Electrophysiological Heterogeneity of Spinally Projecting Serotonergic and Nonserotonergic Neurons in the Rostral Ventromedial Medulla

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Zhang, Liang, Kenneth T. Sykes, Amber V. Buhler, and Donna L. Hammond. Electrophysiological heterogeneity of spinally projecting serotonergic and nonserotonergic neurons in the rostral ventromedial medulla. J Neurophysiol 95: 1853–1863, 2006. First published December 7, 2005; doi:10.1152/jn.00883.2005. This study examined the passive membrane and action potential properties of serotonergic and nonserotonergic neurons in the rostral ventromedial medulla (RVM) of the rat using whole cell patch-clamp recording techniques in the slice. Serotonergic neurons were identified by immunoreactivity for tryptophan hydroxylase (TrpH). Spinally projecting neurons were retrogradely labeled with 1-tritiated 3,3,3-tetramethylindocarbamoyl perchlorate (DiI). Three types of neurons were identified within both spinally projecting serotonergic and nonserotonergic populations. Type 1 neurons exhibited irregular or sporadic spontaneous activity interspersed with periods of quiescence. Type 2 neurons were not spontaneously active and were additionally discriminated by a more negative resting membrane potential and a larger-amplitude action potential. Type 3 neurons fired repetitively without pause. Serotonergic neurons had a higher membrane resistance and greater action potential half-width than their nonserotonergic counterparts and rarely exhibited a fast afterhyperpolarization. Serotonergic type 3 neurons also fired more slowly and regularly than nonserotonergic type 3 neurons. Comparison of electrophysiological and immunohistochemical characteristics suggested that the smallest type 3 serotonergic neurons had an increased risk of immunohistochemical “misclassification” due to failure to detect TrpH, possibly due to more complete dialysis of intracellular contents during lengthy recordings. This risk was minimal for type 1 or 2 serotonergic neurons. The three different types of spinally projecting serotonergic neurons also differed markedly in their responsiveness to the mu opioid receptor agonist D-Ala2,NMePhe4,Gly5-ol-enkephalin. These results provide important new electrophysiological and pharmacological evidence for a significant heterogeneity among spinally projecting serotonergic RVM neurons. They may also provide a basis for resolving the controversy concerning the role of serotonergic RVM neurons in opioid analgesia.

INTRODUCTION

The recent recognition that neurons in the rostroventral medial medulla (RVM) that project to the spinal cord may facilitate, as well as inhibit, nociception (Porreca et al. 2002; Ren and Dubner 2002; Vanegas and Schaible 2004) has renewed interest in developing means to identify these different types of neurons. Two broad classification schemes have been developed. Fields and colleagues used extracellular recording methods to characterize the responses of RVM neurons to noxious stimuli in vivo and proposed that RVM neurons can be assigned to one of three functional classes, termed ON, OFF, and NEUTRAL. (Fields 2004; Fields et al. 1983; Vanegas et al. 1984). Serotonergic cells, which may discharge regularly or irregularly, are also proposed as part of this in vivo classification scheme (Gao and Mason 2001). Others have used responses to iontophoretically applied receptor agonists and antagonists as an additional means to identify these different classes (Haws et al. 1990; Heinricher and Kaplan 1991; Heinricher and McGaraughty 1998; Heinricher and Tortorici 1994; Heinricher et al. 2001). Pan and coworkers used intracellular or whole cell patch-clamp recordings in the slice preparation and proposed that RVM neurons could be categorized as primary or secondary cells based on differences in their input resistance, afferent inputs and the differential responses of these neurons to mu or kappa opioid receptor agonists (Pan et al. 1990, 1997). The broad classification of RVM neurons into ON, OFF, and NEUTRAL cells or into primary or secondary cells belies the pharmaco- and morphological complexity of this region. In addition to serotonergic neurons, the RVM contains neurons that synthesize glutamate, γ-aminobutyric acid, acetylcholine, substance P, enkephalin, or thyrotropin-releasing hormone (Bowker et al. 1983; Finley et al. 1981; Jones et al. 1991; Menetrey and Basbaum 1987). Although many RVM neurons project to the spinal cord (Hama et al. 1997; Skagerberg and Björklund 1985; Wang and Wessendorf 1999), subpopulations project to other brain stem and more rostral nuclei (Clark and Proudftit 1991; Holden and Proudftit 1998; Sim and Joseph 1992; Yeomans and Proudftit 1990). Neurons in the RVM are also implicated in thermoregulation and in autonomic homeostasis (Mason 2001). Given their involvement in several different physiological functions and the paucity of information about neurotransmitter phenotype and functional neuron class, it is surprising that a more comprehensive analysis of RVM neurons has not been undertaken using whole cell patch-clamp methods in the slice. Although this preparation does not permit one to assign function to a neuron, it does enable characterization of the passive membrane properties, action potential properties, excitability, and morphology of RVM neurons. The slice preparation also effectively reduces excitatory and inhibitory afferent influences on RVM neurons and affords an opportunity to characterize the intrinsic membrane properties of different types of neurons. Finally, visualization of retrogradely transported label from axonal termination sites enables study of identified subpopulations of RVM neurons, while post hoc application of immunohistochemical methods permits classification of recorded neurons by neurotransmitter content.

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This study characterized the passive membrane and action potential properties of spinally projecting RVM neurons that were immunohistochemically classified as either serotonergic or nonserotonergic. It also examined their responsiveness to the mu opioid receptor agonist D-Ala\(^2\), NMePhe\(^3\)-Gly\(^5\)-ol-enkephalin (DAMGO), in line with previous studies of the responsiveness of ON and OFF cells (reviewed by Fields 2004) and of primary and secondary cells to mu opioid receptor agonists (Pan et al. 1990). Based on their pattern of spontaneous discharge, three different types of neurons were identified within both serotonergic and nonserotonergic populations of spinally projecting RVM neurons. These neurons differed with respect to membrane capacitance, input resistance, resting membrane potential, and action potential height. Furthermore, the three different types of serotonergic neurons differed markedly in their responsiveness to DAMGO. Although the existence of a heterogeneous population of spinally projecting nonserotonergic RVM neurons was expected, these data provide important new evidence for a significant heterogeneity among spinally projecting serotonergic RVM neurons that may provide a basis for resolving the controversy concerning the role of serotonergic neurons in opioid-mediated antinociception.

**Methods**

These experiments were approved by The University of Iowa Animal Care and Use Committee and were conducted in accordance with the guidelines of the International Association for the Study of Pain and the National Resource Council Guide for the Care and Use of Laboratory Animals.

**Retrograde labeling of spinally projecting neurons in the RVM**

Nine- to 18-day-old male Sprague-Dawley rats (Charles River; Portage, IN) were anesthetized with halothane. Using aseptic methods and sterile tip technique, a midline incision was made over the T\(_1\)-L\(_2\) vertebrae, and one to two vertebrae were removed to expose the lumbar enlargement. The dura was incised vertically and horizontally to make two flaps. A droplet of 2% lidocaine jelly was placed on the spinal cord to facilitate manipulation of the dorsal roots, and additional lidocaine jelly was swabbed over all the exposed muscle and skin edges. The most laterally situated dorsal roots were then gently teased aside dorsomedially to expose the dorsal horn and dorsolateral funiculus. The edge of the dura was pulled to slightly rotate the spinal cord and a 0.5 mm\(^3\) pledget of sterile Gelfoam, which had been dipped in 0.2% 1\(^{-}\)-disoactadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) in 100% DMSO (Molecular Probes, Eugene, OR) and blotted dry, was gently tucked beside the dorsal root in juxtaposition to the dorsolateral aspect of the spinal cord on each side. The muscle and skin were closed as separate layers with 5–0 Vicryl and 6–0 silk sutures, respectively. After subcutaneous injection of 0.5 ml of 5% dextrose in saline, the pup was placed in a warming chamber. After full recovery from anesthesia (~15 min) and complete hemostasis of the incision, all blood was washed from the wound edges and the pup was returned to the dam. Pups in which placement of the pledget caused hindlimb paralysis or in which pinch of the hindpaws did not evoke a normal withdrawal response (<10%) were excluded from the study. Morbidity was greatly decreased when pledges were placed over more caudal lumbar segments of the spinal cord where more space was available in the vertebral canal.

**Slice preparation**

Three to 5 days after surgery, DiI had diffused throughout the dorsoventral extent of the dorsal horn, extending for one to two segments in the rostrocaudal direction. The pups were decapitated at this time, and the brain was rapidly removed and immersed in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM) 126 NaCl, 2.5 KCl, 25 NaHCO\(_3\), 1.2 NaH\(_2\)PO\(_4\), 1.2 MgCl\(_2\), 2.4 CaCl\(_2\), and 11 dextrose and equilibrated with 95% O\(_2\)-5% CO\(_2\). The cerebellum was removed, and the brain stem block was affixed by cyanocrylate glue to a slicing tray that was filled with ice-cold ACSF in which the concentration of MgCl\(_2\) was increased to 5 mM and that of CaCl\(_2\) was decreased to 1 mM. Coronal slices of 160-μm thickness were then cut through the brain stem from the trapezoid body to the inferior olivary nuclei using a vibrating microtome (Leica Microsystems; Bannockburn, IL). Sections were cut in the coronal plane because the dendrites of both serotonergic and nonserotonergic neurons in the RVM are predominantly oriented in the mediolateral plane and are more restricted in the rostrocaudal plane (Gao and Mason 1997; Potrebic and Mason 1993).

**Recording conditions**

After equilibration in oxygenated ACSF for ≥1 h at 34°C, slices were transferred to the recording chamber and continuously perfused with oxygenated ACSF at 32–34°C (4 ml/min); a constant temperature was maintained throughout each recording. Retrogradely labeled neurons were first briefly visualized using a ×40 water-immersion objective and epifluorescent illumination with a rhodamine filter. Figure IA illustrates several such neurons, identified by the presence of bright particles under epifluorescent illumination. These neurons were then viewed under infrared differential interference contrast illumination to guide placement of the patch electrode (Fig. 1B). Recordings were typically restricted to neurons situated within 50–60 μm of the slice surface. Whole cell patch-clamp recordings were made from neurons in the RVM using glass recording pipettes of 3–6 MΩ resistance that contained (in mM) 140 K\(^{+}\) methanesulfonate, 10 HEPES, 2 MgCl\(_2\), 0.6 EGTA, 2 MgATP, and 0.25 Na\(_2\)GTP; pH 7.3, 280–290 mOsm. Bicucullin (0.01%) was included in the pipette to enable subsequent identification of the neurons. Recordings were made with an Axopatch 200B amplifier (Axon Instruments, Union City, CA). Series resistance was compensated by 70–80%. A seal resistance of ≥3 GΩ and access resistance of <30 MΩ (median: 18 MΩ, 25th percentile: 13 MΩ; 75th percentile: 21 MΩ, n = 130) was required. Liquid junction potentials of ~14 mV were uncorrected. A Digidata 1322A board and pClamp 9.0 software (Axon Instruments) were used for data acquisition and analysis.

Passive membrane properties were measured in voltage-clamp mode using the pClamp membrane test before whole cell capacitance and series resistance compensation were made. The holding potential was maintained at ~60 mV. For measurement of membrane resistance and capacitance values, a test pulse of ~10 mV was applied at a frequency that allowed the slow membrane capacitance-induced current transient to decline to a steady-state level. Ten such determinations were made and the average was reported. Resting membrane potentials and action potentials were recorded in current clamp mode, sampled at 10 kHz, and filtered with a 5 kHz low-pass filter. Action potential amplitude was measured from the threshold (onset of the rapidly polarizing phase) to its peak. Action potential half-width was determined as the width of the action potential measured at one-half-peak amplitude. The amplitude of the slow AHP was measured as the difference between resting membrane potential and the negative peak amplitude of the slow AHP. In neurons that did not exhibit spontaneous activity, action potentials were evoked by injection of depolarizing current. It was therefore not possible to measure the amplitude of the slow AHP relative to the resting membrane potential. A similar situation was encountered with a sizable propor-
ELECTROPHYSIOLOGICAL HETEROGENEITY OF RVM NEURONS

Immunohistochemical procedures

At the conclusion of the recording, the brain stem slice was fixed by immersion for 1 h in 4% paraformaldehyde-0.1 M phosphate buffer. Longer fixation times resulted in poorer staining for tryptophan hydroxylase (TrpH). The slices were washed once for 15 min in 0.1 M phosphate buffered saline (PBS), incubated for 30 min in 1% (wt/vol) sodium borohydride, rinsed six times for 15 min each in 0.1 M PBS, and then placed in 0.1 M PBS containing 2% normal goat serum and 0.3% Triton X-100 (PBS-N-T) for 90 min. Sections were then transferred to mouse anti-TrpH (1:500; Oncogene OP71L; Cambridge, MA) in 0.1 M PBS-N-T for 40 h at 4°C. After four rinses for 15 min each in 0.1 M PBS, sections were transferred to a mixture of goat anti-mouse IgG conjugated to Cy5 (1:200, Jackson ImmunoResearch Laboratories, West Grove, PA) and streptavidin conjugated to Cy2 (1:400; Jackson ImmunoResearch Laboratories) in 0.1 M PBS-N-T for 90 min. Some neurons were processed with a Cy2 label on the TrpH and Cy3-conjugated streptavidin. Sections were then rinsed three times in 0.1 M PBS and mounted out of distilled water onto slides. After air-drying for 2–4 h, the sections were coveredslipped with Fluoromount G (Electron Microscopy Services; Hatfield, PA). The sections were examined with a Bio-Rad 1024 confocal microscope using sequential, single laser line scanning methods to obtain serial stacks for each fluorophore throughout the entire Z-axis of the labeled neuron. Neurons that did not colocalize TrpH were determined to be nonserotonergic only when adequately stained neurons could be identified in the immediate vicinity and in the same plane of focus.

Neurons from which recordings were made were often stained less strongly for TrpH than adjacent neurons, mostly likely due to dialysis of the intracellular contents with the contents of the patch electrode. Tryptophan hydroxylase was therefore visualized with Cy5 because this fluorophore has a higher quantum yield than either Cy2 or Cy3. Quenching of one fluorophore by another, particularly under conditions of epifluorescence illumination, can lead to false negative conclusions about the occurrence of double-labeling. Unlike Cy3, the absorption spectrum of Cy5 does not overlap with the emission spectrum of Cy2. Thus this combination of fluorophores was considered optimal. Although an antibody to serotonin provided good staining in fresh-cut tissue, it did not prove suitable for identification of serotonergic neurons in sections that had been subjected to the incubation and recording conditions.

Drugs

(−)-Bicuculline methiodide, strychnine hemisulfate, 5-aminophosphonic acid (APV), 6-cyano-7-nitroquinolinic acid-2,3-dione (CNQX), DAMGO, and naloxone hydrochloride were purchased from Sigma (St. Louis, MO). All drugs were dissolved in distilled water, aliquoted, and stored at −20 or −80°C until use. All drugs were applied by addition to the ACSF and delivered by pump with a BPS-8 channel valve control perfusion system (ALA Scientific Instruments; Westbury, NY). Delivery was controlled by a digital output triggered from pCLAMP. Time between valve opening and delivery of drug to the recording chamber was 50 s with equilibrium concentrations of drug achieved by 90 s.

Statistical analysis

One way-ANOVA was used to compare the passive membrane and action potential properties of the three different populations of neurons. Where assumptions of equal variance or normality were not met, Kruskal-Wallis one-way ANOVA on ranks was used. Newman-Keuls or Dunn’s test, as appropriate, was used for post hoc comparisons among mean values for the individual groups. A Student’s t-test for unpaired samples or Mann-Whitney test was used to compare membrane or action potential properties between DiI and non-DiI labeled neurons within each neuron category. Fisher’s exact test or χ² test was

FIG. 1. A: example of the retrograde labeling of neurons in the rostral ventromedial medulla (RVM) 4–5 days after placement of a small pledget of Gelfoam containing 0.2% (wt/vol) 1-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) on the dorsal surface of the spinal cord. Note that several neurons within a field contain moderate numbers of fluorescent particles within the cytoplasm. B: same field as A examined under infrared differential interference contrast illumination illustrating the positioning of a patch pipette on 1 of the retrogradely labeled neurons. Scale bars are 20 μm.

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used to compare the percentage of neurons among different populations that expressed a particular trait (e.g., presence of spontaneous activity, fast afterhyperpolarization, responsiveness to DAMGO). A $P \leq 0.05$ was considered significant.

RESULTS

Three general types of RVM neurons

Three types of RVM neurons that project to the spinal cord could be discerned on the basis of their spontaneous activity. Type 1 neurons exhibited spontaneous activity interspersed with periods of quiescence that enabled determination of their resting membrane potential (Fig. 2, A and B). Type 2 neurons were not spontaneously active (Fig. 2, C and D). Type 3 neurons were spontaneously active but fired repetitively in a manner that precluded determination of their resting membrane potential (Fig. 2, E and F). All three types were found in similar proportions within both nonserotonergic and serotonergic populations of spinally projecting RVM neurons (type 1: 17.4% (15/86) and 31.8% (14/44), $P = 0.1$; type 2: 40.7% (35/86) and 29.5% (13/44), $P = 0.29$; type 3: 41.8% (36/86) and 38.6% (17/44), $P = 0.86$, respectively; Table 1).

Serotonergic type 2 neurons had a higher membrane capacitance, lower membrane resistance, a more negative resting membrane potential, and a smaller action potential threshold to AHP maximum value than serotonergic type 1 neurons (Table 1). They similarly differed from serotonergic type 3 neurons with the exception that no comparison of resting membrane potential could be made. Nonserotonergic type 2 neurons had a lower membrane resistance, more negative resting membrane potential, and a smaller amplitude action potential than nonserotonergic type 1 neurons. Nonserotonergic type 2 neurons also had a higher membrane capacitance, lower membrane resistance, and a shorter duration action potential than nonserotonergic type 3 neurons. Serotonergic type 1 and type 3 neurons did not differ with respect to mean or median values for their passive membrane and action potential properties. However, the variances of the membrane capacitance ($P < 0.003$; $F$-test) and the membrane resistance ($P < 0.006$; $F$-test) of type 1 and type 3 neurons were different, consistent with the proposal that these are different populations of neurons based on differences in their spontaneous firing pattern and responsiveness to DAMGO (see following text). Nonserotonergic type 1 and type 3 neurons did not differ with respect to mean values for passive membrane and action potential properties, although type 1 neurons had a smaller action potential threshold to AHP maximum than type 3 nonserotonergic neurons.

To determine whether the spontaneous activity of type 1 and type 3 neurons was a consequence of residual excitatory inputs, eight neurons were recorded in slices bathed in ACSF containing 10 $\mu$M CNQX, and 20 $\mu$M APV. All three types of neurons were present under conditions in which fast excitatory inputs were blocked (2 type 1, 2 type 2, and 4 type 3 neurons). To determine whether the lack of spontaneous activity in type 2 neurons or the sparcadic activity of type 1 neurons was a consequence of residual inhibitory inputs, 14 neurons were recorded in slices perfused with ACSF that contained 30 $\mu$M bicuculline and 10 $\mu$M strychnine. All three types of neurons were present under conditions in which fast inhibitory inputs were blocked (3 type 1, 3 type 2, and 8 type 3 neurons). The percentages of type 1, 2, and 3 neurons recorded in the presence of bicuculline and strychnine did not differ from the percentages recorded in the absence of these antagonists ($P = 0.79$, 0.39 and 0.37, respectively). The percentages of type 1, 2, and 3 neurons recorded in the presence of CNQX and APV did not differ from the percentages recorded in the absence of these antagonists ($P = 0.79$, 0.76 and 0.88, respectively). These data support the idea that the different discharge patterns of RVM neurons are not a consequence of excitatory or inhibitory inputs that could persist in the slice preparation.

Bulbospinal serotonergic and nonserotonergic RVM neurons can be distinguished by their electrophysiological profiles

Table 1 also compares the passive membrane and action potential properties of serotonergic and nonserotonergic neurons that project to the spinal cord. The comparison was restricted to spinally projecting neurons so that differences in their electrophysiological properties could be attributed to differences in neurotransmitter content, rather than differences in their efferent projections. As a group, serotonergic spinally-projecting neurons had a significantly higher membrane resistance and greater action potential half-width than did nonserotonergic spinally-projecting neurons (Table 1, Fig. 3). The difference between the action potential threshold and AHP maximum was also larger for serotonergic type 1 and type 3 neurons than their nonserotonergic counterparts. Serotonergic type 2, but not type 1 neurons had a more negative resting membrane potential and, conversely, a larger-amplitude action potential than their nonserotonergic counterparts. Although resting membrane potential could not be determined in type 3 neurons, serotonergic type 3 neurons also had a larger amplitude action potential compared with nonserotonergic type 3 neurons. Finally, fast AHPs were rarely observed in spinally projecting serotonergic neurons (3 of 44; <7%). In contrast, fast AHPs were observed in 47–71% of nonserotonergic spinally-projecting neurons (Table 1, Fig. 3). A significantly higher percentage of nonserotonergic type 1, 2, and 3 neurons exhibited fast AHPs compared with serotonergic neurons of the corresponding type (Table 1).

Type 3 neurons, which fire repetitively, exhibited a wide range of firing frequencies. Immunohistochemically identified
TABLE 1. Passive membrane and action potential properties in serotonergic and nonserotonergic spinally projecting RVM neurons

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Type 1</th>
<th>Type 2</th>
<th>Type 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-S-HT (n=15)</td>
<td>5-HT (n=10)</td>
<td>Non-S-HT (n=14)</td>
</tr>
<tr>
<td>Spontaneous activity</td>
<td>15/15</td>
<td>0/35</td>
<td>0/35</td>
</tr>
<tr>
<td>Membrane capacitance, pF</td>
<td>77.1 ± 9.3</td>
<td>109.5 ± 8.6</td>
<td>102.2 ± 6.2</td>
</tr>
<tr>
<td>Resting membrane potential, mV</td>
<td>-48.1 ± 0.8</td>
<td>-50.7 ± 0.6</td>
<td>-56.6 ± 0.8</td>
</tr>
<tr>
<td>Action potential threshold, mV</td>
<td>-36.5 ± 0.7</td>
<td>-35.2 ± 0.6</td>
<td>-36.1 ± 0.6</td>
</tr>
<tr>
<td>Action potential amplitude, mV</td>
<td>91.9 ± 2.6</td>
<td>97.3 ± 2.1</td>
<td>82.7 ± 1.9</td>
</tr>
<tr>
<td>Action potential half-width, ms</td>
<td>0.77 ± 0.04</td>
<td>1.08 ± 0.04</td>
<td>0.67 ± 0.03</td>
</tr>
<tr>
<td>Slow afterhyperpolarization amplitude, mV</td>
<td>21.1 ± 1.1</td>
<td>21.1 ± 1.0</td>
<td>NA</td>
</tr>
<tr>
<td>Action potential threshold to AHP maximum, mV</td>
<td>32.6 ± 0.9</td>
<td>36.6 ± 1.2</td>
<td>30.3 ± 0.9</td>
</tr>
<tr>
<td>Presence of fast after hyperpolarization</td>
<td>7/15</td>
<td>1/14*</td>
<td>25/35</td>
</tr>
</tbody>
</table>

Values are means ± SE. NA, not applicable; RVM, rostral ventromedial medulla; SHT, serotonin; *P < 0.05 **P < 0.01 compared to corresponding class of nonserotonergic neuron a,b: comparisons among type 1, 2 and 3 nonserotonergic neurons; c,d: comparisons among type 1, 2 and 3 serotonergic neurons. Common superscripts indicate that the values do not differ from one another within that label category (serotonergic or nonserotonergic) as determined by one-way ANOVA or Kruskal-Wallis test.

Possible problems with immunohistochemical methods for classification of neurotransmitter content in neurons subjected to whole cell patch-clamp recordings

Figure 4 illustrates examples of spinally projecting RVM neurons from which patch-clamp recordings were made that were subsequently processed for TrpH immunoreactivity. DI labeling did not withstand the immunohistochemical processing even when care was taken to avoid organic solvent-based mounting media such as DPX (Baker and Reese 1993) and tissue was allowed to air-dry rather than dehydrated by alcohol (Vercelli et al. 2000). This is attributed to the use of Triton X-100 detergent as a permeabilizing agent and NaBH4 to reduce nonspecific staining (Elberger and Honig 1990; Holmqvist et al. 1992). Biocytin filled the cytoplasm and labeled the nucleus. Higher concentrations of biocytin (0.1%) or longer recording times provided for more extensive filling of proximal and distal dendrites (data not shown). Immunoreactivity for TrpH was homogeneously distributed throughout the cytoplasm, excluding the nucleus. In some neurons, there appeared to be comparatively little dialysis of intracellular contents in that immunoreactivity for TrpH was as intense as in adjacent unrecorded neurons (Fig. 4A). However, in other neurons, immunoreactivity for TrpH was much weaker than surrounding neurons, suggesting that substantial dialysis of contents had occurred (Fig. 4C). In merged images, double-labeled neurons exhibited a yellow-red cytoplasm in which a green nucleus was embedded. As previously reported (Beck et al. 2004), the construction of serial confocal images in the z axis was essential for unambiguous identification of double-labeled neurons given that staining for TrpH could be weak.

All neurons for which colocalization of TrpH with biocytin could not be unequivocally demonstrated were examined to determine whether they satisfied two key electrophysiological criteria for classification as serotonergic neurons: having a membrane resistance and an action potential half-width that fell within 1 SD of the mean of neurons that were immunohistochemically classified as serotonergic. Only 1 of 15 type 1 neurons (6.7%) and only 3 of 35 type 2 neurons (8.6%) that were immunohistochemically classified as serotonergic were found to satisfy both criteria for electrophysiological classification as serotonergic. Furthermore, the type 1 neuron and two of the three type 2 neurons lacked a fast AHP as
expected of serotonergic neurons. These data suggest that there is a small risk for immunohistochemical “misclassification” of serotonergic type 1 or type 2 neurons as nonserotonergic. In contrast, 9 of 36 type 3 neurons (25%) that were not immunoreactive for TrpH satisfied both criteria for electrophysiological classification as serotonergic. Of these nine, six lacked a fast AHP suggesting a significant risk existed for immunohistochemical misclassification of this cell type.

Figure 5, A–F, illustrates the frequency distribution of capacitance values for serotonergic and nonserotonergic type 1 and type 3 neurons that project to the spinal cord. It also illustrates the frequency distribution for potentially misclassified type 3 neurons, immunohistochemical examples of which are provided in Fig. 6. Closer examination revealed that seven of the nine (77.7%) nonserotonergic type 3 neurons that were considered to be misclassified had capacitance values ≤50 pF (Fig. 5A). An ancillary experiment was therefore conducted to determine whether limiting the recording time to ≤5 min would increase the percentage of small (≤50 pF) type 3 neurons that were immunoreactive for TrpH. The individual conducting the immunohistochemical analysis was blinded to the recording conditions and neuron type. Three of the four (75%) type 3 neurons with capacitance values of ≤50 pF that were recorded for ≤5 min and that met both electrophysiological criteria for classification as serotonergic were subsequently determined to be immunoreactive for TrpH. Even with this brief period of recording, the intensity of TrpH in the recorded neurons averaged 55 ± 8.3% of the intensity of labeling in neurons immediately adjacent to the recorded neuron. These data suggest that there is a significant risk for immunohistochemical misclassification of serotonergic type 3 neurons and particularly of those with capacitance values of ≤50 pF.

**Additional pharmacological evidence for heterogeneity of spinally projecting serotonergic neurons**

The ability of 1 or 3 μM DAMGO to produce a naloxone-reversible outward current (Fig. 7) was examined in a subset of spinally projecting RVM neurons (n = 65; Table 2). Previous studies established that concentrations of DAMGO ≥1 μM are maximally effective in the locus coeruleus (Osborne and Williams 1995) or thalamus (Brunton and Charpak 1998); this observation was confirmed for the RVM (Zhang and Hammond, unpublished observations). The average peak outward current produced by 1 or 3 μM DAMGO in responsive neurons was 24.5 ± 4.2 pA (n = 11) and 15.7 ± 2.0 pA (n = 9) in serotonergic and nonserotonergic populations; these values did not differ (P = 0.09, t-test). Marked differences were observed in opioid responsiveness among the three types of RVM neurons (Table 2). Slightly more than half (55%, 6/11) of serotonergic type 1 neurons responded to DAMGO with an outward current produced by 1 or 3 μM DAMGO. Twenty-nine percent of serotonergic type 2 neurons were responsive to DAMGO; a similar percentage of nonserotonergic type 2 neurons were similarly responsive to DAMGO. Twenty-nine percent of nonserotonergic type 3 neurons were responsive to DAMGO. Five of 17 (29%) nonserotonergic type 3 neurons were responsive to DAMGO. However, within these 17 neurons were 5 neurons with capacitance values of <50 pF that met the electrophysiological criteria for classification as serotonergic, yet were immunonegative for TrpH. These neurons therefore fit the criteria for assignment as misclassified serotonergic type 3 neurons. None of these neurons were responsive to DAMGO, also consistent with their misclassification. When these misclassified neurons were reallocated to the serotonergic type 3 neurons, the per-
centage of DAMGO-responsive serotoninergic type 3 neurons dropped to 5.8%, whereas the percentage of nonserotonergic type 3 neurons rose to 41.7%.

**DISCUSSION**

**Electrophysiological properties can discriminate serotoninergic neurons in the RVM**

This study provides the first systematic analysis of the passive membrane properties and action potential characteristics of spinally projecting serotoninergic and nonserotonergic RVM neurons. It was prompted by recent reports that serotoninergic neurons in the dorsal raphe nucleus could not be discriminated from nonserotonergic neurons on the basis of their passive membrane properties, action potential characteristics or their response to a 5HT1A receptor agonist (Beck et al. 2004; Kirby et al. 2003; Marinelli et al. 2004). These reports challenged a long-held premise that serotonergic neurons could be identified by their slow tonic discharge, higher input resistance, the presence of a prominent slow AHP, and action potentials of long duration, as well as their hyperpolarization by a 5HT1A receptor agonist (Aghajanian and Vandermaelen 1982; Vandermaelen and Aghajanian 1983). Although earlier studies determined that serotoninergic RVM neurons had a longer duration action potential (Marinelli et al. 2002; Pan et al. 1993), little additional information was provided. This study determined that immunohistochemically identified serotoninergic neurons in the RVM exhibited higher membrane resistance and a greater action potential half-width than nonserotonergic neurons and were additionally characterized by the absence of a fast AHP. Type 2 serotoninergic neurons were further identified by a more negative resting membrane potential and larger-amplitude action potential. Type 3 serotoninergic neurons discharged at a slower rate than their nonserotonergic counterparts. These findings indicate that serotoninergic neurons in the RVM can be discriminated from their nonserotonergic counterparts on the basis of their electrophysiological properties. The present findings are based on recordings in neonatal rats. The absence of an organized arrangement of neurons in the RVM, coupled with poor visibility and viability of these neurons in slices from rats 16–18 days of age, preclude whole cell recordings from RVM neurons in the adult rat. Nonetheless, the results complement an earlier in vivo study in the adult rat that concluded that serotoninergic RVM neurons could be distinguished from nonserotonergic neurons by their highly characteristic pattern of spontaneous activity (Mason 1997). Because serotoninergic neurons comprise only 15–25% of RVM neurons (Moore 1981; Potrebic et al. 1994), the ability to identify them while recording in the slice represents an important advance that can be used to increase the likelihood of sampling from this population and facilitate their further study.

**Serotoninergic neurons in the RVM are heterogeneous**

The finding of three different types of spinally projecting serotoninergic neurons in the RVM that differ in their spontane-
ous firing pattern, passive membrane, and action potential properties and responsiveness to DAMGO strongly suggests that serotonergic neurons in the RVM are heterogeneous. Their occurrence in the slice preparation, in which RVM neurons are effectively deafferented, and under conditions of pharmacological blockade, further suggests that these cell types are not a consequence of residual excitatory or inhibitory inputs. Mason previously proposed that serotonergic neurons were a heterogeneous population on the basis of differences in somatic and dendritic morphology (Gao and Mason 1997, 2000), responsiveness to noxious heating of the tail (Gao and Mason 2000), and their regular or irregular discharge at a slow and steady rate (Gao and Mason 2001; Mason 1997). Unfortunately, those studies could only presume a spinal projection of these neurons by virtue of the caudal trajectory of their axons. The present findings, in which DiI was used as a retrograde label, extend the concept of heterogeneity to include serotonergic neurons that are known to project to the spinal cord. Serotonergic RVM neurons that project to the spinal cord are known to colocalize a number of different neurotransmitters (Bowker et al. 1988; Hokfelt et al. 2000; Millhorn et al. 1987; Sasek et al. 1990).

**Heterogeneity of spinally projecting RVM neurons and opioid actions**

The present data also provide clear evidence that mu opioid receptor agonists do not uniformly affect spinally projecting serotonergic neurons in the RVM. Approximately 60% of type 1 neurons, which discharged intermittently and therefore may correspond to the irregularly firing serotonergic neurons identified by Mason in vivo (Gao and Mason 2001; Mason 1997), were responsive to DAMGO. In contrast, 2/3 of type 2 serotonergic neurons, which were quiescent, were unresponsive to DAMGO. Type 2 serotonergic neurons may correspond to the primary cells of Pan, which contain serotonin, do not discharge spontaneously, have a smaller input resistance, and are largely unresponsive to mu opioid receptor agonists (Pan et al. 1990, 1993). Type 3 spinally projecting serotonergic RVM neurons fired repetitively with a low CV and are likely to correspond to...
to the regularly discharging serotonergic neurons identified by Mason and coworkers (Gao and Mason 2001; Mason 1997). Virtually none of these neurons (1/12) was responsive to DAMGO. These findings provide the first independent confirmation that regularly firing serotonergic neurons in the RVM are unaffected by mu opioid receptor agonists (Gao et al. 1998) and further extend this finding to serotonergic neurons that project to the spinal cord. Based on the relative proportions of the three different types of serotonergic neurons and the percentages of each type that were responsive to DAMGO, it appears that only 25–30% of spinally projecting serotonergic neurons possess functional mu opioid receptors. Approximately 50% of spinally projecting serotonergic neurons in the RVM express mRNA for the mu opioid receptor (Wang and Wessendorf 2002).

Differences in the opioid sensitivity of the different types of spinally projecting serotonergic RVM neurons may underlie some of the controversy concerning the role of serotonergic RVM neurons in nociception and opioid-mediated analgesia (Hammond 1986; Hammond and Proudfoot 2005; Porreca et al. 2002; Suzuki et al. 2004; Vanegas 2004; Vanegas and Schaible 2004). These differences also support some speculation. For example, DAMGO-responsive spinally projecting type 1 serotonergic RVM neurons may correspond to the population of serotonergic pain facilitatory neurons (Zhuo and Gebhart 1991) thought to contribute to development or maintenance of neuropathic or inflammatory pain (Green et al. 2000; Suzuki et al. 2002, 2004). Inhibition of these neurons would be consistent with an anti-hyperalgesic or analgesic effect of mu opioid receptor agonists.

Type 1 and type 2 spinally projecting serotonergic neurons that are not DAMGO responsive are unlikely to possess mu opioid receptors. These neurons may correspond to serotonergic pain inhibitory RVM neurons, the disinhibition of which by opioids acting presynaptically at GABAergic interneurons underlies the production of analgesia. Evidence in support of this postulate includes the findings that serotonergic RVM neurons that project to the spinal cord possess GABA_A receptors (Hama et al. 1997); GABAergic neurons in the RVM express mu opioid receptors (Kalyuzhny and Wessendorf 1998); DAMGO decreases evoked GABA_A receptor-mediated inhibitory postsynaptic currents in spinally projecting RVM neurons, including those that are serotonergic (Finnegan et al. 2004); antagonism of GABA_A receptors in the RVM results in antinociception that is reversed by intrathecal serotonin receptor antagonists (Kaneko and Hammond 1997); and microinjection of DAMGO in the RVM produces antinociception that is reversed by intrathecal serotonin receptor antagonists (Hurley et al. 2003). This postulate leads to the prediction, which remains to be tested, that the in vivo firing rate of certain quiescent type 2 serotonergic neurons or intermittently firing type 1 serotonergic neurons should increase after systemic administration of morphine.

The activity of type 3 regularly firing serotonergic neurons is unaffected by bath application of mu opioid receptor agonists, suggesting that they lack mu opioid receptors. Notably, the firing rate of regularly firing serotonergic neurons recorded in vivo is also unaffected when morphine is administered systemically (Gao et al. 1998). Were type 3 regularly firing serotonergic neurons subject to inhibitory GABAergic input, then systemic morphine should have increased their firing rate in vivo by disinhibition. Collectively, these data suggest that type 3 serotonergic neurons are unlikely to play an important role in opioid analgesia in acute pain states.

### Validity of immunohistochemical methods in the slice preparation

Whole cell recording times often exceed 20 min and can approach 60 min when studying the effects of drugs on presynaptic events or when the neuron must serve as its own control. To our knowledge, an analysis of the sensitivity and specificity of immunohistochemical methods for identification of neurotransmitter content in neurons subjected to lengthy whole cell patch-clamp recordings has not been undertaken. Ideally, such an analysis would compare the results of the immunohistochemical characterization with the predictions of a second, independent measure of serotonergic nature. The current analysis therefore has to be viewed with the understanding that definition of the electrophysiological characteristics of serotonergic neurons was based on the immunohistochemical results.

Immunohistochemical “misclassification” of type 1 and type 2 neurons appeared to be a relatively rare event. In contrast, 25% of nonserotonergic type 3 neurons that were immunonegative for TrpH had electrophysiological characteristics concordant with those of serotonergic type 3 neurons. The majority of these “misclassified” type 3 neurons were small and had capacitances ≤50 pF. Serotonergic neurons are generally smaller than other RVM neurons (Gao and Mason 1997). This observation raised the possibility that their intracellular contents had dialyzed more completely during the 15- to 20-min recording protocol as compared with larger neurons. Indeed, when recording time was limited to ≤5 min, 75% of type 3 neurons with capacitance values of ≤50 pF were immunonegative for TrpH.

<table>
<thead>
<tr>
<th>Type</th>
<th>Serotonergic</th>
<th>Nonserotonergic</th>
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<tbody>
<tr>
<td>Type 1</td>
<td>6/11 (55)</td>
<td>3/5 (60)</td>
</tr>
<tr>
<td>Type 2</td>
<td>2/7 (29)</td>
<td>3/13 (23)</td>
</tr>
<tr>
<td>Type 3</td>
<td>1/12* (8)</td>
<td>5/17 (29)</td>
</tr>
</tbody>
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A neuron was considered responsive if 1 or 3 μM DAMGO produced an outward current ≥10 pA that was reversed by 1 μM naloxone; inward currents were never observed after application of [D-Ala², NMePhe⁵-Gly⁷-ol]-enkephalin (DAMGO). *p < 0.05, **p < 0.01 compared to type 1 neurons within that immunohistochemical classification. Percentage in parentheses.
active for TrpH. Although their mean and median capacitance values (and presumably size) were similar to those of type 3 neurons, type 1 neurons were not subject to misclassification. This finding is most likely due to the fact that a smaller percentage of type 1 neurons had capacitance values of ≤50 pF (21.4% for serotonergic and 26.7% for nonserotonergic; Fig. 5, C and F). The present results suggest that type 1 and type 2 neurons can be reliably categorized as serotonergic or nonserotonergic on the basis of post hoc immunohistochemical determinations as can type 3 neurons with capacitance values of >50 pF. However, recording times >5 min introduce a high risk for immunohistochemical “misclassification” of very small neurons. Collectively, these results suggest that caution should be used when interpreting negative immunohistochemical findings for small neurons from which lengthy recordings are made using whole cell methods.

Summary

The proposal that activation of serotonergic RVM neurons results in an inhibition of nociceptive transmission at the level of the spinal cord and that these neurons play an integral role in morphine-induced analgesia dates back nearly two decades (reviewed by Hammond 1986; Hammond and Proudfoot 2005). However, more recent evidence suggests these neurons also participate in the facilitation of nociceptive transmission, particularly under conditions of nerve injury or inflammation (Porreca et al. 2002; Suzuki et al. 2004) and further questions whether they mediate morphine-induced analgesia (Gao et al. 1998). While much attention has focused on identification of the serotonin receptor subtype and synaptic connections of these neurons in the spinal cord as a basis for their disparate functions in nociception, comparatively little is understood of the neurons themselves. The present results provide important new electrophysiological and pharmacological evidence that serotonergic RVM neurons that project to the spinal cord are a highly heterogeneous population. Moreover, this heterogeneity may underlie some of the controversy concerning the role of spatially projecting serotonergic RVM neurons in opioid analgesia.

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ELECTROPHYSIOLOGICAL HETEROGENEITY OF RVM NEURONS


