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Distinguishing Characteristics of Serotonin and Non-Serotonin-Containing Cells in the Dorsal Raphe Nucleus: Electrophysiological and Immunohistochemical Studies

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Abstract

The membrane properties and receptor-mediated responses of rat dorsal raphe nucleus neurons were measured using intracellular recording techniques in a slice preparation. After each experiment, the recorded neuron was filled with neurobiotin and immunohistochemically identified as 5-hydroxytryptamine (5-HT)-immunopositive or 5-HT-immunonegative. The cellular characteristics of all recorded neurons conformed to previously determined classic properties of serotonergic dorsal raphe nucleus neurons: slow, rhythmic activity in spontaneously active cells, broad action potential and large afterhyperpolarization potential. Two electrophysiological characteristics were identified that distinguished 5-HT from non-5-HT-containing cells in this study. In 5-HT-immunopositive cells, the initial phase of the afterhyperpolarization potential was gradual ($\tau=7.3\pm1.9$) and in 5-HT-immunonegative cells it was abrupt ($\tau=1.8\pm0.6$). In addition, 5-HT-immunopositive cells had a shorter membrane time constant ($\tau=21.4\pm4.4$) than 5-HT-immunonegative cells ($\tau=33.5\pm4.2$). Interestingly, almost all recorded neurons were hyperpolarized in response to stimulation of the inhibitory 5-HT_{1A} receptor. These results suggested that 5-HT_{1A} receptors are present on non-5-HT as well as 5-HT neurons. This was confirmed by immunohistochemistry showing that although the majority of 5-HT-immunopositive cells in the dorsal raphe nucleus were double-labeled for 5-HT_{1A} receptor-IR, a small but significant population of 5-HT-immunonegative cells expressed the 5-HT_{1A} receptor. These results underscore the heterogeneous nature of the dorsal raphe nucleus and highlight two membrane properties that may better distinguish 5-HT from non-5-HT cells than those typically reported in the literature. In addition, these results present electrophysiological and anatomical evidence for the presence of 5-HT_{1A} receptors on non-5-HT neurons in the dorsal raphe nucleus.

Keywords

5-HT; 5-HT_{1A} receptor; intracellular; slice; rat

The dorsal raphe nucleus (DRN) is generally considered a serotonergic nucleus because it is the largest source of 5-hydroxytryptamine (5-HT) terminals in the forebrain. However, substantial evidence points to the heterogeneous nature of the DRN. For example, the proportion of 5-HT-containing cells in the DRN is estimated to range from one to two thirds (Steinbusch et al., 1980; Descarries et al., 1982; Jacobs and Azmitia, 1992; Baumgarten and Grozdanovic, 1997). The remaining non-serotonergic cells contain a variety of other neurotransmitters and neuromodulators including dopamine, norepinephrine, glutamate,

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GABA, enkephalin, substance P, neuropeptide Y, thyrotropin-releasing hormone, vasoactive intestinal polypeptide, cholecystokinin, gastrin and neurotensin (for review, see Kohler and Steinbusch, 1982 or Jacobs and Azmitia, 1992). The heterogeneous nature of the DRN is also reflected by the fact that it is composed of several cell clusters or subdivisions, each of which receive shared as well as selective afferent inputs and give rise to distinct, but overlapping projections (Molliver, 1987). This heterogeneous organization likely underlies the complex variety of behaviors that are influenced by the 5-HT system including sleep, sexual behavior, eating, motor activity, pain, neuroendocrine function, circadian rhythmicity, aggression, anxiety and affective disorders (Jacobs and Azmitia, 1992).

The electrophysiological and pharmacological properties of 5-HT DRN cells have been characterized. Aghajanian and Vandermaelen (1982), using *in vivo* intracellular recording techniques followed by neurochemical identification of the recorded cell, found that 5-HT-containing neurons have a slow, rhythmic firing pattern, a large afterhyperpolarization potential (AHP), gradual interspike depolarization, and a long duration action potential. Later studies using intracellular recording in brain slices examined the characteristics of putative serotonergic neurons in more detail (Vandermaelen and Aghajanian, 1983; Burlhis and Aghajanian, 1987). These cells are either silent or spontaneously active with a slow, regular firing pattern (0.5–2.5 Hz), a high input resistance (150–400 M Ω), and have a long duration action potential (approximately 1.8 ms) followed by a large, slow AHP (amplitude=10–20 mV, duration=200–800 ms). In addition, these cells are hyperpolarized and their firing inhibited by stimulation of inhibitory 5-HT_{1A} autoreceptors. These cellular characteristics and pharmacological profile are frequently cited in the literature as a means of identifying putative 5-HT-containing cells for electrophysiological studies.

A recent study directly compared the properties of 5-HT-containing and non-5-HT-containing neurons in the DRN using *in vitro* intracellular electrophysiological recording techniques followed by neurochemical identification of the recorded cell. Li et al., (2001) found that, morphologically, 5-HT-containing cells were distinguished by their spiny dendrites as compared with non-5-HT-containing cells whose dendrites were primarily aspiny. Electrophysiologically, 5-HT-containing cells had a larger membrane time constant and larger action potential (amplitude and duration) than non-5-HT-containing cells. Small depolarizing current steps elicited a slower and more regular train of spikes in 5-HT-containing cells. Spike trains elicited in non-5-HT-containing cells frequently demonstrated adaptation whereas 5-HT-containing cells did not. No differences in resting membrane potential, input resistance, electrotonic length, or spike threshold were found between the two cell types.

In the current study, we have further characterized the electrophysiological properties of 5-HT and non-5-HT-containing DRN cells in a slice preparation. Additionally, we examined sensitivity of both cell types to a 5-HT_{1A} and an α_1 adrenergic agonist. Finally, dual immunohistochemistry was used to determine the location of 5-HT_{1A} receptors in the serotonergic DRN. By combining these techniques, we have identified additional cell characteristics to distinguish 5-HT-containing from non-5-HT-containing cells and determined that some of the established characteristics may not be discriminatory. Furthermore, we provide electrophysiological and anatomical evidence for 5-HT_{1A} receptors on non-5-HT as well as 5-HT DRN neurons.

Experimental Procedures

Subjects

Adult male Sprague–Dawley rats (Taconic Farms, Germantown, NY, USA) initially weighing 50–100 g were housed two to three per cage on a 12-h light schedule (lights on at 07:00 AM) in a temperature-controlled (20 °C) colony room. Rats were given access to standard rat chow

and water *ad libitum*. Animal protocols were approved by the Institutional Animal Care and Use Committee and were conducted in accordance to the *NIH Guide for the Care and Use of Laboratory Animals*.

Slice preparation

Rats were decapitated, the brain removed and placed in ice-cold saline buffer (4 °C) containing (mM) NaCl (124), KCl (3), NaH₂PO₄ (1.25), MgSO₄ (2), CaCl₂ (2.5), dextrose (10), NaHCO₃ (26). The brain was blocked and coronal slices (500 µm) were cut by vibratome through the entire rostro-caudal extent of the DRN and placed in a holding chamber containing room temperature buffer bubbled with 95% O₂/5% CO₂. The slices were allowed to equilibrate for at least 1 h. For recording, a slice was transferred to a recording chamber where it was perfused with carbogenated (95% O₂/5% CO₂) buffer (35 °C) at a flow rate of 1.5 ml/min, pH 7.4.

Intracellular, sharp electrode recording

Intracellular recordings were conducted according to methods previously described by Beck et al. (1994). Electrodes were pulled from borosilicate capillary tubing and filled with 2 M KCl containing 0.2% neurobiotin (Vector Laboratories, Burlingame, CA, USA) to obtain resistances of 60–100 MΩ. Cells were targeted primarily in the ventromedial and interfascicular subdivisions of the DRN as this region contains the densest population of 5-HT-containing neurons. Cells were impaled and hyperpolarized (1 nA) to facilitate sealing of the cell. Electrical signals were amplified, recorded on a chart recorder, and the data sent to a computer for on-line recording and analysis. Cell characteristics and their response to drugs were collected and analyzed using pCLAMP software (Axon Instruments, Foster City, CA, USA). The cell characteristics measured were resting membrane potential, input resistance, time constant, action potential and AHP characteristics, and voltage-current relationship. Firing rate of spontaneously active cells or cells stimulated by drug treatment was also measured. 5-HT_{1A} receptors were stimulated by bath application of a saturating concentration of 5-carboxamidotryptamine (5-CT, 100 nM; Williams et al., 1988). Although 5-CT is a mixed 5-HT_{1/7} agonist, the hyperpolarization response examined here has been shown to be mediated by the 5-HT_{1A} receptor (Williams et al., 1988; Corradetti et al., 1996). α₁ adrenergic receptors were stimulated by bath application of the selective agonist phenylephrine (PE, 3 µM; Vandermaelen and Aghajanian, 1983; Pan et al., 1994).

Neurobiotin filling and immunohistochemical procedures

After electrophysiological and pharmacological characterization, neurobiotin was iontophoresed into the cell by applying 1 nA current pulses lasting 100 ms at 3.3 Hz for 2–10 min (Takakusaki et al., 1996). The slice was immersion-fixed overnight in 4% paraformaldehyde prepared in 0.1 M phosphate buffer (PB; pH 7.4), and subsequently cryoprotected in 30% sucrose. The 500-µm slice was rapidly frozen with CO₂ and cut into 50-µm coronal sections on a cryostat. The neurobiotin-filled cell was visualized by incubating sections in streptavidin-conjugated Cy3 (1:100; Jackson ImmunoResearch, West Grove, PA, USA) for 60 min at room temperature and 5-HT-containing neurons were visualized using standard immunofluorescence techniques. Sections were incubated with rabbit α 5-HT antibody (1:2000; ImmunoStar Inc., Hudson, WI, USA) for 2 days (4 °C) and then in FITC conjugated donkey α rabbit secondary antiserum (1:100; Jackson ImmunoResearch) for 60 min at room temperature. Between incubations slices were rinsed with PB solutions (3×10 min) and all incubations were done with mild agitation on a shaker.

Localization of 5-HT_{1A} receptor and 5-HT-immunoreactivity in sections through the DRN was accomplished with dual immunofluorescence methods. The rat was deeply anesthetized with pentobarbital (60 mg/kg, i.p.) and perfused transcardially with 100 ml of Ringer's lactate

solution, followed by 1000 ml of 4% paraformaldehyde in PB (4 °C). The brain was removed and stored in the same solution for 90 min (4 °C) and then overnight in a solution of 20% sucrose in 0.1 M PB containing 0.1% sodium azide (4 °C). The brain was rapidly frozen with CO₂ and 30- μ m coronal sections cut on a cryostat. The sections were collected in 0.1 M PB. Sections were first blocked in phosphate buffered saline containing 0.3% Triton-X 100 and 0.5% bovine serum albumin for 30 min. Sections were incubated with rabbit α 5-HT antisera (1:2000; ImmunoStar Inc.) overnight at room temperature and then FITC conjugated donkey α rabbit secondary antiserum (1:100; Jackson ImmunoResearch) for 60 min at room temperature. Sections were then incubated in guinea-pig α 5-HT_{1A} receptor antibody (1:1000; Chemicon International, Temecula, CA, USA) for 2 days (4 °C) and then Cy3 donkey α guinea-pig secondary antiserum (1:100; Jackson ImmunoResearch) for 60 min at room temperature. Between incubations slices were rinsed with PB solutions (3 $\times$ 10 min) and all incubations were done with mild agitation on a shaker.

Sections were mounted on Superfrost plus slides (Fisher Scientific, Pittsburgh, PA, USA) and coverslipped with Vectashield mounting medium (Vector Laboratories). Fluorescein and Cy3 labeled neurons were visualized with fluorescence microscopy using a Leica DMR microscope. Images were captured using a MicroMAX digital camera (Princeton Instruments, Trenton, NJ, USA) and Openlab 2.2.5 software. Images were adjusted for optimal color balance and contrast using Adobe Photoshop, version 6.0.

The specificity of the 5-HT_{1A} receptor antibody was tested by comparing the regional distribution 5-HT_{1A} receptor immunoreactivity (for examples, see Fig. 9) to the pattern described in receptor binding autoradiography (Kung et al., 1995), *in situ* hybridization (Pompeiano et al., 1992; Wright et al., 1995) and immunohistochemistry studies using other 5-HT_{1A} receptor antibodies (Azmitia et al., 1996; Kia et al., 1996; Zhou et al., 1999). For these single-labeling studies, tissue was prepared as described above for dual immunohistochemistry. Sections (30 μ m) were incubated in guinea-pig α 5-HT_{1A} receptor antibody (1:2000; Chemicon International) for 2 days (4 °C) and then biotinylated donkey α guinea-pig secondary antiserum (1:200; Jackson ImmunoResearch) for 90 min at room temperature. Finally, sections were incubated for 90 min in avidin–biotin complex (ABC Elite kit; Vector Laboratories). Sections were visualized by a reaction in 3–3' diaminobenzidine (Sigma Chemical Co., St. Louis, MO, USA) for a brown reaction product. Between incubations slices were rinsed with PB solutions (3 $\times$ 10 min) and all incubations were done with mild agitation on a shaker. Sections were mounted on Superfrost plus slides (Fisher Scientific), dehydrated in a series of alcohols, and coverslipped with Permount. Visualization was performed using a Leica DMR microscope and images were captured using with a Fuji FinePix S1Pro digital camera and Fuji FinePix S1Pro Shooting software. Images were adjusted for optimal brightness and contrast using Adobe Photoshop, version 6.0.

Western blot analysis

Western blotting was also used to evaluate the specificity of the 5-HT_{1A} receptor antibody. Whole protein extracts were prepared from rat DRN tissue as described below. Immunopositive protein bands of 46, 35, and 33 kDa were seen (Fig. 1). The position of the first band corresponds to the 46.5 kDa predicted molecular mass of the native, unmodified 422-amino acid 5-HT_{1A} receptor protein (Albert et al., 1990). The other two bands may reflect the breakdown products of the glycosylated form of the 5-HT_{1A} receptor, which is estimated to range between 63 to 70 kDa (see Zhou et al., 1999 for discussion). For Western blot analysis, rat brains were sectioned and the DRN removed by punch dissection using a 2-mm trephine (Biomedical Research Instruments, Rockville, MD, USA). Protein was extracted in RIPA buffer with added soybean trypsin (25 μ g/ml) and benzamidine (0.1 mM) as described previously (Van Bockstaele et al., 2000). Protein levels were quantified, 25 μ g protein/lane

was separated in a 4–20% Tris-glycine gel (Bio-Rad Laboratories, Hercules, CA, USA) and transferred to Immobilon-P membrane (Millipore, Bedford, MA, USA). Membranes were blocked using tris buffer with saline (TBS) containing 0.1% Tween-20 and 0.5% BSA (BSA TBS-T), incubated in guinea-pig α 5-HT_{1A} receptor antibody (1:2000; Chemicon International), and finally incubated in horseradish peroxidase-conjugated donkey α guinea-pig secondary antibody (1:20,000; Jackson ImmunoResearch). All incubations were performed for 1 h at room temperature and between incubations membranes were rinsed with TBS-T buffer. To visualize bands, membranes were incubated in SuperSignal® West Pico Chemiluminescent Substrate, wrapped in plastic and exposed to X-OMAT film (Eastman Kodak, Rochester, NY, USA). Film was developed using a SJ X-ray developer (Eastman Kodak), scanned using a UMAX Astra scanner (Micro Warehouse, Norwalk, CT, USA) and analyzed using UN-SCAN-IT Software (Silk Scientific, Orem, UT, USA).

Statistical analysis

Cell characteristics of immunohistochemically distinguished cell groups were analyzed for normal distribution using the Kolmogorov-Smirnov test. Student's *t*-test compared groups with normally distributed data. Mann–Whitney Rank Sum Test compared groups with non-normally distributed data. Statistical significance was defined as *P* value of less than 0.05.

Results

Recording in the DRN

Cellular characteristics and pharmacological responses were recorded in 37 neurons in the DRN. Thirty-two cells (86%) were located in the ventromedial/interfascicular subdivision of the nucleus and the remaining five (14%) were located in the dorsomedial subdivision. All cells were obtained from slices located roughly halfway through the rostro-caudal extent of the DRN (c.f. plates 50–52; Paxinos and Watson, 1998). Fig. 2 shows representative examples of neurobiotin-filled neurons in the DRN from which electrophysiological properties were obtained. These neurons were localized in the ventromedial/interfascicular DRN, a subdivision containing numerous 5-HT-immunopositive (IR+) neurons (Figs. 2 and 7; green). Of 37 filled neurons that were immunohistochemically identified, 17 (46%) were 5-HT-IR+ (e.g., Fig. 2A).

Electrophysiological properties of DRN neurons

Table 1 lists the electrophysiological properties of 5-HT-containing and non-5-HT-containing cells in the rat DRN. Many of the characteristics that are frequently noted in the literature to distinguish 5-HT DRN cells (i.e., high-input resistance, long duration action potential and large AHP), were not different between these two cell groups (see Table 1 and Fig. 3). Input resistance appeared to be larger in non-5-HT cells than 5-HT cells, although that difference was not statistically significant and input resistance for both cell types fell within the range reported previously for 5-HT cells [150–400 M Ω (Burlhis and Aghajanian, 1987)]. AHP amplitude and duration of both groups were not statistically different and fell within ranges previously reported for 5-HT cells [amplitude: 10–20 mV, duration: 200–800 ms (Vandermaelen and Aghajanian, 1983)].

Even though the classic characteristics previously noted for 5-HT cells did not differ between the 5-HT-IR+ and 5-HT-immunonegative (IR–) cells, these neurons were distinguished by certain other electrophysiological properties. For example, the time constant (τ) was substantially shorter in 5-HT-IR+ neurons ($U=221.0$, $P<0.05$; Table 1). This was measured as the amount of time it takes for the membrane to charge to 63% of maximum in response to a small hyperpolarizing current pulse (25–50 pA). Fig. 3 shows the time constant calculated in one 5-HT-IR+ (Fig. 3A) and one 5-HT-IR– cell (Fig. 3B).

An additional distinguishing property between 5-HT and non-5-HT neurons was the shape of the initial phase of the AHP. The AHP onset was observed to be more gradual in 5-HT-IR+ cells than in 5-HT-IR- cells. To quantify this characteristic, an exponential function was fit to the initial AHP phase (from the onset of the AHP following an action potential to its maximal level). The time constant of this exponential function is referred to here as the AHP tau. Fig. 4 compares the AHP tau from one 5-HT-IR+ (Fig. 4A) and one 5-HT-IR- cell (Fig. 4B). The AHP tau was significantly longer in 5-HT-IR+ neurons ($U=382.5$, $P<0.05$; Table 1). Fig. 5 illustrates the distribution of the AHP tau data. Fig. 5A, B demonstrates that AHP tau from 5-HT-containing cells were more variable than AHP tau from non-5-HT-containing cells. The majority (17/19) of 5-HT-IR- cells demonstrated a small AHP tau (less than 3 ms). In contrast, one group of 5-HT-IR+ cells (8/17) demonstrated a small and another group (7/17) a very large (greater than 10 ms) AHP tau.

Of 5-HT or non-5-HT neurons recorded, only a few were spontaneously active (3/17 or 18% of 5-HT-IR+ cells; 5/20 or 25% of 5-HT-IR- cells). However, spontaneously active 5-HT-containing cells had a significantly lower firing rate (0.8 ± 0.3 Hz) than did non-5-HT-containing cells (2.9 ± 0.5 Hz; $t(6)=-2.8$, $P<0.05$).

Pharmacological properties of DRN neurons

Table 2 lists the pharmacological properties of 5-HT-containing and non-5-HT-containing cells in the rat DRN. Although the response to activation of 5-HT_{1A} receptors is often cited as a defining feature of 5-HT DRN cells, activation of this receptor by 5-CT hyperpolarized both 5-HT ($N=14$) and non-5-HT-containing cells ($N=15$) and the magnitude of that hyperpolarization was comparable (Table 2; silent cells). Representative examples of this response are shown in Fig. 6 in a 5-HT-IR+ (Fig. 6A) and 5-HT-IR- (Fig. 6B) DRN cell. Both cells hyperpolarized in response to 100 nM 5-CT by 7 mV. Although the magnitude of the hyperpolarization elicited by 5-CT was comparable in silent 5-HT-IR+ and 5-HT-IR- neurons, the effects of 5-CT in spontaneously active neurons was different. Stimulation of 5-HT_{1A} autoreceptors with a saturating concentration of 5-CT completely inhibited the firing ($-100\pm0\%$ change) and hyperpolarized (-11.0 ± 1.4 mV) all three spontaneously active 5-HT cells but only partially inhibited the firing ($-36.0\pm17.5\%$ change) [$t(6)=-2.7$, $P<0.05$] and only minimally hyperpolarized (-1.4 ± 1.1 mV) [$t(6)=-6.2$, $P<0.001$] the five spontaneously active non-5-HT cells. To explore this phenomenon more fully, the adrenergic agonist PE was administered in the perfusion bath of silent cells. PE elicits firing in 5-HT cells by stimulating α_1 receptors, a major excitatory input to 5-HT DRN cells *in vivo* that is severed in the *in vitro* slice preparation (Vandermaelen and Aghajanian, 1983; Pan et al., 1994). PE (3 μ M) elicited firing in both 5-HT ($N=5$) and non-5-HT cells ($N=7$). The “clocklike” pattern of this PE-induced firing was similar in both groups and the firing rate was slightly below the range previously reported for 5-HT cells (0.5–2.5 Hz; Vandermaelen and Aghajanian, 1983). 5-CT completely suppressed PE-elicited firing ($-100\pm0\%$ change) in all three 5-HT and all three non-5-HT cells tested.

Distribution of 5-HT-IR and 5-HT_{1A} receptor-IR neurons in DRN

To examine the anatomical basis of the 5-HT_{1A} receptor-mediated responses of both 5-HT and non 5-HT neurons, we examined the distribution of 5-HT-IR cells in comparison to 5-HT_{1A} receptor-IR cells throughout the DRN. Fig. 7 shows fluorescent photomicrographs depicting 5-HT-IR (green) and 5-HT_{1A} receptor-IR cells (red) in the ventromedial subdivision of the DRN (left panels: low magnification and right panels: high magnification). Consistent with several reports (Weissmann-Nanopoulos et al., 1985; Verge et al., 1986; Sotelo et al., 1990; Miquel et al., 1992), the majority of 5-HT-containing cells also expressed 5-HT_{1A} receptors. Nonetheless, a significant number of single labeled neurons that were 5-HT_{1A}-IR were observed and these were interdigitated with 5-HT-IR neurons.

Quantification of single- and double-labeled cells at different rostro-caudal levels and in different subdivisions of the DRN (Panel A: dorsal subdivision, panel B: lateral wings, panel C: ventral/interfascicular subdivision) is shown in Fig. 8. Most cells were double-labeled (expressing immunoreactivity for both 5-HT and the 5-HT_{1A} receptor) in all divisions of the DRN. The dorsal subdivision and lateral wings were the most heterogeneous such that at caudal levels there was a substantial number of 5-HT-IR only cells and more rostrally there was a substantial number of 5-HT_{1A} receptor-IR only cells. Within the ventromedial subdivision, most cells were double-labeled with very few 5-HT-IR only cells. However, more rostrally, many neurons were 5-HT_{1A} receptor-IR only.

In order to anatomically characterize the 5-HT_{1A} receptor antibody, the distribution of immunoreactivity was analyzed throughout the rat brain. Labeling was seen in regions previously reported to contain 5-HT_{1A} receptors such as the raphe nuclei, septum, hippocampus, cerebral cortex (especially entorhinal cortex), medial habenula, paraventricular nucleus of the hypothalamus, zona incerta, preoptic nucleus, supraoptic nucleus and diagonal band. Fig. 9 shows examples of immunohistochemical labeling for the 5-HT_{1A} receptor in the septum (Fig. 9 A1, A2), supraoptic nucleus (Fig. 9 B1, B2) and medial habenula (Fig. 9C).

Discussion

Both 5-HT-containing and non-5-HT-containing cells were identified in the rat DRN. Forty-six percent of immunohistochemically-identified cells were 5-HT-containing, and 54% were non-5-HT-containing. Characteristics previously used to distinguish 5-HT-containing cells, i.e., high input resistance, long duration action potential, large AHP and membrane hyperpolarization in response to stimulation of 5-HT_{1A} autoreceptors, were not different between the 5-HT-IR+ and 5-HT-IR- cells. Other characteristics, however, did distinguish the two cell types. 5-HT-IR+ cells had a significantly lower time constant (τ) and a slower onset of the AHP than 5-HT-IR- cells. In the majority of recorded neurons which were silent, both 5-HT-IR+ and 5-HT-IR- cells hyperpolarized in response to stimulation of the 5-HT_{1A} receptor, indicating the presence of 5-HT_{1A} receptors on both cell types. Consistent with this was immunohistochemical data showing that a significant number of non-5-HT as well as 5-HT neurons express the 5-HT_{1A} receptor. Together, these results suggest that the electrophysiological and pharmacological characteristics that are typically used to distinguish 5-HT from non-5-HT cells in the DRN are insufficient. Furthermore, these data suggest a greater heterogeneity in the cellular characteristics of 5-HT neurons in the DRN than previously thought. Finally, this report provides evidence for 5-HT_{1A} receptors on non-5-HT cells, suggesting that pharmacological agents that act on 5-HT_{1A} receptors may have more complex effects in the DRN than previously considered.

Interestingly, many of the membrane characteristics that are typically cited in the literature as defining features of serotonergic DRN neurons were indistinguishable between the two cell types in this study. For example, 5-HT neurons are noted for their high input resistance (150–400 M Ω ; Burlhis and Aghajanian, 1987). However, in this study, there was no statistically significant difference in resistance between 5-HT-IR+ and 5-HT-IR- neurons and the average resistance of both cell types fell within that reported range. A long spike duration of approximately 1.8 ms has also been reported to distinguish 5-HT neurons (Vandermaelen and Aghajanian, 1983), though this property was statistically indistinguishable between the two cell types. A large AHP, both in amplitude (10–20 mV) and duration (200–800 ms) has been noted for 5-HT neurons (Vandermaelen and Aghajanian, 1983), though the AHPs from both cell types were large, falling within these ranges. Our findings thus indicate that these membrane characteristics, though accurately descriptive of 5-HT-containing DRN neurons, should not be used as a means of distinguishing 5-HT from non-5-HT-containing cells. For most of these membrane characteristics, data from 5-HT as well as non-5-HT cells ranged

widely with large overlap, hence the group means were not statistically different. From these data, we have concluded that the membrane characteristics of 5-HT DRN cells are significantly more heterogeneous than previously thought, making the cells difficult to distinguish without immunohistochemical labeling. The implications of these findings are that previous intracellular and extracellular recording experiments that have relied on the established characteristics to identify 5-HT cells may also have included a subset of non-5-HT cells as well, thus their results should be interpreted with consideration of this possibility.

In contrast to these other characteristics, the membrane time constant (τ), an estimate of membrane capacitance, did distinguish serotonergic and non-serotonergic cells. Serotonergic cells had a shorter τ than non-serotonergic cells, indicating a shorter time for charge to be distributed across the membrane. Cell surface area and cell morphology are known to affect the τ characteristic, thus these data may indicate that serotonergic cells have a distinct morphology from non-serotonergic cells. Consistent with this, a recent report noted that serotonin-containing DRN cells have dendritic spines whereas dendrites of non-serotonin-containing DRN neurons are aspiny (Li et al., 2001), a distinction that could contribute to the difference in τ .

The time constant of the initial phase of the AHP was identified as an additional characteristic that distinguished 5-HT and non-5-HT-containing DRN cells. 5-HT cells had a long, gradual AHP onset whereas the AHP onset for non-5-HT cells was more abrupt. Since the action potential width was identical between the two cell types, it seems unlikely that any of the ionic currents that underlie the action potential contribute to this AHP τ characteristic. The role of the AHP in neurons is to control their excitability. In DRN neurons the AHP has an early and late component, the latter of which is mediated, as in many other neurons of the brain, by a calcium-dependent potassium current (Crunelli et al., 1983; Pan et al., 1994). The early component of the AHP may contribute to the AHP τ described here. This early-AHP is insensitive to apamin, a neurotoxin that blocks most calcium-dependent potassium currents (Pan et al., 1994). In the future, it will be necessary to use selective channel blockers to characterize the specific ionic mechanisms responsible for this membrane characteristic. Nonetheless, the AHP τ is simple to measure using intracellular recording techniques and may be a useful tool with which to distinguish serotonergic from non-serotonergic neurons in the DRN.

The majority of 5-HT DRN neurons are silent *in vitro* largely because the α -adrenergic tonic input has been severed in the slice preparation. The few spontaneously active 5-HT neurons *in vitro* represent a subset of cells with tonic pacemaker properties (Mosko and Jacobs, 1976; Burlhis and Aghajanian, 1987). Serotonergic spontaneously active cells in this study all had “clocklike” firing patterns whose rates fell within the range reported previously for 5-HT DRN neurons *in vivo* and *in vitro* (0.5–2.5 Hz; Vandermaelen and Aghajanian, 1983). Non-serotonergic spontaneously active cells had a significantly higher firing rate such that the firing rate of 4/5 cells was out of this reported range. Therefore, one characteristic that differentiates 5-HT-IR+ from 5-HT-IR– cells in the slice preparation is the tonic firing rate of spontaneously active neurons. In this spontaneously active subset of DRN cells, 5-HT silenced and hyperpolarized all 5-HT-IR+ and only partially suppressed and minimally hyperpolarized 5-HT-IR– cells. Therefore, serotonergic spontaneously active neurons also appear to be more sensitive to stimulation of 5-HT_{1A} receptors than non-serotonin-containing spontaneously active neurons.

Noradrenergic afferents to the DRN exert a tonic excitatory effect on 5-HT DRN cells through the α_1 receptor (Baraban and Aghajanian, 1980; Yoshimura et al., 1985). Since these afferents have been severed in the slice preparation, activation of the α_1 receptor has been reported *in vitro* to restore the firing of silent serotonergic cells with a pattern and rate that resembles 5-

HT cells *in vivo* (Vandermaelen and Aghajanian, 1983). In this study, the α_1 adrenergic agonist PE had similar effects on serotonergic vs. non-serotonergic silent neurons. PE depolarized all (5/5) silent serotonergic cells and initiated firing in 4/5 of them. PE depolarized 6/7 silent non-serotonergic cells to a similar extent as serotonergic cells and initiated firing in 3/7 of them. The PE-stimulated firing rate in both cell groups was slightly lower than the range reported for serotonergic cells in the literature (see above). These similarities suggest that many non-serotonergic cells also receive tonic excitatory noradrenergic input via the α_1 receptor.

A particularly striking finding was that stimulation of 5-HT_{1A} receptors with 5-CT hyperpolarized silent non-serotonergic cells to a similar extent as serotonergic cells, a property previously considered to be a hallmark of 5-HT-containing DRN neurons. Though surprising, there are some reports of non-serotonergic neurons in the periaqueductal gray (Behbehani et al., 1993) and DRN (Jolas and Aghajanian, 1997; Allers and Sharp, 2001; K. A. Allers, Department of Pharmacology, personal communication) that are inhibited by stimulation of the 5-HT_{1A} receptor. To further examine this finding, we tested 5-CT's effects in cells whose firing had been restored by PE, an attempt to mimic *in vivo* conditions. 5-CT hyperpolarized all PE-stimulated 5-HT and non-5-HT neurons to a similar extent. In addition, those cells induced to fire by PE, regardless of neurochemical identity, were silenced by 5-CT. These findings argue that, except in spontaneously active neurons, the response to 5-HT_{1A} receptor stimulation should not be used to identify presumed serotonergic neurons *in vitro* and caution against the use of this pharmacological characteristic to identify these neurons *in vivo* as well.

The physiological findings suggest that 5-HT_{1A} receptors exist on both 5-HT-IR+ and 5-HT-IR- neurons. The anatomical data presented in this study are consistent with these findings. Previous studies have used selective 5-HT lesions with 5,7-DHT (Weissmann-Nanopoulos et al., 1985; Verge et al., 1986; Miquel et al., 1992) and more direct immunohistochemical evidence using 5-HT-IR and 5-HT_{1A} receptor-IR labeling in adjacent sections (Sotelo et al., 1990) to argue for the presence of 5-HT_{1A} receptors on 5-HT DRN neurons. As expected, we found that the majority of 5-HT-IR+ neurons in the DRN were double-labeled for 5-HT_{1A} receptor-IR. However, there were also clear examples throughout the DRN of single-labeled 5-HT-IR+ cells and single-labeled 5-HT_{1A} receptor-IR+ cells. Interpretation of these anatomical data relies on the selectivity of the 5-HT_{1A} receptor antibody. The antibody used in this study was generated to a sequence present in the third intracellular loop of the 5-HT_{1A} receptor that is not present in other known 5-HT receptor subtypes. Previous work showed that immunohistochemical labeling of hippocampal 5-HT_{1A} receptors was blocked when brain sections were exposed to antibody that had been preincubated with an excess of the antigen (L. M. Foster, Department of Technical Services, Chemicon International, personal communication). Furthermore, this antibody labeled appropriate bands on the Western blot predictive of the native 5-HT_{1A} receptor protein in both its unmodified and glycosylated forms. The distribution of immunohistochemical labeling for the receptor mapped to similar brain regions identified in other autoradiography (Kung et al., 1995), *in situ* hybridization (Pompeiano et al., 1992; Wright et al., 1995) and immunohistochemical studies using other 5-HT_{1A} receptor antibodies (Azmitia et al., 1996). For example, in the septum, other 5-HT_{1A} antibodies have shown similar labeling patterns: dense staining in the neuropil of the lateral septum and lighter labeling of soma in the medial septum (Kia et al., 1996; Zhou et al., 1999). The heavy labeling in supraoptic nucleus and medial habenula was also demonstrated by another 5-HT_{1A} receptor antibody (Pompeiano et al., 1992; Kia et al., 1996).

For three antibodies generated against overlapping portions of the highly selective third intracellular loop of the 5-HT_{1A} receptor protein, there were slightly different subcellular labeling patterns. Kia et al. (1996) used an antibody generated against a synthetic peptide corresponding to amino acid residues 243–268 and demonstrated labeling of soma as well as dendrites. Zhou et al. (1999) used an antibody generated against a fusion protein containing

the amino acid residues 253–327 and demonstrated labeling of soma, dendrites and axons. The antibody used in this study (Chemicon International) was generated against a synthetic peptide corresponding to amino acid residues 248–262 and preferentially labels soma. The nature of the antigens, the sequences employed and their locations within the third intracellular loop of the receptor protein may all contribute to similarities and differences in overall and subcellular labeling patterns between the different antibodies. Sotelo et al. (1990), using the same antibody as Kia et al. (1996), examined 5-HT_{1A} receptor-IR and 5-HT-IR in adjacent sections. These authors reported that every 5-HT-IR+ cell also expressed the 5-HT_{1A} receptor. However, the authors also describe the difficulties of delineating somatic contours in densely packed areas (Sotelo et al., 1990). It is perhaps not surprising that an antibody that labels dendrites as well as soma would make these determinations difficult, especially in areas such as the ventromedial subdivision of the DRN where neurons and dendritic processes are very tightly packed. For this reason, a soma-preferring antibody such as that used in this study may be the ideal tool to distinguish single from double-labeled cells in the DRN. Nonetheless, our data suggest that a minority of serotonergic cells does not express 5-HT_{1A} receptors and some non-serotonergic cells do express these receptors. Consistent with these findings, a recent study in our laboratory demonstrated that though lesions of the 5-HT system with 5,7-DHT significantly reduce 5-HT_{1A} receptor binding in the DRN, a substantial number of binding sites remain (42%) (Kirby et al., 2001). Previous serotonergic lesion studies have shown spared 5-HT_{1A} receptor binding sites or receptor mRNA to range from 10 to 40% (Weissmann-Nanopoulos et al., 1985; Verge et al., 1986; Miquel et al., 1992). It is likely that at least a subset of these spared neurons remain because of their localization on non-5-HT neurons. Therefore, both electrophysiological and immunohistochemical results in this study converge to provide evidence for the presence of functional 5-HT_{1A} receptors on a subpopulation of non-5-HT-containing neurons in the DRN.

An important implication of the present findings is that pharmacological agents thought to be acting at 5-HT_{1A} receptors on 5-HT neurons may be acting on non-5-HT neurons as well to produce their effects. For example, many previous studies have used local injections of 5-HT_{1A} ligands into the DRN or application of 5-HT_{1A} ligands in a DRN brain slice preparation in order to specifically target somatodendritic 5-HT_{1A} autoreceptors and distinguish their functional role from that of distal postsynaptic 5-HT_{1A} receptors (for review, see De Vry, 1995; Barnes and Sharp, 1999; Pineyro and Blier, 1999). Since the present study provides evidence for the existence of proximal postsynaptic 5-HT_{1A} receptors on non-5-HT neurons within the DRN, some of these effects previously attributed to somatodendritic 5-HT_{1A} receptor activation of 5-HT neurons may in fact be due to 5-HT_{1A} receptor activation of non-5-HT neurons. The potential contributions of 5-HT_{1A} receptors on non-5-HT neurons to the effects of 5-HT_{1A} ligands in the DRN should be considered when interpreting the results of the kinds of experiments described above.

Since 5-HT cell bodies account for one to two thirds of all neurons in the DRN, the identity of non-5-HT neurons has been studied extensively. In addition to other neurotransmitters, several peptides, some of which may be co-localized with 5-HT have been identified in the DRN. Our electrophysiological recordings were primarily conducted on cells located in the ventromedial and interfascicular subdivisions of the DRN, particularly along the midline. Dopamine and norepinephrine-containing neurons have been identified in the DRN, though only sparsely scattered throughout the nucleus (Ochi and Shimizu, 1978; Nagatsu et al., 1979), making them unlikely candidates for the identity of the non-5-HT cells recorded in this study. Glutamate-containing cell bodies are localized to the ventromedial DRN (Clements et al., 1991) and GABAergic cells are found densely throughout the DRN (Nanopoulos et al., 1982; Belin et al., 1983), thus they more likely account for some of our non-5-HT cells. Some of the peptides are also potential candidates: enkephalin-containing cells are concentrated along the DRN midline (Moss et al., 1981), substance P-containing cells are also found in large numbers (Moss et al., 1981; Charara and Parent, 1998), and neurotensin-containing cells are found

particularly in the ventromedial subdivision of the nucleus (Shipley et al., 1987). It is possible that these non-5-HT cells represent several different neurochemical classes. In the future, it will be necessary to use triple-labeling immunohistochemical methods to definitively determine their identities.

Conclusion

In summary, both 5-HT-containing and non-5-HT-containing cells in this study conformed to the electrophysiological and pharmacological characteristics (Vandermaelen and Aghajanian, 1983) that have previously been considered unique to 5-HT neurons of the DRN. However, the time constant (τ) and the additional characteristic of the AHP τ have been identified in this study as potentially useful tools with which to electrophysiologically identify 5-HT-containing cells when immunohistochemical methods are unavailable. Nonetheless, these data underscore the heterogeneity of the DRN and of 5-HT neurons within the nucleus. Furthermore, the results of both electrophysiological and immunohistochemical experiments converge to provide evidence for the presence of functional 5-HT_{1A} receptors on non-5-HT as well as 5-HT neurons in the DRN. In the future, experiments using 5-HT_{1A} receptor ligands with effects in the DRN should consider the complexity of their effects on both serotonergic and non-serotonergic cells.

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Abbreviations

AHP	afterhyperpolarization
5-CT	5-carboxamido-tryptamine
DRN	dorsal raphe nucleus
5-HT	5-hydroxytryptamine, serotonin

IR	immunoreactive
PE	phenylephrine
PB	phosphate buffer
S.E.M.	standard error of the mean
TBS	tris buffer with saline

Fig. 1.

Western blot analysis of the 5-HT_{1A} receptor in rat DRN tissue using the 5-HT_{1A} receptor antibody. The antibody correctly labels the native, unmodified 5-HT_{1A} receptor protein with a band at 46 kDa. Two other bands at 35 and 33 kDa likely reflect the breakdown products of the glycosylated form of the receptor, estimated to range from 63 to 70 kDa (see Zhou et al., 1999).

Fig. 2. Immunohistochemistry of a 5-HT-containing (A) and non-5-HT-containing (B) cell in the rat DRN. Fluorescent photomicrograph depicting 5-HT-containing neurons (green) and neurobiotin-filled cell (red). In (A), the cell is double-labeled for both 5-HT and neurobiotin, thus it appears yellow. The neurobiotin-IR cell in (A) is located in the interfascicular subdivision of the DRN (MLF=medial longitudinal fasciculus). The neurobiotin-IR cell in (B) is located in the ventromedial subdivision of the DRN. Scale bar=20 μ m in both panels. Images were adjusted for optimal color balance and contrast using Adobe Photoshop, version 6.0.

Fig. 3. Current-voltage responses and time constant (τ) of a 5-HT-containing (A) and a non-5-HT-containing (B) cell in the rat DRN. The lower panels illustrate the membrane potential responses to current steps (580 ms) from -200 pA to 150 pA increasing in increments of 25 pA. The initial portion (indicated by arrows) of the membrane potential response elicited by a -25 pA current pulse (indicated by asterisk) is amplified in the upper panels. A single exponential function is fit to the data in the upper panel and the τ is defined as the time required to reach 63% of the maximum amplitude (ms).

Fig. 4. AHP tau in a 5-HT-containing (A) and a non-5-HT-containing (B) cell in the rat DRN. The AHP tau cell characteristic describes the initial activation phase of the AHP. The upper panels illustrate a single evoked action potential. The outlined portion of the action potential in the upper panels is magnified in the lower panels. A single exponential function is fit to the initial phase of the AHP (from the start of the AHP to its peak, indicated by crosses on lower panels). The AHP tau is defined as the time required to reach 63% of the maximum AHP amplitude (ms).

Fig. 5. Distribution of AHP tau data in 5-HT-containing ($N=17$) and non-5-HT-containing ($N=19$) cells in the rat DRN. The AHP tau cell characteristic describes the initial phase of the AHP and is defined as the time required to reach 63% of the maximum AHP amplitude (ms). (A) illustrates the distribution of AHP tau data from individual subjects in the two immunohistochemically-distinguished groups. (B) is a frequency histogram of the AHP tau characteristic in the two immunohistochemically-distinguished groups.

Fig. 6.

Similar membrane hyperpolarization response to stimulation of the 5-HT_{1A} receptor in a 5-HT-IR+ (A) and 5-HT-IR- (B) DRN cell. Chart records indicate the effect of the 5-HT_{1A} receptor agonist 5-CT (100 nM) on the membrane potential of two immunohistochemically distinct DRN cells. The length of the line above the chart record indicates the length of time that the drug was added to the perfusion medium. Downward deflections in the chart record indicate changes in membrane potential in response to injection of a current pulse (A: 30 pA, 20 s; B: 20 pA, 20 s) through the recording electrode to monitor changes in membrane resistance. The resting membrane potential is -68 mV in the 5-HT-IR+ cell and -66 mV in the 5-HT-IR- cell.

Fig. 7.

Fluorescent photomicrographs of 5-HT-containing and 5-HT_{1A} receptor expressing cells in 30-μm thick coronal sections through the rat DRN. The upper panels (A, D) show 5-HT-immunoreactivity which appears green. The middle panels (B, E) show 5-HT_{1A} receptor immunoreactivity, which appears red. The bottom panels (C, F) show both 5-HT and 5-HT_{1A} receptor immunoreactivity such that double-labeled cells appear yellow. The left panels illustrate the distribution of these immunoreactive cells in the ventromedial/interfascicular DRN at low magnification (scale bar=100 μm; MLF=medial longitudinal fasciculus). The right panels are higher magnification photomicrographs of the interfascicular region of the DRN from the left panels (scale bar=20 μm). (F) illustrates cells single-labeled for 5-HT_{1A} receptor-IR (denoted by asterisks). The remaining cells are double-labeled for 5-HT-IR and 5-HT_{1A} receptor-IR. Images were adjusted for optimal color balance and contrast using Adobe Photoshop, version 6.0.

Fig. 8. Quantification of 5-HT-containing and 5-HT_{1A} receptor expressing cells throughout the rostro-caudal extent of the DRN. The number of cells in a particular immunohistochemically-distinct group is represented as a percent of total cells counted in a specific subdivision of the DRN (A: dorsal, B: lateral wings, C: ventral/interfascicular). Cells from one subject were quantified throughout the rostro-caudal extent of the DRN represented by stereotaxic coordinates (in mm) relative to the Bregma coordinate on the rat skull surface (Paxinos and Watson, 1998). 5-HT-IR cells are represented by black bars, 5-HT_{1A} receptor-IR cells are represented by white bars, and cells double-labeled for both 5-HT-IR and 5-HT_{1A} receptor-IR are represented by gray bars.

Fig. 9.

Distribution of 5-HT_{1A} receptor-IR labeling in the septum (A1 and A2), supraoptic nucleus (B1 and B2) and medial habenula (C). (A) illustrates the distribution of 5-HT_{1A} receptor-IR labeling in the septum at low (A1; scale bar=300 μ m; LV=lateral ventricle, LS=lateral septum, MS=medial septum) and high magnification (A2; scale bar=50 μ m). (B) illustrates the distribution of 5-HT_{1A} receptor-IR labeling in the supraoptic nucleus at low (B1; scale bar=100 μ m; ox=optic chiasm) and high magnification (B2; scale bar=50 μ m). (C) illustrates the distribution of 5-HT_{1A} receptor-IR labeling in the medial habenula (scale bar=50 μ m; 3V=third ventricle). Arrows in panels A1, A2, B1, and B2 indicate labeled cells in corresponding high

and low magnification photomicrographs. Images were adjusted for optimal brightness and contrast using Adobe Photoshop, version 6.0.

Table 1

Electrophysiological properties of 5-HT-containing and non-5-HT-containing cells in the rat DRN

	5-HT-IR+	5-HT-IR-
Resting membrane potential (mV)	-67.8±1.4 (17)	-65.2±0.7 (20)
Input resistance (MΩ)	241.5±29.2 (15)	325.8±35.7 (20)
Tau (ms)	21.4±4.4 (16)	33.5±4.2[#] (20)
Spike threshold (mV)	-54.9±0.5 (17)	-54.6±0.3 (20)
Spike amplitude (mV)	61.4±2.2 (17)	65.0±2.3 (20)
Spike duration (ms)	2.0±0.1 (15)	1.8±0.1 (17)
AHP amplitude (mV)	15.9±0.9 (17)	16.6±1.4 (19)
AHP duration ($t^{1/2}$; ms)	177.1±21.1 (17)	137.5±8.6 (19)
AHP tau (ms)	7.3±1.9 (17)	1.8±0.6[#] (19)
Firing rate of spontaneously active cells (Hz)	0.8±0.3 (3)	2.9±0.5[*] (5)

Values indicate mean±S.E.M. The group size is indicated within parentheses. Asterisks/pound signs indicate a significant difference between groups by unpaired Student's *t*-test (^{*}) or Mann–Whitney Rank Sum Test ([#]) (*P*<0.05). These distinguishing characteristics are further highlighted by bold print.

Table 2

Pharmacological responses of 5-HT-containing and non-5-HT-containing cells in the rat DRN

	5-HT-IR+	5-HT-IR-
Silent cells		
5-CT elicited hyperpolarization (mV)	8.1±1.6 (14)	8.7±1.8 (15)
PE elicited depolarization (mV)	9.0±2.1 (5)	9.3±1.9 (7)
PE elicited firing rate (Hz)	0.4±0.2 (5)	0.2±0.1 (7)
5-CT elicited hyperpolarization of PE-stimulated cells (mV)	11.7±3.8 (3)	8.4±2.0 (5)
5-CT effect on PE-stimulated firing rate: % change	all cells silenced: -100%±0.0 (3)	all cells silenced: -100%±0.0 (3)
Spontaneously active cells		
5-CT elicited hyperpolarization (mV)	11.0±1.4 (3)	1.4±1.1** (5)
5-CT effect on firing rate: % change	all cells silenced: -100%±0.0 (3)	no cells silenced: -36.0±17.5* (5)

Values indicate mean±S.E.M. The group size is indicated within parentheses. Asterisks indicate a significant difference between groups by unpaired Student's *t*-test ($P<0.05^*$; $P<0.001^{**}$). These distinguishing characteristics are further highlighted by bold print.