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Spinal 5-HT_3 Receptor-mediated Antinociception: Possible Release of GABA

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Although 5-HT is clearly involved in spinal analgesia, its mode of action remains obscure, perhaps because it has multiple and often opposing effects mediated by its multiple receptor subtypes. This investigation uses selective agonists and antagonists directed at the most recently defined class of 5-HT receptors (5-HT_3 receptors) in behavioral and electrophysiological studies of nociception in the spinal cord of rodents. The results demonstrate uniformly inhibitory effects of a selective 5-HT_3 agonist on responses to noxious stimuli. Intrathecally administered 2-methyl 5-HT produced dose-dependent antinociception in the tail-flick test and inhibited behaviors elicited by intrathecally administered agonists for excitatory amino acid and neurokinin receptors, namely NMDA and substance P (SP). All 20 dorsal horn neurons we examined, which projected to the brain and responded to both noxious stimuli and NMDA, were inhibited in a current-related manner by this 5-HT_3 agonist applied iontophoretically. Both the behavioral and electrophysiological effects were blocked not only by the 5-HT_3 antagonists zacopride and ICS 205-930, but also by antagonists to the inhibitory amino acid GABA. Therefore, 5-HT via an action at 5-HT_3 receptors may evoke release of GABA, which may in turn inhibit nociceptive transmission at a site postsynaptic to terminals of primary afferent fibers. If the descending serotonergic analgesic system in humans operates similarly, understanding it may enable the development of new nonopioid, nonaddictive analgesics.

The involvement of 5-HT in pain processing is both profound and perplexing. There exists both theoretical (Basbaum and Fields, 1978; Fields and Basbaum, 1978) and clinical (Tollinson and Kriegel, 1988) evidence that, in the CNS, 5-HT inhibits spinal pain transmission. In the PNS, however, 5-HT appears to *promote* pain in migraine attacks (Fozard and Gray, 1989; Loisy et al., 1985) and in laboratory studies of human skin (Richardson and Engel, 1986). Even studies restricted to the CNS show that spinally administered 5-HT can either inhibit (Wang, 1977; Jordan et al., 1978; Yaksh and Wilson, 1979; Davies and Roberts, 1981; Hylden and Wilcox, 1983; Schmauss et al., 1983) or stimulate (Jordan et al., 1979; Fasmer et al., 1983; Hylden and Wilcox, 1983; Clatworthy et al., 1988; Vaught and Scott, 1988) nociceptive responses, depending on the dose and species tested. On the other hand, stimulation of raphe nuclei releases 5-HT in spinal cord dorsal horn (Yaksh and Tyce, 1979) and elicits inhibition of spinothalamic tract and other neurons (Belcher et al., 1978; Griersmith et al., 1981; Johnston and Davies, 1981) apparently by postsynaptic inhibition of these projection neurons (Giesler et al., 1981). Most traditional 5-HT antagonists have failed to block these descending inhibitory actions consistently or completely (Yezierski et al., 1982). The existence of multiple receptors for 5-HT and the lack, until recently, of highly selective ligands for the *appropriate* receptors may have contributed to these contradictory findings.

In the last decade, at least three distinct 5-HT receptor subtypes have been identified. These subtypes are termed 5-HT_1, 5-HT_2, and 5-HT_3 receptors, with 5-HT_1 receptors further differentiated into A, B, C, and D subtypes (Pedigo et al., 1981; Bradley et al., 1986; Peroutka, 1986, 1988; Fozard, 1987). Studies with selective 5-HT_1 and 5-HT_2 ligands have done little to clarify the role of these subtypes in nociception since *both* pro- and antinociceptive effects can be produced by spinal administration of agonists for either receptor subtype. 5-HT_1 receptors, probably of the B subtype, inhibit nociceptive neurons (El-Yassir et al., 1988), but 5-HT_{1A} receptor agonists promote nociception (Zemlan et al., 1983; Murphy and Zemlan, 1987; Alhaider et al., 1990). Similarly, spinal 5-HT_{1C} and/or 5-HT_2 receptors mediate both pronociceptive (Wilcox and Alhaider, 1990) and antinociceptive effects (Solomon and Gebhart, 1988).

The involvement of 5-HT_3 receptors in nociceptive processing has only recently been studied (Glaum et al., 1989, 1990). Unlike 5-HT_1 and 5-HT_2 receptors, which are coupled to G-proteins (Julius et al., 1988, 1990; Raymond et al., 1989), 5-HT_3 receptors are ligand-gated cation channels (Derkach et al., 1989) and therefore should mediate neuronal excitation. 5-HT_3 receptors, thought to be located on the subcutaneous terminals of primary afferent sensory fibers, may mediate 5-HT–elicited pain in human dermis (Richardson and Engel, 1986). Recent autoradiographical experiments indicate that there is a dense band of 5-HT_3 receptors in superficial dorsal horn where small-diameter primary afferent fibers terminate; the number of binding sites is greatly reduced by neonatal capsaicin treatment (Hamon et al., 1989) or dorsal rhizotomy (LaPorte et al., 1991), suggesting that many are located on primary afferent fibers. However, the

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binding sites remaining after rhizotomy may be located on intrinsic CNS neurons in the dorsal horn; 5-HT_3 binding sites have been found on intrinsic neurons in other parts of the CNS (Kilpatrick et al., 1987). Glaum et al. (1989, 1990) recently reported that in rats 5-HT–induced antinociception in the tail-flick test is mediated by 5-HT_3 receptors. Our own behavioral studies are consistent with the involvement of 5-HT_3 receptors in spinal antinociception and suggest that they are located on intrinsic CNS neurons (Wilcox and Alhaider, 1990). We observed that 2-methyl 5-HT (a selective 5-HT_3 agonist) effectively blocks scratching and biting behavior induced in mice by intrathecally administered substance P (SP) and NMDA, excitants that likely interact with intrinsic spinal neurons (Wilcox, 1988, 1991; Aanonsen et al., 1990).

To test the hypothesis that 5-HT_3 agonists activate *intrinsic* spinal cord neurons, it was first necessary to identify an inhibitory mediator for the antinociceptive effect. A previous study has shown that activation of descending serotonergic systems results in postsynaptic inhibition of spinal projection neurons (Giesler et al., 1981). In that study, electrical stimulation of nucleus raphe magnus (NRM), a medullary nucleus containing large populations of serotonergic cells and raphe–spinal projection neurons, elicited IPSPs in primate spinothalamic tract cells. That these IPSPs were reversed by hyperpolarization or intracellular chloride application (Giesler et al., 1981) suggests that either GABA or glycine is involved in this descending inhibitory action. In addition, $GABA_A$ agonists inhibit excitatory amino acid (EAA)-elicited behavior (Aanonsen and Wilcox, 1989). Furthermore, the $GABA_A$ agonist muscimol inhibits EAA-elicited firing of nociceptive spinal projection neurons, and this effect is similar to that of 2-methyl 5-HT (S. Z. Lei and G. L. Wilcox, unpublished observations). To determine whether the effects of 2-methyl 5-HT were mediated through GABA, we challenged the inhibitory effect of 2-methyl 5-HT by coadministering GABA antagonists.

In this report, we present both behavioral (in mice) and electrophysiological (in rats) evidence that 5-HT_3 receptors on intrinsic spinal cord neurons inhibit nociceptive spinal transmission. The electrophysiological studies of sensory projection neurons in the spinal cord of anesthetized, paralyzed rats were designed to extend the behavioral results and to rule out the possibility that the behavioral effects involve motor rather than sensory systems. Experiments with $GABA_A$ antagonists further suggest that 5-HT_3 receptor activation affects nociception by increasing the release of GABA. Such a mode of action represents a new concept for serotonergic antinociception; if this system is operative in humans, understanding it may enable the development of nonaddictive analgesics that manipulate this nonopioid analgesic system.

Materials and Methods

Subjects and supplies. Subjects for the behavioral studies were 17–27-gm male Swiss–Webster-derived mice (Harlan Sprague–Dawley, Madison, WI) maintained in cages of no more than 10 mice per cage with free access to food and water in the University of Minnesota Research Animal Resources facilities for at least 24 hr before experimentation. Mice were used only once and were killed by exposure to CO_2. Intrathecal injections in mice were carried out as previously reported (Hylden and Wilcox, 1980). Subjects for the electrophysiological experiments were male Sprague–Dawley rats (375–500 gm) from the same supplier maintained in the same facility with no more than two rats per cage. Rats were used only once and were killed by overdose of pentobarbital or urethane (i.v.).

Substance P, *N*-methyl-D-aspartic acid (NMDA), 5-aminovaleric acid hydrochloride (5-AVA), muscimol, picrotoxin, and (−)-bicuculline methiodide were purchased from Sigma Chemical Company (St. Louis, MO). 2-Methyl serotonin, (*RS*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid HBr (AMPA), 3-tropanylindole-3-carboxylate (ICS 205-930), and phaclofen were purchased from Research Biochemical Inc. (Natick, MA). Zacopride and naloxone hydrochloride were kindly donated by A. H. Robins Co. (Richmond, VA) and Du Pont Pharmaceuticals (Wilmington, DE), respectively.

Behavior. We first examined the antinociceptive effect of the selective 5-HT_3 agonist 2-methyl serotonin in mice using the radiant-heat tail-flick test (D'Amour and Smith, 1941). The maximum allowable latency (cutoff) was set at 5 sec; this was approximately three standard deviations above the control mean (2.4 sec) for several initial groups of mice. This cutoff latency also served as a determinant of percent maximum possible antinociceptive effect (% MPE), which was calculated in the usual way as posttreatment latency minus control latency divided by cutoff minus control. Posttreatment latency was determined 5 min after intrathecal injection of the agonist (2-methyl serotonin) and antagonists (zacopride or ICS 205-930) or saline.

The tail-flick test does not allow differentiation between effects mediated by receptors located presynaptically on primary afferent fibers (i.e., presynaptic) and effects mediated by receptors located on intrinsic neurons in the spinal cord (i.e., postsynaptic). To examine the latter possibility, we used stimuli that purportedly act postsynaptically on spinal cord neurons (Wilcox, 1991). These stimuli include intrathecal injection of agonists that mimic the putative excitatory neurotransmitters glutamate and SP. Intrathecally administered NMDA (Aanonsen and Wilcox, 1987) and SP (Cridland and Henry, 1986) elicit hyperalgesia and behavior similar to that observed after exposure to noxious chemical stimuli (Hylden and Wilcox, 1981; Hwang and Wilcox, 1986). Although this is a convenient and informative test, the relevance of the biting and scratching behavior elicited by intrathecally applied SP to nociception has been challenged (Frenk et al., 1988). On the other hand, the ability of most spinally active antinociceptive agents to inhibit this behavior (Wilcox, 1988) supports the utility of the test as an adjunct to thermal tests in studies of spinal antinociception. Behaviors elicited by NMDA (biting; see Fig. 2*A*) and SP (biting and scratching; see Fig. 2*B*) were counted for 1 min following intrathecal administration. 2-Methyl serotonin was coadministered intrathecally with either excitant, and antagonists were given intrathecally as a pretreatment 5 min before. Both zacopride and ICS 205-930 were effective antagonists to 2-methyl serotonin, but we studied zacopride in most experiments because of its higher water solubility.

Statistics. Analysis of variance (ANOVA) with Dunnett's post hoc test was used to analyze the behavioral data and to test the difference between doses and antagonists. Tests of statistical significance with $p < 0.01$ were considered significant.

Electrophysiology. Extracellular recordings were conducted in spinal cord of male Sprague–Dawley rats anesthetized with urethane (1.2gm/kg, i.p.), paralyzed with gallamine (27 mg/kg, i.v.), and artificially ventilated. Adequacy of anesthesia was established by continuous monitoring of heart rate and pupil diameter, and supplementary anesthesia (urethane, 50 mg/ml in 0.2–0.3 ml saline, i.v.) was given when necessary. Temperature was maintained at 37 ± 0.5°C with a hot-water heating pad beneath the rat. End-tidal CO_2 was monitored with a capnometer (model 2200, International Medical Inc., Burnsville, MN) and maintained between 3.5% and 4.5% throughout the experiment by small adjustments in the rate (58–65 breaths/min) and volume (1.8–2.2 ml) of respiration. Laminectomies exposed both the lumbar (for recording) and the cervical (for antidromic activation of lumbar neurons projecting rostrally) enlargements of the spinal cord. The head and vertebral column of the animal were mounted on a rigid frame in a Faraday cage. Tungsten microelectrodes (1 MΩ) glued to 7-barreled borosilicate-glass microelectrodes were used to record from single spinal neurons and to apply drugs iontophoretically. An analog window discriminator was used to detect action potentials that were at least two times larger than noise or spikes from other neurons. Antidromic activation was used at least once every 5 min and during most epochs of inhibition of orthodromic activity to verify constant spike shape and amplitude.

The cells were located at indicated depths between 100 and 700 μm from the dorsal surface, and histological verification of cell location in dorsal horn was possible in seven cases. Once a projection neuron was located by antidromic activation from the cervical spinal cord, the neuron's cutaneous receptive field was characterized by mechanical stim-

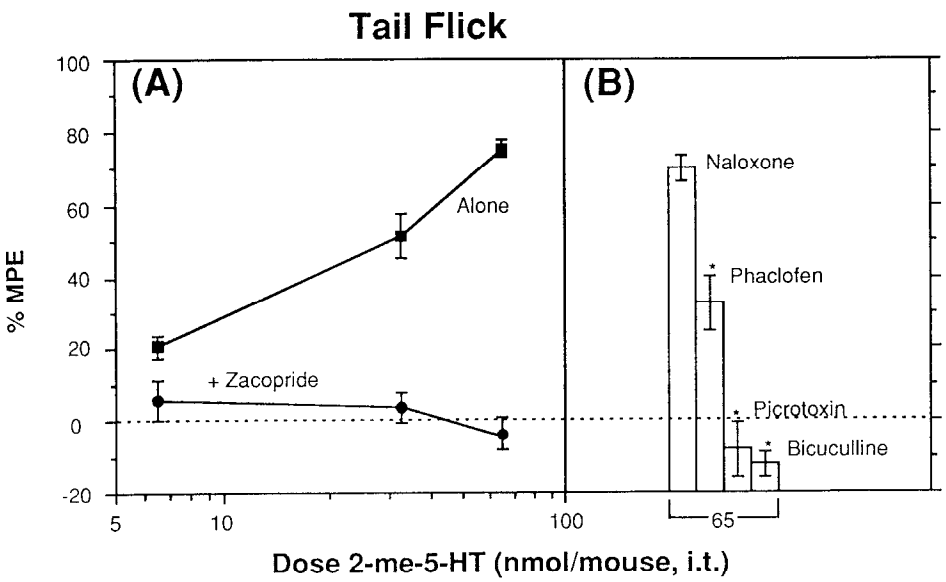

Figure 1. 2-Methyl serotonin (i.t.) produced dose-dependent antinociception in the tail-flick test. The 5-HT_3 antagonists zacopride (*A*) and ICS 205-930 (not shown) and GABA antagonists bicuculline and picrotoxin ($GABA_A$ antagonists) and phaclofen ($GABA_B$ antagonist) (*B*) antagonized the antinociceptive effect of 2-methyl 5-HT. Naloxone failed to block the antinociceptive effect of 2-methyl 5-HT (also in *B*). All antagonists were given intrathecally. *Asterisks* denote significant ($p < 0.01$) post hoc tests. *Error bars* represent SEM.

ulation (brush, pressure, pinch, and squeeze). Cells were classified as follows: (1) low-threshold (LT) cells responded best to either brush or pressure; (2) wide-dynamic-range (WDR) cells responded in a graded manner to brush, pressure, pinch, and squeeze; (3) high-threshold (HT) cells responded best to either pinch or squeeze, with little or no response to brush or pressure. WDR and HT neurons are termed nociceptive.

Action potentials of the neurons evoked by iontophoretic application of excitatory and inhibitory agents and by natural stimulation of the cutaneous receptive field were collected and stored as peristimulus-time histograms (1-sec binwidth) of firing rate using a microcomputer (Aanonsen et al., 1990). The following drugs were used: NMDA (NMDA receptor agonist); AMPA [AMPA receptor (or quisqualate-gated cation channel) agonist]; 2-methyl serotonin (5-HT_3 receptor agonist); zacopride (5-HT_3 antagonist); muscimol and bicuculline ($GABA_A$ agonist and antagonist, respectively). Older nomenclature refers to the AMPA receptor as the quisqualate receptor, but possible confusion with the G-protein–coupled quisqualate receptor mandates this new nomenclature (Watkins et al., 1990). Drugs (5–20 mM) were dissolved in NaCl solution. Sufficient NaCl was added so that the total ionic strength of the solution was 200 mM. NMDA and AMPA solutions were adjusted to pH 8.0, retained with positive current (≈2 nA), and ejected with negative current (2–120 nA); 2-methyl serotonin, zacopride, bicuculline, and muscimol solutions were adjusted to pH 4, retained with negative current (4–10 nA), and ejected with positive current (5–60 nA). NMDA and AMPA were applied regularly and repeatedly in brief pulses. 2-Methyl serotonin, muscimol, bicuculline, and zacopride were ejected with various currents superimposed on repeated NMDA or AMPA pulses.

Our determination of inhibition of firing is based on a reduction of total spikes elicited per excitant epoch (i.e., area under the curve). Stable baseline response rates to NMDA or AMPA were obtained (variability, <20%) before other drugs were applied. Inhibition is reported for reductions of the area under the curve of 40% or more. We felt justified in selecting this artificial cutoff value instead of applying a statistical test for three reasons: (1) examination of several hundred epochs of unit activity indicated that responses of a stable preparation never decreased this much spontaneously, (2) our use of area under the curve instead of peak firing rate is itself a statistical summary that takes account of some natural variability, and (3) the validity of most standard statistical tests, such as ANOVA or *t* tests that we might have used, relies on sample independence that is not clearly met by successive recordings from a single neuron. We defined complete blockade of an inhibitory effect as a return of responses to 100% of control levels, and partial blockade as a return of responses to 80% of control levels.

Results

Behavior

Figure 1 shows that 2-methyl 5-HT (6.5–65 nmol/mouse in saline), in a dose-dependent manner ($F_{2,40} = 15.5$; $p < 0.001$; ANOVA), prolonged tail-flick latency. Zacopride (0.3–6.3 nmol/mouse, i.t. in saline; data shown in Fig. 1*A* for 3 nmol: $F_{1,40} = 182$; $p < 0.001$; ANOVA) and ICS 205-930 (0.3, 1.1, and 3.5 nmol/mouse, i.t.; data not shown) effectively antagonized 2-methyl 5-HT–induced antinociception. These results in mice confirm those obtained by others in rats (Glaum et al., 1989, 1990). The antinociceptive effect of 2-methyl 5-HT was also blocked or reduced by the GABA antagonists (Fig. 1*B*) bicuculline and picrotoxin ($GABA_A$ antagonists) and phaclofen ($GABA_B$ antagonist). At doses that had no effect by themselves, bicuculline (0.02 nmol/mouse, i.t.) or picrotoxin (0.04 nmol/mouse, i.t.) completely blocked the antinociceptive effect of 2-methyl 5-HT, while phaclofen (4 nmol/mouse, i.t.) only partially blocked it. These antagonist effects were significant overall ($F_{3,27} = 34.9$; $p < 0.001$; ANOVA) as well as by individual group post hoc tests. Naloxone (0.3 nmol/mouse, a dose that antagonizes a 90% MPE morphine dose of 1 μg, i.t.) did not block the antinociceptive effect of 2-methyl 5-HT.

2-Methyl 5-HT (0.3–3.3 nmol/mouse) inhibited the biting and scratching behavior induced by both NMDA (0.25 nmol, i.t.) and SP (10 pmol, i.t.) in a dose-dependent manner (Fig. 2). Whereas the NMDA-elicited behavior was completely blocked by 2-methyl 5-HT($F_{4,30} = 82.0$; $p < 0.001$), SP-elicited behavior was reduced by only 65% ($F_{3,28} = 45.7$; $p < 0.001$). Zacopride (0.3–1.0 nmol/mouse) blocked the actions of 2-methyl 5-HT on both SP- ($F_{4,28} = 36.0$; $p < 0.001$) and NMDA-induced ($F_{3,28} = 79.2$; $p < 0.001$) behaviors (Fig. 2*A,B*, insets). Zacopride by itself, at doses below 10 nmol, produced no overt behavioral effects, but at doses over 10 nmol, it induced behavior similar to that induced by NMDA; this result suggests that the effect of some tonic 5-HT release was being removed. In the case of NMDA-induced behavior, the effect of 2-methyl 5-HT was also blocked by bicuculline (Fig. 2*A*, inset; 6–39 pmol/mouse, i.t.; $F_{3,28} = 36.4$; $p < 0.001$) but not by phaclofen (4 nmol/mouse, i.t.) or 5-AVA (0.1 nmol/mouse, i.t.; $GABA_B$ antagonists; data not shown).On the other hand, inhibition of SP-induced behavior by 2-methyl 5-HT was blocked by phaclofen (Fig. 2*B*, inset; 0.12–4 nmol/mouse, i.t.; $F_{4,35} = 58.4$; $p < 0.001$) and 5-AVA (0.1 nmol/mouse, i.t.; data not shown) but not by bicuculline (39 pmol/mouse, i.t.; data not shown). In the absence of 2-methyl 5-HT, these doses of antagonists were without effect.

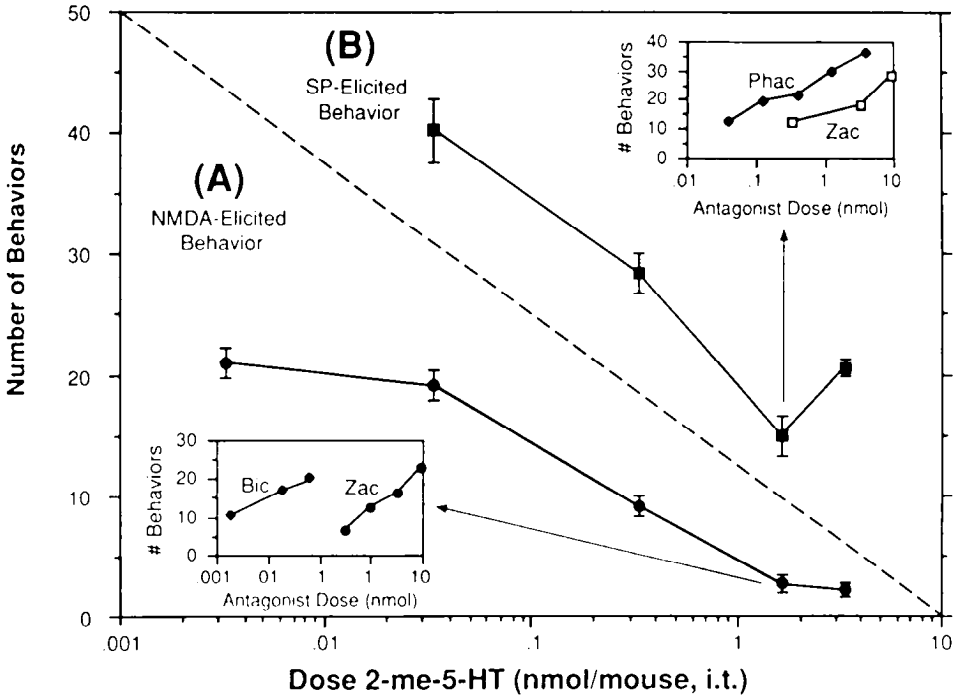

Figure 2. Behaviors elicited by NMDA (i.t.; biting; *A*) and SP (i.t.; biting and scratching; *B*) were inhibited by 2-methyl 5-HT in a dose-related manner. Zacopride (*Zac*) blocked the actions of 2-methyl 5-HT on both SP- and NMDA-induced behaviors (*insets* in *A* and *B*). In the case of NMDA-induced behavior, the effect of 2-methyl 5-HT was blocked by bicuculline (*Bic*; *inset* in *A*; 6–39 pmol/mouse, i.t.), but not by phaclofen (*Phac*; 4 nmol/mouse, i.t.) or 5-AVA (0.1 nmol/mouse, i.t.; $GABA_B$ antagonist; data not shown). On the other hand, inhibition of SP-induced behavior by 2-methyl 5-HT was blocked by phaclofen (*inset* in *B*) and 5-AVA (0.1 nmol/mouse, i.t.; data not shown) but not by bicuculline (39 pmol/mouse, i.t.). In the absence of 2-methyl 5-HT, these doses of antagonists were without effect. *Error bars* represent SEM.

Electrophysiology

Twenty-two spinal cord projection neurons (LT, 2; WDR, 12; HT, 8) were identified in 12 rats. This study was conducted as part of a larger study of 111 spinal projection neurons (Lei and Wilcox, unpublished observations); the responses to natural stimulation, NMDA, and AMPA reported here are typical of the responses to be reported in that larger study. The responses are also consistent with those reported in a previous study from this laboratory; that study showed that nociceptive neurons consistently respond to iontophoretically applied NMDA (Aanonsen et al., 1990). All 22 neurons examined in the present study responded to either NMDA or AMPA, 20 were nociceptive, and 12 responded to both agents. The responses to NMDA and AMPA had short onset latency (<5 sec) and duration (<5 sec after termination). All neurons also responded to cutaneous stimulation of the ipsilateral hindpaw and to antidromic activation from lateral upper cervical white matter.

2-Methyl 5-HT inhibited in a current-related manner NMDA-induced excitation in all 20 neurons excited by NMDA (Fig. 3). The inhibitory effect of 2-methyl 5-HT was blocked by zacopride in all six cells tested (Fig. 3*A*); zacopride alone usually increased NMDA-elicited firing as shown in Figure 3*A*, which is consistent with the behavioral data and suggests the presence of tonic serotonergic activity in these rats. 2-Methyl 5-HT inhibited AMPA-induced excitation less frequently (9 of 14; Fig. 4*B*) than NMDA-induced excitation (20 of 20; Fig. 4*A*). An example of one neuron excited by both excitants is shown in Figure 4, *A* and *B*. In 5 of 6 neurons where the two EAA agonists were compared for susceptibility to 2-methyl 5-HT, NMDA-elicited firing was more sensitive to inhibition by 2-methyl 5-HT than that of AMPA [data, expressed as (% inhibition of NMDA)/(% inhibition of AMPA), for the five neurons for which NMDA activation was more susceptible: 75/26, 77/59, 86/49, 84/68, and 40/30; for the sixth neuron, 57/90]. Muscimol similarly inhibited all EAA-induced excitation (5 of 5 for NMDA-induced excitation; Fig. 4*C*; 3 of 3 for AMPA-induced excitation; data not shown). In all six neurons tested, bicuculline reduced the inhibitory effect of 2-methyl 5-HT on NMDA-elicited excitation, in four completely and two partially (Fig. 4*D*). In summary, nociceptive projection neurons were always inhibited (never excited) by 2-methyl 5-HT, and these inhibitory effects were blocked by either $GABA_A$ or 5-HT$_3$ antagonists.

Discussion

To our knowledge, these results represent the first combined behavioral and electrophysiological examination of the role of the 5-HT$_3$ receptor subtype in 5-HT's spinal antinociceptive action. We confirmed earlier studies showing that activation of 5-HT$_3$ receptors produces thermal antinociception and extended these results to encompass other behavioral tests in another species and electrophysiological measures of sensory neural activity. Our finding of serotonergic inhibition of EAA-induced firing (EAAs are generally thought to act postsynaptically) together with the recordings of IPSPs after activation of a putative serotonergic pathway (Giesler et al., 1981) suggests a postsynaptic site of action in the spinal cord dorsal horn. That this action could be prevented by $GABA_A$, $GABA_B$, or 5-HT$_3$ antagonists indicates the participation of these receptor subtypes in production of this antinociception. Consistency between behavioral and electrophysiological studies diminishes the possibility that drug-induced motor effects are responsible for our behavioral observations. The results of the current study are consistent with the idea that 2-methyl 5-HT releases GABA and are in agreement with the differential involvement of the GABA receptor subtypes in two behavioral tests.

Because 5-HT$_3$ receptors are ligand-gated cation channels, it is unlikely that they exert a direct inhibitory action in the CNS.

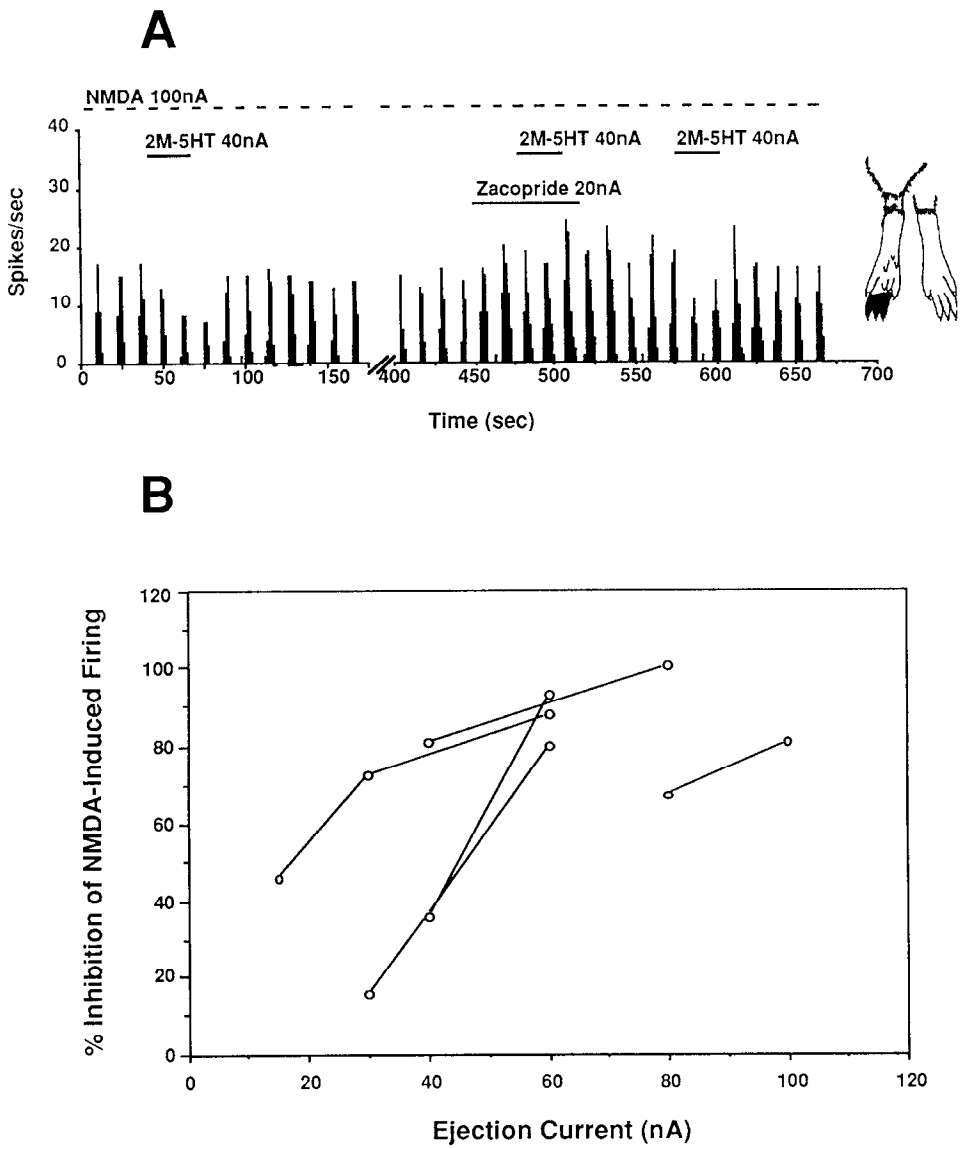

Figure 3. Typical effects of iontophoretically administered drugs (indicated by *horizontal bars*) on NMDA-elicited firing of spinal dorsal horn projection neurons are indicated in peristimulus-time histograms (1 sec/bin). The *ordinate* represents instantaneous firing rates (spikes/sec) of the neurons. *A*, 2-Methyl 5-HT (*2M-5HT*; 40 nA) inhibited NMDA-elicited excitation, and this effect was reversed by zacopride (20 nA); zacopride alone often increased firing slightly, and currents were set low enough to minimize this effect. *B*, 2-Methyl 5-HT inhibition was current dependent in all neurons tested with multiple currents ($N = 5$). All agents were given iontophoretically.

Therefore, the inhibition we observed following activation of these receptors is probably due to *excitation* of an inhibitory neuron. Our finding that GABA antagonists block the action of 5-HT_3 agonists in the spinal cord suggests that endogenously released 5-HT in the cord excites a neuron, which then releases GABA. We have diagrammed schematically how activation of 5-HT–containing descending tracts *might* release spinal GABA and how this GABA *might* inhibit nociceptive projection neurons in Figure 5; this tentative diagram, though not directly supported by the data, could account for both the behavioral and electrophysiological observations reported here. GABAergic interneurons in substantia gelatinosa (Hayes and Carlton, 1990) may be too small to be sampled by our relatively large recording electrode array. Indeed, a previous study using electrodes optimized to record from smaller interneurons found that 5-HT excited a preponderance of neurons in substantia gelatinosa (Todd and Millar, 1983). Previous studies have reported that $GABA_A$ antagonists do not reverse inhibition of spinal neurons elicited by NRM stimulation (Belcher et al., 1978; Griersmith et al., 1981). However, use in that study of nonselective EAA agonists and natural stimulation may account for this negative finding: if activation by the EAA agonists or by the synaptically released glutamate involved AMPA receptors, we would not expect synaptically released 5-HT to inhibit the activation completely. Alternatively, the NRM stimulation parameters used may have recruited nonserotonergic descending pathways.

We attribute the consistency of our results to (1) our use of 2-methyl 5-HT, which selectively activates 5-HT_3 receptors; (2) our use of selective agonists for EAA receptors as excitants (Aanonsen et al., 1990); and (3) our selection of projection neurons. Whereas 5-HT activates all 5-HT receptor subtypes and produces pro- as well as antinociceptive effects in behavioral tests, 2-methyl 5-HT activates one subtype selectively and produces only antinociceptive effects when injected intrathecally in mice (Wilcox and Alhaider, 1990). Although the selectivity of 2-methyl 5-HT for 5-HT_3 receptors is not absolute, its selectivity for 5-HT_3 over 5-HT_2 receptors in some assays approaches 1000:1 (Richardson et al., 1985). Our failure to see any evidence of scratching behavior, which seems to be mediated by 5-HT_2 receptors (Wilcox and Alhaider, 1990), even at high intrathecal

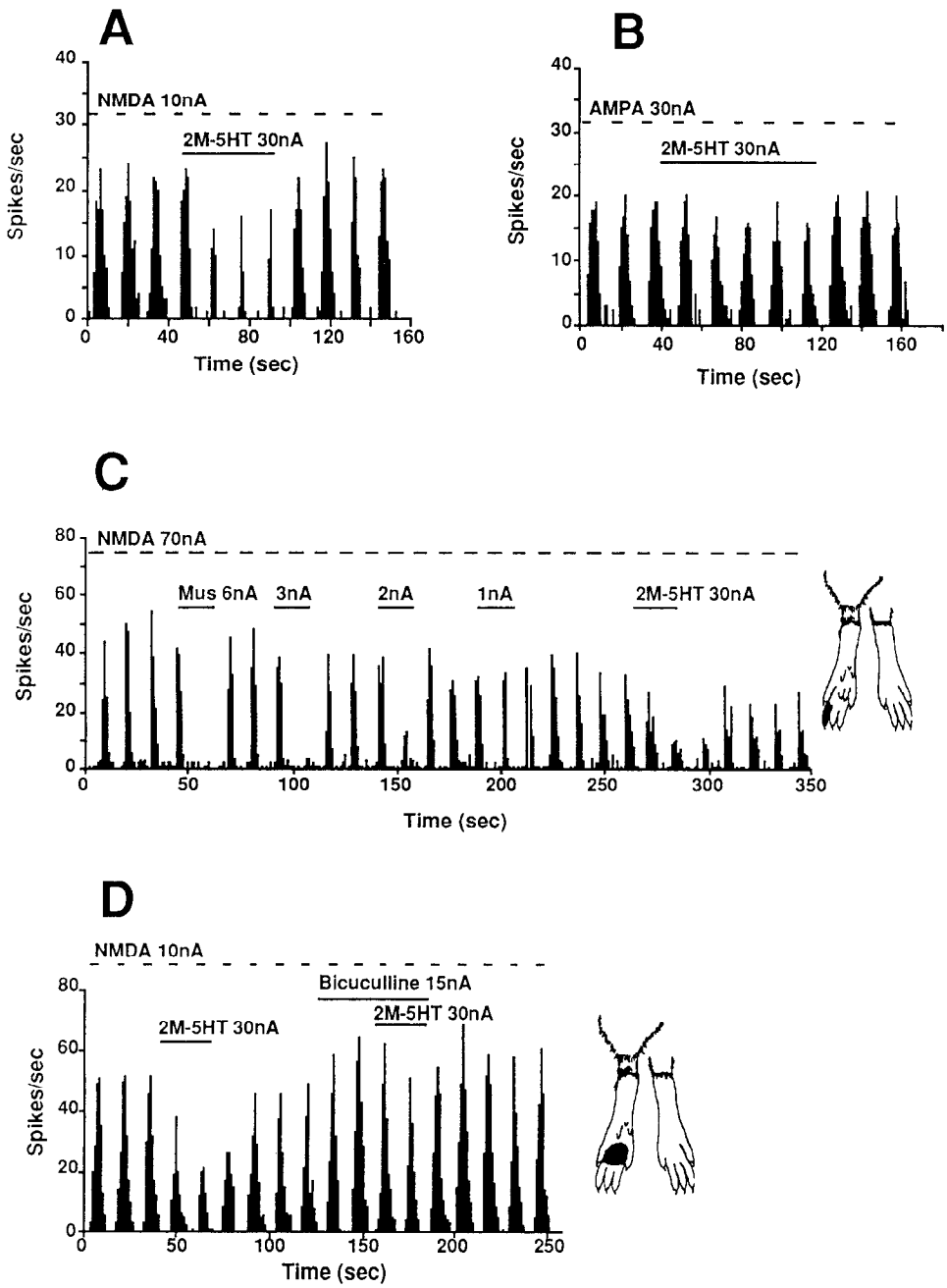

Figure 4. *A* and *B*, For a single WDR neuron, 2-methyl 5-HT (*2M-5HT*) inhibited NMDA-elicited firing (*A*) to a greater degree than AMPA-elicited firing (*B*); this effect was observed in most cases where AMPA was effective. *C*, Muscimol (*Mus*) also inhibited the neurons inhibited by 2-methyl 5-HT. *D*, Bicuculline (15 nA) partially blocked the inhibitory effect of 2-methyl 5-HT (30 nA) on NMDA-elicited firing on another cell; effective currents of bicuculline typically increased firing slightly as shown here. Reports of bicuculline blockade refer to reductions at least this large. All agents were given iontophoretically. Drawings at right describe extent of excitatory receptive fields for each neuron.

doses of 2-methyl 5-HT supports our contention that 2-methyl 5-HT is acting selectively at 5-HT$_3$ receptors in the rodent spinal cord. The apparently pronociceptive action of high doses of zacopride (NMDA-like biting and scratching behavior) may result from disinhibition of neurons that are under the inhibitory influence of tonically released 5-HT. Although we have not excluded the possibility that zacopride activates 5-HT$_2$ receptors as an agonist to produce such behavior, we think this unlikely because zacopride-induced behavior appeared qualitatively more like NMDA-elicited behavior than like behavior elicited by α-methyl 5-HT (a 5-HT$_2$ agonist; Wilcox and Alhaider, 1990).

The effects of 2-methyl 5-HT in the tail-flick test were completely blocked by zacopride and the GABA$_A$ antagonists bicuculline and picrotoxin, only partially blocked by the GABA$_B$ antagonist phaclofen, and not blocked at all by the opioid antagonist naloxone. It appears, therefore, that tail-flick antinociception produced by 2-methyl 5-HT relies more on GABA$_A$ than GABA$_B$ receptors, and that opioid receptors are not involved. Bicuculline also blocked the inhibitory effect of 2-methyl 5-HT on NMDA-induced behavior (Fig. 2*A*, inset), whereas phaclofen was ineffective. In contrast, phaclofen blocked the inhibitory effect of 2-methyl 5-HT on SP-induced behavior (Fig. 2*B*, inset), while bicuculline was inactive. We have previously found that NMDA-induced behavior can be blocked by GABA$_A$ (but not GABA$_B$) agonists (Aanonsen and Wilcox, 1989), while SP-induced behavior can be blocked by GABA$_B$ (but not GABA$_A$) agonists (Hwang and Wilcox, 1989). We therefore conclude from the behavioral studies with GABA antagonists that

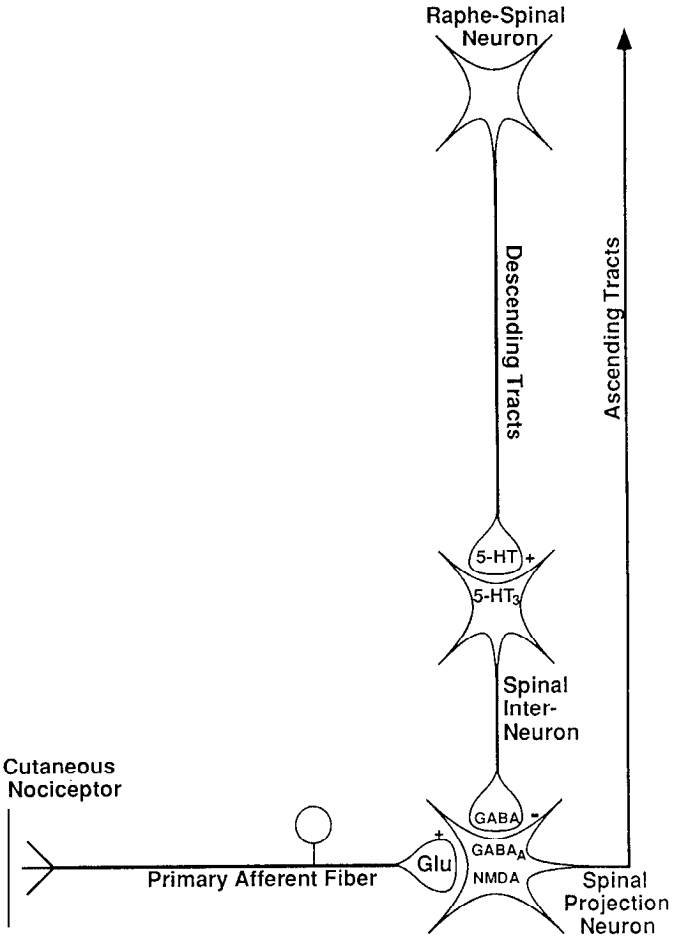

Figure 5. A hypothetical neuronal arrangement that accounts for our experimental findings. Raphe-spinal neurons (e.g., in NRM) presumably release 5-HT in the spinal cord, and the released 5-HT activates 5-HT_3 receptors on GABAergic interneurons. GABA released by these interneurons acts postsynaptically to inhibit nociceptive projection neurons; in the case of NMDA-mediated activation, $GABA_A$ receptors appear to be most effective. Both the behavioral and the electrophysiological data reported here support the synaptic arrangement around the spinal projection neuron. The raphe-spinal connection shown is based on the work of others (Belcher et al., 1978; Giesler et al., 1981; Griersmith et al., 1981), and the selective association between $GABA_B$ and SP receptors suggested by our behavioral data is not diagrammed.

2-methyl 5-HT may facilitate the release of GABA; the released GABA then inhibits NMDA- and SP-induced behavior at $GABA_A$ and $GABA_B$ receptors, respectively.

The 5-HT_3 receptor may represent an important mediator of the descending serotonergic control of nociceptive transmission through the dorsal horn, particularly that component of afferent excitatory transmission mediated through NMDA receptors. We have found that NMDA receptors are important in nociceptive neurotransmission in the dorsal horn (Aanonsen et al., 1990), and others have recently found that this NMDA component may mediate "wind-up" phenomena elicited by repeated stimulation (Mendell, 1966; Davies and Lodge, 1987; Thompson et al., 1990). This wind-up phenomenon may contribute to strong pain sensations in humans (Jorum et al., 1990). We have found that this component of the afferent nociceptive message is particularly susceptible to μ-opioid agonists (Aanonsen and Wilcox, 1987; Lei and Wilcox, 1989); it is well accepted that agents in this class are the most effective and addicting analgesics (Jaffe, 1985). The promising results of the current experiments suggest that intrathecal administration of 5-HT_3 agonists or simultaneous activation of descending serotonergic systems and potentiation of GABAergic neurotransmission may be useful alternatives to addicting opioid analgesics. Further biochemical studies of GABA release and functional studies of interactions between 5-HT_3 receptors and adrenergic antinociceptive systems are warranted.

References

Aanonsen LM, Wilcox GL (1987) Nociceptive action of excitatory amino acids in the mouse: effects of spinally administered opioids, phencyclidine and sigma agonists. J Pharmacol Exp Ther 243:9–19.

Aanonsen LM, Wilcox GL (1989) Muscimol, γ-aminobutyric $acid_A$ and excitatory amino acids in the mouse spinal cord. J Pharmacol Exp Ther 248:1034–1038.

Aanonsen LM, Lei S, Wilcox GL (1990) Excitatory amino acid receptors and nociceptive neurotransmission in rat spinal cord. Pain 41:309–321.

Alhaider AA, Kitto KF, Wilcox GL (1990) Nociceptive nodulation by intrathecally administered 5-HT_{1A} and 5-HT_{1B} agonists in mice. FASEB J 4:A988.

Basbaum AI, Fields HL (1978) Endogenous pain control mechanisms: review and hypothesis. Ann Neurol 4:451–462.

Belcher G, Ryall RW, Schnaffner R (1978) The differential effects of 5-HT, noradrenaline and raphe stimulation on nociceptive and non-nociceptive dorsal horn interneurons in the cat. Brain Res 151:307–321.

Bradley PB, Engel G, Feniuk W, Fozard JR, Humphrey PA, Middlemiss DN, Mylecharance E (1986) Proposals for the classification and nomenclature of functional receptors for 5-hydroxytryptamine. Neuropharmacology 25:563–576.

Clatworthy A, Williams JH, Barasi S (1988) Intrathecal 5-HT and electrical stimulation of the nucleus raphe magnus in rats both reduce the antinociceptive potency of intrathecally administered noradrenaline. Brain Res 455:300–306.

Cridland RA, Henry JL (1986) Comparison of the effects of substance P, neurokinin A, physalaemin and eledoisin in facilitating a nociceptive reflex in the rat. Brain Res 381:93–99.

D'Amour FE, Smith DL (1941) A method for determination of pain sensation. J Pharmacol Exp Ther 72:74–79.

Davies JE, Roberts MHT (1981) 5-Hydroxytryptamine reduces substance P responses on dorsal horn interneurones: a possible interaction of neurotransmitters. Brain Res 217:399–404.

Davies SN, Lodge D (1987) Evidence for involvement of NMDA receptors in "wind-up" of class 2 neurons in the dorsal horn of rat. Brain Res 424:402–406.

Derkach V, Surprenant A, North RA (1989) 5-HT_3 receptors are membrane ion channels. Nature 339:706–709.

El-Yassir N, Fleetwood-Walker SM, Mitchell R (1988) Heterogeneous effects of serotonin in the dorsal horn of rat: the involvement of 5-HT_1 receptor subtypes. Brain Res 456:147–158.

Fasmer OB, Berge O-G, Hole K (1983) Similar behavioral effects of 5-hydroxytryptamine and substance P injected intrathecally in mice. Neuropharmacology 22:485–487.

Fields HL, Basbaum AI (1978) Brainstem control of spinal pain-transmission neurons. Annu Rev Physiol 40:217–248.

Fozard JP (1987) 5-HT: the enigma variations. Trends Pharmacol Sci 8:501–506.

Fozard JR, Gray JA (1989) 5-HT_{1c} receptor activation: a key step in the initiation of migraine? Trends Pharmacol Sci 10:307–309.

Frenk H, Bosut D, Urca G, Mayer DJ (1988) Is SP a primary afferent neurotransmitter for nociceptive input? I. Analysis of pain-related behaviors resulting from intrathecal administration of SP and six excitatory compounds. Brain Res 455:223–231.

Giesler GJ Jr, Gerhart KD, Yezierski RP, Wilcox TK, Willis WD (1981) Postsynaptic inhibition of primate spinothalamic neurons by stimulation in nucleus raphe magnus. Brain Res 204:184–188.

Glaum SR, Proudfit HK, Anderson EG (1989) Reversal of the antinociceptive effects of intrathecally administered serotonin in the rat by a selective 5-HT_3 receptor antagonist. Neurosci Lett 95:313–317.

Glaum SR, Proudfit HK, Anderson EG (1990) 5-HT_3 receptors modulate spinal nociceptive reflexes. Brain Res 510:12–16.

Griersmith BT, Duggan AW, North RA (1981) Methysergide and supraspinal inhibition of the spinal transmission of nociceptive information in the anesthetized cat. Brain Res 204:147–158.

Hamon M, Gallissot MC, Menard F, Gozlan H, Bourgoin S, Vergé D (1989) 5-HT_3 receptor binding sites are on capsaicin-sensitive fibers in the rat spinal cord. Eur J Pharmacol 164:315–322.

Hayes ES, Carlton SM (1990) Ultrastructural analysis of CGRP and GABA terminals on dynorphin cells in primate dorsal horn. Pain [Suppl] 5:S101.

Hwang AS, Wilcox GL (1986) Intradermal hypertonic saline-induced behavior as a nociceptive test in mice. Life Sci 38:2389–2396.

Hwang AS, Wilcox GL (1989) Baclofen, $GABA_B$ receptors and substance P in the mouse spinal cord. J Pharmacol Exp Ther 248:1026–1033.

Hylden JLK, Wilcox GL (1980) Intrathecal morphine in mice: a new technique. Eur J Pharmacol 67:313–316.

Hylden JLK, Wilcox GL (1981) Intrathecal substance P elicits a caudally directed biting and scratching behavior in mice. Brain Res 217: 212–215.

Hylden JLK, Wilcox GL (1983) Intrathecal serotonin in mice: analgesia and inhibition of a spinal action of substance P. Life Sci 33: 789–795.

Jaffe JH (1985) Drug addiction and drug abuse. In: The pharmacological basis of therapeutics, 7th ed (Gilman AG, Goodman LS, Rall TW, Murand F, eds), pp 532–581. New York: Macmillan.

Johnston SE, Davies J (1981) Descending inhibitions from nucleus raphe magnus and adjacent reticular formation to the dorsal horn of the rat are not antagonized by bicuculline or strychnine. Neurosci Lett 26:43–47.

Jordan LM, Kenshalo DR, Martin RF, Willis WD (1978) Depression of primate spinothalamic tract neurons by iontophoretic application of 5-hydroxytryptamine. Pain 5:135–142.

Jordan LM, Kenshalo DR, Martin RF, Haber LH, Willis WD (1979) Two populations of spinothalamic tract neurons with opposite responses to 5-hydroxytryptamine. Brain Res 164:342–346.

Jorum E, Holm E, Lundberg LE, Torebjork HE (1990) Temporal summation in nociceptive systems. Pain [Suppl] 5:S314.

Julius D, MacDermott AB, Axel R, Jessell TM (1988) Molecular characterization of a functional cDNA encoding the serotonin 1c receptor. Science 241:558–564.

Julius D, Huang KN, Livelli TJ, Axel R, Jessell TM (1990) The 5-HT_2 receptor defines a family of structurally distinct but functionally conserved serotonin receptors. Proc Natl Acad Sci USA 87:928–932.

Kilpatrick GJ, Jones BJ, Tyers MB (1987) Identification and distribution of $5HT_3$ receptors in rat brain using radioligand binding. Nature 330:746–748.

LaPorte AM, Lombard MC, Bourgoin S, Gozlan H, Vergé D, Hamon M (1991) Pre- and post-synaptic location of 5-HT_3 receptor in the rat spinal cord. Adv Biosci, in press.

Lei S, Wilcox GL (1989) Opioid inhibition of excitatory amino acid (EAA) agonist-induced excitation of rat spinal projection neurons. Soc Neurosci Abstr 15:733.

Loisy C, Beorchia S, Centonze V, Fozard JR, Schechter PJ, Tell CP (1985) Effects on migraine headache of MDL 7222, an antagonist at neuronal 5-HT receptors. Double-blind, placebo-controlled study. Cephalalgia 5:79–82.

Mendell LM (1966) Physiological properties of unmyelinated fiber projection to the spinal cord. Exp Neurol 16:316–332.

Murphy RM, Zemlan FP (1987) Differential effects of substance P on serotonin-modulated spinal nociceptive reflexes. Psychopharmacology 93:118–121.

Pedigo NW, Yamamura HI, Nelson DL (1981) Discrimination of multiple [^{3}H]5-hydroxytryptamine binding sites by the neuroleptic spiperone in rat brain. J Neurochem 36:220–226.

Peroutka SJ (1986) Pharmacological differentiation and characterization of 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1C} binding sites in rat frontal cortex. J Neurochem 47:529–540.

Peroutka SJ (1988) 5-HT receptor subtypes: molecular, biochemical and physiological characterization. Trends Neurosci 11:496–500.

Raymond JR, Fargin A, Middleton JP, Graff JM, Haupt DM, Caron MG, Lefkowitz RJ, Dennis VW (1989) The human 5-HT_{1A} receptor expressed in HeLa cells stimulates sodium-dependent phosphate uptake via protein kinase C. J Biol Chem 264:21943–21950.

Richardson BP, Engel G (1986) The pharmacology and function of 5-HT_3 receptors. Trends Pharmacol Sci 7:424–428.

Richardson, BP, Engel G, Donatsch P, Stadler PA (1985) Identification of serotonin M-receptor subtypes and their specific blockade by a new class of drugs. Nature 316:126–131.

Schmauss C, Hammond DL, Ochi JW, Yaksh TL (1983) Pharmacological antagonism of the antinociceptive effects of serotonin in the rat and spinal cord. Eur J Pharmacol 90:349–357.

Solomon RE, Gebhart GF (1988) Mechanisms of effects of intrathecal serotonin on nociception and blood pressure in rats. J Pharmacol Exp Ther 245:905–912.

Thompson SWN, King AE, Woolf CJ (1990) Activity dependent changes in rat ventral horn neurons *in vitro*: summation of prolonged afferent evoked postsynaptic depolarizations produce a D-APV sensitive wind-up. Eur J Neurosci 2:638–649.

Todd AJ, Millar J (1983) Receptive fields and responses to iontophoretically applied noradrenaline and 5-hydroxytryptamine of units recorded in laminae I–III of cat dorsal horn. Brain Res 288:159–167.

Tollinson CD, Kriegel ML (1988) Selected tricyclic antidepressants in the management of chronic benign pain. South Med J 81:562–563.

Vaught JL, Scott R (1988) Interactions of substance P antagonists with serotonin in the mouse spinal cord. Peptides 9:909–913.

Wang JK (1977) Antinociceptive effect of intrathecally administered serotonin. Anesthesiology 47:269–271.

Watkins JC, Krogsgaard-Larsen P, Honoré T (1990) Structure–activity relationships in the development of excitatory amino acid receptor agonists and competitive antagonists. Trends Pharmacol Sci 11:25–33.

Wilcox GL (1988) Pharmacological studies of grooming and scratching behavior elicited by spinal substance P and excitatory amino acids. Ann NY Acad Sci 525:228–236.

Wilcox GL (1991) Excitatory neurotransmitters and pain. In: Pain research and clinical management: proceedings of the 6th world congress on pain (Bond M, Woolf CJ, Charlton JE, eds), pp 97–117. Amsterdam: Elsevier.

Wilcox GL, Alhaider AA (1990) Nociceptive and antinociceptive action of serotonin agonists administered intrathecally. Exerpta Med Int Congr Ser 879:205–219.

Yaksh TL, Tyce GM (1979) Microinjections of morphine into the periaqueductal gray evokes the release of serotonin from spinal cord. Brain Res 171:176–181.

Yaksh TL, Wilson PR (1979) Spinal serotonin terminal system mediates antinociception. J Pharmacol Exp Ther 208:446–453.

Yezierski RP, Wilcox TK, Willis WD (1982) The effects of serotonin antagonists on the inhibition of primate spinothalamic tract cells produced by stimulation in nucleus raphe magnus or periaqueductal gray. J Pharmacol Exp Ther 220:266–277.

Zemlan FP, Kow L-M, Pfaff DW (1983) Spinal serotonin receptor subtypes and nociception. J Pharmacol Exp Ther 226:477–485.