Physiological Identification of Pontomedullary Serotonergic Neurons in the Rat

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Mason, Peggy. Physiological identification of pontomedullary serotonergic neurons in the rat. J. Neurophysiol. 77: 1087–1098, 1997. Spinal serotonin is derived entirely from bulbar sources and plays an important role in spinal modulatory processes, including pain modulation. Establishing the electrophysiological properties of serotonergic bulbo spinal neurons in the pontomedullary raphe and reticular formation is critical to understanding the physiological role of serotonin in the spinal cord. Neurons were characterized by their responses to noxious stimulation and their background discharge pattern in the lightly anesthetized rat. Characterized cells were intracellularly labeled with Neurobiotin, which was visualized with a Texas Red fluorophore. Sections containing the labeled cells were processed for serotonin immunocytochemistry with the use of a Bodipy fluorophore. Forty-seven intracellularly labeled cells were tested for serotonin immunoreactivity. The labeled neurons were located in raphe magnus, the nucleus reticularis magnocellularis, and the adjacent reticular and raphe nuclei at levels from the inferior olivary complex to the superior olivary complex. Serotonergic cells were located in the raphe nuclei, in nucleus reticularis magnocellularis pars alpha, and in nucleus reticularis paragigantocellularis lateralis, but not in nucleus reticularis magnocellularis pars beta or nucleus reticularis gigantocellularis. Thirteen intracellularly labeled cells contained serotonin immunoreactivity. The background discharge rate of serotonergic cells averaged 1.8 Hz (range: 0.5–3.1 Hz). Discharge was steady and without sustained pauses or bursts in firing. Most serotonin-immunoreactive cells were affected or slightly excited by pinch and were unaffected by noxious heat. Three serotonergic cells were weakly excited by both noxious pinch and heat, whereas two serotonergic cells were briefly inhibited by these stimuli. Cells that lacked serotonin immunoreactivity were heterogeneous and included ON, OFF, and neutral cells. Nonserotonergic cells differed from serotonergic cells in having an irregular discharge pattern and/or a high mean discharge rate. A linear discriminant function, employing background discharge characteristics as independent variables, was calculated that successfully classified 13 of 13 serotonergic and 32 of 33 nonserotonergic neurons. The probability of misclassification with the use of this discriminant function was estimated to be <10%. Employing the discriminant function on a test group of cells whose immunohistochemical content was unknown revealed a population of serotonergic-like cells that resembled the labeled serotonergic cells in background discharge pattern, response to noxious stimulation, and nuclear location. The discharge of pontomedullary serotonergic neurons is slow and steady, suggesting that these neurons may have a role in the tonic, rather than phasic, modulation of spinal processes.

INTRODUCTION

Serotonin within the spinal cord arises from cells in the caudal brain stem, largely from neurons in the pontomedullary raphe and reticular nuclei (Dahlström and Fuxe 1964; Fuxe 1965; Jacobs and Azmitia 1992). Serotonin-containing axonal terminations in the dorsal horn are concentrated in the superficial laminae, where nociceptors terminate, and arise primarily from neurons in the pontomedullary raphe magnus (RM) and adjacent nucleus reticularis magnocellularis (NRMC) (Jones et al. 1991; Kwiat and Basbaum 1992; Oliveras et al. 1977). Although a large body of evidence suggests that serotonergic neurons in the RM and NRMC are important for descending pain modulation (LeBars 1988; Potrebic et al. 1995; Sawynok 1989), little is known about the physiological characteristics of these cells. The lack of an established method for distinguishing pontomedullary serotonergic neurons from their nonserotonergic neighbors solely on the basis of electrophysiological properties is a major obstacle to understanding the functional role of these cells. The present study was designed to establish electrophysiological criteria that identify pontomedullary serotonergic cells.

Early extracellular studies revealed that most cells located in pontomesencephalic noradrenergic or serotonergic nuclei discharge with a slow and regular pattern, prompting many to speculate that a slow, regular discharge is a marker for monoaminergic neurons (Aghajanian et al. 1968; Aston-Jones and Bloom 1981; Heym et al. 1982b; Moore and Bloom 1979). In a landmark study, Aghajanian and Vandermaelen (1982) demonstrated, in the anesthetized rat, that all intracellularly labeled “slowly, rhythmically firing” midbrain dorsal raphe (DR) and locus coerulescens neurons contained serotonin and norepinephrine histochemistry, respectively. Aghajanian’s study provided convincing evidence that all slow, rhythmic cells in DR and locus coerulescens are monoaminergic. It did not, however, address whether some cells that are not slow and regular are also monoaminergic. To determine whether the discharge of monoaminergic cells is uniformly slow and rhythmic, it is essential to study all neurons that are encountered, regardless of the similarity of their discharge pattern to that described by Aghajanian and Vandermaelen for DR and locus coerulescens cells.

The “slow and regular” standard for monoaminergic cells has been extended to cells in many monoaminergic nuclei, including serotonergic cells in the pontomedullary raphe (Auerbach et al. 1985; Chiang and Gao 1986; Fornal et al. 1990; McCall and Clement 1989; Wessendorf and Anderson 1983; Wessendorf et al. 1981). However, as discussed by Aghajanian and Vandermaelen, it is unclear whether the discharge of serotonergic and noradrenergic cells, located in less homogeneous monoaminergic nuclei than the RD and the locus coerulescens, conforms to the slow and regular pattern.
This issue is particularly important in the rat RM and NRMC (RM/NRMC), a region where no more than 25% of the cells contain serotonin (Moore 1981; Potrebic et al. 1994). In a study in which physiologically characterized and intracellularly labeled RM neurons were directly tested for serotonin immunoreactivity, serotonergic cells were sensitive to noxious stimulation (Potrebic et al. 1994). However, because other neurons that were insensitive to noxious stimulation did not contain serotonin immunoreactivity (Potrebic et al. 1994), this physiological characteristic is insufficient to identify pontomedullary serotonergic cells. The goals of the present study were 1) to characterize the responses of pontomedullary serotonergic cells to noxious stimulation and 2) to develop an electrophysiological method by which pontomedullary serotonergic neurons could be distinguished from their nonserotonergic neighbors. The results presented demonstrate that pontomedullary serotonergic cells have distinct physiological characteristics and can be distinguished from their nonserotonergic neighbors by their background discharge pattern.

**METHODS**

**Experimental protocol**

Male Sprague Dawley rats (250–500 g; Sasco, Madison, WI, n = 95) were used. Rats were pretreated with atropine sulfate (40 µg in 0.1 ml sc) 10 min before anesthetic induction with halothane. A Y tube was inserted into the trachea and anesthesia was maintained with 2% halothane in oxygen during surgery. A posterior craniotomy was made overlying the cerebellum and the exposed dura was cut. Electrodes were inserted bilaterally into the thorax to record the electrocardiogram and into the paraspinal muscles to record the electromyographic activity during tail withdrawal (see below). Core body temperature was maintained at 36–38°C. After surgical preparation, the halothane concentration was reduced to 1% and the animal was allowed to equilibrate at this concentration for 30 min before recording.

Glass micropipettes (resistance 30–80 MΩ) were filled with a solution of 2% Neurobiotin (Vector Laboratories, Burlingame, CA) in 0.1 M tris(hydroxymethyl)aminomethane buffer (pH 7.4) and 0.15 M KCl. Microelectrodes were lowered through the cerebellum and fourth ventricle to reach the brain stem. The dorsal edge of the RM was encountered 8.7–9.0 mm below the cerebellar surface. At this depth, neurons were isolated by their spontaneous activity. When cells were isolated, their waveforms had an initial positive potential of 2–10 mV and resembled those described by Furshpan and Furakawa (1962) as originating from recording sites juxtaposed to the soma of the Mauthner cell. These extracellular recordings never showed any evidence of injury discharge and were stable for up to 20 min. After background cell discharge was recorded for 300 s, each cell was characterized by its responses to noxious stimulation (see next paragraph). After the cell characterization was performed extracellularly, depolarizing current was used to impale the cell. Successful impalement was marked by a large increase in spike height and a membrane potential of −30 to −40 mV. Neurons were intracellularly labeled with Neurobiotin by injecting constant depolarizing current (0.3–3.0 nA) for 30 s to 15 min.

The cell’s responses to noxious heat of the tail (2–5 trials) and noxious pinch of both hind paws and tail were recorded. The noxious heat stimulus consisted of a step to 56°C generated by a radiant heat source focused on the ventrum of the tail. Tail temperature was not monitored; the thermal stimulator was calibrated to heat to 56°C in separate experiments. Noxious pinch was accomplished by squeezing either a digit, in the case of the hindpaws, or the tail with untoothed forceps. The same pinch was judged to be painful when applied to a fold of the investigator’s skin.

Animals were killed with a pentobarbital overdose and perfused with a fixative containing 4% paraformaldehyde and 7% sucrose in 0.1 M phosphate-buffered saline. The brain stem was removed, postfixed for 30–60 min in fixative, and cryoprotected in 30% sucrose in phosphate-buffered saline. Coronal serial 40- to 50-µm sections were cut on a freezing microtome.

**Histological processing**

Sections were incubated in 0.5% Triton X-100 in phosphate-buffered saline for 30 min at room temperature and then transferred to 0.4% Avidin D conjugated to Texas Red (in 0.5% Triton X-100 in phosphate-buffered saline) (Vector Laboratories, Burlingame, CA) for 4 h at room temperature. Sections containing labeled neurons were identified with the use of a fluorescent microscope and processed for serotonin immunocytochemistry as described previously (Potrebic et al. 1994). Briefly, the primary antibody solution consisted of rabbit anti-5HT antibodies (INCStar, Stillwater, MN) that were preabsorbed with 15–20 µg of acetone-extracted rat liver powder and used at a final dilution of 1:5,000 in buffer. The secondary antibody solution consisted of 10 µg/ml of Bodipy goat anti-rabbit antisera (Molecular Probes, Eugene, OR) and was applied for 6–8 h. Mounted sections were covipped with DPX mountant (BDH Laboratory Supplies, Poole, UK) and stored at −4°C. When the primary antibody was omitted from the staining protocol, the Bodipy conjugated secondary antibody produced no specific staining.

For each section with a labeled cell, a control section, taken from the caudal midbrain, was processed for serotonin immunocytochemistry in the same solutions. The control section was viewed before the sections containing the labeled cells. If the control section was judged to be poorly stained for serotonin, compared with previously established standards, the cell was not considered as part of the histological component of this experiment. On the few occasions when sections were poorly stained (6 cells in 4 rats), this could be explained by procedural errors such as accidentally overfixing the tissue.

**Anatomic analysis**

For intracellularly labeled neurons, somata were examined on a Zeiss Axiophot 50 equipped with ×20 and ×63 Plan-NEOFLUAR objectives. The Texas Red label was viewed with the Zeiss filter set 00 and the Bodipy label with an altered filter set 10. Because the Texas Red label was sometimes visible when viewed through filter set 10, this filter set was modified by replacing the transmission filter with a D540/40 filter (Chroma Technology, Brattleboro, VT) which blocks transmission of wavelengths >540 nm. All cells were examined with the use of the ×63 objective, with a numerical aperture of 1.25.

The locations of labeled cells were reconstructed at low magnification by the use of a computer reconstruction system (Neurolucida, MicroBrightField, Chestertown, VT). The locations of all serotonin-immunoreactive cells visible with a ×20 objective were marked on this reconstruction. The rostrocaudal coordinate of each labeled cell was then determined by comparing the cell’s location with the plates in the atlas of Paxinos and Watson (1986). The other two coordinates were determined by measuring the mediolateral distance to the midline and the dorsoventral distance to the top of the basilar artery.

For each intracellularly labeled cell, the cell group containing its soma was identified. Because the distribution of serotonergic somata is restricted, the recorded sites of all serotonergic cells must be within the confines of nuclei that contain serotonergic cells. Therefore the parcellation of pontomedullary raphe and reticular nuclei described by Newman (1985a,b) was adapted (see also...
Kruger et al. (1995). This parcellation resembles that of Kruger et al. (1995) for the rat and that of Taber et al. (1960) for the cat. Newman’s anatomic system represents a departure from previous work in the field, including work by this author. However, if clearly distinguishes nuclei that contain serotoninergic cells from nuclei that do not contain serotoninergic cells, an important advantage in the present study.

The cytarchitectonic boundaries of Newman are illustrated in Fig. 1C. The most important change in this parcellation from that of Paxinos and Watson (1986) and Jones (1995) is that the nucleus located lateral to the RM is termed NRMC (see Fig. 1, C2–C5) instead of nucleus reticularis paragigantocellularis (Basbaum and Fields 1984) or nucleus reticularis gigantocellularis pars alpha (Paxinos and Watson 1986). As seen in Fig. 1, C2–C5, NRMC consists of two subnuclei, a ventrally located pars alpha (NRMCα) and a dorsally located pars beta (NRMCβ). NRMCβ is distinguishable from NRMCα by its larger, more densely packed cells. Nucleus containing serotonin are found in RM, raphe pallidus (RP), raphe obscurus, NRMCα, nucleus reticularis paragigantocellularis lateralis (NRPGl) (see Fig. 1C, dots), and the arcuate nucleus (see Fig. 1, C1–C4, dots within pyramids) (Bowker et al. 1983; Steinbusch et al. 1981). It is important to note that serotoninergic cells are absent from NRMCβ (see Fig. 1, C2–C5).

Electrophysiological analysis

Unit activity and other physiological measures were continuously acquired onto both a VHS tape recorder (Vetter, Rebersburg, PA) and onto a Macintosh Centris 650 (2 kHz) equipped with a 12-bit A-D converter (NB-MIO-16L), a direct memory access board (NB-DMA 2800), and LabView software (National Instruments, Austin TX). Physiological analysis was performed with LabView software (National Instruments, Austin TX). Statistical analyses were performed with SAS (Cary, NC). Illustrations were created with Adobe Photoshop (Adobe Systems, Mountain View, CA) and Igor (WaveMetrics, Lake Oswego, OR).

The background discharge was recorded extracellularly before intracellular impalement. From this record, the mean interspike interval, its SD, and its coefficient of variation (CV = SD/mean) were calculated. All illustrations show the instantaneous discharge, which is defined as the reciprocal of the interspike interval preceding the occurrence of each action potential. This illustration style obviates the need for arbitrarily binning the data. In graphs of instantaneous rate, a point at 50 Hz reflects an action potential that occurred 20 ms after the preceding action potential: it does not reflect the occurrence of 50 action potentials within a bin. Adjacent points are joined by lines and the graph is filled to the zero line.

Responses to noxious stimulation were analyzed off-line and used to classify nonserotonergic cells as ON, OFF, or NEUTRAL (Fields et al. 1983). Cells that were consistently excited by noxious tail heat, before the tail flick withdrawal, were classified as ON cells. Similarly, OFF cells responded to noxious tail heat with large and consistent decreases in discharge rate. Cells that were neither ON nor OFF were classified as NEUTRAL. NEUTRAL cells were either unaffected or weakly affected by noxious stimulation. The responses of serotoninergic cells to noxious pinch and heat were also analyzed off-line, after the immunohistochemical content of the cells was already known. Although serotoninergic cells resembled NEUTRAL cells in their responses to noxious stimulation (see below), their serotonin content is hypothesized to be more critical to their function than is their response to noxious stimulation (see INTRODUCTION). Therefore all intracellularly labeled cells that contained immunoreactive serotonin are referred to simply as serotoninergic cells.

RESULTS

Intracellular labeling of serotoninergic and nonserotonergic cells

Of 119 cells that were physiologically characterized, 47 were successfully labeled and tested for serotonin immunoreactivity. Of these 47, 13 contained serotonin immunoreactivity (see Fig. 2), whereas 34 did not contain serotonin immunoreactivity (see Fig. 3).

As noted in METHODS, a brain was included in the histological sample only if there was adequate staining of serotoninergic cells in a section taken through the midbrain. As a further control for the immunohistochemical process, the number of serotonin-immunoreactive cells was counted in each pontomedullary section that contained an intracellularly labeled cell. Similar numbers of serotonin-immunoreactive cells were seen in sections containing intracellularly labeled cells that were or were not serotoninergic, evidence that the lack of serotonin immunoreactivity in intracellularly labeled nonserotonergic cells was not simply the result of inadequate immunohistochemical processing.

Nuclear location of labeled cells

The locations of intracellularly labeled cells are shown in Fig. 1, A and B (●). Separate panels are used to depict the locations of serotoninergic (Fig. 1A) and nonserotonergic (Fig. 1B) cells and the nuclear boundaries in the region (Fig. 1C). The locations of serotonin-immunoreactive cells, plotted from one representative section at each rostral-caudal level, are depicted by small dots in Fig. 1, A–C. Labeled serotoninergic cells were located in RM (n = 4), NRMCα (n = 4), RP (n = 2), NRPGl (n = 2), and raphe obscurus (n = 1). Labeled nonserotonergic cells were located within regions where serotoninergic cells are present, including RM (n = 9), NRMCα (n = 2), NRPGl (n = 1), and raphe obscurus (n = 1). For example, the intracellularly labeled nonserotonergic cell illustrated in Fig. 3A1 is in very close proximity to the serotonin-immunoreactive neuron shown in Fig. 3A2.

Nonserotonergic cells were also located in regions lacking serotoninergic cells, such as NRMCβ (n = 13) and nucleus reticularis gigantocellularis (n = 8). Although serotonin-immunoreactive cells were observed in the arcuate nucleus, no serotoninergic or nonserotonergic cell was intracellularly labeled in this area.

Responses to noxious stimulation of serotoninergic and nonserotonergic cells

Previous work suggests that serotoninergic cells do not have inhibitory or excitatory responses to noxious tail heat (Potrebic et al. 1994). In line with that report, most serotonin-immunoreactive cells were either unresponsive to noxious pinch and heat (n = 4) or slightly excited by pinch and unaffected by noxious heat (n = 4) (see Fig. 4, A and C). Figure 4 illustrates the range of serotoninergic cell responses to noxious heat (left) and pinch (right). In this figure, the calculated instantaneous discharge rate (see METHODS) is shown in a graph below a raster plot of the same activity. A serotoninergic cell that was insensitive to both noxious heat and pinch is shown in Fig. 4A. An example of a serotoninergic cell that was insensitive to noxious heat but slightly excited by noxious pinch is shown in Fig. 4C. Three serotoninergic cells were weakly excited by both noxious pinch and heat (see Fig. 4D), whereas two were inhibited by both noxious pinch and heat (see Fig. 4D).

Nonserotonergic cells responded to noxious stimulation...
FIG. 1. Location of labeled cells and serotonin-immunoreactive cells within the pontomedullary raphe and reticular cell groups studied. In all columns, the ventromedial brain stem has been enlarged and the locations of serotonin-immunoreactive cells (dots) are illustrated. A1–A5: locations of all intracellularly labeled serotoninergic cells (●) and serotoninergic-like cells (○) are shown in the left column. Serotoninergic-like cells are cells that have the physiological characteristics of serotoninergic cells (as judged by the discriminant function; see text) but that were not directly tested for serotonin immunoreactivity. In A and B, some serotonin-immunoreactive cells near the labeled cells are covered by black (●) or opaque (○) filling. B1–B5: locations of intracellularly labeled nonserotonergic cells (●) and nonserotonergic-like cells (○) are shown. Nonserotonergic-like cells are cells that have the physiological characteristics of nonserotonergic cells (as judged by the discriminant function; see text) but that were not directly tested for serotonin immunoreactivity. C1–C5: nuclear boundaries of the pontomedullary raphe and reticular cell groups studied are shown with the locations of serotonin-immunoreactive cells (dots) overlaid. The anteroposterior level for each trio of sections is as follows (mm caudal to interaural 0): A1–C1: −3.4 to −2.65; A2–C2: −2.6 to −2.45; A3–C3: −2.4 to −2.2; A4–C4: −2.1 to −1.7; A5–C5: −1.6 to −1.1. ION, inferior olivary nuclei; N. VII, facial nucleus; NRGC, nucleus reticularis gigantocellularis; NRMCa, nucleus reticularis magnocellularis pars alpha; NRMCb, nucleus reticularis magnocellularis pars beta; NRPGi, nucleus reticularis paragigantocellularis lateralis; p, pyramid; RD, raphe dorsalis; RM, raphe magnus; RO, raphe obscurus; RP, raphe pallidus.

in patterns that were consistent with previous descriptions of ON, OFF, or NEUTRAL cells (Fields et al. 1983; Leung and Mason 1995). The 34 intracellularly labeled cells that did not contain serotonin immunoreactivity included 4 ON, 6 OFF, and 24 NEUTRAL cells. To determine whether SEROTONERGIC cells physiologically resemble ON and OFF cells, the
noxious heat-evoked responses of SEROTONERGIC cells were compared with those characteristic of nonserotonergic ON and OFF cells (see Fig. 5). As stated above, noxious heat did not evoke a response in most SEROTONERGIC neurons (n = 8) (Fig. 5, A and B). Among SEROTONERGIC cells that responded to noxious heat (n = 5), responses were typically small and transient. The typical inhibitory response of a SEROTONERGIC cell (Fig. 5C) is much weaker and shorter in duration that that of a typical OFF cell (Fig. 5E). Similarly, the typical excitatory response of a SEROTONERGIC cell (Fig. 5D) is also weaker and shorter in duration that that of a typical ON cell (Fig. 5E).

In the SEROTONERGIC cell with the largest excitatory response to heat (see Fig. 4B), the mean discharge rate increased from 3 to 6 Hz. Within 4 s of stimulus termination, this cell’s discharge returned to baseline values. In contrast, in the ON cell illustrated in Fig. 5F, the discharge rate increased from 13 to 26 Hz during the tail heat stimulus and remained elevated for 11 s after stimulus termination. In the SEROTONERGIC cell with the greatest inhibitory response to heat (see Fig. 4D), the cell was briefly inhibited by tail heat but resumed firing during the stimulus application. Noxious tail heat evoked a stronger inhibition in a typical OFF cell (see Fig. 5E) that outlasted the stimulus. This OFF cell did not resume discharging for ≥ 3–4 s after tail heat termination and firing remained below baseline rates for another 10–15 s after stimulus termination.

As typified by the examples above, the responses of nonserotonergic ON and OFF cells to noxious stimulation are stronger and more sustained than those of SEROTONERGIC cells. Thus, judging from their responses to noxious stimulation alone, all SEROTONERGIC cells would be classified as NEUTRAL cells because they did not respond at all or responded only weakly to noxious tail heat. However, in the present paper, all intracellularly labeled and serotonin-immunoreactive cells are grouped separately from nonserotonergic NEUTRAL cells, and are called SEROTONERGIC cells (see METHODS).

Discharge pattern of SEROTONERGIC cells

Previous studies of SEROTONERGIC and other monoaminergic cells have emphasized these cells’ slow and regular discharge patterns (see INTRODUCTION). To test whether the discharge patterns of SEROTONERGIC and nonserotonergic cells were different, 5 min of background discharge was recorded and analyzed. SEROTONERGIC cells discharged slowly and without prolonged bursts or pauses in firing (see Fig. 6A), a pattern that resembles that previously described for SEROTONERGIC RD cells.

The mean discharge rate for the 13 SEROTONERGIC cells was 1.8 Hz, with a range of 0.5–3.1 Hz (see Table 1, Figs. 6A and 7A). Although SEROTONERGIC cells did not alternate between inactivity and bursts of activity, they were not “clocklike” either (see Fig. 6A). To measure regularity, the SD of the interspike intervals and the CV$_{ISI}$ were calculated for each cell. The CV$_{ISI}$ for SEROTONERGIC cells ranged from 0.23 to 0.74 and averaged 0.40 (see Table 1, Fig. 7B).

To determine whether there was a systematic relationship between physiological properties and anatomic location, measures of rate (mean interspike interval) and regularity (CV$_{ISI}$) for each cell were plotted against every coordinate value of...
somantic location (rostrocaudal, mediolateral, and dorsoventral; see METHODS). A least-squares linear regression analysis revealed that no significant correlations existed between background discharge properties and cellular location.

Discharge characteristics of nonserotonergic cells

The background discharge of nonserotonergic cells was also recorded and analyzed quantitatively. ON and OFF cells had low discharge rates averaging 5.8 and 4.5 Hz, respectively (see Table 1). The discharge of both ON and OFF cells contained pauses and bursts in activity that measured tens of seconds to minutes in duration. This discharge irregularity was evident in both groups having a mean CV_{ISI} of >1 (see Table 1). As illustrated in Fig. 6B, the intraburst discharge of both ON and OFF cells was also irregular. Thus ON and OFF cells can be distinguished from SEROTONERGIC cells by the irregularity of their discharge.

The background discharge of NEUTRAL cells was heterogeneous with 9 cells having a regular discharge pattern and 15 NEUTRAL cells having an irregular discharge pattern (Leung and Mason 1995). Regularly discharging NEUTRAL cells discharged at a mean rate of 20.5 Hz and had a mean CV_{ISI} of 0.24 (see Table 1). A typical example of such a cell is illustrated in Fig. 6B. Because the regularly discharging NEUTRAL cell with the lowest rate of discharge fired at a rate of 10.0 Hz, these cells were easily distinguished from SEROTONERGIC cells by their relatively high discharge rate. Irregularly discharging NEUTRAL cells (n = 15) fired at a mean rate of 7.6 Hz and had a mean CV_{ISI} of greater than unity (see Table 1). These NEUTRAL cells could be distinguished from SEROTONERGIC cells by their irregular discharge pattern and in some cases by their high mean discharge rate (see Figs. 6B and 7A).

Both the mean interspike interval and the CV_{ISI} of SEROTONERGIC cells overlapped with those of nonserotonergic cells. However, when the mean interval and CV_{ISI} are plotted on a two-dimensional graph (see Fig. 7B), there is no overlap between SEROTONERGIC and nonserotonergic cells. A two-way analysis of variance showed that the mean interspike interval and CV_{ISI} for SEROTONERGIC and nonserotonergic cells were significantly different [F(2,43) = 7.885, P = 0.001]. Thus nonserotonergic cells discharged more rapidly and/or more irregularly than SEROTONERGIC cells (see below).

Discriminant analysis of SEROTONERGIC and nonserotonergic discharge patterns

The nonoverlapping distributions of background discharge properties for known SEROTONERGIC and nonserotonergic
cells suggested that a function could be described that would separate the two cell groups. The method used for the development of this function was a linear discriminant analysis (Morrison 1990). This analysis was performed on the discharge data from 13 SEROTONERGIC and 33 nonserotonergic cells. This data set, which was used to develop the discriminant function, is termed the "training" data set. The discharge pattern of one nonserotonergic cell was not recorded on tape before impalement and was therefore not included in this analysis.

As independent variables, the mean interspike interval ($\bar{x}$, in ms) and the SD of the intervals ($s$, in ms) were chosen. The SD was used in place of the CV of ISI because its use led to a better discrimination. The discriminant analysis characterizes each unit by the linear sum, $y_i = b_1 \bar{x}_i + b_2 s_i$. The mean values of $y_i$ for the SEROTONERGIC and nonserotonergic cells are called $y_p$ and $y_n$, respectively. The coefficients, $b_1$ and $b_2$, are then chosen to maximize the difference between the values of $y_p$ and $y_n$. The constant, $b_0$, is set to $(y_p + y_n)/2$. For the training data set containing 46 known SEROTONERGIC and nonserotonergic cells, the resulting discriminant function is

$$y(x, s) = 146 - \bar{x} + 0.98s$$ \hspace{1cm} (1)

The straight line representing $y(x, s) = 0$ defines the optimal linear boundary between the two groups and is illustrated in Fig. 7A. When plotted in log-log coordinates, a curved line results. If the value of this function is $<0$ [$y(x, s) < 0$, points below the line in Fig. 7A], the cell is likely to be SEROTONERGIC, and if the value of the function is $>0$ [$y(x, s) > 0$, points above the line in Fig. 7A], the cell is likely to be nonserotonergic. The discriminant function defines two conditions that must be met for a cell to be classified as SEROTONERGIC: 1) because $s$ must be $>0$, then $x > 146 \text{ ms}$ or, equivalently, the discharge rate must be $<7 \text{ Hz}$; and 2) the discharge must be sufficiently regular such that $x < x$ by $\approx 146 \text{ ms}$.

All 13 SEROTONERGIC neurons fell below the line (all • are below the function line), whereas 32 of 33 nonserotonergic cells are located above the line. One nonserotonergic
FIG. 6. Background discharge of SEROTONERGIC and nonserotonergic cells. Each trace is 1 min in length and is taken from a 5-min recording, from which the mean discharge rate and the coefficient of variation printed above each trace was calculated. A: discharge of 4 SEROTONERGIC cells. B: discharge of 4 nonserotonergic cells.

cell was misclassified as a SEROTONERGIC cell (see ○ below the line in Fig. 7A). This cell had a mean interspike interval of 5,640 ms (<0.2 Hz) and an SD of 4,650 ms, suggesting that the discriminant function may not be appropriate for cells with extremely low discharge rates. The value of the function for several other cells was very close to 0. However, for each of these cells the value of the function (and the center of the symbol in Fig. 7) was consistent with the serotonin immunoreactivity of the cell. The discharge rate and regularity for nonserotonergic ON (△), OFF (▽), and NEUTRAL (○) cells overlapped (see Fig. 7A).

Only 1 of 46 cells independently tested for serotonin immunoreactivity was misclassified, suggesting a 2% probability of misclassification when using the training data set. To employ this discriminant function on cells that will be recorded in the future, it is important to estimate the probability of misclassification for cells whose serotonin content is not known. One would expect that the misclassification rate would increase because future cells are not part of the training data set that was used to calculate the discriminant function. To estimate this error rate, a cross validation procedure was performed (Lachenbruch and Mickey 1968). In this procedure, 1 of the 46 identified cells was removed from the sample and a new discriminant function was calculated from the remaining 45 cells. The new discriminant function was then used to classify the removed cell. This process was repeated for each cell in the sample. When this procedure was performed on the present data set, 2 of 33 (6.1%) nonserotonergic cells were misclassified as SEROTONERGIC cells and 1 of 13 (7.7%) SEROTONERGIC cells was misclassified as nonserotonergic cells. This result suggests a misclassification rate of 5–10%.

Application of the discriminant function to an unknown sample

One method for assessing the discriminant function’s validity is to apply the function to a “test” sample and compare the resulting cell classes with those in the original, training sample. Therefore the discriminant function was used to classify 72 cells that were subjected to the same physiological protocol as above but were not immunocytochemically tested for serotonin because they were not intracellularly labeled (n = 43), because the immunocytochemistry was inadequate (n = 6), or because they were processed with diaminobenzidine and their serotonin content was not determined (n = 23). These cells with unknown immunocytochemical content constitute a sample of test cells. The discriminant scores

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>N</th>
<th>Mean Discharge Rate</th>
<th>Range of Discharge Rate</th>
<th>Mean CV ISI</th>
<th>Range of CV ISI</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEROTONERGIC</td>
<td>13</td>
<td>1.8 ± 0.2</td>
<td>0.5–3.1 Hz</td>
<td>0.40 ± 0.04</td>
<td>0.23–0.74</td>
</tr>
<tr>
<td>ON</td>
<td>4</td>
<td>5.8 ± 2.4</td>
<td>0.6–12.1</td>
<td>1.08 ± 0.25</td>
<td>0.43–1.65</td>
</tr>
<tr>
<td>OFF</td>
<td>6</td>
<td>4.5 ± 2.5</td>
<td>0.2–13.9</td>
<td>1.35 ± 0.30</td>
<td>0.76–2.63</td>
</tr>
<tr>
<td>NEUTRAL-regular</td>
<td>9</td>
<td>20.5 ± 3.7</td>
<td>10.0–45.5</td>
<td>0.24 ± 0.04</td>
<td>0.12–0.52</td>
</tr>
<tr>
<td>NEUTRAL-irregular</td>
<td>14</td>
<td>7.6 ± 1.7</td>
<td>0.2–22.2</td>
<td>1.05 ± 0.18</td>
<td>0.36–2.22</td>
</tr>
<tr>
<td>All nonserotonergic</td>
<td>33*</td>
<td>10.3 ± 1.8</td>
<td>0.2–45.5</td>
<td>0.88 ± 0.12</td>
<td>0.12–2.63</td>
</tr>
</tbody>
</table>

Values are means ± SE. * The data from one nonserotonergic NEUTRAL (irregular type) cell is not included here because its background discharge was not recorded on tape.
were calculated, from Eq. 1, for the cells in the test sample. To determine the accuracy of the classification procedure, the responses to noxious stimulation and nuclear locations of test cells with a negative value were then compared with those of known serotoninergic neurons. Similarly, test cells with a positive value were compared with known nonserotonergic neurons.

Of the 72 test cells, 18 had negative scores and were classified as serotoninergic-like neurons. The scores for all other test cells were positive and these cells are referred to as nonserotonergic-like neurons. The proportion of serotoninergic-like neurons in the test sample (18 of 72, 25%) was similar to the proportion of serotoninergic neurons in the training sample (13 of 47, 28%).

The test sample of serotoninergic-like and nonserotonergic-like units resembled their training counterparts in their
responses to noxious stimulation. As was the case for positively identified serotonergic cells, most serotonergic-like cells did not respond to either pinch or heat (n = 9) or were slightly excited by both pinch and heat (n = 5). The remaining serotonergic-like units were slightly excited by pinch but unaffected by heat (n = 2) or were inhibited by both pinch and heat (n = 2). Nonserotonergic-like cells included 7 ON, 11 OFF, and 36 neutral cells; these three cell classes were present in similar proportions in the training and test samples. As with cells in the training sample, the responses of nonserotonergic-like ON and OFF cells were positive value. Because units located outside of the circumventricular organs have been misidentified as nonserotonergic in previous studies, it is therefore possible that serotonergic neurons in the pontomedullary raphe and reticular nuclei located in RM do not contain serotonin-immunoreactive cells. Eighteen nonserotonergic-like units were labeled. These cells were located in RM (n = 8), NMRCβ (n = 5), NMRCα (n = 2), nucleus reticularis gigantocellularis (n = 2), and NRPG1 (n = 1) (see Fig. 1A, C).

As dictated by the discriminant analysis, the background discharge patterns of serotonergic-like units resembled those of serotonergic cells. Figure 7B shows the distribution of X and CV SI for both the training and test cells. The line in Fig. 7B represents the line given by y = (146/X) − 1 + 0.98 CV SI, which is the discriminant function (Eq. 1) in terms of X and CV SI. The mean interval and CV SI of the serotonergic-like cells overlapped with those of serotonergic cells (see Fig. 7B, A and B). In addition, the mean interval and CV SI of nonserotonergic-like neurons overlapped those of nonserotonergic cells (see Fig. 7B, open symbols with and without center dot), evidence that the intracellularly labeled and immunocytochemically tested sample contains cell types that are representative of the pontomedullary population.

Limitations of the current study

There are three limitations to the present study. First, it is possible that some nonserotonergic cells contained low concentrations of serotonin that were below the detection threshold of the immunocytochemical procedure. Second, no cells, serotonergic or not, located within the pyramids (the arcuate nucleus) were successfully labeled. It is therefore unclear whether the arcuate serotonergic cells have physiological characteristics similar to those of serotonergic cells located in other pontomedullary raphe and reticular nuclei.

The most serious limitation to the present study is the limited context, i.e., the halothane-anesthetized rat, for which the present findings provide direct evidence. Future studies are needed to compare the discharge pattern of serotonergic-like cells in anesthetized and unanesthetized conditions. Despite this need, there is some evidence that serotonergic cells discharge with a similar pattern when recorded under a variety of anesthetics as well as in the absence of anesthesia. Serotonergic cells recorded in the halothane-anesthetized rat in the present study resemble pontomedullary raphe cells in awake cat (Auerbach et al. 1985; Fornal et al. 1985; Heym et al. 1982a; Jacobs and Azmitia 1992) and in isoflurane-anesthetized rats (P. Mason and C. G. Leung, unpublished observations). Furthermore, cells with a slow and regular discharge in the awake cat RD, a sample that likely included
at least some serotoninergic cells, did not alter their dis-charge by >20% after administration of a sedative bolus of
the anesthetic chloral hydrate, evidence that the physiologi-cal characteristics of candidate serotoninergic cells in the
anesthetized state resemble those during the unanesthetized
waking state (Heym et al. 1984).

**SEROTONERGIC neurons are not ON or OFF cells**

Although the classification of ON and OFF cells is adm-ittedly crude, the responses of serotoninergic cells to noxious
pinch and/or heat appear different from those of ON and OFF
cells and are similar to those of neutral cells. First, the
discharge of most serotoninergic neurons was unaffected by
noxious tail heat. Second, the serotoninergic cells that did
respond to noxious tail heat had weak responses that did not
outlast the stimulus. The present results are consistent with
the previous findings that RM/NRMC ON and OFF cells are
nonserotonergic (Potrebic et al. 1994) and that physiologi-cally presumed serotoninergic cells are weakly sensitive or
insensitive to noxious stimulation (Auerbach et al. 1985;
Chiang and Gao 1986; Wessendorf and Anderson 1983).

**SEROTONERGIC neurons are unlikely to be necessary for
RM antinociception**

Several lines of evidence, accumulated over the last 25
years, have led to the hypothesis that serotoninergic bulbo-
spinal neurons are important mediators of RM-mediated in-hibition of spinal nociceptive transmission (LeBars 1988;
Potrebic et al. 1994; Sawynok and Reid 1987). Because OFF
cells are the hypothesized pain inhibitory cells of the RM,
there was an expectation that OFF cells would contain sero-tonin (Fields et al. 1991). However, the present study confirms
a previous report that OFF cells do not contain serotonin
(Potrebic et al. 1994). In support of the idea that serotonin-
ergic cells do not mediate the descending inhibition of noc-ci-ceptive transmission, the optimal RM stimulation parameters
for evoking serotonin release differ significantly from those
for eliciting antinociception. This was most clearly demon-
strated by Sorkin et al. (1993), who showed that high-fre-
quency stimulation of RM at intensities that suppress noci-
ceptive neuronal responses does not evoke serotonin release.
In contrast, stimulation of the RM at slower rates evokes
serotonin release in the dorsal horn but does not inhibit
spinal nociceptive transmission (Bowker and Abhold 1990;
Hammond et al. 1985; Sorkin et al. 1993).

**Functional implications**

Phasic pain modulation, such as that which occurs after
noxious stimulation, has been the focus of intensive investi-gation (Basbaum and Fields 1984). Serotoninergic cells are
unlikely to participate in such phasic modulation because
they are not strongly inhibited or excited by noxious stimula-tion. The phasic responses of nonserotonergic ON and OFF
cells are better suited to participation in the pain modulation
that follows noxious stimulation. In support of this idea,
brief noxious stimulation evokes an inhibition of OFF cells,
an excitation of ON cells, and an increase in subsequent
nociceptive sensitivity (Ramirez and Vanegas 1989).

Serotoninergic cells that discharge slowly and steadily are
likely to serve a tonic function. All spinal functions, includ-ing
somatosensory (both nociceptive and nonnociceptive),
autonomic, and motor processes, are modulated in ac-cordance with behavioral state (Kleitman 1963). Given that
serotonin has a net inhibitory effect on pain transmission
(Belcher et al. 1978; Duggan 1992; Yaksh 1985; Yaksh and
Wilson 1979) and that at least some serotoninergic cells are
tonically more active during waking than sleeping (see above),
then one can hypothesize that nociceptive sensitivity will be
greater during sleeping than during waking. Indeed, rats withdraw from noxious paw heat more rapidly during slow-wave sleep than during waking (Escobedo and Mason 1995). In further support of a waking “analgesia” mediated by monoamines, systemic methysergide, a serotonin antago-nist, causes dorsal horn neurons that only responded to in-noxious stimulation to become responsive to noxious pinch
and heat in awake cats (Saito et al. 1990). Future experi-ments are required to determine whether the state-dependent
discharge of serotoninergic cells is sufficient to explain the
state-dependent changes in nociceptive sensitivity.

**Conclusions**

In the anesthetized preparation, serotoninergic cells dis-
charge slowly and steadily and are not strongly affected by
noxious stimulation. The slow tonic discharge of pontomed-ullary serotoninergic cells resembles that of serotonin-
ergic cells in pontomesencephalic nuclei as well as that reported
for other monoaminergic cells (Jacobs and Azmitia 1992;
Monti 1993; Moore and Bloom 1979). The finding that se-rotonergic cells have a tonic discharge pattern has impor-tant implications for the functional role of these cells. Like other monoaminergic cells, serotoninergic cells are
likely to function in the modulation of spinal processes dur-
ing tonic behavioral or social states.

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