Monoamine-Dependent, Opioid-Independent Antihypersensitivity Effects of Intrathecally Administered Milnacipran, a Serotonin Noradrenaline Reuptake Inhibitor, in a Postoperative Pain Model in Rats

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ABSTRACT

The neurotransmitters serotonin (5-HT) and noradrenaline (NA) have important roles in suppressing nociceptive transmission in the spinal cord. In the present study, we determined the efficacy and nature of the antihypersensitivity effects of milnacipran, a 5-HT and NA reuptake inhibitor (SNRI), in the spinal cord in a rat model of postoperative pain. Sprague-Dawley rats were used in all experiments. An incision was made on the plantar aspect of the hind paw. Mechanical hypersensitivity was measured by determining the withdrawal threshold to von Frey filaments applied to the paw. Drugs were administered intrathecally 24 h after paw incision. Microdialysis studies of the dorsal horn of the lumbar spinal cord were also performed to measure 5-HT and NA levels after systemic injection of milnacipran. Milnacipran (1–30 μg) produced dose-dependent antihypersensitivity effects. The effect lasted 6 h after the 30-μg injection. Doses of 30 μg or less produced no abnormal behavior. The peak antihypersensitivity effect of 10 μg of milnacipran was blocked by intrathecal pretreatment with antagonists of the α₂-adrenoceptor (idazoxan; 30 μg) or 5-HT receptors (methysergide; 30 μg). Intrathecal pretreatment with 30 μg of naltrexone, a μ-opioid receptor antagonist, did not reverse the effect of milnacipran. Isobolographic analysis indicated antinociceptive synergism between milnacipran and morphine. Microdialysis studies revealed that milnacipran increased both 5-HT and NA levels in the spinal dorsal horn. These findings suggest that the antihypersensitivity effect of intrathecal milnacipran in the postoperative pain model is monoamine-mediated. Combined administration of an SNRI with morphine might be a promising treatment to suppress postoperative hypersensitivity.

Introduction

Bulbospinal descending noradrenaline (NA) and serotonin (5-HT) systems suppress nociceptive signals from primary afferent neurons to the spinal dorsal horn neurons. Intrathecal administration of adrenoceptor agonists and 5-HT receptor agonists produces antinociceptive effects on acute pain in rodents (Yaksh and Wilson, 1979; Reddy et al., 1980) and suppresses allodynia in a rat model of neuropathic pain (Yaksh et al., 1995; Obata et al., 2001). Antidepressants have antinociceptive effects and are widely used for the treatment of chronic pain. Antidepressants might inhibit chronic pain in the spinal cord by blocking NA or 5-HT reuptake (Sindrup et al., 2005). Among antidepressants, tricyclic antidepressants (TCAs) and 5-HT and NA reuptake inhibitors (SNRIs) are recommended for the management of neuropathic pain (Dworkin et al., 2007). The mechanisms of the antinociceptive effects of TCAs are complicated. TCAs inhibit the reuptake of NA and 5-HT at neuronal terminals (Sindrup et al., 2005). TCAs have antagonistic action at N-methyl-D-aspartate (NMDA) receptors (Reynolds and Miller, 1988), and inhibit adenosine reuptake (Phillis and Wu, 1982). Furthermore, most TCAs have affinity for opioid (Isenberg and Cicero, 1984), NA, 5-HT, histamine, and muscarinic acetylcholine receptors (Hall and Ogren, 1981). In contrast, SNRIs selectively inhibit the reuptake of NA and 5-HT without relevant affinity for any other receptors or ion channels (Sindrup et al., 2005). One representative SNRI is milnacipran, which is...
Microdialysis studies were performed to test whether the effect of milnacipran in the postoperative pain model involved \( \alpha_2 \)-adrenergic receptors (idazoxan), 5-HT receptors (methysergide), muscarinic receptors (atropine), and \( \mu \)-opioid receptors (naloxone). Saline or 30 \( \mu \)g of each antagonist was administered intrathecally 15 min before milnacipran injection. The dose of the antagonist was selected according to previous studies (Obata et al., 2005b). Drugs were administered intrathecally in a volume of 5 \( \mu \)l, followed by a 10-\( \mu \)l saline injection to flush the catheter. All drugs were dissolved in normal saline. Milnacipran was donated by the Asahi Kasei Corporation (Osaka, Japan). Other drugs were purchased from Sigma-Aldrich (St. Louis, MO).

Dose-response curves were generated from the peak effect at each dose after conversion of withdrawal thresholds to percentages of maximum possible effect (%MPEs), where %MPE = 100 \times (postdrug response − baseline)/preincision threshold − baseline). The area under the time course curves (AUCs) for the %MPE was then calculated from individual scores at each time point using the trapezoidal rule and divided by the maximal score that could be obtained over the observation period (% maximal possible AUC). Isobolographic analysis was performed to determine the type of interaction between milnacipran and morphine. The dose producing a 50% MPE (ED\(_{50}\)) was calculated from dose-response curves for each drug that reduced postoperative hypersensitivity. For drug combination dose responses, a fixed ratio combination of milnacipran and morphine was administered. The fixed ratio combination was based on the ratio of the ED\(_{50}\) value of milnacipran and morphine (1:0.018 w/w).

**Microdialysis Studies.** Microdialysis studies were performed with normal rats and rats with paw incision (24 h after incision). Anesthesia was induced with urethane (1.2–1.5 kg/kg i.p.) and maintained with 0.5% isoflurane in 100% oxygen through a nose cone. The left femoral vein was cannulated for drug injections. Rectal temperature was maintained at 37–38°C by a heating pad placed beneath the animal. The \( L_3-L_4 \) level of the spinal cord was exposed by a thoracolumbar laminectomy, and the rat was placed into a stereotaxic apparatus. After opening of the dura, a dorsal root that enters the spinal cord above the level of the recording sites was lifted using a glass retractor, so that a microdialysis probe could be advanced into the superficial layer of the dorsal horn. The probe was inserted from just lateral to the dorsal root and advanced at a 15° angle to a depth of 1 mm by using a micromanipulator (model WR-88, Narishige, Tokyo, Japan). The surface of the spinal cord was covered with mineral oil. Microdialysis probes were composed of a 1-mm length of hairpin-shaped dialysis membrane (o.d., 0.22 mm; i.d., 0.20 mm), and the membrane was attached to a 1-cm-silica double lumen.
tube (o.d., 0.35 mm; Eicom Co., Kyoto, Japan). The microdialysis probe was perfused with Ringer’s solution (147 mM NaCl, 4 mM KCl, and 2.3 mM CaCl₂) at a constant flow rate (1 μL/min) by using a syringe pump (ESP-64; Eicom Co.). After 120 min of constant perfusion, two consecutive samples were collected to determine basal NA and 5-HT concentrations in the dialysate. Milnacipran was administered intravenously to stably increase the drug concentration in the spinal cord. Saline (0.5 ml) or milnacipran (10 mg/kg) was administered through a femoral vein cannula, and the 15-min perfusate fractions were collected into an auto injector (EAS-20; Eicom Co.). Samples (15 μl) were automatically injected and analyzed for NA and 5-HT concentration by using high-performance liquid chromatography with electrochemical detection by an HTEC-500 analyzing system (Eicom Co.). The chromatographic conditions were as follows: the mobile phase consisted of 0.1 M ammonium acetate buffer, pH 6.0, and methanol [7:3 (v/v)] containing 0.05 M sodium sulfonate, and 50 mg/l EDTA-2Na. The column was an EICOMPAC CAX (2.0 × 200 mm; Eicom Co.). The detection limit of the assay is under 30 fentograms per injection (Eicom Co.).

Statistics. Data from behavioral and microdialysis studies were normally distributed and are shown as mean ± S.E.M. The time course data were analyzed using a two-way analysis of variance (ANOVA). Post hoc tests were completed for between-group comparisons at time points using a Student-Newman-Keuls post hoc test for multiple comparisons. A one-way ANOVA, followed by a Student-Newman-Keuls post hoc test was used for comparison of AUCs. The ED₅₀ value was calculated by linear regression analysis. The isobolograms were constructed as described previously (Tallarida et al., 1989). In isobolographic analysis, the theoretical additive points lie on a line connecting the ED₅₀ values of the each drug. Experimental values that lie on or near that line are considered to be generated by additive interactions. Values that lie below and to the left of this additive line are considered to be synergistic, whereas values that lie above and to the right of that line demonstrate a less than additive interaction. The difference between the theoretical additive point and the experimentally determined value was compared using the Student’s t test. A P value of less than 0.05 was considered to indicate statistical significance.

Results

Behavioral Studies. Intrathecal administration of milnacipran (1–30 μg) produced antihypersensitivity effects in a dose-dependent manner (P < 0.01 by two-way ANOVA; Fig. 1). The threshold increased within 15 min, and a peak effect was attained approximately 30 min after injection. The effect continued for 6 h after administration of 30 μg compared with the saline-treated group (P < 0.05). No adverse behavioral effects, such as motor effects, sedation, or agitation, were observed. The antihypersensitivity effect of 10 μg of milnacipran administered intrathecally was attenuated by idazoxan (P < 0.01; Fig. 2B). Although methysergide attenuated the peak effect of milnacipran (P < 0.01; Fig. 2A), it did not decrease the AUC of 10 μg of milnacipran (Fig. 2B). Intrathecal administration of idazoxan and methysergide alone at these doses produced slight agitation but did not alter the withdrawal threshold, which was 5.4 ± 0.5 g before idazoxan with a nadir of 4.9 ± 0.7 g after idazoxan, or 5.6 ± 0.5 g before methysergide with a nadir of 5.0 ± 0.8 g after methysergide. Neither atropine nor naloxone affected the antihypersensitivity effect of 10 μg of milnacipran (Fig. 2). Intrathecal administration of morphine also produced a dose-dependent antihypersensitivity effect at doses from 0.03 to 0.3 μg, and the effect of the maximal dose of morphine was completely reversed by intrathecal pretreatment with nalox-
one ($P < 0.01$ by two-way ANOVA; Fig. 3). The peak antihypersensitivity effects of intrathecal injection of the fixed ratio combination of the two drugs were observed 30 min after injection (Fig. 4A). Dose responses at the time of peak antihypersensitivity (30 min after injection) for milnacipran, morphine, and their combination are shown in Fig. 4B. The ED$_{50}$ (95% confidence interval) for milnacipran was $2.93$ ($1.93$–$4.09$)$\mu g$ and that for morphine was $0.053$ ($0.018$–$0.089$)$\mu g$. The ED$_{50}$ value of their combination was $0.41$ $\mu g$ (the ED$_{50}$ value for milnacipran and morphine was $0.40$ and $0.0074$ $\mu g$, respectively), which was only $27.9\%$ of the theoretical additive total dose ($1.47$ $\mu g$; $P < 0.05$). Isobolographic analysis indicated a synergistic interaction between milnacipran and morphine (Fig. 5).

**Microdialysis Study.** Baseline NA concentration before drug injection was not different between normal rats and rats with paw incision ($0.64 \pm 0.083$ pg/15 $\mu l$ in normal rats and $0.54 \pm 0.058$ pg/15 $\mu l$ in rats with paw incision, respectively). Baseline 5-HT concentrations before drug injection were also similar ($0.45 \pm 0.072$ pg/15 $\mu l$ in normal rats and $0.40 \pm 0.12$ pg/15 $\mu l$ in rats with paw incision, respectively). In the saline-treated group, NA and 5-HT concentrations in the dialysates did not change over time (Fig. 6). In the milnacipran (10 mg/kg i.v.)-treated group, NA concentrations increased within 30 min after the drug injection and reached approximately $300\%$ of the baseline value in normal rats, and the increase continued for more than 4 h after drug injection. The increase in NA concentration was smaller in rats with paw incision compared with the normal group ($P < 0.05$ by two-way ANOVA; Fig. 6A). The concentration of 5-HT also increased, but peaked 30 min after drug injection and then gradually decreased within 2 h. There was no difference between the normal and paw incision groups (Fig. 6B).

**Discussion**

The present study demonstrated that intrathecal administration of the SNRI milnacipran mediated a dose-dependent antihypersensitivity effect in a rat postoperative pain model. The maximal dose of milnacipran (30 $\mu g$) produced a long-lasting reversal of hypersensitivity with no adverse behavioral effects. The antihypersensitivity effects were atten-
ated by intrathecal pretreatment with an $\alpha_2$-adrenoceptor antagonist. The peak effect of milnacipran was also attenuated by a 5-HT receptor antagonist. Direct measurements of NA and 5-HT from the spinal dorsal horn with microdialysis revealed that milnacipran increased both NA and 5-HT levels. Spinal $\mu$-opioid receptors are not involved in the antihypersensitivity effect of milnacipran. Isobolographic analysis revealed that milnacipran interacts synergistically with intrathecal morphine.

Emerging literature suggests that postoperative pain exhibits a unique pharmacology of analgesia compared with other sustained pain models. For example, although spinal NMDA receptor antagonists attenuate hypersensitivity in most models of persistent pain, they are not effective for treating hypersensitivity after paw incision (Zahn and Brennan, 1998). In contrast, intrathecal administration of non-NMDA receptor antagonists (Zahn et al., 1998) or neurokinin-1 receptor antagonists (Yamamoto and Sakashita, 1999) are effective. Dorsal horn neurons are sensitized after incision, but this sensitization is completely reversed by intrathecal pretreatment with an $\alpha_2$-adrenoceptor antagonist. The peak effect of milnacipran was also attenuated by intrathecal pretreatment with methysergide attenuated the peak effect of milnacipran to a similar degree as idazoxan. These findings suggest that the antihypersensitivity effects of intrathecally administered milnacipran mainly rely on increased levels of NA in the spinal cord. Increased 5-HT may also play a role in the early phase of antihypersensitivity effects of milnacipran. This speculation is supported by the observations from the microdialysis studies that 10 mg/kg milnacipran injected intravenously preferentially inhibited NA reuptake in the spinal cord. In the present study, the NA increase after milnacipran injection in the postoperative pain model was smaller than that of normal rats. This result was contrary to findings in a rat model of neuropathic pain study showing increased basal NA level and noradrenergic axon sprouting in the spinal dorsal horn (Hayashida et al., 2008); however, sensitization and reorganization of the spinal cord are associated not only with neuropathic pain but also with postoperative pain. One possible cause of the smaller NA increase observed in the postoperative pain model is the disinhibition of the noradrenergic descending inhibitory system may occur after acute activation of the inhibitory system by paw incision.

A previous study demonstrated that muscarinic receptors are involved in the antihypersensitivity effects of intrathecally administered milnacipran in a neuropathic pain model, because atropine completely reversed the effects of milnacipran (Obata et al., 2005b). Activation of the spinal muscarinic receptors produces antinoceptive effects (Eisenach, 1999). The stimulation of cholinergic systems by intrathecal injection of $\alpha_2$-adrenoceptor agonists is widely documented. For example, intrathecal clonidine increases acetylcholine concentrations in the cerebrospinal fluid (De Kock et al., 1997). The reliance of clonidine-induced antinoception on

Fig. 6. Microdialysis to detect increased spinal NA (A) and 5-HT (B) levels. Normal rats ($n = 6$) or rats with paw incision ($n = 6$) received intravenous saline or milnacipran (10 mg/kg). Data are presented over time as a percentage of the baseline. *: $P < 0.05$ compared with saline-treated group. #: $P < 0.05$ compared with milnacipran-treated paw incision group (two-way ANOVA).
this spinal cholinergic interaction varies between normal and nerve-injured animals. Intrathecal clonidine-induced antinocepción to acute thermal stimuli in normal rats is unaffected by intrathecal atropine (Paqueron et al., 2003), but the reversal of hypersensitivity by intrathecal clonidine after nerve-injury is completely blocked by intrathecal atropine (Pan et al., 1999). Intrathecally administered 5-HT receptor agonists also interact with muscarinic receptors to inhibit neuropathic pain (Obata et al., 2003). In the present study, however, intrathecal pretreatment with atropine did not reverse the antihypersensitivity effect of milnacipran in the postoperative pain model. The result is consistent with a previous study showing that antihypersensitivity derived from selective spinal reuptake inhibition of NA is not reversed by intrathecal atropine (Obata et al., 2005a). We speculate that the muscarinic dependence of spinal NA/5-HT inhibitory systems increases progressively from postoperative hypersensitivity to nerve-injury-induced chronic hypersensitivity.

Recent studies suggest that the opioidergic system is involved in antidepressant-induced antinociception. For example, several types of systemically administered antidepressants produce antinociception that is reversed by systemically administered naloxone (Ardid and Guilbaud, 1992; Gray et al., 1998). Although direct and indirect actions of antidepressants on opioid receptors are suggested (Ardid and Guilbaud, 1992), the mechanisms and sites of this interaction are not clear. In the behavioral studies, we injected all drugs intrathecally in a small volume of saline (5 µl) to examine spinal mechanisms of milnacipran for postoperative pain. Although we cannot exclude drug effects at brain or dorsal root ganglia locations, drugs mainly act at the spinal level after intrathecal injection (Yaksh and Rudy, 1976). The data from the present study suggest that the antihypersensitivity effect of milnacipran does not involve the opioidergic system, least in the spinal cord, for two reasons. First, naloxone failed to reverse the antihypersensitivity effect of milnacipran; the dose of naloxone used in the present study (30 µg) was sufficient because pretreatment with naloxone completely reversed morphine-mediated antihypersensitivity. Second, the combination of milnacipran and morphine produced a synergistic interaction. Synergy usually indicates that the two drugs have different final pathways to produce their effect.

Interactions between milnacipran and morphine may also be important for a practical reason. Morphine is administrered epidurally or spinally to treat acute pain during the perioperative period (American Society of Anesthesiologists Task Force on Acute Pain Management, 2004). Because morphine therapy often induces adverse effects, such as pruritus, urinary retention, respiratory depression, nausea, and vomiting, the current observation of synergistic interactions between milnacipran and morphine suggest that the morphine dose and potentially its adverse effects could be reduced by the addition of milnacipran.

In conclusion, simultaneous inhibition of NA and 5-HT reuptake in the spinal cord by milnacipran effectively suppressed mechanical hypersensitivity after paw incision in rats. The antihypersensitivity effect of milnacipran in the postoperative pain model depends on monoaminergic, but not opioidergic, systems and differs from its effect in the neuropathic pain model. Isosoblographic analysis revealed that milnacipran interacted synergistically with morphine. Therefore, intrathecal or epidural injection of an SNRI combined with morphine might be a promising treatment for postoperative pain.

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References


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