Heterogeneous Biophysical Properties of Frog Dorsal Medullary Nucleus (Cochlear Nucleus) Neurons

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Yang S, Feng AS. Heterogeneous biophysical properties of frog dorsal medullary nucleus (cochlear nucleus) neurons. J Neurophysiol 98: 1953–1964, 2007. First published August 8, 2007; doi:10.1152/jn.00427.2007. The cochlear nucleus (CN) in mammals, or its counterpart in birds, has multiple subdivisions each containing distinct morphological and functional (i.e., temporal discharge patterns and biophysical properties) cell types that project to different auditory nuclei in the brain stem in parallel. The analogous structure in frogs, the dorsal medullary nucleus (DMN), is a single phylogenetically older structure with no subdivision. Similar to the CN, the DMN has complex cytoarchitecture and contains neurons with diverse morphological phenotypes, but whether these cell types possess distinct biophysical characteristics, like their counterparts in mammals and avians, is unclear. Here we show that DMN neurons in young adult northern leopard frogs (Rana pipiens pipiens) possess heterogeneous biophysical properties. There are four major biophysical phenotypes on the basis of the unit’s response (i.e., its temporal firing pattern) to depolarizing currents: onset, phasic-burst, sustained-chopper, and adapting. These cells have distinct membrane input resistances and time constants, spike shapes, current-voltage relationships, first-spike latencies, entrainment characteristics, and ionic compositions (i.e., low-threshold potassium current, \(I_h\), and hyperpolarization-activated current, \(I_h^+\)). Furthermore, these phenotypes correspond to cells’ dendritic morphologies, and they bear similarities and differences to those found in the mammalian CN. The similarities are remarkable considering that amphibians are a distinct evolutionary lineage from birds and mammals.

INTRODUCTION

The dorsal medullary nucleus (DMN) in the amphibian caudal brain stem is analogous to the cochlear nucleus (CN) in mammals. It is the first auditory station in the brain receiving a direct projection from the auditory nerve (Feng 1986; Wilczynski and Endepols 2006). Although the DMN is a single auditory nucleus, its neurons exhibit diverse temporal discharge patterns in response to tone bursts at the units’ characteristic frequencies (Feng and Lin 1994; Hall and Feng 1990, 1991), as observed in the mammalian and avian CN (Koppl and Carr 2003; Sullivan 1985; see reviews in Rhode and Greenberg 1992). These temporal discharge patterns are distinct from those observed in auditory nerve fibers, reflecting the transformation taking place in the DMN and the signal processing therein. DMN neurons having different temporal discharge patterns show varying entrainment abilities when presented with amplitude-modulated stimuli. For example, the phasic-burst and phasic neurons have the highest entrainment ability, i.e., showing time-locked firing to amplitude-modulated stimuli ≈250 Hz (Feng and Lin 1994; Hall and Feng 1991). The DMN also contains a number of morphological cell types with distinct somatic and dendritic characteristics (Feng and Lin 1996)—these show remarkable resemblances to the morphological cell types observed in the mammalian and avian CN (Cant 1992; Carr and Boudreau 1991, 1993; Soares et al. 2002).

Although the different morphological cell types in the mammalian CN (mostly in neonates) have been shown to possess distinct biophysical properties and temporal discharge patterns (Manis and Marx 1991; Oertel 1999; Rhode and Greenberg 1992), virtually nothing is known about the biophysical properties of DMN neurons and their underlying cellular mechanisms or their structure–function relationships. In this study, we used whole cell patch recording from brain slices of young adult northern leopard frogs (Rana pipiens pipiens) to investigate the biophysical properties of single DMN neurons. The cells were filled with biocytin to study their morphological characteristics and topographical profiles and structure–function relationships. We found that DMN neurons consist of four major phenotypes based on their intrinsic membrane biophysical properties and dynamic response properties, i.e., firing patterns in response to depolarization currents and entrainment ability in response to repetitive depolarization pulses. In addition, pharmacological studies, using specific blockers for hyperpolarization-activated current (\(I_h\)) and low-threshold potassium current (\(I_h\)), revealed their differential expressions in these cell types. These biophysical phenotypes generally corresponded to cells having distinct somatic and dendritic morphologies. The resemblances in the biophysical and morphological characteristics of neurons in the frog DMN and those of mammalian and avian CN suggest the possibility of shared neural coding strategies across the different vertebrate classes.

METHODS

Slice preparation and electrophysiological recordings

To prepare frog brain slices, young adult northern leopard frogs (R. pipiens pipiens) weighing 5–20 g were anesthetized in 0.2% tricaine methanesulphonate (MS-222, Sigma, St. Louis, MO) and decapitated. Once the brain was extracted, it was immediately placed in an appropriate oxygenated (95% O₂-5% CO₂) Ringer solution (in mM): 112 NaCl, 2 KCl, 3 MgCl₂, 24.2 d-glucose, 17 NaHCO₃, and 3 CaCl₂ (pH 7.4) (Malayev and Debski 1998). After removal of the dura and pia mater, the tissue was embedded in a 3.3% solution of low-melting-point agarose (Sigma) dissolved in Ringer solution. After hardening and cooling the agarose block in the freezer (−12°C) for 5–7 min, the block was trimmed to obtain desired slice orientation. The brain stem

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was sectioned along the transverse plane into 250 to 260 μm slices using a vibratome (Ted Pella, 1,000 plus); only one or two of these brain slices contained the posterior branch of the eighth cranial nerve that innervates the auditory organs in the inner ear. Brain slices were immersed in oxygenated Ringer solution in a tissue chamber for 1–2 h at room temperature before the electrophysiological recording; the chamber was continuously perfused with Ringer solution at a rate of 2 ml/min. A fixed stage microscope (Zeiss Axioskop-2FS) equipped with differential interference contrast optics and a ×63 water-immersion objective was used to visualize individual neurons in the slices. The experimental protocols were reviewed and approved by the University of Illinois Institutional Animal Use and Care Committee (Protocol 02033).

Patch electrodes were fabricated from 1.5-mm-diameter borosilicate glass micropipettes and had impedance of 3–8 MΩ when backfilled with the following solution (in mM): 117 K-gluconate, 13 KCl, 1.0 MgCl2·6H2O, 0.07 CaCl2·2H2O, 0.1 EGTA, 10 HEPES, 3 ATP-Mg, 0.3 GTP-Na, and 0.3% biocytin (pH 7.4–7.6; osmolarity = 260–270 mOsm). The recording electrode was slowly advanced into the slice until the tip encountered a cell surface under visual monitor. After tight seal formation, the whole cell configuration was obtained by application of a negative pressure to the patch pipette. The initial membrane potential was corrected for a measured junctional potential of –10 mV. The resting membrane potential (RMP) was measured from the voltage change induced by small hyperpolarizing current pulses (10 to 20 ms duration) when backfilling of the patch pipette. The initial access resistance typically ranged from 10 to 15 MΩ and remained stable during the recording session. Under the current-clamp mode, recordings were accepted when cells had a resting membrane potential between –58 and –70 mV and a series resistance of 9–15 MΩ stable during the recording session. Under the current-clamp mode, the membrane potential was monitored by a voltage-clamp amplifier (pCLAMP software) (Molecular Devices, Sunnyvale, CA). Data were collected and analyzed by means of pCLAMP software (Molecular Devices).

**Biocytin labeling**

To identify the locations of the recorded neurons in the DMN and the cells’ morphological characteristics, the cells were filled with biocytin while they were held in whole cell patch mode. Because the cell group spans 400 μm mediolaterally and 300 μm dorsoventrally (Fig. 1) (Feng and Lin 1996), we selected DMN neurons from a depth of 300 μm of the dorsal margin of the brain slice. To avoid inadvertent recording from the dorsal extension of the medial vestibular nucleus, the extreme lateral and medial extensions of the DMN were not recorded.

At the end of a recording session, we placed the slice(s) in 4% paraformaldehyde overnight. Slices were reacted with avidin-biotin-peroxidase complex (ABC Elite, Vector Labs) (Cox et al. 1996), mounted, and cover slipped with permount (Fisher Scientific, Pittsburgh, PA). Labeled neurons were examined under a light microscope and drawn with the aid of a camera lucida drawing attachment. To obtain the mean soma diameter, two measurements of the major (long) axis and the minor (short) axis were averaged and indicated.

**Chemicals**

Concentrated stock solutions of pharmacological agents were prepared and diluted in Ringer solution to a final concentration before use: ZD 7288 (N-ethyl-1,6-dihydro-1,2-dimethyl-6-methyliminoo-N-phenyl-4-pyrimidin amine, 50 μM), a specific blocker for Ih, was purchased from Tocris (Ellisville, MO), α-DTX (a blocker for Ikᵢ), 50 nM) from Alomone Labs (Israel), and TEA (a blocker for Iₙᵢ, 2 mM) from Sigma. These drugs were bath applied in final concentrations.

**Data analysis**

The resting membrane potential (RMP) was measured from the voltage trace 1 min after whole cell configuration was achieved; the potential was corrected for a measured junctional potential of –10 mV. The membrane time constant was represented by the elapsed time for the voltage to reach 63% of the peak value and determined by fitting exponential curves to hyperpolarizing voltage responses. The cell’s input resistance was calculated from its steady-state membrane potential, i.e., from the voltage change induced by small hyperpolarizing current pulses (–10 to –50 pA). We used the peak amplitude of the first spike (relative to the after-spike membrane potential) at the lowest depolarizing current as the spike amplitude. The first-spike latency at threshold was the time from the onset of a stimulus to the time of occurrence of the peak of the first spike at threshold depolarizing current. The spike half-width was the width of an action potential at the half-maximum amplitude (re: threshold). Numerical results are given as means ± SD in Table 1. Differences of the means

**Table 1. Biophysical and membrane characteristics of the major cell types in the DMN**

<table>
<thead>
<tr>
<th>Membrane Properties</th>
<th>Onset I</th>
<th>Onset II</th>
<th>P-burst</th>
<th>S-chopper I</th>
<th>S-chopper II</th>
<th>Adapting</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMP, mV</td>
<td>–62.6 ± 2.5&lt;sup&gt;1&lt;/sup&gt;</td>
<td>–63.7 ± 2.4&lt;sup&gt;1&lt;/sup&gt;</td>
<td>–60.3 ± 2.0&lt;sup&gt;1&lt;/sup&gt;</td>
<td>–61.9 ± 2.7&lt;sup&gt;1&lt;/sup&gt;</td>
<td>–59.8 ± 1.0&lt;sup&gt;1&lt;/sup&gt;</td>
<td>–62.7 ± 2.5&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Input resistance, Ω</td>
<td>44 ± 17&lt;sup&gt;1&lt;/sup&gt;</td>
<td>142 ± 30&lt;sup&gt;1&lt;/sup&gt;</td>
<td>156 ± 67&lt;sup&gt;2,3&lt;/sup&gt;</td>
<td>306 ± 116&lt;sup&gt;2&lt;/sup&gt;</td>
<td>2579 ± 484</td>
<td>208 ± 75&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Time constant, ms</td>
<td>1.3 ± 0.8&lt;sup&gt;1&lt;/sup&gt;</td>
<td>4.0 ± 1.6&lt;sup&gt;1&lt;/sup&gt;</td>
<td>5.1 ± 2.4&lt;sup&gt;2&lt;/sup&gt;</td>
<td>12.9 ± 5.5&lt;sup&gt;3&lt;/sup&gt;</td>
<td>79.9 ± 26.2</td>
<td>7.2 ± 4.8&lt;sup&gt;2,3&lt;/sup&gt;</td>
</tr>
<tr>
<td>*First-spike amplitude, mV</td>
<td>61.3 ± 12.0&lt;sup&gt;3&lt;/sup&gt;</td>
<td>45.5 ± 16.6&lt;sup&gt;1&lt;/sup&gt;</td>
<td>20.0 ± 6.6&lt;sup&gt;1&lt;/sup&gt;</td>
<td>53.2 ± 11.7&lt;sup&gt;3&lt;/sup&gt;</td>
<td>55.0 ± 13.0&lt;sup&gt;1&lt;/sup&gt;</td>
<td>37.3 ± 15.9&lt;sup&gt;1,2&lt;/sup&gt;</td>
</tr>
<tr>
<td>*First-spike latency, ms</td>
<td>3.7 ± 2.1&lt;sup&gt;1&lt;/sup&gt;</td>
<td>3.6 ± 1.2&lt;sup&gt;1&lt;/sup&gt;</td>
<td>6.8 ± 2.6&lt;sup&gt;1&lt;/sup&gt;</td>
<td>16.3 ± 5.7&lt;sup&gt;3&lt;/sup&gt;</td>
<td>146 ± 49</td>
<td>9.9 ± 8.2&lt;sup&gt;2,3&lt;/sup&gt;</td>
</tr>
<tr>
<td>*Spike Half–width, ms</td>
<td>0.60 ± 0.08&lt;sup&gt;1&lt;/sup&gt;</td>
<td>1.03 ± 0.27&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1.0 ± 0.2&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.1 ± 0.8&lt;sup&gt;2,3&lt;/sup&gt;</td>
<td>3.0 ± 1.0</td>
<td>0.97 ± 0.40&lt;sup&gt;2,3&lt;/sup&gt;</td>
</tr>
<tr>
<td>N</td>
<td>15</td>
<td>14</td>
<td>15</td>
<td>16</td>
<td>7</td>
<td>8</td>
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</table>

Values are means ± SD of various measures. Superscripts with the same numbers show means that are not significantly different at the 0.05 level (Tukey multiple comparison ANOVA test); tests yielding significant differences in the means are without superscripts. DMN, dorsal modullary nucleus; RMP, resting membrane potential. *Measurement with unit’s first spike at near threshold level.
among groups were evaluated using Tukey multiple comparison ANOVA test; difference was significant if $P < 0.05$ (SAS program).

To assess the regularity of firing in neurons displaying sustained discharges, we measured the CVs from their interspike interval histograms. Neurons having a CV of $>0.3$ were defined as adapting neurons, and those having a CV of 0.3 or lower as chopper neurons (Peruzzi et al. 2000).

To gain insight into the strength of hyperpolarization-activated current ($I_h$) in the different functional cell types, inward currents were induced in voltage-clamp mode in 10 mV steps from $-50$ to $-130$ mV. The $I_h$ magnitude was measured by subtracting the peak current amplitude with the peak current amplitude after application of ZD 7288. All negative current values ($\text{pA}$) obtained for maintaining the negative potentials ($-50$ to $-130$ mV) were converted into positive values ($\text{pA}$) and plotted.

RESULTS

The DMN is situated in the dorsolateral region of the medulla at the entrance of the eighth cranial nerve (Fig. 1A) (Butler and Hodos 1996; Feng and Lin 1996; Wilczynski and Endepols 2006). As a reference, the various cell types and their topographical distributions within the DMN are shown in Fig. 1B. We performed intracellular recordings (in whole cell patch mode) from 75 DMN neurons in brain slices of 58 young adult $R.\ p.\ p.$ Under current-clamp mode, DMN neurons displayed four dominant temporal discharge patterns in response to depolarizing currents (250 ms): onset, phasic-burst, sustained-chopper, and adapting. 

Onset neurons

Onset neurons fired one spike with short latencies in response to depolarizing currents, irrespective of the current magnitude between 200 and 800 pA (Fig. 2, A and D). Of the 29 onset neurons recorded, only two cells produced two spikes at the maximum suprathreshold depolarization. To observe the subthreshold voltage transients (or humps) of onset neurons, we applied fine step currents with 25 pA increments, following the procedure described for the mammalian and avian CN (Golding et al. 1999; Zhang and Trussell 1994). The current increments induced stepwise humps in onset neurons (Fig. 2A, inset), and eventually triggered the firing of an action potential.

In response to hyperpolarizing current, these neurons showed an overt depolarization sag, and for 15 of the 29 onset neurons (i.e., the onset type-I neurons), there was a rebound spike when the current was withdrawn (Fig. 2B). In light of the depolarization sag, the cell’s current-voltage (or $I-V$) response curves for its transient and steady-state responses to hyperpolarizing currents showed a divergence (Fig. 2C). The depolarization sag is attributed to the strong presence of a hyperpolarization-activated current ($I_h$); as to be described later (see Fig. 10), $I_h$ was abolished with bath application of ZD 7288 (an $I_h$ blocker).

Onset type I neurons had the lowest input resistance ($44 \pm 17 \text{ M}\Omega$) among all functional phenotypes (Table 1), presumably because of the widespread distribution of $I_h$ channels that are active even at resting membrane potentials and the comparatively large cell size. On average, these cells had a mean soma diameter of $18.8 \pm 3.7 \mu\text{m}$ ($n = 6$)—this was 46% larger than that of the other cell types ($12.5 \pm 5.1 \mu\text{m}$, $n = 16$). These neurons also had the shortest time constant ($1.3 \pm 0.8\text{ ms}$) and first-spike latency ($3.7 \pm 2.1\text{ ms}$).

All but two biocytin-filled onset neurons were large bushy cells (Fig. 2Ei); the two exceptions were octopus cells (Fig. 2Eii) having a soma shaped like an octopus’s head and two to three primary dendrites arising from two corners of its soma (Feng and Lin 1996). The majority of onset neurons were found in the dorsomedial region of the DMN.

For the remaining 14 onset cells (i.e., the onset type-II neurons), there was no rebound spike even in response to strong ($>300$ pA) hyperpolarizing currents (Fig. 3B). Similar to type I, type II onset neurons fired only one spike, followed by a brief hyperpolarizing undershoot. Compared with onset type I neurons, the input resistances of type II onset neurons were markedly higher ($142 \pm 30 \text{ M}\Omega$), and their time constant ($4.0 \pm 1.6\text{ ms}$) was longer, but the first-spike latency was different (Table 1). Onset type II neurons also displayed a strong depolarization sag and divergent $I-V$ curves in response to hyperpolarizing currents (Fig. 3B and C). Onset type II neurons were concentrated in the medioventral region of the DMN instead of the dorsomedial region where the onset type I cells resided. Their somatic morphology resembled that of small bushy cells ($11.1 \pm 1.3 \mu\text{m}$, $n = 5$) having ovoidal somata; their dendritic arborization was characterized by several primary dendrites radiating out of different poles of the
Phasic-burst neurons

Phasic-burst neurons also fired only one spike in response to weak depolarizing currents, but unlike onset neurons, application of stronger currents (>300 pA) consistently elicited two to four spikes at increasing interspike intervals (Fig. 4A). A further increase in depolarization current typically suppressed the late spikes, reducing the spike count from four to three to two, without affecting the early spikes or the first-spike latency (Fig. 4D). Phasic-bursts also differed from onset neurons in that they exhibited depolarization sag that was smaller but more rapid than that of onset neurons (Fig. 4B). As such, their transient and steady-state responses to hyperpolarizing currents generally also showed a slight divergence (Fig. 4C). Phasic-bursts had the smallest first-spike amplitude (20.0 ± 6.6 mV) among all functional phenotypes. They had a short first-spike latency (6.8 ± 2.6 ms), a narrow spike half-width (1.0 ± 0.2 ms), and a membrane time constant (5.1 ± 2.4 ms) that ranked second shortest behind the onset neurons.

Inspection of biocytin-filled phasic-burst neurons showed that they showed appearance of small bushy cells with axons projecting to the superior olivary nucleus (SON), either ipsilaterally (Fig. 4Ei), contralaterally (Fig. 4Eii), or bilaterally (Fig. 4Eiii).

Sustained-chopper neurons

The most broadly distributed functional phenotype in the DMN was sustained-choppers. In response to depolarizing currents, these cells showed regular discharges at constant interspike intervals (CV < 0.3) over the duration of the current injection (Fig. 5A). Sustained-choppers (n = 23) could also be divided into two subcategories based on the cell’s input resistance and the presence/absence of depolarization sag. Type I sustained-choppers (n = 16) had input resistances of <500 MΩ (average = 306 ± 116 MΩ; Table 1). The spikes of type I cells displayed overt undershoot and rapid repolarization compared with type II sustained-choppers (Fig. 5A, inset). In response to hyperpolarizing currents, they showed a depolarization sag (Fig. 5B) and hence a divergence of the transient and steady-state responses to hyperpolarization currents (Fig. 5C). About one half of type I sustained-choppers exhibited a progressively higher spike count, showing a higher frequency of firing and a shorter interspike interval with increasing current level, i.e., monotonic rate-level functions (Fig. 5D). The remainder of type I sustained-choppers displayed nonmonotonic rate-level functions (data not shown). Biocytin-filled type I sustained-
Adapting neurons

Adapting neurons were characterized by a marked firing adaptation (CV > 0.3), i.e., increasing interspike intervals over time with depolarizing current injections (Fig. 7A). These neurons generally showed nonmonotonic rate-level functions (5/7 cells; Fig. 7D). Like phasic-burst neurons, in response to hyperpolarization currents, these cells displayed a small depolarization sag and thus a slight divergence in the hyperpolarization responses of their I-V curves (Fig. 7, B and C). Adapting neurons had intermediate membrane input resistance (208 ± 75 MΩ) and time constant (7.2 ± 4.8 ms). Biocytin-filled adapting neurons showed radiating dendritic arborization patterns (Fig. 7E) (Feng and Lin 1996), similar to cartwheel cells in the mammalian CN (Golding and Oertel 1997).

Entrainment characteristics of DMN neurons

To gain insight into the functional significance of DMN neurons with different biophysical properties, we studied their entrainment ability in response to depolarizing pulse trains at different repetition rates, from 5 to 500 pulses per second (pps). We found that, with near-threshold stimulations, phasic-burst neurons exhibited the strongest entrainment ability with 1–1 firing up to a few hundred pulses per second (Fig. 8A). In contrast, sustained-chopper neurons showed the lowest entrainment ability, with some having difficulties following the pulse train even at 20 pps (Fig. 8A); the absolute position of the aborted spike varied with repetition rate and from cell to cell.
Adapting neurons, respectively) are shown in Fig. 8A. The pooled data for near threshold stimulations (n = 7, 6, 6, 3, and 3 for onset neurons, phasic-burst, type I sustained-choppers, type II sustained-choppers, and adapting neurons, respectively) are shown in Fig. 8B. The differences in the entrainment ability among cell types are statistically significant (P < 0.01, ANOVA test; Fig. 8B).

Adapting and onset neurons had intermediate entrainment abilities (Fig. 8A). The obtained data for near threshold stimulations (n = 7, 6, 6, 3, and 3 for onset neurons, phasic-burst, type I sustained-choppers, type II sustained-choppers, and adapting neurons, respectively) are shown in Fig. 8B. The differences in the entrainment ability among cell types are statistically significant (P < 0.01, ANOVA test; Fig. 8B).

The differential entrainment ability for the different functional cell types was maintained over a wide range of stimulation levels, from near- to supra-threshold. Because of the broad range of input resistances for these cell types, the absolute currents that could be applied to them were different and thus are normalized in Fig. 8B (low, mid, and high referred to 1–1.2, 1.3–1.5, and 1.6–2 times threshold currents, respectively). The maximum following frequency for phasic-bursts averaged 388 ± 55 pps; those for onset neurons, adapting neurons, type I sustained-choppers, and type II sustained-choppers were 255 ± 18, 333 ± 00, 250 ± 00, and 166 ± 00 pps, respectively.

Strength and role of hyperpolarization-activated current (I_h)

To determine the presence of I_h and its functional role in the different cell types, we applied 50 μM ZD 7288 to the bath and examined the units’ responses to membrane polarizations under current-clamp and voltage-clamp modes. All phasic-burst neurons tested showed a robust depolarization-sag when membrane was hyperpolarized, indicating the presence of I_h (Fig. 9Aii); I_h was stronger with increasing membrane hyperpolarization (Fig. 9Aii). Application of ZD 7288 abolished the depolarization sag in the current clamp data and the hyperpolarization-activated inward current in the voltage clamp data (Fig. 9Aii). Drug application also hyperpolarized the cell’s resting membrane potential by 10 ± 2.7% and increased the cell’s input resistance by 101 ± 27%. Note that I_h remained active at membrane potential of between –60 and –70 mV, around which the entrainment took place.

To assess the contribution of I_h to the cell’s entrainment ability, we performed the entrainment tests before and during bath application of ZD 7288. We found that drug application lowered phasic-burst cell’s entrainment ability; under current-clamp mode, the drug elevated the threshold for 1:1 firing (Fig. 9Bii) and reduced the number of spikes (500 pps) at suprathereshold levels (Fig. 9Bii), without any effect on the response latency (Fig. 9Bii). The jitter of response latency (the elapsed time from the onset of stimulation to the peak of the
action or subthreshold potential) was measured by calculating the SD of the average response latency to the five current pulses.

Of all the functional cell types, onset neurons showed the strongest ZD 7288–sensitive $I_h$ ($n = 4, 315 \pm 41$ pA at $-100$ mV; Fig. 10, A and B). In contrast, at the same membrane potential ($-100$ mV), type I sustained-choppers ($n = 2$) showed generally weak $I_h$, averaging $38 \pm 20$ pA (Fig. 10, A and B), with phasic-bursts having an intermediate $I_h$ ($n = 3, 179 \pm 37$ pA; Fig. 10). The magnitude of $I_h$ (to $-100$ mV holding potential) was significantly different between cell types ($P < 0.01$, ANOVA test). In mammals, $I_h$ shows a sigmoidal function of the membrane potential—it begins to activate near the RMP, and its activity is saturated at approximately $-110$ mV (Pape 1996). In frog DMN, however, $I_h$ was well fitted with an exponential (rather than sigmoidal) curve even to membrane potential of $-130$ mV.

Roles of low-threshold ($I_{kl}$) and high-threshold potassium current ($I_{h}$)

To assess the magnitude and role of $I_{kl}$ in the different cell types, we applied 50 nM $\alpha$-DTX, an $I_{kl}$ blocker, into the bath. We found that application of $\alpha$-DTX transformed the firing patterns of onset type I and type II neurons as well as that of phasic-bursts. Under $\alpha$-DTX, onset type I neurons showed sustained firing at suprathreshold depolarization, onset type II gave transient-chopper discharge (with successively decreasing spike amplitude) followed by rippled membrane oscillation, and phasic-bursts exhibited sustained rhythmic firing of mini-spikes (Fig. 11A). In contrast, application of $\alpha$-DTX did not change the firing pattern of type II sustained choppers; the unit’s responses to depolarization current overlapped completely before and after $\alpha$-DTX application (Fig. 11A).

We also examined the effect of $\alpha$-DTX on the entrainment ability of DMN neurons to depolarization pulse trains. The action potentials of phasic-bursts were too small for them to be distinguished from the subthreshold responses, and thus these data were not analyzed further. During the control (i.e., no drug) condition, onset neurons tested ($n = 5$) exhibited 1–1 firing (having different thresholds) to pulse trains from 166 at a low depolarization current to 250 pps at high currents (Fig. 11B). Application of $\alpha$-DTX increased the spike count at all current levels and lowered the threshold for 1–1 firing, thereby enabling this neuron to give 1–1 firing to pulse train at 333 pps at smaller depolarization currents (Fig. 11, B and Bii). Whereas the neuron’s ability to fire 1–1 was strengthened on $\alpha$-DTX application, its jitter was more pronounced (showing variable response latencies at low current levels; Fig. 11, Bi and Biii). These data suggest that $I_h$ plays an important role in membrane excitability and precision of temporal coding in the frog DMN.
In contrast to α-DTX, TEA had a minor effect on the excitability and time coding. Figure 12 shows the effects of TEA on two onset neurons. Application of 2 mM TEA that blocked $I_{kh}$ broadened the spike half-width (from ~0.7 to ~3.0 ms) and increased the spike amplitude (from ~73 to ~79 mV; Fig. 12, A and B). Application of TEA reduced the units’ entrainment ability, mostly because of broadening of their spikes and increasing of spike amplitude (Fig. 12C)—these results suggest the importance of $I_{kh}$ in determining the shape of action potentials.

**Topographical distribution of DMN neurons**

The majority of onset type I and phasic-burst neurons occupied the dorsomedial region of the DMN. Relative to the above, type II onset neurons showing no rebound spike were distributed more centrally and ventrally. In contrast, sustained-chopper neurons occupied throughout much of the DMN, except the extreme dorsal and medial region of the nucleus. Adapting neurons were found in the medioventral region of the DMN.

**DISCUSSION**

This study represents the first comprehensive investigation of the biophysical characteristics of central auditory neurons in anurans, providing insight into the cellular basis of central auditory processing. Previous in vitro intracellular studies of frog central auditory neurons have focused primarily on describing their connectivity patterns and basic responses to stimulation of their ascending and descending afferent sources.
(Christensen-Dalsgaard and Walkowiak 1999; Ende pols and Walkowiak 1999, 2001; Luksch and Walkowiak 1998; Luksch et al. 1996). Results of our patch-clamp experiments showed that DMN neurons exhibit four basic temporal discharge patterns in response to membrane depolarizations, onset, phasic-burst, sustained-chopper, and adapting, similar to what is observed in the CN of birds and mammals that contains multiple subdivisions (Fukui and Ohmori 2004; Manis and Marx 1991; Oertel 1999; Oertel et al. 2000; Ostapoff et al. 1994; Rhode and Greenberg 1992; Soares et al. 2002). These four functional cell-types possess distinct intrinsic membrane properties (Table 1), morphological characteristics, and ionic compositions.

**Onset neurons**

Onset neurons in the DMN typically elicit a single spike at the beginning of depolarization current, and they have a very short time constant, very low input resistance, narrow spike half-width, and very short first-spike latency (Table 1). These properties resemble those of bushy and octopus neurons in the ventral cochlear nucleus in mammals and of principal neurons in the avian magnocellular nucleus (Carr and Soares 2002; Ferragamo and Oertel 2002; Golding et al. 1999; Manis and Marx 1991; Oertel et al. 2000; Rothman and Manis 2003; Schwarz and Puil 1997). Interestingly, labeled DMN onset neurons also have large cell bodies with appearance of bushy cells or octopus cells (Feng and Lin 1996).

As is in the avian magnocellular nucleus (Fukui and Ohmori 2004), onset neurons in the frog DMN consist of two subpopulations with distinct biophysical characteristics. Onset type I shows a single spike in response to depolarizing currents and a rebound spike immediately after withdrawal of hyperpolarization currents. The onset type II features an onset spike in response to depolarizing currents but shows no rebound spike even in response to strong hyperpolarizing currents (that induces −120 mV anti-peak response); these cells have higher input resistances compared with the first population. All onset neurons that were successfully labeled appear to be projection neurons within the transverse plane of the brain slice. Axons project either along the dorsal or the ventral acoustic tract (Figs. 2E and 3E); the destination of axonal projection cannot be determined, however, because of the limited thickness of the slice, but in one case, the axon can be seen to project to the contralateral SON.

The larger soma size and the low input resistance of onset neurons implicate that a large conductance is needed for initiating an action potential. As suggested previously (Carr and Soares 2002; Ferragamo and Oertel 2002; Oertel et al. 2000), this feature is important for detecting the depolarizations at synapses with the afferent auditory nerve fibers and for reducing the voltage fluctuations. In the DMN, type I onset neurons exhibit linear I-V relationship in response to depolarizing currents—they can thus summate multiple inputs linearly. Indeed, onset neurons give finely graded voltage humps (indicative of low-threshold potassium conductance, \(I_{kl}\)) to stepwise increasing depolarizing currents because of their low input resistance (Fig. 2A, inset), as shown also for octopus cells in the CN of mammals and nucleus magnocellularis neurons in the avian (Bal and Oertel 2001; Golding et al. 1999; Oertel et al. 2000; Zhang and Trussell 1994).

Results of \(\alpha\)-DTX experiments suggested that \(I_{kl}\) plays a role in determining the cell’s temporal discharge pattern in response to membrane depolarization. We found that blocking of \(I_{kl}\) transforms a single spike response of onset type I neurons into chopper-like pattern or of onset type II neurons into transient-chopper pattern (Fig. 11). \(I_{kl}\) generally promotes rapid repolarization and thus rapid adaptation of firing. We were therefore surprised that \(\alpha\)-DTX application does not markedly prolong the spike width or increase the spike amplitude; these two parameters under the drug condition (0.73 ± 0.15 ms and 76 ± 2.3 mV) are not significantly different from the control condition (0.72 ± 0.16 ms and 76 ± 1.8 mV; \(P > 0.05\), paired Student’s \(t\)-test, \(n = 5\)). Blocking of \(I_{kl}\) merely reduces the amount of positive current required to reach spike threshold, and increases the spike count, indicating an increase in membrane excitability. In contrast, bath application of 2 mM TEA mostly broadens the cell’s spike half-width and increases the spike amplitude. Thus it seems that, for onset neurons, \(K_{l1}\) is responsible for the cell’s temporal precision and \(K_{l3}\) is involved in determining the spike shape. In the mammalian CN, however, \(K_{l1}\) seems to be responsible for shaping the peak and half-width of action potentials (Bal and Oertel 2001).

**Phasic-burst neurons**

Phasic-burst neurons feature the smallest spike amplitude and a very short time constant (Table 1), and they exhibit the highest entrainment ability in response to repetitive current stimulation (Fig. 8, phasic-burst). These firing properties and intrinsic membrane characteristics are similar to those of spherical bushy cells in the anteroventral cochlear nucleus (AVCN) and principal neurons in the bird nucleus magnocellularis (Fukui and Ohmori 2004; Manis and Marx 1991; Oertel 1999; Schwarz and Puil 1997; Trussell 1999)—these cells are well suited for rapid signal transmission and phase locked discharges.

In the frog DMN, the second and third spikes in phasic-burst neurons generally have lower amplitudes (\(n = 13/15\); Fig. 4A), as reported previously for spherical bushy cells in the AVCN (Schwarz and Puil 1997). In the AVCN, the reduced amplitudes are shown to be caused by Na\(^+\) inactivation and/or voltage-activated K\(^+\) conductance. Na\(^+\) inactivation together with voltage-activated K\(^+\) conductance is also responsible for suppressing the firing duration (Rothman and Manis 2003; Schwarz and Puil 1997). In the frog DMN, application of blocker of \(I_{kl}\) transforms phasic-burst into sustained-chopper (Fig. 11), suggesting that low-threshold potassium conductance alone (without Na\(^+\) inactivation) can account for the phasic-burst pattern.

Like onset neurons, phasic-burst neurons also show a robust \(I_h\), as shown by their responses to hyperpolarization voltage steps, despite exhibiting only a small depolarization sag in response to hyperpolarizing current (Fig. 9A). The small size of depolarization sag is likely attributed to the depolarization effect of \(I_h\) (shown by membrane hyperpolarization after the application of ZD7288 that abolishes the \(I_h\)) as well as the sizable ZD-nonsensitive current (Fig. 10A).

Earlier studies have suggested that \(I_h\) improves temporal precision of firing by shortening the cell’s time constant and reducing temporal summation, suggesting (but without empirical validation) that these would enhance the cell’s entrainment.
ability (Bal and Oertel 2000; Koch and Grothe 2003). The result of our ZD experiment shows directly that blocking of $I_h$ reduces the cell’s entrainment ability at threshold and suprathreshold levels (Fig. 9Bii)—this is the first direct evidence for the importance of $I_h$ on cell’s entrainment ability. However, in agreement with the results of a recent study (Leao et al. 2006a), ZD7288 has no effect on the precision of the response latency of AVCN neurons (Fig. 9Bii).

**Sustained chopper neurons**

Sustained-choppers show sustained and regular firing patterns over the duration of stimulation, and they have the highest input resistance, the longest first-spike latency, and the longest time constant (Table 1). Type I sustained-choppers show depolarization sag in response to hyperpolarization currents; they have an $I_h$ of $38 \pm 20$ pA at $-100$ ml. These neurons closely resemble tonic I neurons in the avian nucleus angularis (Soares et al. 2002), and stellate neurons in the mammalian CN (Ostapoff et al. 1994). As shown in Fig. 8, in response to a train of depolarization pulses, type I sustained choppers show generally inferior entrainment ability compared with onset and phasic-burst neurons.

Type II sustained-choppers have unusually high-input resistance (Table 1). These cells differ from type I sustained-choppers, having different membrane properties and morphologies. Type I and type II sustained-choppers likely serve different functional roles. The very high-input resistance of type II sustained-choppers indicates that they probably have small cell size; anatomical recovery of type II neurons validates this hypothesis, showing that they correspond to small neurons in the DMN (Feng and Lin 1996).

In the CN of mammals, there are also two subtypes of sustained-choppers: stellate-D and stellate-T cells; they differ in their biophysical characteristics and in their innervation patterns and axonal/dendritic morphology (Oertel et al. 1999; Rodrigues and Oertel 2006). The input resistance for stellate-T cells is higher than that for stellate-D cells, but both input resistances are $<100$ M\(\Omega\)—these are more than one order lower than that of type II sustained choppers in the DMN. Although type II tonic neurons in the nucleus of angularis in birds show a higher input resistance than that of stellate-T and -D cells, having a value in the range of 200–250 M\(\Omega\) (Soares et al. 2002), this is still one order below that of type II sustained-choppers in the DMN. Also, neither the stellate-T and stellate-D cells in mammals nor type II tonic cells in avians show the very low entrainment ability exhibited by type II sustained-choppers in the DMN (Fig. 8). It thus seems that type II sustained-choppers are unique, having no obvious biophysical counterpart in other vertebrates.

It is possible that the differences simply reflect the disparities in the age group and myelination pattern of the experimental animals (neonatal in birds and mammals vs. young adult in frogs) or in the absolute cell size (type II sustained choppers in the DMN are very small neurons). It is noted, however, that there are many tiny cells (e.g., granule and golgi cells) in the mammalian CN; these have not been carefully sampled, and it is possible that they may share similar biophysical properties to those reported for type II sustained choppers in the frog DMN.

In response to repetitive depolarization pulses, sustained-choppers exhibit the worst entrainment ability (Fig. 8A1). Therefore coding time-varying signals using a temporal code is problematic, particularly at high rates; they are presumably involved in transforming the auditory input into a rate code, as suggested previously (Peruzzi et al. 2000). Thus in contrast to onset and phasic-burst neurons, which are well suited for temporal processing, sustained-choppers most likely process nonlocalization tasks in the ascending auditory pathways, e.g., processing of intensity cues. Interestingly, in vivo extracellular studies from the DMN have also shown that the entrainment ability of DMN neurons varies among DMN neurons (Feng and Lin 1994). In particular, neurons exhibiting phasic-burst and a class of primarily-like (i.e., PL-3) tone-evoked discharge patterns display superior time-locked responses to a wide range of AM frequencies compared with neurons showing other firing patterns. Whether phasic-burst biophysical phenotype in vitro corresponds to phasic-burst and PL-3 tone-evoked temporal discharge patterns in vivo is unclear.

**Adapting neurons**

Adapting neurons are characterized by a rapid burst of action potentials in response to onset of depolarizing current, followed by spikes whose interspike interval increases over time. Other than the invariance in spike amplitude, these neurons have similar biophysical characteristics as those of phasic-bursts (Table 1), i.e., short time constant, low input resistance, little depolarization sag in response to hyperpolarizing current, and short first-spike latency. Adapting neurons are also found in much the same topographical locations in the DMN as phasic-bursts. However, in addition to having a different temporal firing pattern, adapting neurons differ from phasic-bursts in their somatic and dendritic morphology. Adapting neurons have the appearance of radiate cells (Fig. 7) (Feng and Lin 1996). Adapting neurons presumably account for the diversity of tone-induced primary-like firing patterns found in the frog DMN (Feng and Lin 1994; Hall and Feng 1990), e.g., primary-like type 1 to 3 with differing adaptation rates, from slow to very fast.

Adapting neurons in the DMN are similar to the radiate (type III tonic) neurons in the avian nucleus angularis (Soares et al. 2002); they show linear J-V relationship and they fire a train of overtly adapting spikes in response to strong depolarizing currents. To date, there is no published report of adapting neurons in the mammalian CN; adapting neurons have been observed at lateral lemniscus nuclei and the inferior colliculus (Peruzzi et al. 2000; Zhao and Wu 2001). However, a recent study showed that cartwheel cells in the dorsal CN exhibit marked adaptations in response to depolarizing currents (J Mancilla and PB Manis, unpublished observations). Thus adapting neurons are present in the first central auditory center across all vertebrates after all.

**Comparisons with avian and mammalian CN**

As described above, despite the fact that amphibians arise from a different evolutionary lineage, the frog DMN shares many similar coding strategies with the CN of birds and mammals. The similarities suggest that the cell-specific biophysical characteristics in amphibians, avians, and mammals might have shared a common ancestral origin or alternatively evolved independently. Characterization of the
biophysical properties of the first-order central auditory neurons in fishes is therefore crucial for determining the evolutionary origins of such properties in higher vertebrates.

There are specific differences in physiological and morphological features among the different vertebrate classes, however. First, octopus neurons are observed in the mammalian CN and the frog DMN but not in the avian CN. At the same time, the planar neurons that are found in the avian nucleus angularis (having a damped firing pattern; Carr and Soares 2002; Soares et al. 2002) are not observed in the frog DMN or the CN in mammals (Cant 1992). Second, neurons having a pause-build up firing pattern observed in the mammalian dorsal CN (pyramidal neuron; Kanold and Manis 1999), and a tonic II pattern observed in the avian nucleus angularis (radiate cell; Soares et al. 2002) have yet to be uncovered in the frog DMN. This discrepancy may simply be caused by an age difference in the preparations—in nucleus angularis of the chicken P-build-up pattern disappears after hatching (Fukui and Ohmori 2003). Third, as described previously, type II sustained choppers in the frog DMN have no counterparts in avians or mammals.

At this time, it is unclear whether these differences are attributed to the phylogeny of these different vertebrate classes or to the difference in the absolute cell size or the age of animals for slice preparations (i.e., young adult frogs vs. embryonic/neonatal organisms for avians and mammals). A recent study (Bortone et al. 2006) showed that the expressions of potassium channels change in developing rat auditory brain stem, with some showing a monotonic increase in the mRNA levels during early development and others showing the opposite trend. In the main nucleus of trapezoid body of mice, sodium persistent and resurgent currents are not present at postnatal day 7 but are robust at postnatal day 14 (Leao et al. 2006b). It is therefore possible that the age differences in the slice preparations can account for some of the differences in the findings in amphibians, avians, and mammals. Future research is necessary to pin down the origins of these discrepancies. In mammals, tone-induced temporal discharge patterns have been shown to correspond to neuronal morphology, albeit the correspondence is not one-to-one (Rhode and Greenberg 1992); the cell morphology also gives rise to distinct intrinsic membrane properties that again do not bear one-to-one relationships (as described earlier). The absence of 1–1 correspondences is understandable given the heterogeneity of channel expression and synaptic innervation patterns. At this time, it is unclear how the different in vitro biophysical cell types in the DMN correspond to the temporal discharge patterns in response to acoustic stimulation in vivo. Future research involving in vivo intracellular recordings is necessary to establish such correspondences definitively.

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