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# Activation of Serotonergic Neurons in the Raphe Magnus Is Not Necessary for Morphine Analgesia

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A wealth of pharmacological and behavioral data suggests that spinally projecting serotonergic cells mediate opioid analgesia. A population of medullary neurons, located within raphe magnus (RM) and the neighboring reticular nuclei, contains serotonin and is the source of serotonin in the spinal dorsal horn. To test whether serotonergic neurons mediate opioid analgesia, morphine was administered during recordings from medullary cells that were physiologically characterized as serotonergic ($5HT_p$) by their slow and steady discharge pattern in the lightly anesthetized rat. Selected $5HT_p$ cells ($n = 14$) were intracellularly labeled, and all contained serotonin immunoreactivity. The discharge of most $5HT_p$ cells was not affected by an analgesic dose of systemic morphine. In a minority of cases, $5HT_p$ cells either increased or decreased their discharge after morphine administration. However, morphine altered the discharge of some $5HT_p$ cells in the absence of producing analgesia and conversely did not alter the discharge of most $5HT_p$ cells in cases in which analgesia occurred. RM cells with irregular discharge patterns and excitatory or inhibitory responses to noxious tail heat were classified as ON and OFF cells, respectively. All ON and OFF cells that were intracellularly labeled ($n = 9$) lacked serotonin immunoreactivity. All ON cells were inhibited, and most OFF cells were excited by systemic morphine. Because $5HT_p$ cells do not consistently change their discharge during morphine analgesia, they are unlikely to mediate the analgesic effects of morphine. Instead, nonserotonergic cells are likely to mediate morphine analgesia in the anesthetized rat. In light of the sensitivity of morphine analgesia to manipulations of serotonin, serotonin release, although neither necessary nor sufficient for opioid analgesia, is proposed to facilitate the analgesic effects of nonserotonergic RM terminals in the spinal cord.

*Key words: pain modulation; nociception; antinociception; monoamines; serotonin; discharge pattern; morphine*

Behavioral and pharmacological studies have led to the idea that serotonin is important in the generation of opioid analgesia (LeBars, 1988; Sawynok, 1989). Serotonin in the spinal dorsal horn is derived almost entirely from serotonergic cells located in the medullary raphe magnus (RM) and adjacent nucleus reticularis magnocellularis (NRMC) (Dahlstrom and Fuxe, 1964; Oliveras et al., 1977). This region also contains both opioid peptides and opioid receptors that are responsive to exogenous morphine (Khachaturian et al., 1983; Satoh et al., 1983; Williams and Dockray, 1983; Bodnar et al., 1988; Bowker and Dilts, 1988). The analgesia evoked by systemic or supraspinal morphine is attenuated by inactivation of RM and NRMC neurons or by neurotoxic depletion of serotonergic terminals in the spinal cord (Deakin and Dostrovsky, 1978; Mohrland and Gebhart, 1980; Vasko et al., 1984). Consistent with the idea that morphine-evoked serotonin release in the spinal cord mediates opioid analgesia, the analgesia evoked by systemic opioids is partially attenuated by serotonin antagonists administered intrathecally (Wigdor and Wilcox, 1987; Milne and Gamble, 1990). Furthermore, morphine administration can evoke serotonin release in the spinal cord (Shiomi et al., 1978; Matos et al., 1992), where serotonin has a strong and specific inhibitory effect on dorsal horn nociceptive transmission (Belcher et al., 1978; Yaksh and Wilson, 1979).

The above studies have led to the "textbook" mechanism for opioid analgesia: opioids, in addition to their direct effects on spinal opioid receptors, activate RM serotonergic cells that release serotonin within the dorsal horn, thereby inhibiting spinal nociceptive transmission. However, there is little physiological evidence to support this hypothesis. Instead, physiological experiments provide indirect evidence that the RM cells whose discharge increases during opioid analgesia are nonserotonergic. RM and NRMC contains two physiological cell types that are affected by opioids. OFF cells, characterized by their inhibitory response to noxious stimulation, are excited by analgesic doses of opioids (Fields et al., 1983; Barbaro et al., 1986). ON cells, in contrast, are characterized by an excitatory response to noxious stimulation and are inhibited by opioid administration. Although we have demonstrated recently that neurons, characterized as ON and OFF cells by their responses to noxious heat, lack serotonin immunoreactivity (Potrebic et al., 1994; Mason, 1997), neurons that exhibit an opioid response have never been directly tested for serotonin content.

Using measures of discharge rate and regularity, a discriminant function was derived recently that distinguishes serotonergic from nonserotonergic cells (Mason, 1997). This function makes possible a direct test of whether opioid administration activates serotonergic or nonserotonergic cells in the anesthetized rat. Therefore, the discharge of serotonergic and nonserotonergic

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cells was recorded in lightly anesthetized rats in response to systemic administration of several doses of morphine.

## MATERIALS AND METHODS

*Experimental protocol.* Male Sprague Dawley rats (Sasco, Madison, WI) were used. Rats were anesthetized initially with halothane and maintained on 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. A catheter was inserted into either the femoral or brachial artery for recording of arterial blood pressure. Core body temperature was maintained at 36–38°C. After surgical preparation, the anesthetic concentration was reduced to 1.0–1.2%, and the animal was allowed to equilibrate at this concentration for ≥30 min before a recording was made.

A recording microelectrode was inserted into the region of the RM/NRMC (posterior −1.5 to −2.6 mm, lateral 0.0–1.0 mm, and ventral 9.0–10.5 mm from the cerebellar surface). Both glass micropipettes and Pb-plated metal electrodes were used for recording. Glass micropipettes were filled with a solution of 2% neurobiotin in 0.1 M Tris buffer, pH 7.4, and 0.5 M KCl, and had a tip resistance of 40–70 MΩ.

The background discharge of isolated cells was recorded for 5 min in the absence of any purposeful stimulation. After the background discharge was recorded, tail heat stimuli were administered every 3–5 min. After two to five baseline tail withdrawals, morphine sulfate (0.3 ml, s.c.) was then administered at doses of 0.5–10 mg/kg. After the tail withdrawal was suppressed for two to three tail heat trials, naloxone (0.4 mg in 1 ml, i.p.) was administered during recordings from most cells ($n$ = 33). In some animals ($n$ = 6), 0.3 ml of saline was administered subcutaneously after the baseline tail heat trials and before the morphine administration; in these cases, an additional two tail heat stimulations were recorded between the saline and morphine injections. After completion of the protocol and when recording with metal electrodes, the recording site was lesioned with 20 nA negative direct current for 4 min.

When glass micropipettes were used, cells were initially recorded extracellularly. The extracellular waveforms were very large positive-going action potentials that did not show any evidence of injury discharge and were stable for periods of up to 3 hr. After completion of the above protocol, most cells recorded in this manner could be impaled by injecting depolarizing current (≤1.5 nA). Successful impalement was marked by a large increase in spike height, a graded increase in spike frequency, and a hyperpolarized membrane potential. Neurobiotin was then injected with constant depolarizing current (0.3–1.5 nA) applied for 30 sec to 10 min.

During recordings of almost all cells (43/45), one of three doses of morphine was used. In most experiments, a 1 mg/kg dose was used because it consistently produces antinociception in the anesthetized rat but does not produce nonspecific effects on motor and cortical activity in the awake rat. A low dose, 0.5 mg/kg, was used to try to dissociate the antinociceptive and cardiovascular effects of morphine. Finally, some rats received a high dose of morphine, 10 mg/kg, to compare the results with pharmacological studies that consistently report an increase in serotonin release evoked by high doses of opioids (Tao and Auerbach, 1994).

In eight animals, a second cell was recorded at a minimum of 90 min, but typically 220–250 min, after the previously recorded cell. In all such cases, a tail withdrawal was present before the second morphine administration.

*Analysis: cell classification.* All cells were physiologically characterized as serotonergic ($5HT_p$) or nonserotonergic (non-$5HT_p$) 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 mean and SD of the interspike intervals (ISIs) were calculated from the recording of background discharge. 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 $5HT_p$ if the function value was <0 and non-$5HT_p$ if the function value was >0 (Mason, 1997).

Non-$5HT_p$ cells were further classified as ON or OFF cells by their response to repeated trials of noxious tail heat. Cells that were consistently excited by noxious tail heat were considered ON cells, and cells that were consistently inhibited by noxious tail heat were considered OFF cells.

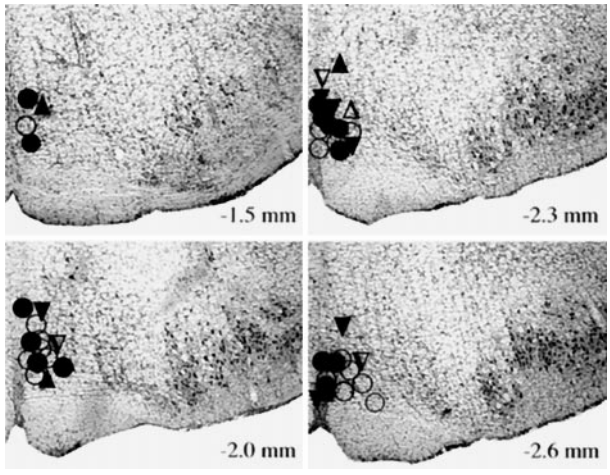


*Figure 1.* Recording sites on nissl-stained coronal sections of the ventromedial medulla. Recording sites for $5HT_p$ (○), $5HT_{p/ir}$ (●), ON cells (*upward triangles*), and OFF cells (*downward triangles*). $5HT_p$ cells were identified by physiological criteria alone, whereas $5HT_{p/ir}$ cells were initially identified physiologically and then were labeled and found to contain serotonin immunoreactivity. ON and OFF cells that were immunochemically confirmed to be nonserotonergic are shown as *filled symbols*. The *number* below each section is the location of that section relative to interaural zero (in millimeters).

NEUTRAL cells were not recorded in this study. Cells that were classified as $5HT_p$ and were affected by noxious tail heat were not considered ON or OFF cells. As described previously (Mason, 1997), serotonergic cells are distinguished from nonserotonergic cells by their discharge pattern but not by their responses to noxious stimulation. Because of the importance of serotonin in nociceptive modulation, the function of serotonergic cells is likely to be distinct from that of nonserotonergic ON and OFF cells. Therefore, serotonergic cells, regardless of their response to noxious stimulation, were classified in a single physiological class.

*Analysis: criterion for a "response."* For the 60 sec before each tail heat trial, the mean and SD of the discharge rate, heart rate, and blood pressure were calculated. The average of the discharge rates calculated from the baseline period was then considered as the mean baseline discharge rate. All discharge rates after drug administration (saline or morphine) were expressed as a proportion of the baseline discharge rate. Although $5HT_p$ cells discharge steadily, there is a slow, low amplitude oscillatory variation in the discharge of many such cells (our unpublished observations). This variation is well described by the coefficient of variation of the interspike interval ($CV_{ISI}$) of the cell. Therefore, $5HT_p$ discharge was considered to be altered by drug administration if it changed by a proportion greater than or equal to the baseline $CV_{ISI}$.

*Histology.* The animals were perfused with saline and 500 ml of fixative. Coronal serial sections (50 μm) were cut on a freezing microtome. Appropriate medullary sections were stained for neurobiotin and serotonin immunoreactivity as described previously (Mason, 1997).

## RESULTS

### Characterization of serotonergic cells

All $5HT_p$ cells ($n$ = 32) were recorded from RM and NRMCα, regions that contain serotonin-immunoreactive cells (Fig. 1). During the 5 min unstimulated period, $5HT_p$ cells had background discharge rates of 0.7–3.6 Hz (mean 1.6 ± 0.1 Hz) and a mean coefficient of variation of the interspike interval ($CV_{ISI}$) of 0.45 ± 0.03 (Fig. 2). The $5HT_p$ cells were unaffected ($n$ = 23), excited ($n$ = 7), or inhibited ($n$ = 2) by noxious tail heat. Because serotonergic cells are distinguished from nonserotonergic cells by their discharge pattern, but not by their response to noxious heat, all cells that had a negative value in a previously described discriminant function (see Materials and Methods), regardless of their noxious-evoked responses, were classified as $5HT_p$ cells.

As a confirmation of the serotonergic identity of the recorded

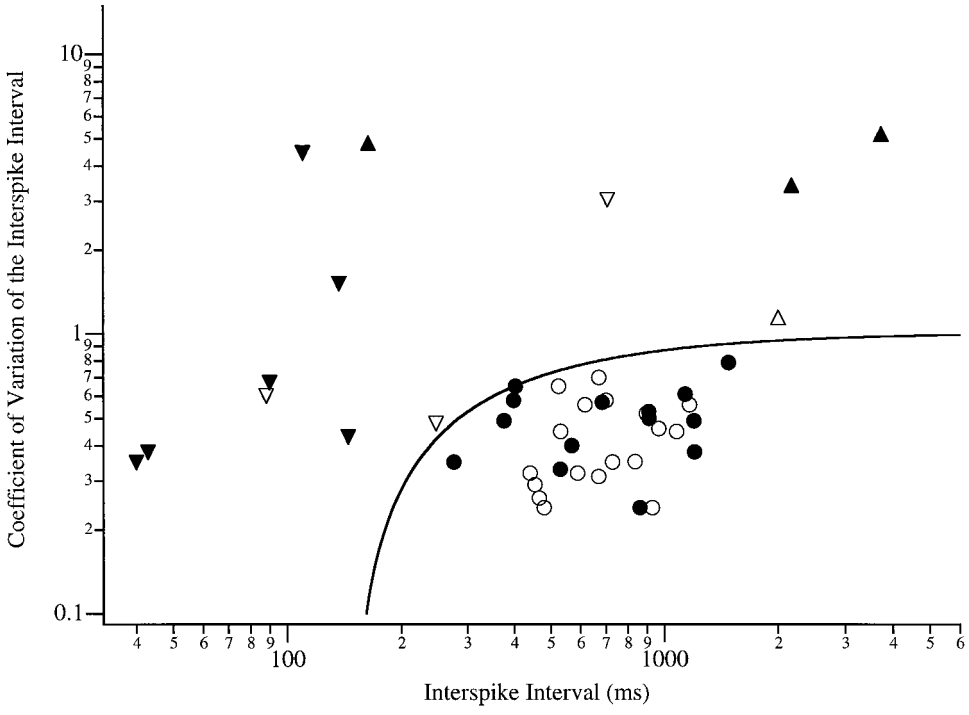


*Figure 2.* Physiological characteristics of recorded cells. The coefficient of variation of the interspike interval ($CV_{ISI}$) is plotted against the mean interspike interval for a 5 min period of background discharge. A *line* representing the optimal linear boundary between serotonergic and nonserotonergic cells is illustrated on this same graph. $5HT_p$ (○); 5HTp/ir (●); ON cells (*upward triangles*); and OFF cells (*downward triangles*). ON and OFF cells that were immunochemically confirmed to be nonserotonergic are shown as *filled symbols*.

cells, 14 $5HT_p$ cells were intracellularly labeled and tested for serotonin immunoreactivity. All 14 cells contained serotonin immunoreactivity and are referred to as $5HT_{p/ir}$ cells (Fig. 3*A–D*). Because there were no differences between the $5HT_p$ and $5HT_{p/ir}$ cells, the two groups will be discussed together below and referred to as $5HT_p$.

### Effects of morphine on motor and autonomic measures

The tail flick withdrawal evoked by noxious heat was unaffected by a saline injection ($n = 6$) but was blocked by morphine in 44 of 45 cases. Morphine decreased heart rate in a dose-dependent manner and had variable effects on blood pressure (Table 1). Morphine also blunted or eliminated the tachycardiac and hypertensive reactions that were typically evoked by noxious heat (see Fig. 6).

### Effect of morphine on serotonergic cells

The mean discharge rate of $5HT_p$ cells after an intraperitoneal injection of saline (103 ± 8% of baseline; $n = 5$) was not different from that after morphine administration (106 ± 10% of baseline; $n = 32$; unpaired $t$ test). When analyzed individually, the discharge rate of most $5HT_p$ cells ($n = 20$) was unaffected by the administration of morphine (Fig. 4*A,C*). Figure 5*A* shows an example of a $5HT_p$ cell whose discharge rate and pattern was unaffected by morphine administration. The discharge of a minority of $5HT_p$ cells ($n = 12$) changed after systemic administration of morphine (Figs. 4*B,D*, 5*B,C*); six of the affected cells decreased their discharge rate and six increased their discharge rate after morphine administration. The discharge of most affected cells changed only transiently after morphine administration, typically returning to baseline values within 3–15 min of the morphine injection and before naloxone administration (Fig. 5*B,C*). Four cells that altered their discharge after morphine administration were recorded after a second injection of morphine. None of the four cells was affected by the second administration of morphine.

Cells that changed their discharge after morphine administration had significantly higher values of $CV_{ISI}$ (0.55 ± 0.04) than cells that were unaffected by morphine administration (0.40 ± 0.03; unpaired $t$ test; $p = 0.007$). In addition, morphine doses of ≥2 mg/kg (7/11) were more likely to alter the discharge of $5HT_p$ cells than were doses of ≤1 mg/kg (5/21; $\chi^2$ test; $p = 0.03$).

There was no consistent relationship between the effect of tail heat and the effect of morphine. For instance, of seven $5HT_p$ cells that were excited by tail heat, two increased, two decreased, and three did not change their discharge rate after morphine administration. Morphine attenuated the heat-evoked responses of $5HT_p$ cells that were responsive to noxious tail heat (Fig. 6*B*).

### Relationship between morphine-evoked analgesia and changes in serotonergic cell discharge

Among $5HT_p$ cells that changed their discharge after morphine administration, changes in discharge were not correlated with suppression of the noxious-evoked tail withdrawal (Fig. 4). Figure 5, *B* and *C*, shows two cells that were transiently affected by morphine. In both cases, the peak effect of morphine on neuronal discharge occurred at a time when tail heat still elicited a withdrawal response.

The relationship between morphine-evoked analgesia and changes in $5HT_p$ cell discharge was examined further by comparing the discharge rate from individual time points, with and without a tail flick response. For each time point, the discharge rate was normalized as a percentage of the baseline discharge value (see Materials and Methods). As shown in Table 2, the morphine-evoked change in discharge was not different at time points when the tail withdrawal was suppressed or not suppressed.

Four cells, which were recorded in response to two injections of morphine, provide further confirmation that changes in $5HT_p$ cell discharge were not related to the presence of analgesia. The

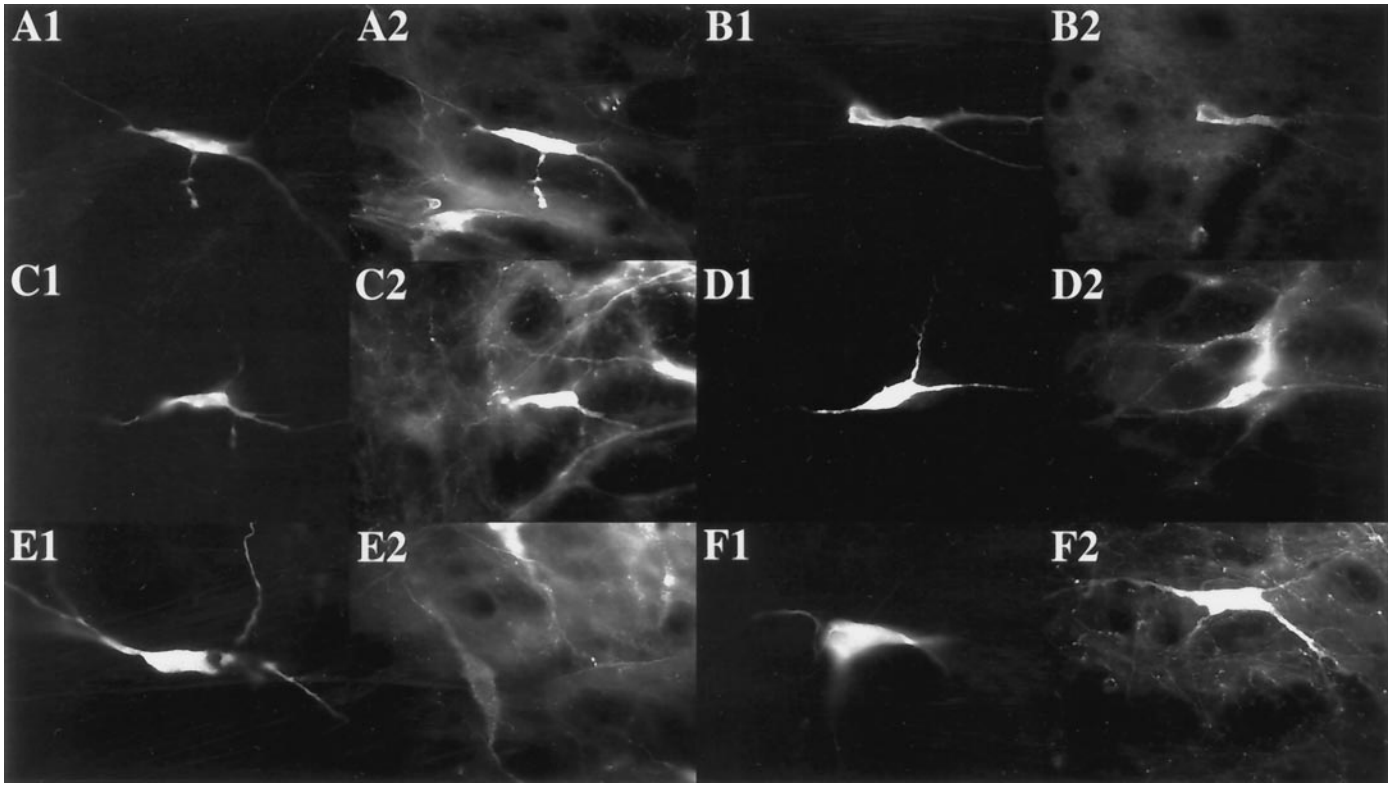


*Figure 3.* Serotonin immunoreactivity in intracellularly labeled cells. The intracellular label visualized with Texas Red (*A1–F1*) and serotonin immunoreactivity visualized with Bodipy (*A2–F2*) are shown for serotonergic (*A–D*) and OFF (*E, F*) cells.

**Table 1. Effect of morphine on blood pressure and heart rate expressed as a change from baseline in beats/minute or mm Hg**

| Morphine dose | Heart rate (bpm) | Blood pressure (mm Hg) |
|---|---|---|
| 0.5 mg/kg | −7 ± 13 (3) | −1 ± 4 (3) |
| 1 mg/kg | −21 ± 4 (18) | −3 ± 1 (15) |
| 10 mg/kg | −47 ± 6 (7) | −5 ± 2 (7) |

Each number is the mean ± SE.

initial dose of morphine evoked a change in cell discharge in all four cases and suppressed the tail flick withdrawal in three of the four cases. In contrast, the second injection of morphine had no effect on cell discharge but suppressed the tail flick withdrawal in all cases.

### Relationship between morphine-evoked bradycardia and changes in serotonergic cell discharge

In the cases of the four cells that changed their discharge rate after an initial dose of morphine but not after a second injection, the second injection of morphine also had no effect on blood pressure or heart rate. Because the first dose of morphine that changed $5HT_p$ cell discharge rate also evoked a bradycardia, an analysis of the relationship between a change in heart rate and a change in $5HT_p$ cell discharge was performed. In response to an initial dose of morphine, cells were more likely to change their discharge (12/24) when a bradycardia of ≥15 beats per minute (bpm) was evoked than when the heart rate changed by <15 bpm (0/7) ($\chi^2$ test; $p = 0.02$). There was no correlation between the magnitude of the bradycardia and the magnitude of the discharge change.

### Characterization of nonserotonergic cells

Non-$5HT_p$ cells were classified as ON ($n = 4$) or OFF ($n = 9$) cells according to their response to noxious tail heat (Fig. 6*C–D*). To confirm previous studies that ON and OFF cells do not contain serotonin (Potrebic et al., 1994; Mason, 1997), six OFF and three ON cells were intracellularly labeled, and none were found to contain serotonin (Fig. 3*E,F*).

### Effect of morphine on nonserotonergic cells

Administration of morphine at doses of 1 mg/kg ($n = 2$) and 10 mg/kg ($n = 2$) inhibited all four ON cells tested. Morphine inhibited the background discharge of ON cells by 75–100% and completely blocked the noxious heat-evoked responses (Fig. 6*D*).

Administration of morphine at doses of 1 mg/kg ($n = 4$) and 10 mg/kg ($n = 5$) increased the background discharge of three OFF cells by >100% and five OFF cells by >25% and did not affect one OFF cell. In agreement with previous observations (Leung and Mason, 1995; C. Leung and P. Mason, unpublished data), the OFF cell that was unaffected by morphine had a regular pattern of background discharge ($CV_{ISI} = 0.43$). After morphine administration, there was a large increase in the number of ISIs that were ≤100 msec in six of nine OFF cells. The noxious evoked responses of OFF cells were attenuated or completely blocked by morphine administration, an effect that was reversed by naloxone (Fig. 6*C*).

## DISCUSSION

### Identification of serotonergic cells

All $5HT_p$ cells were characterized using a previously described algorithm developed from an analysis of physiologically characterized, intracellularly labeled, and immunocytochemically tested cells (Mason, 1997). The reliability of the classification scheme is

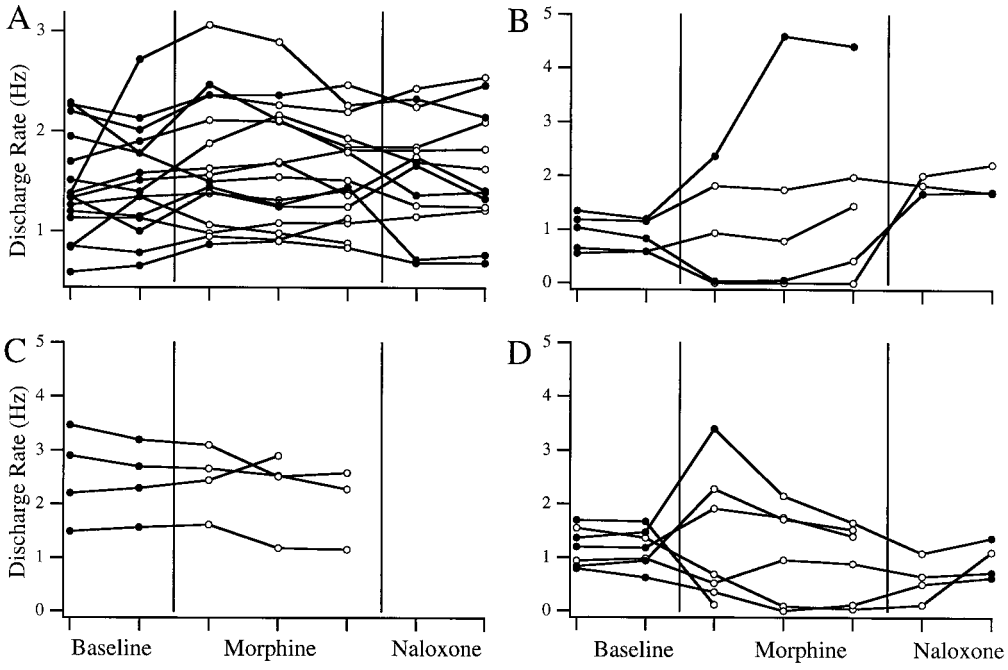


*Figure 4.* Effect of morphine on $5HT_p$ cell discharge. The average discharge rate for each $5HT_p$ cell, during the 60 sec before each tail heat stimulation, is shown for baseline, morphine, and naloxone conditions. Tail flick withdrawals occurred at time points marked with a *filled circle* and were suppressed at time points marked with an *open circle*. *A*, $5HT_p$ cells whose discharge was unaffected by administration of ≤1 mg/kg morphine. *B*, $5HT_p$ cells whose discharge was affected by administration of 1 mg/kg morphine. *C*, $5HT_p$ cells whose discharge was unaffected by administration of >1 mg/kg morphine. *D*, $5HT_p$ cells whose discharge was affected by administration of >1 mg/kg morphine.

supported by the current observation that 14 physiologically characterized $5HT_p$ cells contained serotonin immunoreactivity and that nine physiologically characterized non-$5HT_p$ cells lacked serotonin immunoreactivity. In total, of 31 cells that have been physiologically characterized as $5HT_p$ and tested for serotonin content since the original derivation of the classification algorithm, 30 have contained serotonin immunoreactivity (Gao and Mason, 1997; Gao et al., 1997; our unpublished observations). Furthermore, the similarity between the background discharge pattern, response to noxious stimulation, and nuclear location of $5HT_p$ cells recorded in the current study and those of intracellularly labeled serotonergic cells recorded previously (Mason, 1997) strengthens our confidence in the validity of this procedure for characterizing immunochemically untested cells.

### Serotonergic cells are not activated during opioid analgesia

The present study demonstrates that the serotonergic cell population is not excited by analgesic doses of opioids in the anesthetized rat. This is consistent with previous reports that RM cells with "slow and regular" discharge patterns and/or slow conduction velocities, a population that presumably includes mostly serotonergic cells (Mason, 1997), are insensitive to opioid administration (Auerbach et al., 1985; Chiang and Pan, 1985). Thus, morphine does not alter the discharge of the serotonergic cell population in any consistent manner.

The discharge of a minority of serotonergic cells was affected by morphine administration, with some cells being excited and others inhibited. The effect of morphine on $5HT_p$ cell discharge was not related to the response of a cell to noxious tail heat. Because the effect of morphine on ON and OFF cells is strongly related to the responses of these cells to tail heat, these results provide further evidence that $5HT_p$ cells should not be considered ON or OFF cells, even when they respond to tail heat.

The inconsistent opioid effects on a minority of serotonergic cells may be attributable to direct activation of opioid receptors located on serotonergic cells, an idea that is supported by a recent report that raphe magnus serotonergic neurons express $\mu$ opioid receptor-like immunoreactivity (Kalyuzhny et al., 1996). However, this idea is inconsistent with physiological studies using raphe magnus slices that have demonstrated that cells that contain serotonin-like immunoreactivity do not respond directly to $\mu$ opioid receptor agonists (Pan et al., 1993). The finding that morphine-evoked serotonin release is blocked by deep anesthesia (see below) is additional evidence that opioids are unlikely to affect serotonergic cells directly (Tao and Auerbach, 1994).

An alternate explanation for the inconsistent opioid effects on serotonergic cell discharge is that the changes in discharge are secondary to the profound effect of morphine on cardiovascular tone. In support of cardiovascular-related discharge in serotonergic cells, baroreceptor activation increases the number of *fos*-immunoreactive serotonergic neurons in the raphe magnus and pallidus (Erickson and Millhorn, 1994). Although physiological studies have failed to demonstrate discharge related to baroreceptor activation or sympathetic nerve activity in serotonergic cells of the caudal raphe nuclei (McCall and Clement, 1989; King and McCall, 1992), we have observed recently that many serotonergic cells respond to peripherally evoked changes in blood pressure and heart rate (Genzen et al., 1997). Furthermore, the magnitude of the discharge change evoked by morphine was comparable to the magnitude of discharge variation observed during spontaneous, very slow oscillations in blood pressure (Genzen et al., 1997). Therefore, the morphine-evoked change in the discharge of some serotonergic cells may be attributable at least partially to changes in cardiovascular-related afferent input.

### Effects of morphine on serotonergic cell discharge and serotonin release

The finding that the population discharge of medullary serotonergic cells is unaffected by morphine seems to be at odds with previous reports that opioid administration increases the release of serotonin in the spinal cord and medulla (Shiomi et al., 1978; Yaksh and Tyce, 1979; Vasko et al., 1984; Matos et al., 1992). In most studies that report an opioid-evoked increase in spinal serotonin, a high dose of morphine (≥10 mg/kg) is administered systemically (Shiomi et al., 1978; Rivot et al., 1988; Tao and

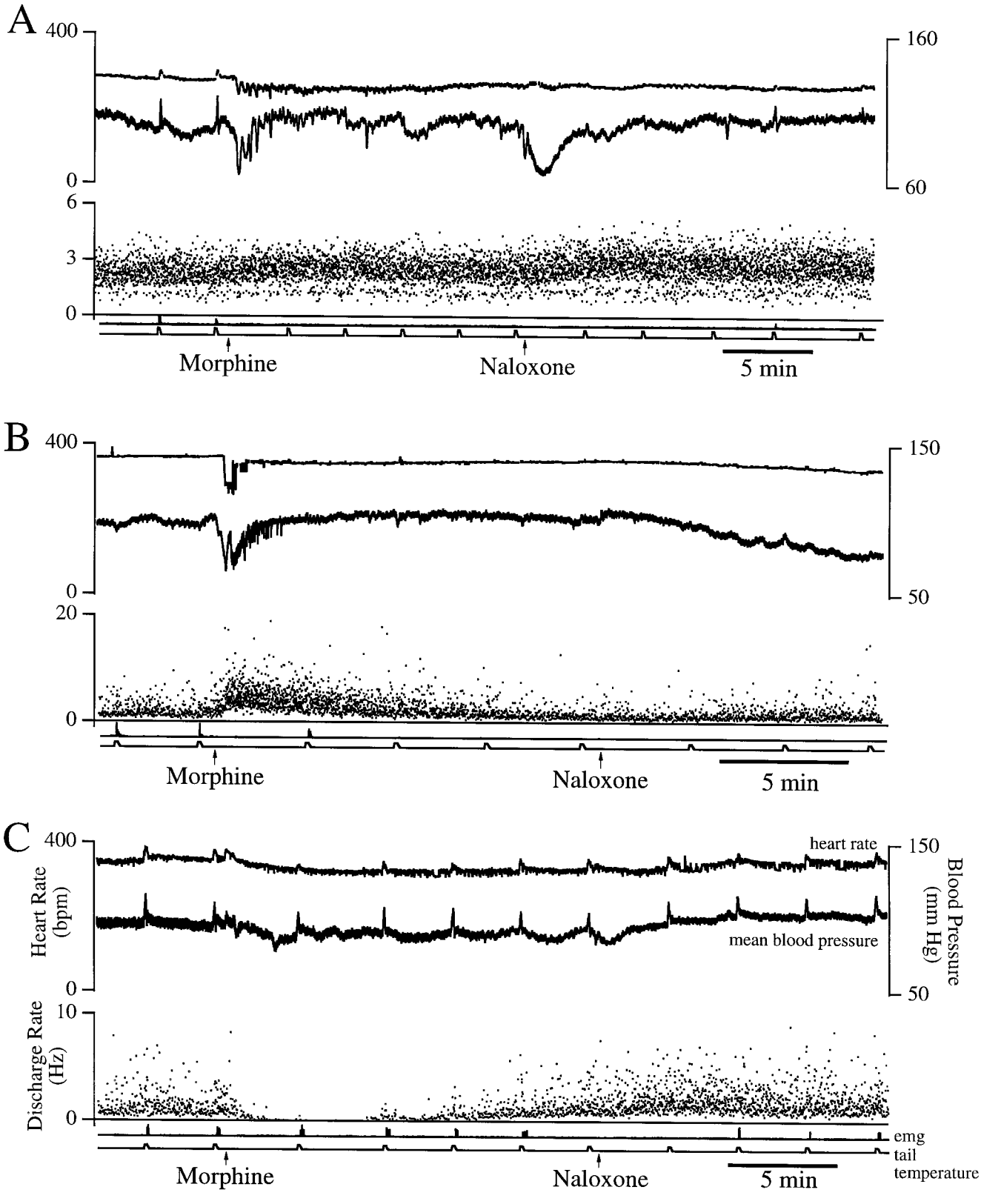


*Figure 5.* Representative recordings from RM and NRMC serotonergic cells before and after morphine administration. The traces are labeled in *C* and are (*top* to *bottom*) heart rate, mean arterial blood pressure, neuronal discharge rate, rectified paraspinous EMG, and thermal tail stimulus. The scales for the neuronal discharge and heart rate (in bpm) are on the *left*, and the scale for blood pressure (in mm Hg) is on the *right*. Injections of morphine and naloxone were administered at times indicated by the labeled arrows below the tail stimulus trace. *A*, Continuous record from a $5HT_{p/ir}$ cell that was unaffected by 1.0 mg/kg morphine and 1 mg/kg naloxone. *B*, Continuous record from a $5HT_p$ cell that transiently increased its discharge after 2 mg/kg morphine and was unaffected by 1 mg/kg naloxone. *C*, Continuous record from a $5HT_{p/ir}$ cell that transiently decreased its discharge after 1 mg/kg morphine and was unaffected by 1 mg/kg naloxone.

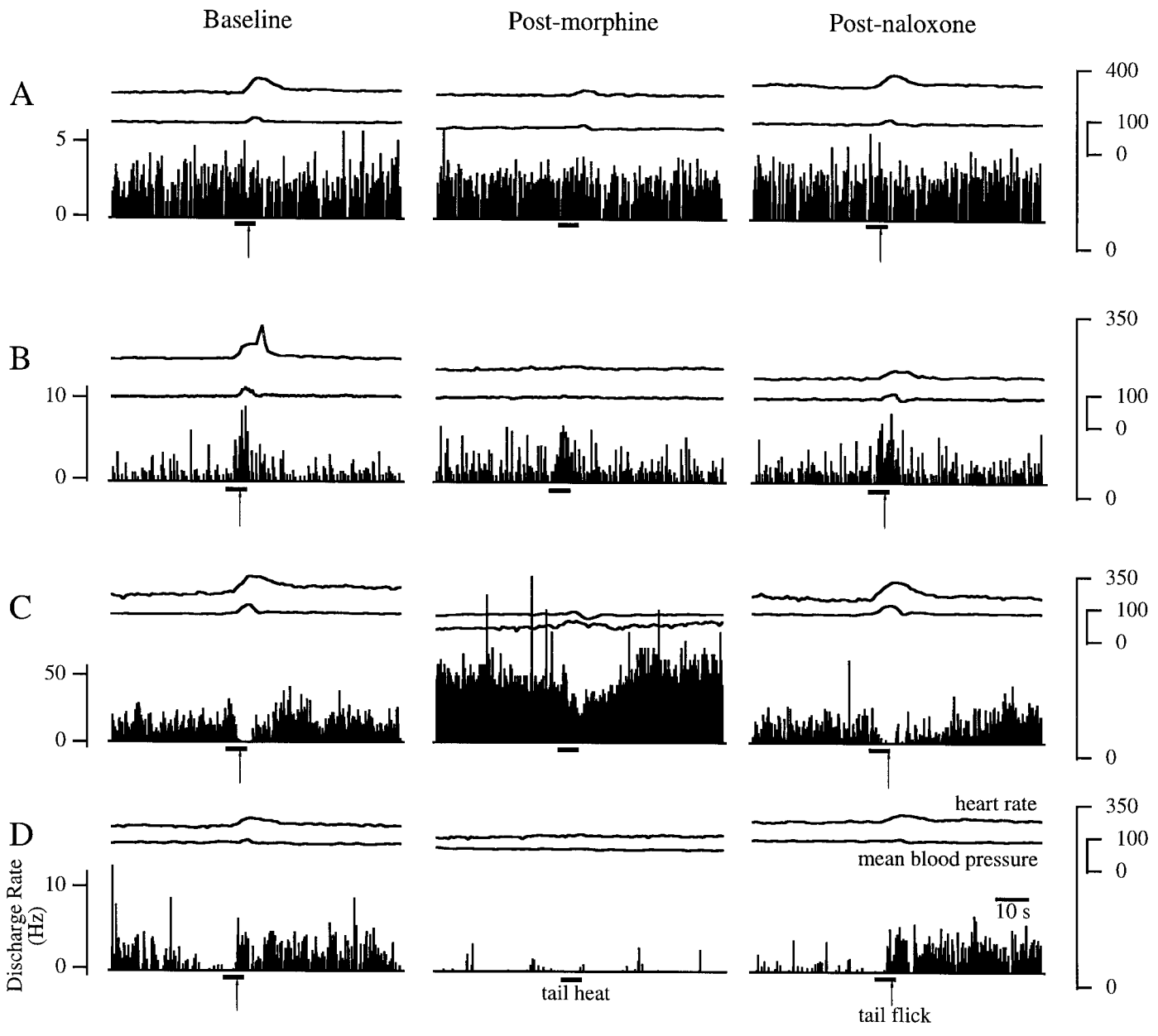


*Figure 6.* Evoked responses from recorded cells before and after morphine and naloxone. The *bottom trace* represents instantaneous discharge rate of the cell. The *middle trace* represents mean arterial blood pressure, and the *top trace* shows the instantaneous heart rate. The scale bar for the neuronal discharge rate (in Hz) is on the *left*. The small scale on the *right* is for blood pressure (0–100 mmHg), and the large scale on the *right* (bpm) is for heart rate. The *bars* below the unit trace indicate the application of noxious tail heat, and the *arrows* indicate the time of the withdrawal. In cases in which the animal did not withdraw, there is no arrow. Baseline, post-morphine, and post-naloxone responses are shown in the *left, middle*, and *right columns*, respectively. Each trace is 100 sec in duration. *A*, A $5HT_p$ cell that was unresponsive to noxious heat. *B*, A $5HT_{p/ir}$ cell that was excited by noxious heat. *C*, An OFF cell. *D*, An ON cell.

Auerbach, 1994). Such doses produce nonspecific effects, including both motoric hyperactivity and catatonia (Silva et al., 1971; Chaillet et al., 1983; Winters et al., 1988) and may be inappropriate for the study of the serotonin dependence of opioid analgesia. In addition, morphine at doses ≥10 mg/kg evokes serotonin release from serotonergic terminals located in various regions, both related and unrelated to pain (Commissiong, 1983; Crisp and Smith, 1989). Even centrally administered morphine, at a dose as low as 5 μg, microinjected into the midbrain periaqueductal gray (PAG), produces a motor hyperactivity that is followed by a quiescent catatonia (Jacquet and Lajtha, 1974). Therefore, the in-

**Table 2. Effect of morphine or saline on serotonergic cell discharge at time points when the tail flick is or is not suppressed**

| Drug | Tail flick suppression | No tail flick suppression | *p* |
|---|---|---|---|
| Saline | | 103 ± 8% | |
| Morphine (≤1 mg/kg) | 107 ± 4% | 114 ± 6% | n.s. |
| Morphine (≥2 mg/kg) | 95 ± 11% | 190 ± 65% | n.s. |

The numbers are the mean ± SE of the serotonergic cell discharge, expressed as a percentage of baseline. *p* values are from unpaired *t* tests. A value of $p < 0.05$ is considered significant. Nonsignificant differences are marked n.s.

crease in intrathecal serotonin evoked by microinjection of 5 μg of morphine into the PAG (Yaksh and Tyce, 1979) may be secondary to an effect on motor or autonomic modulatory neurons.

In the present study, there was no correspondence between the effect of morphine on serotonergic cell discharge and on tail flick withdrawal. This result is consistent with previous reports that changes in the release of serotonin within the spinal cord are not tightly correlated with behavioral analgesia after opioid administration. Most importantly, analgesia can occur in the absence of an increase in serotonin release (Chiang and Xiang, 1987; Matos et al., 1992). These findings provide evidence that an increase in serotonin release is neither necessary nor sufficient for the analgesic effect of opioids.

### The role of serotonergic cells in opioid analgesia

Rivot showed that the voltammetric increase in RM serotonin, evoked by morphine microinjection into the RM, is blocked by chloral hydrate anesthesia (Rivot et al., 1988). Similarly, the increase in serotonin release from dorsal raphe terminals evoked by high doses of systemic morphine is blocked by deep anesthesia, evidence that it is unlikely to be caused by a direct effect on opioid receptors (Tao and Auerbach, 1994). Instead, behavioral state or autonomic status, processes that are suppressed by deep anesthesia, may be important in mediating the opioid-evoked release of serotonin.

In light of the finding that anesthesia blocks the effect of morphine on serotonin release, it is possible that morphine affects behavioral state, which in turn affects serotonin release. Morphine blocks desynchronized sleep and attenuates the time spent in slow wave sleep, whereas it increases the time spent in a state of alert rigidity (Kay et al., 1979). It has been well established that neurons with slow and steady discharge patterns, which are likely to correspond to serotonergic cells, have state-dependent discharge patterns in the unanesthetized animal (Trulson and Jacobs, 1979). These cells, including units in RM and NRMC, discharge at their highest rates during the most active periods of waking and at lower rates during slow wave sleep and are often inactive during desynchronized sleep (Trulson and Jacobs, 1979; Fornal et al., 1985). Therefore, a morphine-evoked increase in serotonin release may be secondary to a primary opioid effect that increases the time spent in the waking behavioral state. This possibility would explain the nonspecific distribution of morphine-evoked serotonin release and the lack of correlation between serotonin release and analgesia (see above).

### Non-serotonergic cells mediate opioid analgesia

The present study is the first direct demonstration that RM and NRMC cells that respond to morphine are nonserotonergic. The opioid activation of OFF cells, neurons that are hypothesized to inhibit dorsal horn nociceptive transmission, is likely to occur indirectly through a disinhibition mediated by the direct inhibition of ON cells (Fields et al., 1991). The present results combined with our previous findings that antinociceptive stimulation in the PAG excites nonserotonergic but not serotonergic cells at short latency (Mason et al., 1988; Gao et al., 1997) provide strong evidence that nonserotonergic cells are the predominate RM mediators of PAG-mediated and opioid-mediated analgesia.

The OFF cell neurotransmitter or neurotransmitters that contribute to opioid suppression of nociceptive transmission remain unknown. RM neurons, including nonserotonergic cells, contain a wide variety of neuropeptides as well as putative amino acid neurotransmitters. Because the action potential frequency required for the release of neuropeptides is typically greater than that required for the release of amino acid transmitters (Iverfeldt et al., 1989; Verhage et al., 1991; Franck et al., 1993), it is intriguing that morphine increased OFF cell discharge at frequencies of ≥10 Hz. Morphine may not only increase OFF cell release of nonpeptide neurotransmitters but may also elicit the release of an additional peptidergic transmitter that is not released by background OFF cell discharge.

## Conclusions

In light of our finding that the medullary serotonergic cell population is not activated by analgesic doses of morphine, it may seem paradoxical that intrathecally administered serotonin antagonists attenuate morphine analgesia. A possible resolution of this paradox arises if serotonin modulates the nociceptive modulatory actions of other neurotransmitters. For instance, serotonin may enhance the antinociceptive actions of transmitters released from nonserotonergic RM OFF cells, making the resulting antinociception sensitive to serotonin antagonists. This effect of serotonin is not dependent on a change or an increase in serotonin release but simply on the presence of a tonic level of serotonin. In support of this idea, intrathecal administration of serotonin uptake inhibitors potentiates the analgesic effects of morphine (Larsen and Christensen, 1982; Taiwo et al., 1985). Furthermore, morphine analgesia is sensitive to serotonin receptor antagonists in anesthetized, awake, and stressed states, conditions during which serotonergic cells are tonically discharging (Hammond and Yaksh, 1984; Barbaro et al., 1985; Crisp and Smith, 1989; Gamble and Milne, 1989; Alhaider and Wilcox, 1993).

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