SEROTONERGIC Pontomedullary Neurons Are Not Activated by Antinociceptive Stimulation in the Periaqueductal Gray

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The antinociceptive and cardiovascular effects of midbrain periaqueductal gray (PAG) stimulation are mediated through a relay in the pontomedullary raphe magnus (RM) and adjacent nucleus reticularis magnocellularis (NRMC). To test whether the neurons important in mediating PAG-evoked effects are SEROTONERGIC, the responses of pontomedullary SEROTONERGIC-LIKE cells to PAG stimulation were tested. SEROTONERGIC-LIKE neurons (n = 21) were recorded extracellularly in halothane-anesthetized Sprague Dawley rats. Serotonergic-like neurons were distinguished by their slow and steady background discharge. Two neurons that were physiologically characterized as SEROTONERGIC-LIKE were intracellularly labeled and processed for serotonin immunoreactivity; both cells tested contained immunoreactive serotonin. Train stimulation of sites within the midbrain PAG, at intensities of ≤50 μA, suppressed the tail withdrawal from noxious heat and evoked changes in blood pressure and heart rate. No SEROTONERGIC-LIKE cells were activated by single-pulse or short-train (two to five pulses) stimulation of the PAG at antinociceptive intensities. In most cases, SEROTONERGIC-LIKE cells were unaffected by long-train stimulation (5–6 sec) of the PAG, which produced antinociception and cardiovascular changes. In contrast, >50% of the cells in two nonserotonergic-like cell classes were activated at short latency by such PAG stimulation. In conclusion, monosynaptic excitation of SEROTONERGIC cells in RM/NRMC is unlikely to be necessary for the nociceptive and autonomic modulatory effects of PAG stimulation.

Key words: raphe magnus; serotonin; monoamine; pain modulation; autonomic modulation; antinociception
also contains bulbospinal serotonergic cells (Mason and Fields, 1989; Schenberg and Lovick, 1995).

If serotonergic cells mediate the nociceptive and/or cardiovascular modulatory effects of PAG stimulation, then PAG stimulation would be expected to activate RM and NRMC serotonergic cells. Although previous work has demonstrated that long-train (10 sec) stimulation of the PAG excites RM/NRMC on and off cells (Vanegas et al., 1984), two types of nonserotonergic cells in the rat (Vanegas et al., 1984; Potrebic et al., 1994; Mason, 1997), the response of RM/NRMC serotonergic cells has not been tested directly. Recently an electrophysiological method for identifying pontomedullary serotonergic cells in the rat has been developed (Mason, 1997), enabling a direct test of the effects of PAG stimulation on RM and NRMC serotonergic-like cells.

MATERIALS AND METHODS

Experimental protocol. Male Sprague Dawley rats (Sasco, Madison, WI, or Harlan, Indianapolis, IN) were used. Rats were pretreated with atropine sulfate (40 μg in 0.1 ml, s.c.) 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 paraspinous muscles to record the electromyographic activity during tail withdrawal. In some animals, an arterial catheter was inserted into the femoral artery to record blood pressure. 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.

Six stainless steel microelectrodes tip-plated with platinum were used to stimulate three regions of the PAG [anterior (A), 0.3–3.3 mm from interaural zero; lateral (L), 0–2.0 mm; ventral (V), 5.0–7.0 mm from cerebral surface] bilaterally. The array of six monopolar stimulating electrodes was inserted at an angle of 30º rostral to the frontal plane. To ensure correct placement of the stimulation electrodes, suppression of the tail flick (TF) reflex evoked by noxious heat was confirmed using train stimulation of the PAG (∼50 μA, 200 μsec pulses, 5–6 sec trains, 300 Hz) for a period 1–2 sec longer than the baseline TF latency (2–4 sec) and beginning ≤1 sec before the tail heat stimulus.

A recording microelectrode was inserted into the region of the RM/NRMC [posterior (P), −1.5 to −2.6 mm; L, 0.0–1.0 mm; V, 9.0–10.5 mm from the cerebellar surface]. Both glass micropipettes and stainless steel electrodes were used for recording. Stainless steel microelectrodes were tip-plated with platinum. Glass micropipettes were filled either with a solution of 0.1 M Tris buffer, pH 7.4, and 0.5 M KCl and broken to a final tip resistance of 0.5–5.0 MΩ or with 2% Neurobiotin in the above solution and used with a tip resistance of 40–70 MΩ. Cells were isolated and discriminated using a slope-triggered two-point discriminator (Bak Electronics, Germantown, MD).

All cells were characterized as serotonergic-like or nonserotonergic-like using a previously described algorithm that makes use of quantitative differences between the two populations of cells in the rate and variability of the interspike intervals recorded during background conditions (Mason, 1997). A cross-validation procedure estimated the probability of misclassification using this discriminant function to be <10%. Therefore, in the present study the background discharge of isolated cells was recorded for 5 min, and the mean and SD of the interspike intervals was calculated from this recording. For each cell, the value of the function:

\[ y(x, s) = 146 - x + 0.98 s \]

was calculated, where \( x \) is the mean interspike interval (in milliseconds), and \( s \) is the SD of the intervals (in milliseconds). Cells were classified as serotonergic-like if the function value was <0 and as nonserotonergic-like if the function value was >0 (Mason, 1997). All cells were further characterized by their responses to noxious tail heat and noxious pinch of the hindpaws and tail, as described previously.
After characterization, cells were tested for their responses to PAG stimulation at all six sites in the electrode array at a current of 50–500 μA with single shocks or short trains (300–500 Hz) of two to six pulses. Selected cells were then tested during long trains (5–6 sec) of PAG stimulation that were effective in suppressing the TF withdrawal or in producing a cardiovascular change. To determine whether a cell responded to tail heat or long-train PAG stimulation or both, the cell discharge rate before, during, and after the stimulus was calculated for 6 sec bins. In the absence of stimulation, the variation in the number of discharges before, during, and after the stimulus was calculated for 6 sec bins. In the absence of stimulation, the variation in the number of discharges before, during, and after the stimulus was calculated for 6 sec bins. In the absence of stimulation, the variation in the number of discharges before, during, and after the stimulus was calculated for 6 sec bins.

RESULTS

The histologically verified sites of PAG stimulation were located bilaterally in the caudal (0.3–0.7 mm rostral to interaural zero), middle (1.7–2.3 mm), and rostral (2.7–3.3 mm) thirds of the PAG. Most stimulation sites in the caudal and middle thirds of the PAG were concentrated in the ventral half of PAG, whereas those of the rostral third of PAG were located throughout the dorsoventral range within PAG (Fig. 1A). In each experiment, train stimulation (4–6 sec, 300 Hz, 200 μsec, ±50 μA) of two to six PAG sites was effective in suppressing the withdrawal evoked by noxious tail heat.

A total of 21 SEROTONERGIC-LIKE cells from 18 animals were recorded and analyzed. Histological sites for the recorded and analyzed cells were found to be in RM and NRMCα, regions that contain serotonin-immunoreactive cells (Fig. 1B). In two cases, physiologically characterized SEROTONERGIC-LIKE cells were intracellularly labeled and tested for serotonin immunoreactivity. In both cases, the SEROTONERGIC-LIKE cells contained serotonin immunoreactivity (Fig. 2). Because there were no differences between the SEROTONERGIC-LIKE and serotonergic cells, the two groups will be discussed together below and referred to as SEROTONERGIC-LIKE.

SEROTONERGIC-LIKE cells had background discharge rates of 0.4–2.9 Hz (mean 1.4 ± 0.2 Hz) (Fig. 3). The mean coefficient of variation of the interspike interval was 0.52 ± 0.03, with a range of 0.18–0.80. SEROTONERGIC-LIKE cells were unaffected (n = 13) or slightly excited (n = 8) by noxious tail heat and unaffected (n = 14) or slightly excited (n = 7) by noxious pinch.

Single- and double-pulse stimulation of the PAG, at intensities of up to 500 μA, did not alter the discharge rate of any SEROTONERGIC-LIKE cell tested (n = 21) (Fig. 4A). Short-train (three to six pulses) stimulation of PAG, at intensities of 50–150 μA, was ineffective in altering the discharge of any SEROTONERGIC-LIKE cell. Short-train stimulation, at a stimulation intensity of 500 μA, of 24 PAG sites was tested on four SEROTONERGIC-LIKE cells. At such currents, two cells were excited by stimulation of only four PAG sites (Fig. 4B–D). The latency from the first PAG shock to peak excitation of these two RM/NRMC SEROTONERGIC-LIKE cells was 18–23 msec. The latency to the recorded response was highly variable, making it unlikely that this represents a monosynaptic connection. Furthermore, the calculated conduction velocities for a monosynaptic connection, assuming a synaptic delay of 0.5 msec, were 18–23 msec. The latency to the recorded response was highly variable, making it unlikely that this represents a monosynaptic connection. Furthermore, the calculated conduction velocities for a monosynaptic connection, assuming a synaptic delay of 0.5 msec, were 18–23 msec. The latency to the recorded response was highly variable, making it unlikely that this represents a monosynaptic connection. Furthermore, the calculated conduction velocities for a monosynaptic connection, assuming a synaptic delay of 0.5 msec, were 18–23 msec.

During PAG-evoked suppression of the TF (50 μA, 300 Hz, 6 sec), the discharge of most SEROTONERGIC-LIKE cells did not change (Fig. 5). In two cases, there were small increases in discharge associated with the PAG train stimulation (Fig. 5F). These increases always had a latency of several seconds. During PAG-evoked changes in blood pressure, the discharge of SEROTONERGIC-LIKE cells did not change (Fig. 5B,D,F).
No SEROTONERGIC-LIKE cells were antidromically activated by PAG stimulation at any site.

Although the PAG stimulation was effective in evoking PAG suppression of the noxious-evoked TF, additional confirmation of the efficacy of PAG stimulation was examined by recording from nonserotonergic-like cells in RM and NRMC, which have previously been reported to respond to PAG stimulation (see introductory remarks). Nonserotonergic-like cells were recorded in the same animals as the SEROTONERGIC-LIKE cells discussed above. The background discharge of nonserotonergic-like cells was faster and/or more irregular than that of SEROTONERGIC-LIKE cells (Fig. 3). Nonserotonergic-like cells were characterized further as ON (n = 15), OFF (n = 10), and NEUTRAL (n = 47) cells, as described previously (Leung and Mason, 1995; Mason, 1997). Single-pulse or short-train (two to six pulses) stimulation, with currents of ≤200 μA, evoked a short latency excitation in 64% of the ON cells, 56% of the OFF cells, and 27% of the NEUTRAL cells. The latency for PAG excitation of nonserotonergic RM/NRMC cells varied from <2 msec to >10 msec. A small proportion of ON (27%) and NEUTRAL (15%) cells were antidromically activated by PAG stimulation at latencies of 0.8–3.5 msec.

DISCUSSION

Summary

The current study provides little evidence for a monosynaptic excitatory connection between PAG and RM/NRMC SEROTONERGIC cells. Short-train stimulation of sites located throughout the rostrocaudal extent of the midbrain PAG, at intensities that suppressed the noxious-evoked TF, failed to activate any of the 21 SEROTONERGIC-LIKE cells tested. Furthermore, PAG suppression of the TF occurred in the absence of SEROTONERGIC-LIKE cell activation. In the two cases in which PAG suppression evoked an increase in SEROTONERGIC-LIKE cell discharge, this activation was likely attributable to oligo- or polysynaptic rather than monosynaptic pathways (see below).

Two SEROTONERGIC-LIKE cells were excited at short latency by PAG train stimulation at an intensity of 500 μA. The latency of these excitations (18–23 msec) is evidence that if a monosynaptic excitatory connection exists between PAG and RM/NRMC SEROTONERGIC cells, the conduction velocity would have to be very slow, ≤0.4 m/sec. Shah and Dostrovsky (1980) reported a mean conduction velocity of 4.1 m/sec for PAG units that project to the RM in the rat; only 3 of 29 PAG units had conduction velocities in the unmyelinated range (<1 m/sec), the slowest of which conducted at 0.4 m/sec.

The activation of two SEROTONERGIC-LIKE cells by train stimulation at an intensity of 500 μA is likely attributable to current spread or activation of oligosynaptic pathways or both. Responses were evoked by short-train stimulation only at intensities of ≥500 μA. At such high intensities, somata and myelinated fibers that are ≥750 μm and 1.5 mm, respectively, from the electrode are likely to be stimulated (Ranck, 1975). In response to long-train (6 sec) stimulation at intensities of 50 μA, two cells responded with a small increase in discharge rate that had a latency of several seconds. This long latency suggests that the response may be secondary to a gross stimulation effect (Depaulis and Bandler,

Figure 3. Graph of the background discharge characteristics of recorded cells. The coefficient of variation (CV) of the interspike interval is plotted against the mean interspike interval for a 5 min period of background discharge. A line representing the discriminant function \[ y(x, s) = 0 \] defines the optimal linear boundary between SEROTONERGIC and nonserotonergic cells and is illustrated on this same graph. SEROTONERGIC-LIKE (open circles) and SEROTONERGIC (filled circles) cells are all located below the discriminant line. Nonserotonergic-like cells that respond (asterisks) or do not (plusses) respond to PAG stimulation are all located above the discriminant line. Nonserotonergic-like cells with a CV >3.5 (n = 16) are not shown on this graph.
1991). It is less likely that these long-latency effects are attributable to monosynaptic connections.

All SEROTONERGIC-LIKE cells were characterized using a previously described algorithm developed from an analysis of more than 45 physiologically characterized, intracellularly labeled and immunocytochemically tested cells (Mason, 1997). As mentioned above, the probability of misclassification using this discriminant function is likely to be <10%. The similarity between the background discharge pattern, response to noxious stimulation, and nuclear location of the cells recorded in the current study and those of intracellularly labeled SEROTONERGIC cells allows some confidence in the use of this discriminant function on immunocytochemically untested cells. Moreover, the validity of the classification scheme was supported further by the observation that two physiologically characterized, SEROTONERGIC-LIKE cells contained serotonin immunoreactivity.

Functional implications
The current findings suggest that the fast glutamatergic input that mediates the antinociceptive effects of PAG activation (see introductory remarks) is likely to act on RM/NRMC nonserotonergic cells. This is consistent with the previous observation that intracellularly labeled RM cells that receive a monosynaptic EPSP...
from PAG stimulation do not contain serotonin immunoreactivity in the cat (Mason et al., 1988).

In light of the anatomical evidence that PAG cells project to serotonin-containing neurons in RM, the current finding that pontomedullary serotonergic-like cells do not respond physiologically to PAG stimulation is puzzling. It is possible that PAG-derived synaptic input to serotonergic RM and NRMC cells is not of sufficient strength to change the discharge rate recorded extracellularly. It is also possible that PAG stimulation inhibits serotonergic cells; any inhibition lasting less than the mean interspike interval (i.e., 300–2000 msec) would be difficult to detect in the present study. Finally, serotonergic cells in RM and NRMC are a heterogeneous population with regard to physiology, morphology, and neurochemistry (Bowker et al., 1982; Mason, 1997; K. Gao and P. Mason, unpublished observations). It is possible therefore that a subset of the serotonergic cell population in RM and NRMC is monosynaptically excited by PAG activation but was not recorded in the present study.

The lack of a strong physiological input from PAG to pontomedullary serotonergic-like cells is also puzzling because of the large body of evidence that the antinociceptive and cardiovascular modulatory effects of PAG stimulation are mediated, at least in part, by the spinal release of serotonin. Pontomedullary serotonergic cells are the primary, if not the only, source of serotonin

Figure 5. An example of a serotonergic cell during PAG suppression of the noxious-evoked TF. The bottom trace illustrates the instantaneous discharge rate (= the reciprocal of the interspike interval; left axis) of the unit. It is important to note that in graphs of instantaneous rate, a point at 10 Hz reflects an action potential that occurred 100 msec after the preceding action potential; it does not reflect the occurrence of 10 action potentials within a bin. Adjacent points are joined by lines, and the graph is filled to the zero line. The middle trace represents the systemic blood pressure, and the top trace shows the instantaneous heart rate. The bar below the trace indicates where the heat stimulus was applied, and the arrow shows the time of the animal’s withdrawal. A, Instantaneous discharge rate during a control trial of noxious tail heat. B, Discharge during suppression of the noxious heat-evoked TF by stimulation in the rostral right PAG (300 Hz, 200 μsec, 50 μA; dashed line under graph). C, Discharge during a control trial of noxious tail heat obtained 3 min after the suppression test shown in B. D, Discharge during suppression of the noxious heat (solid bar under graph)-evoked TF by stimulation in the rostral left PAG (300 Hz, 200 μsec, 50 μA; dashed line under graph). E, Discharge during a control trial of noxious tail heat obtained 3 min after the suppression test shown in D. F, Discharge during suppression of the noxious heat (solid bar under graph)-evoked TF by stimulation in the caudal left PAG (300 Hz, 200 μsec, 50 μA; dashed line under graph). G, Discharge during a recovery trial of noxious tail heat (solid bar under graph) obtained 3 min after the suppression test shown in F. The scale bar on the left of each trace represents the instantaneous discharge frequency; the scale bar on the right of each trace represents 0–100 for the blood pressure and 0–400 for the heart rate. H, This serotonergic cell was intracellularly labeled. The somatodendritic arbor is illustrated. The arrow points at the soma.
in the spinal cord (Dahlstrom and Fuxe, 1964; Oliveras et al., 1977). As described in the introductory remarks, a number of studies have demonstrated that the local application of serotonin receptor antagonists attenuate the antinociceptive and cardiovascular modulatory effects of PAG stimulation. A tonic release of serotonin within the spinal cord, as suggested by a number of studies (Rivot et al., 1987; Duggan, 1992; Peng et al., 1996), is the most parsimonious explanation for the paradox of serotonergic cells not being activated by PAG stimulation, whereas PAG stimulation evokes serotonin-sensitive modulatory effects on nociception and cardiovascular tone. In support of this idea, local application of serotonin antagonists alters the background discharge of dorsal horn and preganglionic sympathetic cells (Kadziela, 1983; Rivot et al., 1987; Peng et al., 1996). Local application of serotonin or serotonin agonists excites preganglionic sympathetic neurons and has primarily inhibitory effects on the nociceptive responses of dorsal horn cells (el Yassir et al., 1988; Bras et al., 1989). Serotonin antagonists facilitate nociceptive transmission (Rivot et al., 1987; Peng et al., 1996) and inhibit sympathetic activity (Huangfu et al., 1994) in the anesthetized rat. Serotonergic cells discharge tonically (Mason, 1997), steadily releasing serotonin within the dorsal horn and the intermediolateral cell column in this same condition. During PAG activation, nonserotonergic cells in RM, NRMC, and RVL are activated (Hilton and Smith, 1984; Vanegas et al., 1984; Mason et al., 1988; Gao and Li, 1993), presumably releasing nonserotonergic transmitters and neuropeptide modulators within both the dorsal horn and the intermediolateral cell column. Serotonin may then modulate the electrophysiological effects of neuropeptides and amino acid neurotransmitters released from bulbospinal terminals (Murase et al., 1990). During PAG stimulation, local application of serotonin antagonists would then attenuate the serotonin-mediated modulation of nonserotonergic neurotransmitters and neuromodulators. In this way, the effects of PAG stimulation on nociceptive transmission and cardiovascular control would be sensitive to serotonin antagonists (McCall, 1984; Peng et al., 1996).

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