Intrathecal Morphine Reduces Alloodynia after Peripheral Nerve Injury in Rats via Activation of a Spinal A1 Adenosine Receptor

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Background: The degree to which intrathecally administered morphine can alleviate hypersensitivity in animals after peripheral nerve injury is controversial, and the mechanisms by which morphine works in these circumstances are uncertain. In normal animals, morphine induces adenosine release, and in vitro data suggest that this link is disrupted after peripheral nerve injury. Therefore, using a controlled, blinded study design, the authors tested intrathecal morphine efficacy in rats with peripheral nerve injury and the role of spinal A1 adenosine receptors in the action of morphine.

Methods: Male rats underwent intrathecal catheter implantation and lumbar spinal nerve ligation, resulting in hypersensitivity to tactile stimulation of the paw. Intrathecal morphine alone or with naloxone or the specific A1 adenosine receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), was administered, and withdrawal threshold to von Frey filament application to the hind paw was determined.

Results: Intrathecal morphine (0.25–30 μg) dose-dependently reversed mechanical hypersensitivity after spinal nerve ligation, with an ED50 of 0.79 μg. The effect of morphine was blocked by intrathecal naloxone. Intrathecal DPCPX alone had no effect on withdrawal threshold after spinal nerve ligation but completely reversed the effect of morphine, with an ID50 of 5.6 μg.

Conclusions: This study is in accord with two recent reports that support short-term efficacy of intrathecal morphine to reverse hypersensitivity to mechanical stimuli in animal models of neuropathic pain. Despite previous studies demonstrating that morphine releases less adenosine after peripheral nerve injury, the current study suggests that the antihypersensitivity effect of morphine in these conditions is totally reliant on A1 adenosine receptor activation.

THE use of opioids to treat chronic pain remains controversial, in part because of concerns over addiction, tolerance, and opioid-induced hyperalgesia with long-term treatment. Surprisingly, opioid efficacy even with short-term administration has been questioned in patients with neuropathic pain and in animal models of neuropathic pain. For example, early clinical reports showed poor efficacy from normal doses of opioids to treat neuropathic pain, but opioids have subsequently been demonstrated to have excellent efficacy in rigorously controlled clinical trials.2 Similarly, early laboratory studies reported near complete lack of efficacy of intrathecal morphine, in doses active in normal animals, to alleviate hypersensitivity to mechanical stimuli in animals after peripheral nerve injury.5,6 whereas recent reports claim complete efficacy with much lower morphine doses.5,6 Although the reasons for this discrepancy in laboratory studies are unclear, we recently suggested that experimenter bias could have played a role.7 Therefore, one purpose of the current study was to test, using a blinded, controlled study design, the dose response for intrathecal morphine to reduce hypersensitivity to mechanical stimuli in rats with peripheral nerve injury.

A second purpose of the current study was to test whether intrathecal morphine reduces hypersensitivity by an action involving spinal adenosine release and A1 adenosine receptor activation. In support of this hypothesis, morphine induces adenosine release in the normal rat spinal cord,8 and morphine-induced antinociception in normal rats is reversed by A1 adenosine receptor antagonists.9,10 In addition, intrathecally administered adenosine lacks antinociceptive efficacy in normal animals but can be used effectively to treat hypersensitivity after spinal nerve ligation in rats.11,12 On the other hand, we recently demonstrated a right shift in the morphine concentration–dependent release of adenosine from spinal cord synaptosomes from rats after spinal nerve ligation13 and suggested that this decreased potency to release adenosine after nerve injury could underlie the lack of efficacy of morphine in this setting. To test the relevance of adenosine release and receptor activation in the effect of morphine in animals after spinal nerve ligation, we coadministered a highly selective A1 adenosine receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), with morphine.

Materials and Methods

Surgical Preparation
After approval by the Animal Care and Use Committee (Wake Forest University School of Medicine, Winston-Salem, North Carolina), male Sprague-Dawley rats weighing 200–250 g (Harlan, Indianapolis, IN) underwent spinal nerve ligation as previously reported.14 Briefly, during halothane anesthesia, the L5 and L6 spinal nerves were exposed and tightly ligated using 6-0 silk sutures distal to the dorsal root ganglion. Muscle layers and skin were closed using 4-0 silk sutures. After surgery, animals...
were housed individually in plastic cages in a climate-controlled room under a 12 h–12 h light–dark cycle, with free access to food and water. One week after nerve ligation, an intrathecal catheter was inserted as previously described.15 During halothane anesthesia, a 32-gauge polyethylene catheter (ReCathCo, Allison Park, PA) connected to a piece of Tygon external tubing (Saint-Gobain Performance Plastics, Akron, OH) was inserted 7.5 cm through the cisterna magna until the tip lay near the lumbar enlargement. Rats showing neurologic deficits were immediately euthanized by an overdose of pentobarbital. The remaining rats were allowed to recover 1 week before behavioral testing. After all of the behavioral experiments were completed, 10 μl lidocaine, 2%, was injected in some animals to confirm correct catheter position, as indicated by transient bilateral hind limb paralysis.

Behavioral Testing
Withdrawal threshold of the left paw was measured in response to application of von Frey filaments, using an up–down method.16 Hypersensitivity to tactile stimulation was confirmed 2 weeks after surgery, and animals with a withdrawal threshold greater than 4 g were excluded (2 of 25 rats). All behavioral testing was performed with the experimenter blinded to drugs and doses being administered.

On the day of the experiment, rats were placed in clear plastic boxes above a wire mesh floor. After acclimation for at least 20 min, calibrated von Frey filaments in log increments of force (0.69, 1.20, 1.48, 2.04, 3.63, 5.50, 8.51, 11.75, 15.14, and 28.84 g) were applied for a duration of 5 s to the midplantar aspect of the left paw, beginning with the 1.48-g filament. In addition to withdrawal threshold testing, animals were observed for overall behavior with special attention to any reduction in overall or hind limb movements.

Drugs and Drug Administration
All drugs for intrathecal injection were made freshly immediately before the experiments by an individual not performing the behavioral assessments. Morphine was diluted with saline. DPCPX was dissolved in dimethyl sulfoxide to a final concentration of 20 μg/μl as a stock solution and then diluted in saline just before injection. Dimethyl sulfoxide, 10%, was used as vehicle. Under gentle restraint, intrathecal drug injections were performed using a 100-μl Hamilton syringe. The drug or vehicle injection (10 μl) was followed by a 10-μl saline flush. Each group consisted of six to eight rats, and each rat received a maximum of four drug injections, with experiments separated by 1 week. Rats were randomly divided into at least two groups in each series to test the effects of vehicle, DPCPX (10 μg), morphine (0.25, 0.5, 1, 2.5, and 5 μg), and combinations (2.5 or 5 μg morphine with 3, 5.5, and 10 μg DPCPX). In addition, some animals received 5 μg intrathecal naloxone 15 min before 5 μg morphine injection. Finally, to test whether higher doses of morphine alone produced excitation rather than inhibition, 16 rats were randomly assigned to receive intrathecal saline (n = 2), 10 μg morphine (n = 6), and 30 μg morphine (n = 8). Withdrawal threshold was determined before and every 30 min for 4 h after injection. Dose–response curves were constructed at the time of peak effect and were converted to percentage maximal possible effect (%MPE) according to the formula %MPE = (post drug threshold – baseline threshold)/(presurgery threshold – baseline threshold) × 100.

Morphine sulfate was purchased from Astra Pharmaceutical Inc. (Westborough, MA). DPCPX was purchased from RBI Inc. (Natick, MA). Dimethyl sulfoxide and the remaining chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Statistical Analysis
Withdrawal thresholds and %MPE were normally distributed and are presented as mean ± SE. Sigmoidal nonlinear regression curve fitting for dose–response data was performed, and the dose of morphine producing a 50% maximum efficacy (ED_{50}) or that of DPCPX producing a 50% maximum inhibition of morphine (ID_{50}) and their 95% confidence limits were calculated. Time-course and dose–response effects of morphine and DPCPX were analyzed using two-way repeated-measures analysis of variance with the Student-Newman-Keuls post hoc test. P < 0.05 was considered significant.

Results
Before spinal nerve ligation, the withdrawal threshold was 20.4 ± 0.4 g (n = 23). Two weeks after nerve ligation, the withdrawal threshold decreased significantly to 1.9 ± 0.2 g and remained unchanged over the maximum 4 weeks of study.

Efficacy of Intrathecal Morphine
Morphine dose-dependently reversed hypersensitivity to mechanical simulation induced by spinal nerve ligation, with a peak effect 30 min after injection, whereas vehicle had no effect (fig. 1, left). Two-way repeated-measures analysis of variance showed that all doses except 0.25 μg morphine increased withdrawal threshold compared with vehicle 30 min after injection. The dose response for morphine was sigmoidal in the log rhythmic domain, with an ED_{50} of 0.79 μg (95% confidence interval, 0.75–0.88 μg; fig. 1, right). The just maximum effective dose of morphine was 2.5 μg (fig. 1, right). In separate studies with higher doses of morphine, saline was ineffective, whereas 10 μg morphine produced a 95 ± 3% MPE effect and 30 μg morphine produced a 100% MPE effect.
When preceded by vehicle injection, 5 \( \mu \text{g} \) intrathecal morphine increased withdrawal threshold from 2.1 \( \pm \) 0.9 to 22.3 \( \pm \) 0.8 g, whereas when preceded by 5 \( \mu \text{g} \) naloxone, this same dose of morphine did not alter withdrawal threshold (1.9 \( \pm \) 0.9 g before and 2.9 \( \pm \) 1.9 g after morphine). Thus, naloxone completely prevented the antihypersensitivity effect of a dose of morphine (5 \( \mu \text{g} \)) twice the just maximum effective dose.

Reversal of Effects of Morphine by DPCPX

DPCPX dose responses were performed against a just maximum effective dose of morphine (2.5 \( \mu \text{g} \)) and a dose twice this large. Against the 2.5-\( \mu \text{g} \) dose, all doses of DPCPX reduced the efficacy of morphine (fig. 2, left), whereas all but the lowest dose of DPCPX (3 \( \mu \text{g} \)) reduced the effect of 5 \( \mu \text{g} \) morphine (fig. 2, right). Maximum inhibition achieved was 70–80% (fig. 3), and the ID\(_{50}\) for DPCPX was 5.6 \( \mu \text{g} \) (95% confidence interval, 4.8–6.6 \( \mu \text{g} \)) against the 2.5-\( \mu \text{g} \) dose of morphine and 7.4 \( \mu \text{g} \) (95% confidence interval, 6.5–8.9 \( \mu \text{g} \)) against the 5-\( \mu \text{g} \) dose of morphine. These ID\(_{50}\) doses differ by \( t \) test \((P < 0.05)\). None of the treatments altered gross or hind limb movements, and animals ambulated normally when removed from the testing chamber.

Discussion

The potency and efficacy of morphine in the current study surprised us because we observed only minor efficacy (< 30% MPE) after a large intrathecal morphine dose (30 \( \mu \text{g} \)) in a previous study.\(^{17}\) Although it is theoretically possible that this large dose, also used in other laboratory studies in which morphine did not alter withdrawal threshold after spinal nerve ligation,\(^{3,4}\) could have induced excitatory phenomena or acute hyperalgesia, hiding the efficacy of morphine, this was not observed in the current study. Rather, the current results are similar to recent reports of intrathecal morphine efficacy after nerve injury. In rats younger than those studied in the current protocol (140–160 g), the ED\(_{50}\) for morphine was 0.65 \( \mu \text{g} \),\(^{5}\) and the ED\(_{50}\) for morphine in the spared nerve injury model in adult rats was similar, at 0.52 \( \mu \text{g} \).\(^{6}\) Although there are methodologic differences among these studies, the ED\(_{50}\) values for morphine to relieve hypersensitivity to tactile mechanical stimuli are remarkably consistent, and the values obtained in these previous studies are within the confi-

Fig. 1. (Left) Withdrawal threshold to von Frey filament application to animals before spinal nerve ligation (time before 0) and after injection, at time 0 of 0.25–5.0 \( \mu \text{g} \) intrathecal morphine. Morphine significantly increased withdrawal thresholds for all doses except 0.25 \( \mu \text{g} \). Each symbol represents the mean \( \pm \) SE of six to eight rats. (Right) Dose response for intrathecal morphine to alleviate hypersensitivity after spinal nerve ligation, calculated as the percent maximum possible effect (%MPE), which is the percent return to presurgical threshold. The line represents the sigmoidal fit of the data \((R = 0.97, P < 0.001)\).

Fig. 2. Withdrawal threshold to von Frey filament application to animals before spinal nerve ligation (time before 0) and after injection, at time 0 of intrathecal DPCPX, 10 \( \mu \text{g} \) alone or in doses of 3–10 \( \mu \text{g} \) with 2.5 \( \mu \text{g} \) (left) or 5 \( \mu \text{g} \) (right) morphine. DPCPX alone did not affect withdrawal threshold, morphine significantly increased withdrawal threshold, and DPCPX reduced the effect of morphine. Each symbol represents the mean \( \pm \) SE of six to eight rats.

Fig. 3. Dose response for intrathecal DPCPX to inhibit the action of morphine on withdrawal threshold after spinal nerve ligation. The lines represent the sigmoidal fit of the data \((R = 0.92–0.96, P < 0.001)\).

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We previously noted that inhibition of adenosine metabolism in the spinal cord increased the efficacy of intrathecal morphine in rats with spinal nerve ligation,\(^1\) consistent with the hypothesis that morphine’s mechanism of action to reduce hypersensitivity in these animals involves adenosine synthesis or release in the spinal cord. This follows logically from observations that morphine increases adenosine release from spinal cord synaptosomes from normal animals,\(^8\) primarily from capsaicin-sensitive primary afferent terminals,\(^21\) and that antinociception from intrathecal morphine is blocked by adenosine receptor antagonists.\(^22\) In addition, spinal adenosine seems to play a key role in hypersensitivity states. Therefore, in normal rats and mice, intrathecal injection of adenosine receptor antagonists induces hypersensitivity,\(^22,23\) and intrathecal injection of adenosine reduces hypersensitivity, including mechanical allodynia with neuropathic pain, in rats and humans.\(^11,12,24–26\)

The current observation, that the effect of intrathecal morphine in animals with spinal nerve ligation is essentially abolished by intrathecal DPCPX, adds significantly to these observations. We chose to probe A1 adenosine receptors using DPCPX because this subtype is involved in spinal antinociception in normal and nerve-injured animals.\(^27,28\) DPCPX produced a dose-dependent inhibition of the effect of morphine in the current study, with an ID\(_{50}\) that increased with increasing dose of morphine, consistent with physiologic antagonism. This profound reliance of the action of morphine on spinal activation of adenosine receptors is unexpected, given our previous results in spinal cord synaptosomes of decreased potency of morphine to release adenosine after spinal nerve ligation. Several explanations are possible. Because the morphine concentration response to release adenosine in synaptosomes was shifted to the right after spinal nerve ligation without an effect on slope,\(^13\) it is conceivable that this effect is overcome by the very high local tissue concentrations of morphine after intrathecal injection. Alternatively, intrathecal morphine could result in adenosine receptor activation via an indirect pathway or circuit not measured in vitro in synaptosomes.

Finally, we did not observe increased hypersensitivity with intrathecal injection of DPCPX alone, unlike observations in normal animals.\(^22,25\) These previous observations suggested a tonic inhibitory function of spinal adenosine in the normal condition, and one might expect this inhibitory function is further enhanced after spinal nerve ligation because in vitro studies of G-protein signaling indicate an increased basal inhibitory G-protein coupling due to tonic A1 adenosine receptor activation after spinal nerve ligation.\(^29\) It is conceivable that the small dynamic range of von Frey filaments used at the very small forces in the hypersensitive animals after spinal nerve ligation reduced the sensitivity to observe further enhanced hypersensitivity from DPCPX to test this hypothesis.

In summary, this study confirms recent observations that intrathecal morphine reduces hypersensitivity to mechanical stimuli in rats after spinal nerve ligation with a potency similar to that in models of inflammation and postoperative pain. Morphine works via opioid receptor activation and relies essentially on direct or indirect release of adenosine, which acts on an A1 adenosine receptor. These data suggest that intrathecal opioids may be effective short-term treatments for neuropathic pain and that drugs that reduce adenosine uptake or metabolism may further increase opioid potency in such patients.

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