The pronociceptive dorsal reticular nucleus contains mostly tonic neurons and shows a high prevalence of spontaneous activity in block preparation

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1Instituto de Biologia Molecular e Celular, Porto, Portugal; 2Departamento de Biologia Experimental, Faculdade de Medicina, Universidade do Porto, Porto, Portugal; and 3Centro de Matemática, Universidade do Porto, Porto, Portugal

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Sousa M, Szucs P, Lima D, Aguiar P. The pronociceptive dorsal reticular nucleus contains mostly tonic neurons and shows a high prevalence of spontaneous activity in block preparation. J Neurophysiol 111: 1507–1518, 2014. First published January 15, 2014; doi:10.1152/jn.00440.2013.—Despite the importance and significant clinical impact of understanding information processing in the nociceptive system, the functional properties of neurons in many parts of this system are still unknown. In this work we performed whole cell patch-clamp recording in rat brain stem blocks to characterize the electrophysiological properties of neurons in the dorsal reticular nucleus (DRt), a region known to be involved in pronociceptive modulation. We also compared properties of DRt neurons with those in the adjacent parvicellular reticular nucleus and in neighboring regions outside the reticular formation. We found that neurons in the DRt and parvicellular reticular nucleus had similar electrophysiological properties and exhibited mostly toniclike firing patterns, whereas neurons outside the reticular formation showed a larger diversity of firing patterns. Interestingly, most of one-half of the neurons also showed spontaneous activity. While the general view of the reticular formation is a loosely associated mesh of groups of neurons with diverse function, and earlier reports suggest more electrophysiological heterogeneity, we showed that this is indeed not the case. Our results indicate that functional difference of neurons in the reticular formation may mostly be determined by their connectivity profiles and not by their intrinsic electrophysiological properties. The dominance of tonic neurons in the DRt supports previous conclusions that these neurons encode stimulus intensity through their firing frequency, while the high prevalence of spontaneous activity most likely shapes nociceptive modulation by this brain stem region.

dorsal reticular nucleus; pronociception; whole cell patch-clamp; tonic activity; spontaneous activity

THE DORSAL RETICULAR NUCLEUS (DRt, also called subnucleus reticularis dorsalis) is located in the dorsolateral quadrant of the medulla oblongata, surrounded by the cuneate nucleus, the nucleus tractus solitarius, motor nuclei of the vagal (10N) and hypoglossal (12N) nerves, the spinal trigeminal nucleus and the caudal ventrolateral reticular formation. The DRt is part of the medullary dorsolateral reticular formation and extends from the spinomedullary junction rostrally, up to the level of the rostral border of the area postrema (Andrezik and Beitz 1985; Newman 1985). DRt neurons were shown to be preferentially (partial nociceptive convergent neurons) or exclusively (total nociceptive convergent neurons) activated by noxious stimulation conveyed by Aδ and C fibers from the entire body (Lima and Almeida 2002; Villanueva et al. 1988, 1989). The number of brain and spinal areas projecting to the DRt are extensive (Lima and Almeida 2002), including projections from the somatosensory cortex (Almeida et al. 1999; Desbois et al. 1999; Valverde 1962), locus coeruleus and nucleus raphe magnus (Velo et al. 2013) and bilateral projections from the spinal cord with a predominance of those originated in the dorsal horn (Lima 1990; Villanueva et al. 1991). Although the reticular formation also relays signals from the eyes and ears to the cerebellum, DRt neurons do not seem to respond to visual, auditory or proprioceptive stimuli (Villanueva et al. 1996). There is also strong evidence that DRt may support a spinoreticulo-thalamo-cortical nociceptive pathway providing whole body nociceptive information to large areas of the cortex (Almeida et al. 2002).

A critical functional aspect of the DRt is its involvement in a positive feedback loop, activated by nociceptive inputs and exerting excitatory effects within the superficial and deep laminae of the spinal dorsal horn in rodents (Almeida et al. 1993, 2000; Bernard et al. 1990; Lima 1990; Raboisson et al. 1996; Soto and Canedo 2011; Tavares and Lima 1994; Villanueva et al. 1991; Villanueva and Le Bars 1995). This loop supports the hypothesis that the DRt exerts pronociceptive actions in spinal dorsal horn nociceptive neurons by potentiating their response to peripheral stimulation (Lima and Almeida 2002; Monconduit et al. 2002), and gives to the DRt an important role in the integration and control of nociceptive information.

Action of this positive feedback loop is apparent from saturation of DRt responses to increasing noxious thermal stimulations in the presence of spatial summation (Villanueva et al. 1989) or to mechanical stimuli by increasing the size of the stimulated area (Villanueva et al. 1994). Local administration of glutamate in the DRt induces long-lasting increase in the responsiveness of nociceptive spinal neurons (Dugast et al. 2003), while lidocaine leads to the suppression of responses (reviewed by Lima and Almeida 2002). DRt neurons were also shown to encode the intensity of noxious stimuli and to be associated with diffuse noxious inhibitory controls (Le Bars 2002; Roy et al. 1992; Villanueva et al. 1989).

While the anatomical connections (Velo et al. 2013) and possible functional roles of the DRt are relatively well described in rodents, mostly in rat (Almeida et al. 2002; Lima and Almeida 2002), to the best of our knowledge, very little is known about the basic electrophysiological properties of DRt neurons. Furthermore, the available electrophysiological data were obtained from a different species, cat (Soto and Canedo 2011).

It is also not known whether electrophysiological properties of DRt neurons are different from that of neurons in the
adjacent brain stem areas, such as the parvicellular reticular nucleus (PCRt). While these two adjacent regions are both part of the meshlike neuronal column of the reticular formation, their input is clearly different. Ascending axons from the spinal dorsal horn to the reticular formation could not be traced further than the rostral border of DRT (Raboisson et al. 1996), while PCRt receives projections from the trigeminal mesencephalic nuclei neurons and contains the dorsal group of interneurons that integrate and coordinate activity of the oral motor nuclei (Zhang and Luo 2003).

Thus the primary focus of this work was 1) to characterize electrophysiological properties of DRT neurons in the rat using intracellular recordings; 2) to establish functional classes; and 3) to identify electrophysiological features that would distinguish DRT neurons from neurons in adjacent reticular formation and neighboring brain stem regions.

MATERIALS AND METHODS

Ethical guidelines. Laboratory Wistar rats [postnatal day (P) 14–P18] were killed in accordance with the Portuguese guidelines (Direccão Geral de Veterinaria, Ministério da Agricultura) after anesthesia by intraperitoneal injection of pentobarbital sodium (30 mg/kg) and subsequent check of pedal withdrawal reflexes. The experiments were approved and carried out according to the guidelines laid down by the institution’s animals ethics committee (Comissão de Ética do Instituto de Biologia Molecular e Celular). The number of animals used in this study was 124.

Preparation. The animal was quickly decapitated at the cervical level, and the head was immersed in oxygenated artificial cerebrospinal fluid (ACSF) at room temperature. The parietal and occipital bones were removed, and the brain along with the brain stem was carefully dissected out from the cranial base. The brain stem was exposed by cutting away first the cerebral cortex, followed by the cerebellum and finally the tectum. After the remaining pia mater was removed, transverse cuts were performed with a razorblade roughly at the level of the transition between the open and the closed part of the fourth ventricle and 2–3 mm away caudally from this point. The caudal surface of the resulting brain stem block was glued, with cyanoacrylate adhesive, to a 0.7-mm-thick golden plate and transferred to the recording chamber. The metal plate provided mechanical stability for the preparation throughout the recording and labeling procedure.

Neurons on the cut surface were visualized using the oblique infrared light-emitting diode illumination technique (Szucs et al. 2009). The infrared light-emitting diode (L850F-02U; Marubeni) was positioned outside the solution meniscus present in the recording chamber. The images obtained with a digital charge-coupled device camera (C4742–95; Hamamatsu) were adjusted using Adobe Image Ready software.

Histological processing and reconstruction. After fixation (4% paraformaldehyde; 4°C for a least 12 h), the brain stem block was embedded in agar with the recorded side facing upward, and transverse serial 100-μm-thick sections were prepared with a vibrating microtome (Leica VT 1000S). To reveal the biocytin, the free-floating sections were permeabilized with 50% ethanol, treated according the avidin-biotinylated horseradish peroxidase method (ExtrAvidin-peroxidase, diluted 1:1000), and the histochemical reaction was completed with a diaminobenzidine chromogen reaction. Sections were counterstained with 1% toluidine blue, to help in determining location of the cells during evaluation, and mounted with EUKITT (Fluka, Buchs, Switzerland). Photomicrographs were taken with a Primo Star (Zeiss) equipped with a Guppy (Allied Vision Technologies, Tsaadtroda, Germany) digital camera. Contrast and brightness of the images used for the figures were adjusted using Adobe Image Ready software.

Location of the neurons was determined using a ×4 or a ×10 objective. When the soma was located in an incomplete section (in most cases, due to the uneven cut surface of the block), the subsequent serial sections were also checked to establish the borders of landmark structures (nuclei of cranial nerves X and XII).

Complete three-dimensional reconstruction of a labeled cell, showing characteristic morphological features of reticular formation neurons, along with contours of the brain stem and borders of landmark structures was performed with a ×40 objective, using Neurolucida (MBF Bioscience, Williston, VT) as described in Szucs et al. (2013).

Spontaneous activity analysis. Suprathreshold spontaneous activity data (i.e., action potentials at a membrane potential with zero injected current), acquired in current clamp mode, was analyzed using custom-made scripts developed in MATLAB 7.9 (MathWorks). Interspike intervals were calculated from the time points of the peaks of the spontaneously occurring spikes (detected using a threshold algorithm). Fast Fourier transforms, power spectra and autocorrelations were also calculated using custom scripts developed in MATLAB.
**Postsynaptic event analysis.** Excitatory and inhibitory postsynaptic potentials (PSPs) were counted on three consecutive 850-ms-long current clamp traces corresponding to the −20-, −10- and 0-pA steps of the current protocol used to establish the firing pattern of the neurons. Action potentials that could mask subthreshold PSPs are usually absent from these traces, and whenever a trace contained action potentials it was excluded. In case of cells with more than one firing pattern protocol, the PSP count was averaged. Excitatory and inhibitory postsynaptic currents (EPSCs and IPSCs, respectively) were automatically detected in voltage-clamp traces, using the calculated membrane resting potential as the holding potential, and quantified using the Mini Analysis Program 6.0.7 (SynaptoSoft, Decatur, GA). All detected events were inspected visually to exclude false PSCs. Plotting and data analysis were performed using MATLAB.

The current noise was 1.7 pA in our experiments; thus this minimum amplitude threshold satisfied the criteria of being more than three times the measured current noise (Santos et al. 2009). The current noise magnitude was calculated as the root mean square of membrane potential fluctuations in voltage-clamp (Diba et al. 2004; Jacobson et al. 2005).

**Subthreshold membrane potential distributions.** As an in-depth analysis of electrophysiological properties, membrane potential distributions were calculated for neurons exhibiting spontaneous activity to provide information about membrane current dynamics, (bi-)stability, multiple membrane states (e.g., up/down), changes in membrane equilibrium potential and balance between excitation and inhibition. The membrane potential distributions were obtained from the membrane potential histograms of long recordings (typically 100 s) in current-clamp mode with zero injected current. Signals were low-pass filtered at 3 kHz before digitization at 10 kHz. The bin size used in the histograms was 0.5 mV for experimental data and 0.1 mV in the histograms for simulation results. Although membrane potential distributions were calculated for the whole range of membrane potentials, the time spent in the suprathreshold interval was negligible compared with the time spent in subthreshold. As a consequence, the cumulative probability mass above −40 mV was always very small and therefore excluded from the figures. For each neuron, the membrane potential median was calculated and defined as a median potential $V_{\text{med}}$. This potential value, $V_{\text{med}}$, often corresponded to the baseline potential in the voltage traces and especially in the simulations.

**Computer simulations.** Biophysically detailed mathematical models of neuronal dynamics were used to: 1) recreate the firing properties of DRT neurons; 2) study the possible membrane currents giving rise to the after-hyperpolarization (AHP) variability observed experimentally; and 3) explore the mechanisms shaping the membrane potential histograms under spontaneous activity conditions. All simulations were performed in NEURON 7.2 (Hines and Carnevale 1997). The questions addressed in this work were not focused on space-related properties; therefore, a single compartmental model was used to study firing properties and temporal features of the membrane dynamics of DRT neurons. The Hodgkin-Huxley formalism (Hodgkin and Huxley 1952) was used to describe the ionic currents present in the model: a persistent sodium current ($I_{\text{h}}$), a delayed-rectifier potassium current ($I_{\text{Kdr}}$), a voltage-dependent calcium current ($I_{\text{Ca}}$, L-type high threshold), and a calcium-activated potassium current ($I_{\text{KCa}}$, or $I_{\text{AHP}}$ given its role in shaping the AHP). The mathematical descriptions for each of these ionic currents followed the models combined in Aguiar et al. (2010). The neuron model also included a description for the internal calcium concentration dynamics (Destexhe et al. 1993). Synapses were modeled using dual exponential conductance profiles and were introduced for the sole purpose of introducing and analyzing the contribution of membrane fluctuations. The parameterization of the synaptic inputs was set to produce a zero net change in membrane potential (Aguiar et al. 2010). Stochastic network activity was simulated by adding random excitatory and inhibitory synaptic inputs with predefined mean times. In some simulations, membrane potential fluctuations were generated by injecting normally distributed current noise to the neuron model. The model implementation in NEURON is available through ModelDB (Hines et al. 2004) under accession number 151949.

**RESULTS**

A total of 100 neurons were recorded in the dorsomedial quadrant of brain stem block preparations. Morphological recovery that allowed identification of the neuron or its location was achieved in 64 cases, and the dataset was further reduced to 43 cells by removing neurons that did not remain stable for sufficient time to perform adequate electrophysiological characterization. The locations of these cells are shown in two schematic representations of the brain stem block surfaces (at the level of PCrT and of DRT; Fig. 1A). From the 43 neurons, 30 were located within the reticular formation, 16 in the DRT, while 14 more rostrally, in the adjacent PCrT. The remaining 13 neurons were located in the neighboring solitary nucleus complex (Sol) and in the nucleus of the 10th cranial nerve (10N) (Fig. 1B and C). Neurons in the DRT and PCrT had a mean soma cross-sectional area of 385 ± 32 μm² and 398 ± 39 μm², respectively, and issued in most cases two to five slender long dendrites (Fig. 1, D and E) that extended both mediolaterally and rostrocaudally. Neurons recovered outside the reticular formation (Sol and 10N) had larger somata (mean cross-sectional area: 491 ± 49 μm²) and more dendrites with frequent branching (not shown).

**Passive and active membrane properties.** Passive and active membrane properties of the recorded neurons are shown in Table 1. Passive membrane properties ($E_{\text{med}}$, $R_{\text{in}}$, and $\tau_{\text{m}}$) of the neurons in the four regions did not show any significant difference, although the combination of smallest $\tau_{\text{m}}$ and a high $R_{\text{in}}$ in case of DRt neurons suggests somewhat smaller somata in this group. Action potential amplitude and overshoot, measured on voltage traces corresponding to the first suprathreshold depolarizing current step, was the highest in DRt neurons (Table 1). The same tendency was observed when measuring several hundreds of action potentials in neurons discharging spontaneously with zero injected current; action potentials of DRt neurons had the highest mean overshoot (25.1 ± 0.7 μV; $n = 2,100$), while the overshoot was smaller in action potentials of PCrT (15.1 ± 1.2 μV; $n = 2,987$) and non-Rt (15.6 ± 1.1 μV; $n = 1,492$) neurons. Interestingly, non-Rt neurons had the slowest spikes (neurons in 10N in particular), which was evident from the significantly larger half-width of the action potentials.

**Firing patterns.** Neurons in the reticular formation (DRt and PCrT) responded, almost exclusively, with tonic firing profiles to depolarizing current steps; the single exception being a DRt neuron that showed gap-firing. Neurons outside the reticular formation, especially those in 10N, presented a larger repertoire of firing patterns: tonic, gap-firing, and single-spike (Fig. 2A). The single-spike pattern was only present in neurons of 10N. The response curves for the tonic neurons from the three recorded regions are presented in Fig. 2B. Given the firing...
pattern (tonic) and the implicit assumption of rate coding, the response curves were measured as firing-frequency as a function of input current. The response profile is similar for the tonic neurons from the three regions, but DRt and PCRt neurons apparently have a wider dynamic range (non-Rt tonic neurons, on average, saturate at a smaller frequencies). The rheobase current (minimal current to elicit a response) for the three regions was (mean ± SD): 14.0 ± 9.9 pA in DRt, 17.9 ± 25.5 pA in PCRt, and 12.9 ± 21.4 pA in non-Rt tonic neurons.

Representative traces of all the recorded firing patterns for each group are presented in Fig. 2, C–E. The single DRt neuron showing gap-firing (Fig. 2C) deviated only marginally from a tonic profile, as opposed to the distinctive gap-firing pattern of non-Rt neurons. Changes in the membrane’s equilibrium potential during spiking, similar to those reported in the DRt of the cat (Morisset and Nagy 1999), were frequently observed, independent of the location (DRt, 81%; PCRt, 57%; non-Rt, 85%) and firing pattern of the given neuron.

Since the firing pattern of DRt neurons in our sample were dominantly tonic, we investigated what factors influence the tonic pattern. First, the contribution of calcium channels to the firing profile of DRt neurons was assessed experimentally by selectively blocking these channels with equimolar substitution of Ca²⁺ in the ACSF by Co²⁺ (n = 15). Blocking calcium channels did not alter the tonic firing pattern, although it slightly increased firing frequencies in response to the same depolarizing current steps (Fig. 3), suggesting that calcium-dependent potassium currents may be present in these neurons. Changes in the membrane’s equilibrium potential during spiking were not eliminated by blocking Ca²⁺ currents.

### Table 1. Passive and active membrane properties of neurons in the recorded regions

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<th>Passive</th>
<th>Active</th>
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<tr>
<td></td>
<td>Eₐₐ, mV</td>
<td>τₐₐ, ms</td>
</tr>
<tr>
<td>DRt</td>
<td>16</td>
<td>-68 ± 2</td>
</tr>
<tr>
<td>PCRt</td>
<td>14</td>
<td>-68 ± 1</td>
</tr>
<tr>
<td>Sol</td>
<td>7</td>
<td>-66 ± 3</td>
</tr>
<tr>
<td>10N</td>
<td>6</td>
<td>-67 ± 1</td>
</tr>
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</table>

Values are means ± SE; n, no. of neurons. DRt, dorsal reticular nucleus; PCRt, parvicellular reticular nucleus; Sol, solitary nucleus; 10N, nucleus of the 10th cranial nerve; Eₐₐ, resting membrane potential; τₐₐ, membrane time constant; Rₐₐ, input resistance; Amp, spike amplitude; Peak, spike overshoot; Hw, half-width spike duration.
While firing responses to large depolarizing current steps were consistently tonic, analysis of the responses to near-threshold depolarizing steps revealed some variability in the amplitude, duration and number of phases of AHP of action potentials (Fig. 4, A1–A4). Both slow (above 100 ms) and fast (tens of milliseconds) AHPs were observed (Fig. 4, B1 and B2). While AHP in some neurons was monophasic (Fig. 4, B1 and B2), others showed a biphasic AHP consisting of an early fast and a delayed slower component (Fig. 4, B3 and B4).

Next, we tried to reproduce firing response properties of DRt neurons using a biophysically detailed mathematical model of the neuronal membrane dynamics, to identify a minimal set of ionic currents giving rise to the experimentally observed firing patterns. In addition to the canonical voltage-gated transient sodium channels ($I_{Na}$) and delayed rectifier potassium channels ($I_{Kdr}$), the model also included a $I_{K,Ca}$ and a $I_{Ca}$, necessary to

![Fig. 3. Contribution of calcium conductances to the tonic firing pattern of DRt neurons. Voltage responses of a typical DRt neuron to increasing suprathreshold current pulses (bottom line; left to right: 10, 20 and 120 pA) in control condition (top line of traces) and in the presence of 2 mM Co$^{2+}$ (middle line of traces) are shown. The response profile of DRt neurons was relatively unaffected after selectively blocking calcium channels with cobalt.](image1)

![Fig. 2. Firing-pattern profiles of the recorded neurons. A: types of firing patterns, and associated number of recorded neurons, in the three examined regions [DRt, PCRt and non-reticular nucleus (non-Rt)]. B: response curves, firing frequency as a function of injected current, for the tonic neurons in the three regions. The injected current is presented as rheobase current (Rheo) plus additional current. Dark solid lines represent the mean response curve, while dashed lines represent the mean ± SD confidence intervals. Individual neuronal response curves are represented in gray. C: representative membrane potential traces (for three input current levels) for the tonic (C1) and the only gap firing (C2) DRt neurons. D: PCRt neuron responses were exclusively tonic. E: neurons in the non-Rt region exhibited a larger variety of firing-pattern profiles, such as tonic (E1), gap (E2) and single-spike (E3). The three current steps evoking the depicted potential profiles are indicated on the bottom in each case.](image2)

While firing responses to large depolarizing current steps were consistently tonic, analysis of the responses to near-threshold depolarizing steps revealed some variability in the amplitude, duration and number of phases of AHP of action
A (Fig. 6, APPENDIX), while the parameters for the remaining three model. From the minimal set of four ionic currents, (Prinz et al. 2004) was assessed using the same computational ession that the different types of AHP may arise simply from electrophysiological features of reticular neurons. The hypoth- notion that these four currents are sufficient to capture the main variations, which is associated with a Poisson process. The third distribution approximates a negative complementary analyses, including interspike interval distributions, membrane potential fast-Fourier power spectra and autocorrelograms (Fig. 6, B–D). A summary of the prevalence of rhythmic and irregular spontaneous activity is presented in Table 2.

The mean action potential frequency of spontaneously active, rhythmic DRt neurons was 3.0 ± 1.0 Hz (n = 3; range, 1.6–3.5 Hz). Despite the wider range of recorded frequencies (1.4–11 Hz), mean frequency of spontaneous action potentials fired by rhythmic PCRt neurons was very similar, 3.4 ± 1.5 Hz (n = 5). The mean frequency of non-Rt neurons was 5.6 ± 1.5 Hz (n = 6; range, 2–12 Hz).

To investigate whether spontaneous firing activity was associated with membrane bistability, we analyzed firing rate change in response to 1-s-long hyperpolarizing and depolarizing current steps that were applied during the spontaneous activity. In spontaneously active DRt neurons (n = 7), the mean firing rate was calculated, from 10-s-long voltage traces during long periods (180 s; Fig. 7A), as well as before and after a 1-s-long depolarizing (Fig. 7B) or hyperpolarizing current step (Fig. 7D) and also without a test pulse (Fig. 7C). These measurements were performed immediately after stabilization of the cell following the transient perturbation associated with the membrane rupture during the establishment of whole cell configuration. While the mean firing rate of the spontaneous activity showed small fluctuations along time (Fig. 7A), it remained constant when comparing the periods immediately before and after the test stimuli, independent of its depolarizing or hyperpolarizing character.

**Fig. 5.** Firing pattern and different AHP types of DRt neurons reproduced by a biophysically detailed neuronal model. A: superimposed firing patterns in response to a 500-ms-long suprathreshold current pulse, produced by a single compartment model simulation with currents $I_{\text{Na}}$ (sodium), $I_{\text{KCa}}$ (delayed-rectifier potassium), $I_{\text{Ca}}$ (calcium) and $I_{\text{KCa}}$ (calcium-activated potassium) and recorded experimentally (gray). B: modifications in the $I_{\text{KCa}}$ parameters (channel density and current kinetics) in the model were sufficient to recreate (black traces) the AHP variability obtained experimentally (gray traces from Fig. 4, B1–B4). Arrows indicate the first AHP component.

drive calcium inflow. We found that experimentally observed tonic firing patterns could be recreated with a high degree of approximation by the theoretical model (Fig. 5A), enforcing the notion that these four currents are sufficient to capture the main electrophysiological features of reticular neurons. The hypothesis that the different types of AHP may arise simply from small variations on the same functional set of ionic currents (Prinz et al. 2004) was assessed using the same computational model. From the minimal set of four ionic currents, $I_{\text{KCa}}$ alone was subject to modifications (see model 2 parameterization in the APPENDIX), while the parameters for the remaining three currents were kept unchanged. Small changes in the parameters controlling channel density and current kinetics were sufficient to reproduce the types of AHP profiles observed experimentally (Fig. 5B).

**Spontaneous activity.** More than one-half of all recorded neurons (24 of 43) showed spontaneous activity (i.e., discharged action potentials spontaneously) with zero injected current. The prevalence of this spontaneous activity was slightly higher in the case of DRt than in PCRt cells (Table 2), while the highest percentage of spontaneously active cells was observed in Sol. When analyzing spontaneous activity in the reticular formation, some cases appeared to be rhythmic (Fig. 6A, left and middle), while in other cases it looked irregular (Fig. 6A, right). Thus, to better assess the rhythmicity, we used

Table 2. Number of neurons with different types of spontaneous activity in the recorded regions

<table>
<thead>
<tr>
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<th>Rhythmic</th>
<th>Irregular</th>
<th>Total</th>
<th>No SA</th>
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<tbody>
<tr>
<td>DRt</td>
<td>3</td>
<td>6</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>PCRt</td>
<td>5</td>
<td>2</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Sol</td>
<td>3</td>
<td>3</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>10N</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
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</table>

SA, spontaneous activity.

**Fig. 6.** Rhythmic spontaneous activity in DRt neurons. A: membrane potential trace of different DRt neurons showing rhythmic (left and middle columns) or irregular (right column) spontaneous activity. B: histograms of the interspike intervals associated with the traces in A. The first two distributions show clear peaks around 0.53 and 0.44 s. The third distribution approximates a negative distribution which is associated with a Poisson process. C: fast-Fourier power spectrum showing a peak (marked with a *"*”) at 1.6 Hz and at 2.3 Hz in case of the first two neurons. D: autocorrelograms confirming the modulating frequencies of the neurons with rhythmic spontaneous activity: 0.601 s (1.66 Hz) and 0.417 s (2.40 Hz), marked with a "*".  

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Most DRt neurons are tonic and spontaneously active in vitro

Since EPSCs could drive the spontaneous activity observed in all cell groups, we further analyzed their kinetic parameters and frequency. The mean amplitude of EPSCs was 14.3 ± 7.1 pA in DRt, 18.5 ± 4.4 pA in PCrt and 19.5 ± 3.9 pA in Sol neurons. Neurons in all three regions received largely variable EPSCs with overlapping distributions of their rise and decay time constant parameters (Fig. 8, F–H). While mean rise time and decay time of DRt and PCrt neurons were similar, EPSCs received by Sol neurons had rise times that were markedly faster (Fig. 8H), suggesting the involvement of different glutamate receptors. Given that rise times were nevertheless still within the same order of magnitude, this points to possible differences in the types of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors (as opposed to differences in types, or contributions, of N-methyl-D-aspartate or metabotropic glutamate receptors).

To assess regularity of EPSCs interpeak interval (IPI) analysis was performed. Normalized histograms of the IPIs (Fig. 8, C–E; distributions on the right) showed exponential distribution in all three groups, indicating that occurrence of synaptic input followed a Poisson process in all cases. The steep exponential distribution in case of Sol neurons reflects the higher mean frequency of EPSCs.

Membrane potential distribution profiles. To further characterize the electrophysiological properties of spontaneously active (firing) neurons, in which $E_m$ could not be reliably measured, we analyzed membrane potential distributions of current-clamp traces with zero injected current (see MATERIALS AND METHODS). These distributions are shaped by the local network input activity and by the intrinsic membrane properties, and provide a fingerprint of the neuron’s electrophysiology as they reflect the relative time spent at different membrane potentials.

Subthreshold membrane potential distributions showed several distinct histogram profiles that could be grouped in two major classes and were present in all three neuronal populations. The first class was characterized by unimodal (symmetric) or skewed unimodal (asymmetric) distributions with the peak centered at $V_{mem}$ (Fig. 9A). Independent of the region, this profile was more often found in neurons that showed irregular spontaneous activity ($n = 5$) than in the ones with rhythmic spontaneous activity ($n = 3$). Interestingly, while all DRt neurons of this class had symmetric distributions, PCrt cells in our sample had asymmetric distributions.

The second class of membrane potential distributions was characterized by polimodal (often bimodal or quasi-bimodal) distributions with $V_{mem}$ not necessarily matching the mode of the distribution (Fig. 9, B1 and B2). Neurons, in all regions, that showed rhythmic spontaneous activity presented this profile more frequently ($n = 7$) than irregular ones ($n = 4$). The mean firing frequency of rhythmic neurons giving rise to this type of distributions was in the order of 1–3 Hz.

The most prevalent histogram profiles in neurons from all regions showing spontaneous activity belonged to the polimodal class. Unimodal type was most prevalent in non-Rt (Sol and 10N) neurons that probably reflected the higher number and frequency of spontaneous input in these neurons (Fig. 9C).

Hypothetical mechanisms giving rise to the types of membrane potential distribution encountered were assessed using a theoretical approach. The DRt tonic neuron model previously described was used to calculate the membrane potential distributions produced under different model parameterizations (see...
In the theoretical model, it was possible to define explicitly a resting membrane potential, corresponding to the reversal potential of the leakage current ($I_L$) ($-70$ mV) in the passive properties, and this value was used as the reference in the distributions.

It was hypothesized that the membrane potential distribution profile of neurons at low firing frequencies would either show a symmetric distribution due to relatively balanced excitatory and inhibitory synaptic inputs, or an asymmetric distribution in case of unbalanced synaptic inputs (class I, unimodal distribu-
balanced (excitatory/inhibitory) synaptic input, negligible polymodal (class II) distribution in case of significant under-

Fig. 9. Membrane potential distribution profiles in neurons of the three recorded regions. Probability density function of the membrane potential values are shown. A: examples for symmetric (DRt and non-Rt) and asymmetric (PCRt) unimodal distributions. B1–B2: typical polymodal distributions in neurons from the three regions. C: number of neurons with uni- and polymodal distributions in the recorded regions. Dark vertical lines indicate $V_{\text{med}}$, the median membrane potential; uni, unimodal; poly, polymodal.

At higher firing frequencies, the membrane potential trace continuously cycles between values close to the $I_{K\text{dr}}$ reversal potential and the action potential on-set, limiting the influence from random fluctuations driven by intrinsic and extrinsic sources. Thus instead of a unimodal distribution, further peaks would be obtained related to three mechanisms: AHP, reflects the rectifier mechanism of the $I_{K\text{dr}}$ combined or not with $I_{K,Ca}$ close to the potassium reversal potential; leakage, the passive leak equilibrium potential; and plateau, noteworthy changes in the membrane’s equilibrium potential during spiking, the membrane potential generated by prolonged depolarizing currents (e.g., plateau generating $I_{Ca}$ values or persistent $I_{Na}$ values, the later one not recreated in the model) (Fig. 10A). These mechanisms, however, would only lead to a polymodal (class II) distribution in case of significant underlying currents.

To test the above-mentioned scenarios, we first applied balanced (excitatory/inhibitory) synaptic input, negligible $I_{K,Ca}$ conductance, and constant injected current leading to 1-Hz rhythmic activity. A unimodal symmetric synthetically distribution could be generated (Fig. 10B). When omitting inhibitory input with the same parameterization, the unimodal distribution became asymmetric (Fig. 10C). Balanced synaptic input, negligible $I_{K,Ca}$ conductance, and constant injected current leading to 10-Hz rhythmic activity also showed a slightly asymmetric unimodal distribution (Fig. 10D). Polymodal (class II) distributions only became apparent when balanced synaptic noise and constant injected current leading to 1-Hz rhythmic activity were accompanied by increased $I_{Ca}$ (Fig. 10E) or increased $I_{K,Ca}$ (Fig. 10F) conductance. The membrane potential distributions generated by these parameterizations matched well the distributions observed experimentally.

DISCUSSION

DRt is known to be involved in the establishment of a modulatory positive feedback loop activated by nociceptive inputs and affecting neuronal activity at the superficial and deep laminae of spinal dorsal horn in rodents (Lima and Almeida 2002). Thus basic electrophysiological properties that
patch-clamp recordings in the reticular formation. Given the fact that the nervous system of rats at P14–P18 may be the difference in the age of the experimental animals, since rats used in our experiments (P14–P18) were older than the ones in the article of Venugopal et al. (2010), and some firing patterns (e.g., spike-adaptive) were proposed to represent immature neurons (Brocard et al. 2006). This suggests that, with the maturation of the animal, input integrated by reticular formation neurons in considerable proportions (Venugopal et al. 2010). One explanation for this discrepancy may be the difference in the age of the experimental animals, since rats used in our experiments (P14–P18) were older than the ones in the article of Venugopal et al. (2010), and some firing patterns (e.g., spike-adaptive) were proposed to represent immature neurons (Brocard et al. 2006). This suggests that, with the maturation of the animal, input integrated by reticular formation neurons is increasingly relayed in a rate-coded manner to the particular target of the given area. However, our finding that tonic DRt (as well as PCrt) neurons exhibit different membrane potential distribution profiles means that they may still respond differently to the same input stimuli. Given the fact that the nervous system of rats at P14–P18 may still not be fully matured, some electrophysiological differences may exist compared with the adult system. The choice over the age of the animals used in our experiments (P14–P18) results from a balance between using animals as close as possible to a fully matured neuronal system while still allowing patch-clamp recordings in the reticular formation.

Our results with blockers of $I_{Ca}^{2+}$ values suggest that, while Ca$^{2+}$ conductances may contribute to regulation of the firing-frequency, they do not play a critical role in establishing the tonic response behavior of DRt neurons.

The observed AHP variability is in agreement with electrophysiological data of DRt neurons in the cat (Soto and Canedo 2011). Based on our modeling experiments, we nevertheless argue that this AHP variability should not be associated with different functional classes within the DRt (and also PCrt) neuronal population and instead reflects the fact that channel conductances inside the same neuronal class are themselves subject to variability (Prinz et al. 2004). In other words, the main functional/computational features of these neurons are the same, despite the observed range of AHP characteristics resulting, most likely, from some degree of variability on channel densities.

An unexpected observation in our study was the frequent occurrence of spontaneous firing among DRt neurons, contradicting previous reports stating that the great majority of DRt neurons in the rat did not exhibit spontaneous activity (Villanueva et al. 1996). However, in a more recent in vivo work, although in a different species, the cat, a large fraction of DRt neurons showed spontaneous activity (Soto and Canedo 2011). Neurons in reticular formation areas involved in generating rhythmic motor activity, such as the pre-Bötzinger complex, are known to present spontaneous rhythmic firing (Koizumi et al. 2013) and these, through their collaterals (Koizumi et al. 2013), could be driving spontaneous activity in neighboring regions like Sol, DRt and PCrt. Independent of the source of the extrinsic synaptic drive, since all neurons in DRt are thought to be involved in nociception (Villanueva et al. 1988), the prevalence of spontaneous activity (regular and irregular, ~60%) certainly affects the nociceptive information modulation provided by these neurons. Due to the limitations of the in vitro approach used in our study, it was impossible to determine whether DRt neurons fire spontaneously also in vivo and with intact descending and ascending input. The brain stem block preparation used in this study has, nevertheless, the advantage of preserving, relatively unaffected, the local connections (as opposed to a slice preparation).

Compared with the neighboring regions, the PCrt and Sol, the number of synaptic inputs (both excitatory and inhibitory) were smallest in DRt cells, suggesting that they receive and integrate less input from local sources than PCrt and Sol cells. This fits the earlier reports that, although the reticular formation relays signals from the eyes and ears to the cerebellum, DRt neurons do not seem to respond to visual, auditory or proprioceptive stimuli (Villanueva et al. 1996). While both PCrt and Sol (Li and Yang 2007) are known to receive caudal and rostral inputs, this underlies the earlier proposition that the loop from the spinal cord is an important drive of DRt neurons (Lima and Almeida 2002). The low number of inhibitory synaptic input suggests that even local inhibitory interneurons targeting DRt neurons are under the control of ascending/descending projections.

The IPI analysis showed that EPSCs were randomly distributed in all investigated cell groups. Thus it is not likely that the observed rhythmic spontaneous firing is driven by suprathreshold rhythmic synaptic input (e.g., from the pre-Bötzinger complex).

Taking all this together, we conclude that neurons in the adjacent reticular formation regions, DRt and PCrt, do not differ significantly in their passive and active electrophysiological parameters, being mostly tonic firing neurons that support the idea that they code stimulus intensity by their firing rate (rate coding hypothesis). Furthermore, we demonstrated a high prevalence of spontaneous activity, both intrinsic and synaptic-input-driven, that may not be apparent in vivo but may have influence on nociceptive information processing and integration, especially in case of the DRt neurons that are known to be involved mostly in nociceptive modulation.

**APPENDIX**

All simulations were performed in NEURON 7.2 using a single compartmental model. The model is available in ModelDB, under accession number 151949. The Hodgkin-Huxley formalism (Hodgkin and Huxley 1952) was used to describe the ionic currents present in the model. The mathematical descriptions for each of these ionic currents followed the models in Aguiar et al. (2010). The following currents were present in the model: persistent $I_{Na}$, $I_{Kc}$; voltage dependent $I_{Ca}$, (L-type high threshold); $I_{K,Ca}$ (or $I_{AHP}$ given its role in shaping the AHP); and $I_{L}$. Injected currents were modelled by:

\[
I_{Na} = m^3 h g_{Na} (E_{Na} - V)
\]
\[
I_{K} = n^4 g_{K} (E_{K} - V)
\]
\[
I_{Ca} = m^2 p_{Ca} GHK(V, [Ca]^i, [Ca]^o)
\]
\[
I_{K,Ca} = n^3 g_{K,Ca} (E_{K,Ca} - V)
\]
where $V$ is membrane potential; $g$ is maximum ion conductance; $E$ is reversal potential; and $n$ and $m$ are activation gating variables; $h$ is the inactivation gating variable; $P_{Ca}$ is maximum calcium permeability; $[Ca]_{i}$ is intracellular calcium concentration; $[Ca]_{o}$ is extracellular calcium concentration; and GHK is the Goldman-Hodgkin-Katz equation. The gate-variables kinetics are available in ModelDB. The following sections summarizes all of the model parameters, according to the type of simulation.

Model 1: Fitting firing-pattern. The parameters for model 1 are as follows:

- $I_{Na}$: $g = 0.05 \mu S$, $E = 50 \text{ mV}$, $V_{th}$ = $-65 \text{ mV}$.
- $I_{K,na}$: $g = 0.06 \mu S$, $E = -77 \text{ mV}$.
- $I_{K,ca}$: $g = 0.02 \mu S$, $E = -77 \text{ mV}$.
- $I_{Ca}$: $g = 1 \times 10^{-5} \mu S$, $E = -70 \text{ mV}$.
- $I_{K,dr}$: $P_{Ca}$ = $2.76 \times 10^{-4} \text{ cm/s}$.
- $[Ca]_{i}$: $\tau_{pump}$ = $10 \text{ ms}$, $[Ca]_{i}$ = $5 \times 10^{-5} \text{ mM}$.
- Size: $L = 40 \mu m$, $diam = 25 \mu m$.
- $I_{clamp}$: $del = 95 \text{ ms}$, $dur = 500 \text{ ms}$, $amp = 0.13 \text{ nA}$.
- Temp: $25^\circ \text{C}$.

Model 2: Fitting APH variability. The parameters for model 2 are as follows:

- $I_{Na}$: $g = 0.02 \mu S$, $E = 50 \text{ mV}$, $V_{th}$ = $-63 \text{ mV}$.
- $I_{K,ca}$: $g = 0.06 \mu S$, $E = -77 \text{ mV}$.
- $I_{K,dr}$: $g = 0.02 \mu S$, $E = -77 \text{ mV}$.
- $I_{Ca}$: $g = 1 \times 10^{-5} \mu S$, $E = -70 \text{ mV}$.
- $I_{K,dr}$: $P_{Ca}$ = $2.76 \times 10^{-4} \text{ cm/s}$.
- $[Ca]_{i}$: $\tau_{pump}$ = $10 \text{ ms}$, $[Ca]_{i}$ = $5 \times 10^{-5} \text{ mM}$.
- Size: $L = 40 \mu m$, $diam = 25 \mu m$.
- $I_{clamp}$: $del = 95 \text{ ms}$, $dur = 500 \text{ ms}$, $amp = 0.67 \text{ nA}$.
- Temp: $25^\circ \text{C}$.

Model 3: Explore membrane potential histograms under spontaneous activity conditions. The parameters for model 3 are as follows:

- $I_{Na}$: $g = 0.01 \mu S$, $E = 50 \text{ mV}$, $V_{th}$ = $-55 \text{ mV}$.
- $I_{K,ca}$: $g = 0.06 \mu S$, $E = -77 \text{ mV}$.
- $I_{K,dr}$: $g = 0.02 \mu S$, $E = -77 \text{ mV}$.
- $I_{Na}$: $g = 1 \times 10^{-5} \mu S$, $E = -70 \text{ mV}$.
- $I_{K,dr}$: $P_{Ca}$ = $2.76 \times 10^{-4} \text{ cm/s}$.
- $[Ca]_{i}$: $\tau_{pump}$ = $2 \text{ ms}$, $[Ca]_{i}$ = $5 \times 10^{-5} \text{ mM}$.
- Size: $L = 40 \mu m$, $diam = 25 \mu m$.
- $I_{clamp}$: $del = 95 \text{ ms}$, $dur = 500 \text{ ms}$, $amp = 0.67 \text{ nA}$.
- Temp: $36^\circ \text{C}$.

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MOST DRT NEURONS ARE TONIC AND SPONTANEOUSLY ACTIVE IN VITRO


