unknown. Indeed, a physiological characterization of RAm is an essential step toward understanding how it interacts with different targets in both the cord and XIIts and, ultimately, how it transmits information from the telencephalon and respiratory centers to mediate song.

METHODS

Subjects

Experiments were performed using 75 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 and the University of Auckland Animal Ethics Committee.

Brain slices

These experiments used electrophysiological techniques that have been described extensively in previously published studies (Sturdy et al. 2003). Briefly, transverse brain slices from the medulla of adult male zebra finches were cut at 400 µm and transferred to a humidified interface-type holding chamber (room temperature). Intracellular recordings were made using an interface-type chamber (30°C; Medical Systems). Artificial cerebrospinal fluid (ACSF) consisted of (in mM) 119 NaCl, 2.5 KCl, 1.3 MgCl₂, 2.5 CaCl₂, 1 NaH₂PO₄, 26.2 NaHCO₃, and 11 glucose and was equilibrated with 95% O₂-5% CO₂. Equimolar sucrose was substituted for NaCl during the tissue preparation stage.

In vitro electrophysiology

Sharp electrodes (borosilicate glass, BF100-50-10, Sutter Instrument, Novato, CA) were pulled to yield a resistance of 100–200 MΩ when filled with 2 M K-acetate and 2% neurobiotin (Vector Laboratories) or 2% biocytin (Sigma). A motorized microdrive (model 860A, Newport Scientific, Irvine, CA) was used to lower electrodes into the nucleus. RAm and PAm can separately be identified in counterstained material, but this distinction was not possible in the slice preparation. However, because our recording sites were primarily limited to more caudal medullary sections that often included the commissura infima located caudal to the obex, they were probably located within RAm rather than PAm (Reineke and Wild 1998), and henceforth are referred to as RAm. XIIts was visible under epi-illumination as a circular structure situated ~0.5 mm from the midline, and RAm was identified as a pale structure extending in an arc ventrolaterally from XIIts (see Wild 1993b). Brief (~1 ms) capacitance overcompensation was used to “ring” the electrode to achieve entry into the cell. An AxoClamp 2B intracellular amplifier (Axon Instruments, Foster City, CA) was used in bridge mode to record intracellular membrane potentials, which were low-pass filtered at 3 kHz, digitized at 10 kHz, and stored on a PC hard drive. All custom software for this study was written by M. Rosen, F. Livingston, and R. Neumann (Duke University).

Hyperpolarizing responses to negative current pulses (~0.1 or ~0.5 nA; measured during steady state) were collected to estimate input resistance, and instantaneous and mean firing rates were calculated in response to positive current pulses (~0.2- to 1-nA steps, 1-s duration). Concentric bipolar stimulating electrodes (Frederic Haer, Bowdoinham, ME) were positioned in XIIts or Ram, and synaptic potentials or antidromic action potentials were evoked using brief (100 µs) currents of 50–600 µA. Once data collection was completed, cells were filled with neurobiotin or biocytin using positive currents (~0.5–1 nA, 500 ms at 1 Hz or 50 ms at 10 Hz). After injection of the tracer, slices were kept in the recording chamber under oxygenated ACSF for ~20 min, after which they were fixed by immersion in 4% paraformaldehyde (PF) in 0.1 M phosphate buffer (PB) overnight and processed as described below.

To search for synaptically coupled neurons between RAm and XIIts, we first obtained a stable recording from a XIIts motor neuron and advanced a second recording electrode into RAm. Action potentials were elicited in turn from each neuron in the pair by passing brief (~10 ms) depolarizing current pulses (~0.5–1 nA) through the recording electrode. A software threshold peak detector was used to generate spike-triggered averages (STAs) of the membrane potential of the partner cell in the pair. STAs were plotted in reference to the time of the trigger spike; note that the zero time for the STA corresponds to the action potential peak and that we suspect that the resultant STAs might be slightly leftward-shifted with respect to the actual onset of transmitter release.

In vitro intrinsic data analysis

A variety of measurements was used to define the intrinsic properties of the individual cells (Fig. 1), including instantaneous spike frequency (ISF), resting membrane potential ($V_{rest}$), input resistance at rest ($R_{input}$), threshold (Th), amplitude of the action potentials (APs), half-width of the action potential (APw), afterhyperpolarization (AHP), afterdepolarization (ADP), hyperpolarization-related voltage sag (VSag), voltage at action potential peak (AP), and number of action potentials after +200- and +400-pA depolarizing pulses (AP200 and AP400). A software threshold event detector was used to count the number of action potentials and to measure instantaneous spike rates over defined time intervals. Action potential half-height
Multivariate analysis

Qualitative neuronal classification based on intrinsic electrical properties was complemented by cluster analysis and data reduction by principal components analysis (PCA; SPSS v. 11.5, SPSS, Chicago, IL) to aid in the classification scheme and to uncover structure in the data set. An initial PCA analysis was performed using all continuous variables, varimax rotation, and listwise exclusion of missing values. Factors with eigenvalues $>1$ were used for the analysis. A two-step cluster analysis using log-likelihood distance and Bayesian information criterion (BIC) was employed as an alternative classification method. An initial PCA analysis was performed incorporating all continuous variables. In the case of highly correlated variables, the one with the larger communality was kept for the subsequent analysis. The chosen variables were used for a final PCA and two-step cluster analysis. Both analyses yielded similar results.

Histology

All tissue was cryoprotected in 30% sucrose in 0.1 M PB and cut in a freezing microtome at 30–50 μm. The tissue was preincubated in 3% H$_2$O$_2$ in 1:1 PBS:methanol for 10 min to block the activity of endogenous peroxidase.

To visualize cells filled with neurobiotin or biocytin, the tissue was incubated with either Alexa coupled streptavidin (Molecular Probes) or with a horseradish peroxidase (HRP)-conjugated streptavidin (1:1,000) dissolved in 0.4% Triton X-100 in PBS (0.01 M) and developed using a Chromagen solution consisting of PBS, 0.25 mg/ml diaminobenzidine tetrahydrochloride (DAB), and 0.018% H$_2$O$_2$. In some cases, 0.02% cobalt chloride was added to the Chromagen solution to render the reaction product black. All steps in this and all other incubation procedures were separated by washes in the incubation buffer. The tissue was mounted onto subbed slides and coverslipped with Citiﬁlm (for light microscopy). Some of the sections were incubated for immunocytochemistry for parvalbumin (monoclonal antibody from SWANT) to visualize the RA terminal field (Wild et al. 2001), to confirm the location of the cells within RA. The tissue was incubated overnight at room temperature in the primary antibody in the presence of 2.5% normal serum and 0.4% Triton X-100 in PBS. Sections were incubated in the appropriate secondary antibody for 1–2 h at room temperature. For light microscopy, a biotinylated secondary antibody was used, followed by streptavidin-HRP (1:1,000; SA-HRP, Molecular Probes) for 1–2 h at room temperature, and developed with a biotinylated secondary antibody for 1–2 h at room temperature. For light microscopy, an Alexa-conjugated antibody (Molecular Probes) was used instead of streptavidin-HRP, after which sections were washed, mounted, and coverslipped with Citiﬁlm (Agar Scientific).

Light microscopy

For light microscopy, a brown and/or blue/black reaction product indicated positive staining. The material was photographed using a Kontron ProgRes 3008 digital camera attached to a Leica DMRA microscope and was captured using Adobe PhotoShop with the appropriate plug-in. The images were processed with Adobe PhotoShop v.5.5 software to produce the final figures.

Confocal microscopy

Confocal imaging was performed using a Leica TCS SP2 confocal laser-scanning microscope. Data were collected sequentially for each channel. Image formats employed were either 1024 × 1024 or 2048 × 2048. To image the fluorochrome Alexa350, excitation at 350/364 nm was employed, and the emission wavelength range set for collection was 400–550 nm. To visualize labeling with the fluorochrome Al-

widths were measured from the shoulder of the spike, where the membrane potential described a sharp positive inflection (Fig. 1A). The amplitudes of spike AHPs were measured from the spike shoulder to the trough of the hyperpolarization after the spike. Similarly, the amplitudes of spike ADPs were measured from the trough of the spike AHP to the peak of the spike ADP. Resting membrane potential was determined by subtracting any DC offset observed after electrode withdrawal. Input resistance measurements were calculated by measuring the steady-state voltage caused by injecting small ($\pm 0.1$ or $\pm 0.5$ nA) hyperpolarizing current pulses. $V_{\text{sag}}$ values were measured as the maximum hyperpolarization seen during negative current injection minus the membrane voltage at the offset of the hyperpolarizing pulse (steady state; Fig. 1B). All values are reported as means ± SE unless otherwise stated. Statistics and tests for statistical significance are reported in RESULTS.

FIG. 1. Schematic showing the parameters measured for each cell. $A$: $V_{\text{pp}}$, voltage at action potential peak; $V_{\text{pa}}$, action potential amplitude; $V_{\text{th}}$, threshold, established as the point of inflection of the voltage trace; $V_{\text{pwr}}$, width of the action potential at half-amplitude; $V_{\text{AHPr}}$, afterhyperpolarization; $V_{\text{ADP}}$, afterdepolarization. $B$: $V_{\text{sag}}$, voltage difference between the peak of the hyperpolarization and steady state. $C$: schematic of the slice showing relative location of hypoglossal motor nucleus (XIIts) and medullary nucleus retroambigualis (RAm) and recording and stimulation sites.
exa488, argon laser excitation at 488 nm was used, and the emission wavelength range set for collection was 500–600 nm. To visualize labeling with Alexa594, green helium-neon excitation at 543 nm was used, and the emission wavelength range set for collection was 555–700 nm. A series of optical sections was collected through the entire structure being imaged. Optimal pinhole settings were employed. The data sets were subsequently merged and z-projections were made, when required, using Leica Confocal Software version 2.5.1104.

RESULTS

Electrophysiological characterization of RAm neuron types

To characterize the electrophysiological properties of RAm neurons, we made intracellular recordings in RAm in transverse brain stem slices (Fig. 1C; see METHODS). The membrane potential responses of the neurons to the injection of depolarizing and hyperpolarizing current pulses were used to establish an initial qualitative classification into two cell types (types I and II), which we independently validated using PCA and cluster analysis techniques. In addition, XIIIt-projecting RAm neurons were identified by using electrical stimulation in XIIIts to antidromically activate them, by intracellular staining with neurobiotin or biocytin, and by making paired intracellular recordings in RAm and XIIIts to search for synaptically coupled cell pairs.

We recorded from 131 neurons in RAm. Neurons could be qualitatively classified into two cell types based on the shape of their action potential and their distinct responses to depolarizing current pulses (Fig. 2). The action potentials of type I cells were characteristically broad, lacked an ADP, and showed a long AHP. In these type I cells, DC-evoked firing rates were relatively low, and spike frequency and spike height adaptation occurred over the duration of the injected current pulse. In contrast, the action potentials of type II cells were characteristically narrower and exhibited a very clear ADP. These type II cells displayed higher average firing frequencies than did type I cells, and after a brief period of spike frequency adaptation, maintained a steady firing rate for the duration of the current pulse and relatively constant action potential amplitudes. Although type I cells were less frequently impaled, the different types could be encountered in a single pass of the electrode through the slice and were even sometimes adjacent to one another.

Type I RAm neurons

We classified 22 cells as type I (Fig. 2, A–F), which represented 17% of all cells in this study (Table 1). Most type I cells (16/22) responded to depolarizing pulses of current with a sustained and relatively low frequency train of action potentials that accommodated markedly throughout the depolarizing pulse (Figs. 2 and 3A, black circles). In all type I cells, the mean firing frequency increased linearly as a function of the injected current amplitude (40 Hz/nA, \( r^2 = 0.999 \); Figs. 2, B and E, and 3B). The mean resting potential of type I neurons showing these different degrees of spike accommodation was similar (−46.1 ± 2.0 vs. −46.7 ± 2.9 mV), and although the

FIG. 2. Examples of the DC-evoked responses and action potential shapes of type I (A–F) and type II (G–I) neurons. A, D, and G: DC-evoked responses after +0.2 (black) or −0.2 nA (gray) of injected current. B, E, and H: instantaneous frequency vs. spike interval for type I (B and E) and type II (H) neurons. Typical action potentials of type I (C and F) and type II (I) neurons.
TABLE 1. Intrinsic properties of type I and type II RAm cells

<table>
<thead>
<tr>
<th>Type</th>
<th>Type I</th>
<th>Type II</th>
<th>Significance (2-Tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number</td>
<td>22</td>
<td>109</td>
<td></td>
</tr>
<tr>
<td>Percentage</td>
<td>16.8%</td>
<td>83.2%</td>
<td></td>
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<tr>
<td>$V_m$</td>
<td>$-46.3 \pm 1.6$</td>
<td>$-59.7 \pm 0.9$</td>
<td>0.001*</td>
</tr>
<tr>
<td>$R_m$</td>
<td>95.3 ± 7.0</td>
<td>81.1 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>APs</td>
<td>61.8 ± 3.4</td>
<td>60.4 ± 1.0</td>
<td>0.705</td>
</tr>
<tr>
<td>APw</td>
<td>0.6 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>0.001*</td>
</tr>
<tr>
<td>AHP</td>
<td>26.1 ± 1.4</td>
<td>16.5 ± 0.5</td>
<td>0.001*</td>
</tr>
<tr>
<td>ADP</td>
<td>N/A</td>
<td>4.1 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>V_sag</td>
<td>4.3 ± 3.9</td>
<td>4.1 ± 4.7</td>
<td>0.847</td>
</tr>
<tr>
<td>APp</td>
<td>21.5 ± 4.4</td>
<td>11.9 ± 1.2</td>
<td>0.044</td>
</tr>
<tr>
<td>Threshold</td>
<td>$-38.3 \pm 2.5$</td>
<td>$-48.4 \pm 0.9$</td>
<td>0.001*</td>
</tr>
<tr>
<td>AP200</td>
<td>9.3 ± 4.0</td>
<td>42.5 ± 1.9</td>
<td>0.001*</td>
</tr>
<tr>
<td>AP400</td>
<td>18.0 ± 1.6</td>
<td>66.5 ± 3.0</td>
<td>0.001*</td>
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</tbody>
</table>

N/A, not applicable. Data are means ± SE. *denotes statistical significance.

mean input resistance of the more phasic cells was slightly lower, this difference was not significant (mean input resistance: 101.2 ± 9.0 and 80.1 ± 6.7 MΩ, respectively, $P < 0.6$, Mann-Whitney rank test). All type I cells were characterized by relatively broad action potentials (0.6 ± 0.0 ms) that lacked an ADP but possessed a large, long-lasting AHP (Fig. 2, $C$ and $F$; Table 1). In response to hyperpolarizing current pulses, 13/22 type I neurons displayed a “sag” in the membrane potential (V_sag), suggestive of a hyperpolarization-activated inward current.

Type II RAm neurons

We classified 109 cells as type II RAm neurons, representing 83% of all cells in this study (Table 1). In response to depolarizing current injection, type II neurons fired action potential trains that accommodated rapidly from very high frequencies over the first several spike intervals, then maintained a stable firing frequency throughout the remainder of the current pulse (Figs. 2, $G$–$J$, and 3A, open circles). The mean firing rate increased linearly as a function of the applied current with a rate of increase that was steeper than that observed for type I cells (110 Hz/nA, $r^2 = 0.989$; Fig. 3B, open circles). Numerous features of the DC-evoked response distinguished type II from type I neurons (Table 1). Type II cells had narrower action potential half-widths, and unlike type I cells, exhibited a spike ADP (Fig. 2I; Table 1). In addition, type II cells had more negative resting membrane potentials ($−59.7 ± 0.9$ vs. $−46.3 ± 1.6$ mV; $P < 0.01$), a smaller AHP, a lower action potential threshold, and much higher instantaneous and average DC-evoked firing rates (see Table 1 for mean quantitative comparisons and significance, as well as Figs. 2 and 3).

As with most type I cells, 50 of 103 type II cells tested exhibited a “sag” in membrane potential during the initial part of the hyperpolarizing current pulse (Fig. 2G). Type II cells did not differ from type I cells in their mean input resistance, mean action potential amplitude, or mean voltage sag in response to prolonged hyperpolarizing current injection (Table 1).

About one-half (53/109) of the type II neurons exhibited an action potential “burst” associated with the release from hyperpolarized membrane voltages (Fig. 4A). This bursting behavior could occur at the offset of a hyperpolarizing current pulse and at the onset of a short depolarizing pulse applied during tonic membrane hyperpolarization (Fig. 4, $A$ and $C$). These bursts were typically composed of two to five action potentials whose amplitude decremented throughout the burst. Figure 4B shows the instantaneous frequency of the first 10 action potentials after a +0.4-nA current pulse in nonbursting (triangles) and bursting (black and gray circles) type II cells. The mean instantaneous frequency of bursting type II cells as a function of spike number also was analyzed when these cells were under conditions that were either permissive (i.e., prior membrane hyperpolarization) or nonpermissive for DC-evoked bursting (Fig. 4B; permissive: black circles; nonpermissive, gray circles). In the nonpermissive conditions, the spike frequency behavior of type II “bursting” neurons was indistinguishable from that of type II neurons that could not be induced to burst by prior membrane hyperpolarization. Bursts were characterized by a very high initial instantaneous frequency ($323 ± 98$ Hz at 0.4 nA), which rapidly decayed by the fourth spike interval to levels comparable with those of the same cell under nonpermissive conditions injected with the same amplitude depolarizing current. No other differences in the intrinsic properties of bursting versus nonbursting type II cells were observed.

Classification of cells by PCA and cluster analysis

We used a PCA to classify RAm neuron types more objectively. PCA indicated that electrophysiological properties

FIG. 3. A: mean instantaneous frequency vs. interval number in type I (○) and type II (□) neurons in response to +0.2 nA of current injection. Data points are mean ± SD. Data tend to be more affected by high-frequency firing cells at larger interval numbers, because only high-frequency firing cells have large interval numbers. B: mean number of action potentials vs. injected currents for type I (○) and type II (□). Data points are mean ± SD. Regression functions (model I regression) for type I, $y = 0.04x + 1.06$ ($r^2 = 0.999$), and for type II, $y = 0.11x + 16.97$ ($r^2 = 0.989$).
could be associated with two major neuronal classes in RAm, and these two PCA-determined classes corresponded to types I and II as distinguished more qualitatively (Table 2; Fig. 5). The PCA uncovered three principal components that were differentially correlated with intrinsic properties of the cells (Table 2). Component 1 was positively correlated with $V_{m}$, APw, and AHP and negatively correlated with ADP and AP400; component 2 was positively correlated with APa and V$sag$; and component 3 was positively correlated with $R_{m}$ (Fig. 5A). When the scores for each of the variables were plotted, two major clusters emerged that corresponded to the cell types arrived at by our initial qualitative classification (Fig. 5B). The same result was obtained with cluster analysis (data not shown). Thus PCA and cluster analysis support the grouping of RAm neurons into two major cell types based on their intrinsic electrophysiological properties.

Type II and not type I RAm neurons innervate XIIts: antidromic and morphological characterization

Antidromic stimulation and intracellular staining were used to further characterize type I and type II neurons with respect to their possible projections to XIIts. Electrical stimulation in XIIts evoked a response in only 1 of the 10 type I neurons tested, and this response consisted of an inhibitory postsynaptic
potential. Action potentials were never (0/10) elicited in type I cells by electrical stimulation of XIIts, suggesting that the axons of type I cells do not travel into or through XIIts. Furthermore, six type I cells were reconstructed morphologically, and no projections to XIIts were found after examination of the filled material. In one case where the axon of a filled cell could be more fully reconstructed, it was seen to course laterally and dorsally, away from XIIts, before leaving the plane of the slice close to the root of the X nerve. These electrophysiological and morphological observations suggest that type I RAm neurons do not innervate XIIts.

In contrast, electrical stimulation in XIIts evoked short-latitude action potentials and/or synaptic responses (excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs)) in many (26/48) type II cells. Of the 26 type II neurons that responded to electrical stimulation in XIIts, 14 of 22 tested were bursting type II neurons and 12 of 26 tested were nonbursting type II neurons. The evoked responses consisted of action potentials and/or postsynaptic responses (PSPs) that could be either depolarizing or hyperpolarizing in nature (Fig. 6A). In most (20/26) cases, action potentials were elicited, whereas in six cases, only a subthreshold PSP (4 EPSPs and 2 IPSPs) was observed. Collision tests were used to identify antidromically elicited action potentials. In five type II cells (2 bursting and 3 nonbursting), the occurrence of a spontaneous action potential within a short time (∼1 ms for the cell in Fig. 6A) before applying electrical stimulation in XIIts prevented the appearance of the electrically evoked spike (Fig. 6A, bottom trace). These positive collision tests suggest that the axons of both bursting and nonbursting type II neurons terminate in or travel through XIIts.

We also found morphological evidence that type II RAm neurons innervate XIIts. Intracellular staining with neurobiotin in four bursting and four nonbursting type II RAm neurons resulted in labeling of axonal processes within XIIts. These axonal processes were marked by distinct puncta in XIIts, consistent with presynaptic specializations. We also noted that such axon terminal labeling could be detected in both the ipsilateral and contralateral XIIts after filling of a single type II RAm neuron, suggesting that individual type II neurons provide bilateral innervation to XIIts (Fig. 6B).

However, because the axons of these individual type II RAm neurons could not be traced in complete continuity from their origin in RAm to their terminations in XIIts, it could not be determined by which of several possible routes these axons reach the contralateral XIIts (see Discussion). In any case, the morphological results presented here, together with the antidromic stimulation experiments, indicate that XIIts is inner-
vated exclusively by a distinct subset of RAm neurons, namely the type II neurons identified here by their intrinsic electrophysiological properties.

**Physiological inputs onto XIIIts**

We also made dual intracellular recordings to characterize further synaptic interactions between type II neurons in RAm and MNs in XIIIts. Spike-triggered averaging (see METHODS) was used to determine whether DC-evoked action potentials in the RAm neurons were associated with subsequent membrane potential changes in the XIIIts MNs. Paired recordings were done in 42 RAm and 81 XIIIts cells. Postsynaptic hyperpolarizing responses in the XIIIts cell were observed in five pairs, and, in all cases, the RAm neuron could be classified as a type II neuron that exhibited hyperpolarization-dependent bursting. Notably, single action potentials in the RAm cell were ineffective in eliciting a postsynaptic response in XIIIts, whereas action potential bursts in the type II RAm neuron were followed by a membrane potential hyperpolarization in the XIIIts neuron (Fig. 7). These results suggest that bursting type II RAm neurons provide inhibitory input onto XIIIts motor neurons. In no cases did action potentials in the XIIIts neuron evoke a membrane potential deflection in the RAm neuron, consistent with prior anatomical observations that XIIIts neurons lack axon collaterals (Sturdy et al. 2003).

**DISCUSSION**

The RAm/PAm neuronal complex of birds is a functionally and anatomically diverse structure that, through its synaptic connections with a variety of targets, is postulated to play an important role in calling, singing, and respiration (Fig. 8) (Reinke and Wild 1997, 1998; Wild 1993a,b, 1994, 2004).

RAm seems to be directly analogous, perhaps homologous, to NRA of mammals, whereas PAm may be functionally equivalent to a more rostral, inspiratory-related part of the ventral respiratory group (Reinke and Wild 1997; Wild 1994). Initially shown to project to many of the motor neurons involved in the final common path for vocalization, including spinal motor neurons innervating expiratory muscles (Holstege 1989; Jürgens 2002), NRA also more recently has been shown to be the source of a variety of other bulbo-spinal projections involved in a wider set of behaviors generally associated with reproduction (Gerrits and Holstege 1999; Gerrits et al. 2000, 2004; VanderHorst and Holstege 1995, 1996, 1997; Vander-Horst et al. 1997, 2000). Because song is a major component of reproductive behavior in songbirds, it is possible that RAm is also involved more generally in the control of reproductive behavior, in addition to its involvement in vocalization and expiration.

Regardless of the specific role of the NRA in reproductive behaviors other than song, the involvement of RAm and PAm...
neurons in distinct patterns and types of respiratory and vocal activity suggests that they may be specialized in their intrinsic properties. Here we used in vitro intracellular methods to obtain a physiological description of cell types in RAm of the zebra finch as a first approach toward dissecting the role of this integratory nucleus in the formation of patterned vocal motor output. These recordings reveal two major classes of RAm neurons (type I and II), and variable expression of bursting behavior in type II neurons, underscoring physiological heterogeneity within RAm. Furthermore, type II but not type I neurons were found to innervate XIIts, suggesting that physiological specializations within the RAm neurons may relate in part to the nature of the targets they innervate.

Electrophysiological cell types in RAm

We found two cell types in RAm that differed in their intrinsic physiological properties, specifically in the shape of their action potentials and in their responses to depolarizing current pulses. Type I neurons had relatively broad action potentials and generated rapidly adapting spike trains in response to depolarizing currents. In contrast, type II neurons had narrower action potentials and generated sustained and highly regular action potential trains when injected with depolarizing current pulses. This difference in spike rate accommodation suggests that type I and type II neurons may translate tonic excitation from their afferents into different patterns of activity. We could also further subdivide type II neurons based on the presence or absence of action potential bursting activity on release from hyperpolarizing membrane potential states.

Therefore RAm neurons in zebra finches comprise several distinct electrophysiological cell types.

In both birds and mammals, RAm or NRA neurons exhibit expiratory-related activity (Merrill 1970; Wild 1993b), and therefore may constitute an integral part of the respiratory network. Inspiratory and expiratory mammalian neurons have been classified into different subtypes based on their discharge patterns in relation to the respiratory cycle (for reviews, see Ballanyi et al. 1999; Bianchi et al. 1995; Iscoe 1998). Most mammalian studies in the caudal medulla, however, have been performed under conditions that maintain the rhythmic respiratory activity, making it difficult to dissociate the discharge features that are the result of the intrinsic properties of the cell and those that are imposed by the respiratory circuit. A low voltage-activated Ca\(^{2+}\) current expressed in some mammalian respiratory neurons seems to underlie their ability to exhibit respiratory-related rhythmic activity (Bianchi et al. 1995; Onimaru et al. 2003; Pierrefiche et al. 1999; Ramirez and Richter 1996), whereas inhibitory inputs seem to shape their discharge pattern (Ramirez et al. 1997). Although the location of the respiratory pattern generator has yet to be determined in the songbird, and the ionic mechanisms underlying bursting in type II neurons are unknown, bursting type II RAm neurons exhibit properties similar to those observed in mammalian respiratory neurons that could facilitate the respiratory-related rhythmical activity.

Projection patterns of the two major RAm neuron types

This study provides several lines of evidence that some type II neurons provide synaptic input onto XIIts. First, electrical
neurons other than those we encountered in paired recordings. Though the identity of those RAm neurons providing the inhibition of RAm neurons can drive excitatory as well as inhibitory earlier study showing that either electrical or chemical activation onto XIIts, these results extend those obtained in an intracellular fills of both bursting and nonbursting type II neurons. By identifying a cell type in RAm that provides inhibitory input onto XIIts motor neurons. By identifying a cell type in RAm that provides inhibitory input onto XIIts motor neurons (Sturdy et al. 2003). Finally, because only about one-half of the type II RAm neurons we encountered were activated by electrical stimulation in XIIts, this study cannot rule out the possibility that other type II RAm neurons innervate other known targets of RAm, including expiratory neurons in the ventral horn of the spinal cord and neurons in the contralateral RAm.

Although predominantly ipsilateral retrograde labeling results from unilateral injections of tracer into either XIIts or RAm (Wild et al. 2000, 2001), we observed that individual type II RAm neurons can make bilateral terminal arborisations in XIIts. This pattern of terminations is reminiscent of the bilateral projections that individual neurons in or near the mammalian NRA make onto laryngeal motor neurons n. ambiguus, and these bilateral projections of NRA neurons are thought to be important in coordinating vocal fold activity during vocalization (Holstege 1989; Shiba et al. 1997). In the zebra finch, which possesses a bipartite vocal organ like all songbirds, XIIts and RAm receive almost exclusively ipsilateral projections form the vocal control nucleus RA (Wild 1993a; Wild et al. 2000). Therefore the bilateral projections from RAm to XIIts likely provide a synaptic substrate for determining the extent to which the two sides of the syrinx are coordinated during phonation (Wild et al. 2000).

In contrast to type II neurons, the type I neurons identified in this study formed only a small minority of the total number of RAm neurons examined, and their postsynaptic targets remain uncertain. Antidromic stimulation experiments and intracellular staining indicate that type I neurons do not project on XIIts, and intracellular staining suggests instead that the axons of these cells leave the medulla, possibly through the vagus nerve. Apropos, a group of large, multipolar vagal neurons are associated with the ventrolateral aspect of RAm. Despite their more peripheral location in the nucleus, these vagal neurons lie within the terminal field of projections descending from the telencephalic nucleus RA, suggesting that they are associated with the respiratory–vocal system (Wild 2004). If the type I cells are actually vagal neurons, their peripheral targets are not only unknown, but may be challenging to identify, given the extensive distribution of vagal projections throughout the body (but see Kubke et al. 2004).

**Bimodal behavior in type II neurons: switching from quiet respiration to singing**

The importance of prior membrane hyperpolarization to the bursting behavior of type II RAm neurons is reminiscent of the state-dependent bursting behavior described in thalamocortical relay neurons, wherein membrane hyperpolarization deactivates low threshold calcium currents (Llinas and Jahnens 1982). Such similar behavior in type II RAm neurons suggests that prior activation of inhibitory inputs onto these neurons could switch them from nonbursting to bursting modes of firing. Indeed, some RAm neurons express glycine receptors on stimulation in XIIts antidromically activates type II and not type I RAm neurons, consistent with the idea that type II neurons extend their axons into or through XIIts. Second, intracellular fills of both bursting and nonbursting type II neurons showed that either type could make bilateral projections onto XIIts, although the route by which individual axons reach the contralateral nucleus could not be determined (see RESULTS). Indeed, extracellular injections of tracer in RAm labeled RAm axons that reach the contralateral medulla by a variety of routes: many destined to terminate in the contralateral RAm cross the midline through the ventral medulla, whereas others cross dorsal to XIIts in the commissura infima, and still others between XIIts and the supraspinal nucleus (Wild et al. 2000). Finally, in a small number of cases, paired recordings in RAm and XIIts indicated that bursting type II RAm neurons can provide inhibitory input onto XIIts motor neurons. By identifying a cell type in RAm that provides inhibition onto XIIts, these results extend those obtained in an earlier study showing that either electrical or chemical activation of RAm neurons can drive excitatory as well as inhibitory responses in XIIts motor neurons (Sturdy et al. 2003). Although the identity of those RAm neurons providing the excitatory input to XIIts remains to be determined, one possible source of excitation onto XIIts from RAm are type II neurons other than those we encountered in paired recordings.
their cell bodies (M.F.K., personal observations), and given the absence of a direct projection from XIlls to RAm, the IPSPs evoked in RAm neurons by XIlls stimulation indicate that the axons of XIlls-projecting RAm neurons must extend local collaterals that synapse on other RAm neurons. Although the sources and types of synaptic inputs onto RAm neurons have yet to be characterized, earlier studies and the results obtained here suggest that one source of inhibition could arise from other type II RAm neurons, which can provide inhibitory input onto XIlls motor neurons.

**Functional significance of burst firing in type II RAm neurons**

In singing birds, high-frequency burst firing typifies activity in the RA-projection neurons that innervate the brain stem, and this bursting activity likely provides a highly precise timing signature for vocal and respiratory muscle activity necessary for song (Hahnloser et al. 2002; Lewicki 1996; Studny et al. 2003; Yu and Margolis 1996). In addition to bursting activity at the level of RA, the hyperpolarization-dependent bursting behavior of type II RAm neurons may enable them to switch from a linear behavior during quiet respiration to a nonlinear behavior during singing. Notably, this study provides preliminary evidence that bursting in type II neurons is necessary to drive inhibitory responses in XIlls neurons. Therefore the presence or absence of bursting activity in the type II neurons may influence the functional connectivity between respiratory and vocal areas in the songbird’s brain stem. Furthermore, because some RAm neurons are capable of generating burst firing, at least when primed by prior membrane hyperpolarization, the brain stem respiratory–vocal network may introduce additional nonlinearity to the motor pattern rather than acting simply as a linear follower of song premotor activity transmitted from RA.

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