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Nerve Injury Induces a Tonic Bilateral μ-Opioid Receptor–mediated Inhibitory Effect on Mechanical Allodynia in Mice

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Background: **Mice lacking the μ-opioid receptor gene have been used to characterize the role of μ-opioid receptors in nociception and the analgesic actions of opioid agonists. In this study, the authors determined the role of μ-opioid receptors in neuropathic pain behaviors and the effectiveness of μ- and κ-opioid receptor agonists on this behavior in mice.**

Methods: **The authors studied the behavioral responses of μ-opioid receptor knockout and wild-type mice to thermal and mechanical stimuli before and after neuropathic pain induced by unilateral ligation and section of the L5 spinal nerve. Response to mechanical stimuli was evaluated by determining the frequency of hind paw withdrawal to repetitive stimulation using a series of von Frey monofilaments. Thermal hyperalgesia was assessed by determining the paw withdrawal latencies to radiant heat and frequency of hind paw withdrawal to cooling stimuli. The effects of systemic morphine, the κ-opioid agonist U50488H, and naloxone on responses to mechanical and thermal stimuli were also studied in spinal nerve–injured mice.**

Results: **After spinal nerve injury, wild-type mice developed increased responsiveness to mechanical, heat, and cooling stimuli ipsilateral to nerve injury. μ-Opioid receptor knockout mice not only had more prominent mechanical allodynia in the nerve-injured paw, but also expressed contralateral allodynia to mechanical stimuli. Hyperalgesia to thermal stimuli was similar between μ-opioid knockout and wild-type animals. Morphine decreased mechanical allodynia dose dependently (3–30 mg/kg subcutaneous) in wild-type mice—an effect that was attenuated in the heterozygous mice and absent in the homozygous μ-opioid knockout mice. The κ-opioid agonist U50488H (3–10 mg/kg subcutaneous) attenuated mechanical allodynia in wild-type, heterozygous, and homozygous μ-opioid mice. Naloxone in wild-type mice resulted in enhanced ipsilateral and contralateral allodynia to mechanical stimuli that resembled the pain behavior observed in μ-opioid receptor knockout mice.**

Conclusions: **The authors' observations indicate that (1) unilateral nerve injury induces a bilateral tonic activation of endogenous μ-opioid receptor–mediated inhibition that attenuates mechanical allodynia but not thermal hyperalgesia, (2) both μ- and κ-opioid agonists attenuate neuropathic pain in mice, and (3) the antihyperalgesic actions of morphine are mediated primarily *via* μ-opioid receptors.**

NEUROPATHIC pain has been reported to respond poorly to treatment with opioids, a phenomenon that is in contrast to the enhanced effect of opioid agonists in inflammatory pain.[1–3] Recent clinical studies, however, indicate that neuropathic pain is not resistant to opioids, and patients with peripheral and central neuropathic pain may benefit from therapy with oral opioids.[4–6] Animal studies have reported both increased and decreased effects of opioids on neuropathic pain.[7–11] These discrepancies in pharmacologic studies may in part be due to lack of *in vivo* selectivity of various opioid compounds. Hence, it is difficult to exclude that the analgesic effects of opioids considered to be highly selective for a given receptor are not confounded by their actions on other receptor subtypes *in vivo*.[12]

Recently, mice lacking μ-, δ-, or κ-opioid receptors have been produced by homologous recombination. Studies in these animals have provided new insights on the roles of the different opioid receptors. The analgesic effects of morphine have been shown to be mediated primarily by μ-opioid receptors as determined on various acute thermal and mechanical nociceptive tests.[13–15] κ-Opioid receptor–mediated analgesia is reported to be unaffected in μ-opioid receptor knockout mice.[15,16] In contrast, δ-opioid receptor agonist–mediated analgesia has been shown to be reduced in μ-opioid receptor knockout mice, indicating an interaction between μ- and δ-opioid receptors.[15–17]

Phenotypic changes in dorsal root ganglia and spinal and supraspinal neurons have been observed after inflammation and peripheral nerve injury.[18,19] After inflammation and polyarthritis, an increase in density of spinal opiate receptors, synthesis of opioid peptides, and functional activity of opiate receptors have been reported.[20–22] Persistent inflammation results in increased endogenous opioid tone and enhanced antihyperalgesic and antinociceptive effects of exogenous opioid agonists.[23,24] It is unclear whether similar alterations in opioid receptor activity plays a role in the mechanisms of neuropathic pain resulting from nerve injury.

Spinal nerve injury in rats results in a neuropathic pain–like behavior characterized by hyperalgesia to mechanical and thermal stimuli.[25,26] This animal model has been used to study the underlying mechanisms of neuropathic pain and to help develop rational therapies for clinical neuropathic pain. To better understand the role

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of μ-opioid receptors in neuropathic pain, we compared the neuropathic pain behavior that develops after spinal nerve injury in μ-opioid receptor knockout and wild-type control mice. Complementary pharmacologic studies with systemic administration of naloxone were conducted in wild-type mice to determine whether nerve injury results in opioid receptor–mediated tonic inhibitory activity. In addition, to assess the effectiveness of opioid receptor agonists on neuropathic pain, we studied the effects of morphine and the κ-opioid agonist, U50488H, on neuropathic pain behavior.

Materials and Methods

The experiments were performed using μ-opioid receptor knockout homozygous (n = 19), heterozygous (n = 17), and wild-type (n = 27) mice. Mice deficient in μ-opioid receptor were generated by gene targeting as described earlier.[14] The animals were maintained in a 12-h light–dark cycle and were provided with food and water *ad libitum*. The μ-opioid receptor knockout animals were grossly normal developmentally, were fertile, and seemed healthy. Knockout animals could not be separated from the wild-type mice by their appearance. Animals were allowed to habituate for 5 days to the testing environment and for 15 min each day before the behavioral studies were started. The studies were performed with the investigators blinded to the genotype of the mice. The research protocol was approved by the Johns Hopkins Animal Care and Use Committee (Baltimore, Maryland).

Surgery

The animals underwent surgery under halothane anesthesia. The skin on the back was shaved and cleaned with a 10% povidone–iodine solution. After a dorsolateral skin incision on the back, the muscle layer was incised. After the transverse process of L6 was identified, it was freed of its muscular attachments and removed with forceps. The exposed L5 spinal nerve was isolated, ligated with a 6-0 silk suture, and transected just distal to the knot. After confirming hemostasis, the muscle layer was closed with a 6-0 silk suture, and the skin was stapled. Throughout surgery, the animals were kept warm with use of a heating pad. The animals were returned to their cages to recover.

Withdrawal Frequencies to Mechanical Stimuli

Mechanical withdrawal frequencies were measured by calibrated von Frey monofilaments (Stoelting Co., Wood Dale, IL). Mice were placed in a clear plastic chamber on an elevated mesh screen. The von Frey monofilaments were applied to the plantar surface of each hind paw in ascending series. The monofilaments used in this study produced forces of 0.06, 0.20, 0.52, 1.15, 2.86, and 4.43 g. Every monofilament was applied to the hind paw for approximately 1 s. Each hind paw was studied with ascending series of monofilaments five times. A positive withdrawal response was defined as a mechanical stimulus–related withdrawal of the tested paw. The frequency of paw withdrawal (percent) was calculated for each monofilament.

Behavioral Responses to Thermal Stimuli

Radiant Heat. To measure withdrawal latency to radiant heat, mice were placed on a glass plate preheated to a constant temperature of 30°C, surrounded by a Plexiglas chamber (model 336 Analgesia Meter; IITC Inc./Life Science Instruments, Woodland Hills, CA). Radiant heat was applied from below to the plantar surface of each hind paw, and the withdrawal latency was measured by an electronic timer.[27] Three measurements were performed in each hind paw, spaced at least 1 min apart to determine mean withdrawal latency. A cutoff time of 20 s was used for the heat stimulus to avoid damage to cutaneous tissues.

Cooling Stimuli. To measure withdrawal frequencies to cold stimuli, mice were placed in a Plexiglas chamber on an elevated mesh screen. A small bubble of acetone was formed at the tip of polyethylene tubing connected to a syringe, which was applied to the plantar surface of the foot.[26] The acetone bubble was applied to each hind paw five times, with at least 2-min intervals between applications. Brisk foot withdrawal and licking of the treated paw were considered positive responses. The frequency of paw withdrawal (percent) was calculated for both paws.

Experimental Protocol

Baseline behavioral responses to heat, cold, and mechanical stimuli were measured 1 day before surgery. After the L5 spinal nerve section, behavioral responses were determined on days 1, 7, 14, and 28 after surgery.

Effects of Systemic Naloxone on Neuropathic Pain Behavior of Wild-type Mice

To examine the effects of endogenous opiate systems on nerve injury–induced mechanical and thermal hyperalgesia, the effects of naloxone (0.1 and 1.0 mg/kg subcutaneous, n = 8) and saline were tested in wild-type and μ-opioid knockout mice. The selection of the dose of naloxone was based on our previous studies indicating that the higher dose was adequate to block the effects of both μ- and κ- opioid agonists.[15] Paw withdrawal frequencies to the 0.52-g von Frey hair were tested before and 5 or 6 days after spinal nerve injury and normal saline or naloxone were administered. Similar studies were conducted using radiant heat stimuli to examine the effects of naloxone on heat hyperalgesia in wild type mice.

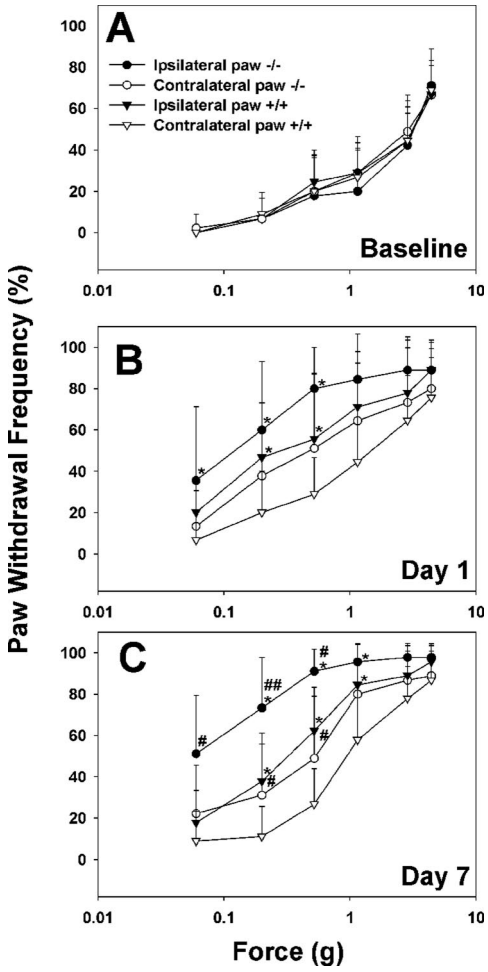

Fig. 1. Paw withdrawal frequencies to mechanical stimuli of wild-type and μ-opioid receptor knockout mice before and after spinal nerve injury. (*A*) Before injury; (*B*) 1 day after nerve injury; (*C*) 1 week after nerve injury. Whereas preinjury mechanical withdrawal frequencies were similar in the two groups of mice, paw withdrawal frequencies increased significantly after nerve injury in both wild-type (+/+) and homozygous knockout (−/−) mice. The increase in mechanical withdrawal responses on the side of nerve injury was enhanced on day 7 in the knockout mice compared with the wild-type mice (# $P < 0.05$, ## $P < 0.01$). Knockout mice also expressed contralateral allodynia at all time points studied. Wild-type, n = 9; homozygous, n = 9. * $P < 0.05$, ipsilateral *versus* contralateral paw. *Error bars* = SDs.

Effects of Systemic Morphine or U50488H on Neuropathic Pain Behavior

The effect of morphine (on postoperative day 20) or the κ-opioid receptor agonist, U50488H (on postoperative day 23), on mechanical withdrawal frequencies after spinal nerve injury was tested in wild-type, heterozygous, and homozygous μ-opioid receptor knockout mice (n = 7–9/group). The effects of morphine on the response to cooling and heat stimuli were also examined in a separate group of animals 8 and 13 days after nerve injury, respectively (n = 8–10/group). To test the effects of morphine and U50488H, a cumulative drug-dosing regimen was used (saline, 3, 10, 30 mg/kg morphine or saline, 3, 10 mg/kg U50488H). The interval between drug injections was 40 min. The drugs were administered subcutaneously, and at the end of the cumulative dose regimen, animals received a subcutaneous injection of naloxone (1 mg/kg). Responses to mechanical and heat stimuli were tested 20 min after each injection of a drug dose and immediately before administration of the next drug dose.

Statistical Analysis

Data on frequency of paw withdrawals to mechanical and cold stimuli were analyzed using nonparametric tests, Friedman two-way analysis of variance by ranks, and Kruskal Wallis tests followed by Wilcoxon matched-pairs and Mann–Whitney U tests. Radiant heat–evoked latencies were analyzed with repeated-measures analysis of variance followed by the Tukey test. All statistical analyses were performed using the statistical software package Statistica (StatSoft, Inc., Tulsa, OK). $P < 0.05$ was considered significant. Data are presented as mean ± SD unless otherwise indicated.

Results

Altered Responses to Mechanical Stimuli after Nerve Injury

Before nerve injury, withdrawal frequencies to von Frey hairs (range, 0.06–4.43 g) were similar in both hind paws and were not significantly different in the three genotypes, homozygous and heterozygous μ-opioid receptor mutant mice and wild-type mice ($P > 0.05$, Mann–Whitney U test;

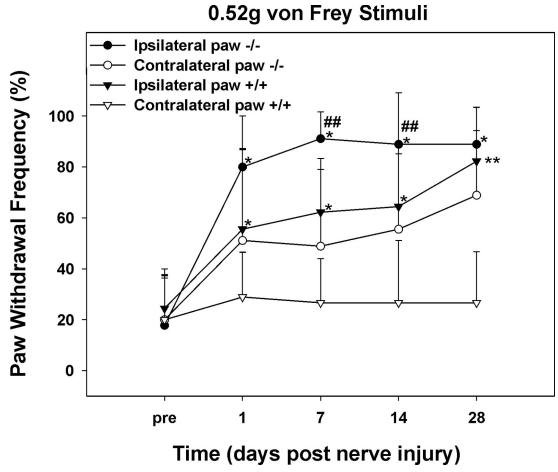

Fig. 2. Time course of allodynia to mechanical stimuli after spinal nerve injury. Preinjury withdrawal frequencies to the 0.52 g–force von Frey hair were similar in homozygous μ-opioid receptor knockout (+/+) and wild-type (−/−) mice. Mechanical withdrawal frequency increased significantly in both genotypes after nerve injury compared with preinjury values. Mechanical hyperalgesia was enhanced in the homozygous mice, which also expressed a contralateral increase in paw withdrawal frequencies. Wild type, n = 9; homozygous, n = 9. ## $P < 0.01$, homozygous *versus* wild type. * $P < 0.05$, ipsilateral *versus* contralateral paw. *Error bars* = SDs.

figs. 1 and 2; data for heterozygous not shown). After the L5 spinal nerve injury, withdrawal frequencies increased ipsilateral to nerve injury starting from day 1 in all groups compared with presurgery values and with the contralateral side ($P < 0.05$, Wilcoxon matched-pairs test or Mann–Whitney U test; figs. 1 and 2). Increased responsiveness to mechanical stimulation lasted until the end of the 4-week observation period.

In the homozygous μ-opioid receptor knockout group, mechanical withdrawal frequencies ipsilateral to injury were significantly greater than in the wild-type ipsilateral hind paw at all time points studied (postoperative days 1, 7, 14, and 28; $P < 0.05$, Mann–Whitney U test; fig. 2). Also, in the homozygous μ-opioid receptor knockout mice, withdrawal frequencies in the *contralateral* paw increased significantly from baseline values ($P < 0.05$, Wilcoxon matched-pairs test; figs. 1 and 2). Of note, in homozygous mice, allodynia to mechanical stimuli contralateral to nerve injury was as severe as allodynia in the ipsilateral paw of wild-type mice ($P > 0.05$, Mann–Whitney U test; figs. 1 and 2). In the wild-type mice, an increase in withdrawal frequency to mechanical stimuli of the contralateral hind paw was observed only with the higher von Frey forces on day 7 (fig. 1). The mechanical hyperalgesia in heterozygous μ-opioid mutant mice was intermediate between the μ-opioid knockout and wild-type mice but was restricted to the paw ipsilateral to the nerve injury (data not shown).

Altered Responses to Radiant Heat after Nerve Injury

Latencies to radiant heat were not significantly different between different genotypes or ipsilateral *versus* contralateral paws before nerve injury ($P > 0.05$, analysis of variance; fig. 3). Response latencies to radiant heat decreased significantly in the ipsilateral but not contralateral paw after nerve injury in all three groups of mice ($P < 0.05$, analysis of variance; fig. 3). In contrast to mechanical hyperalgesia, hyperalgesia to heat stimuli returned to baseline on day 28. The reductions in withdrawal latencies in the paw ipsilateral to nerve injury were similar in the three genotypes of mice at all time points (days 1, 7, 14 and 28; $P > 0.05$, Tukey test; fig. 3).

Altered Responses to Cooling Stimuli after Nerve Injury

Before the nerve injury, withdrawal frequencies to acetone application were not significantly different between the different genotypes and between the ipsilateral and contralateral paws ($P > 0.05$, Mann–Whitney U test; fig. 4). Response frequencies to acetone application increased ipsilateral to nerve injury starting from day 1 in all three groups of mice compared with presurgery values and with the contralateral side ($P < 0.05$, Wilcoxon matched-pairs test or Mann–Whitney U test; fig. 4). Increased responses were measured in all groups until the end of the 4-week testing period. Cold allodynia was similar in homozygous, heterozygous, and wild-type mice.

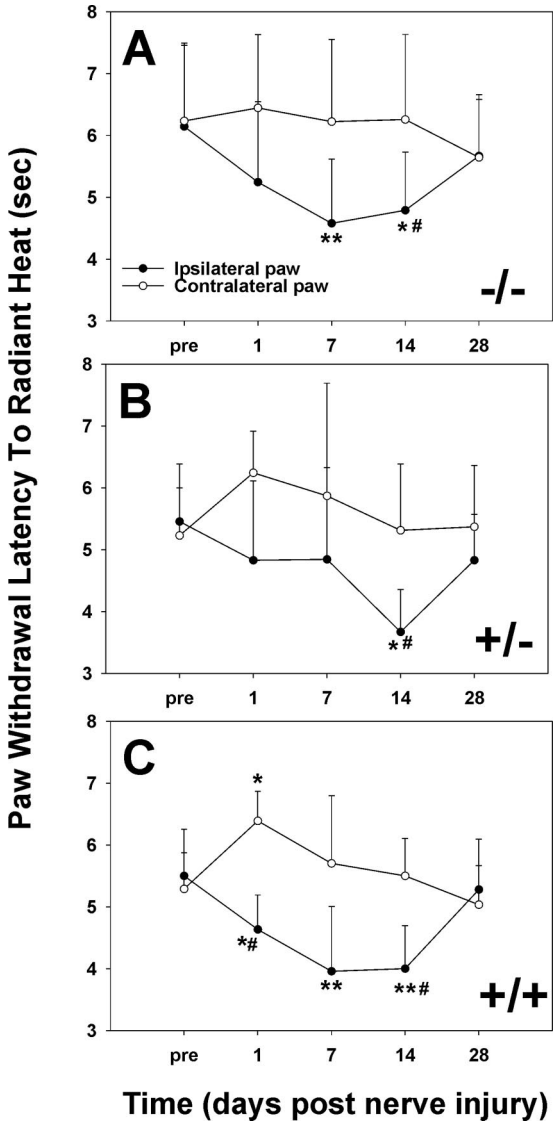

Fig. 3. Paw withdrawal latencies to radiant heat in (*A*) homozygous (−/−) and (*B*) heterozygous (+/−) μ-opioid receptor knockout and (*C*) wild-type (+/+) mice. Paw withdrawal latencies were not different between the three genotypes before nerve injury. Nerve injury resulted in a similar decrease in the withdrawal latency to radiant heat (thermal hyperalgesia) in all three genotypes of mice. Wild type, n = 9; homozygous, n = 9; heterozygous, n = 7. * $P < 0.05$, ** $P < 0.01$ compared with presurgery values. # $P < 0.05$, injured side (ipsilateral) *versus* contralateral paw. *Error bars* = SDs.

Effects of Naloxone on Nerve Injury–induced Neuropathic Pain Behavior

Systemic administration of naloxone (0.1 and 1 mg/kg), 5 days after spinal nerve section, increased the mechanical withdrawal frequencies in the paw ipsilateral to the nerve injury in wild-type mice (fig. 5A) but not in μ-opioid receptor knockout mice. Allodynia to mechanical stimuli was also observed in the contralateral

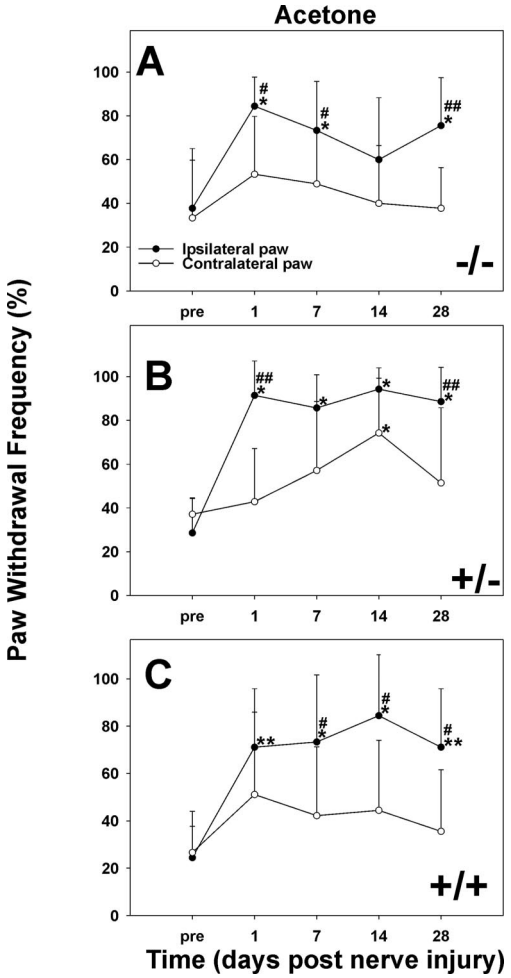

Fig. 4. Paw withdrawal frequency to acetone drop was increased after nerve injury in (*A*) homozygous (−/−) and (*B*) heterozygous (+/−) μ-opioid receptor knockout and (*C*) wild-type (+/+) mice. Wild type, n = 9; homozygous, n = 9; heterozygous, n = 7. * $P < 0.05$, ** $P < 0.01$ compared with presurgery values. # $P < 0.05$, ## $P < 0.01$, injured side (ipsilateral) *versus* contralateral paw. *Error bars* = SDs.

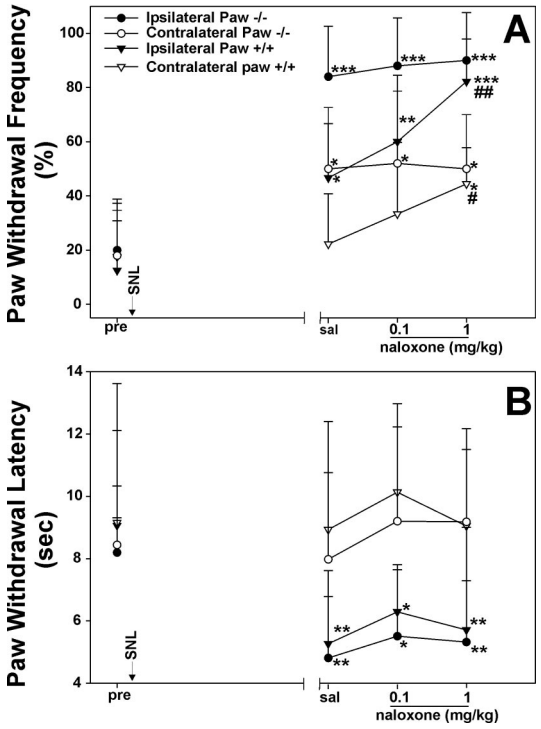

Fig. 5. Effects of systemic naloxone administration on paw withdrawal frequencies to mechanical and heat stimuli in wild-type and homozygous μ-opioid receptor knockout mice. Frequencies of paw withdrawal to a 0.52 g–force von Frey hair (*A*) and paw withdrawal latencies to radiant heat (*B*). Testings were performed 5 and 6 days after the spinal nerve injury, respectively. Stimuli were presented immediately before and 20 min after each subcutaneous dose of naloxone. The paw withdrawal frequencies to mechanical stimuli in the injured and contralateral, uninjured paws were increased after naloxone in wild-type mice but not the μ-opioid receptor knockout mice. Naloxone had no significant effect on the paw withdrawal latencies to radiant heat in either genotype of mice. n = 8 or 9. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with baseline. # $P < 0.05$, ## $P < 0.01$ compared with values after normal saline. *Error bars* = SDs; sal = saline; SNL = spinal nerve ligation and section.

paw of wild-type mice. The bilateral allodynia to mechanical stimuli in the wild-type mice after the high dose of naloxone resembled the pain behavior observed in the μ-opioid knockout mice. Systemic administration of naloxone, 6 or 7 days after spinal nerve section, had no significant effects on the paw withdrawal latencies to radiant heat in wild-type mice ipsilateral to the nerve injury (fig. 5B). The lack of an effect of naloxone on response to heat stimuli is consistent with a lack of observed difference in wild-type and μ-opioid knockout mice in heat hyperalgesia.

Effects of Morphine on Mechanical Withdrawal Frequencies

Systemic administration of morphine (3–30 mg/kg subcutaneous) or naloxone (1 mg/kg subcutaneous) did not have significant effects on the mechanical withdrawal frequencies in homozygous μ-opioid knockout mice (fig. 6). In the heterozygous μ-opioid knockout mice, morphine significantly attenuated the mechanical withdrawal frequencies only at a dose of 30 mg/kg (fig. 6). The response frequencies ipsilateral to nerve injury were still significantly higher compared with the contralateral side after 30 mg/kg morphine, indicating that morphine did not completely reverse mechanical allodynia in the heterozygous mice. In the wild-type mice, morphine was effective in attenuating mechanical withdrawal frequencies dose dependently, starting from the lowest dose of 3 mg/kg subcutaneous (fig. 6). Attenuation of mechanical withdrawal responses were seen bilaterally after morphine administration. With the highest dose of morphine (30 mg/kg subcutaneous), the responses ipsilateral to nerve injury were not significantly different from the contralateral side, indicating that this dose was able to abolish mechanical allodynia. Attenuation of mechanical withdrawal responses after morphine administration in

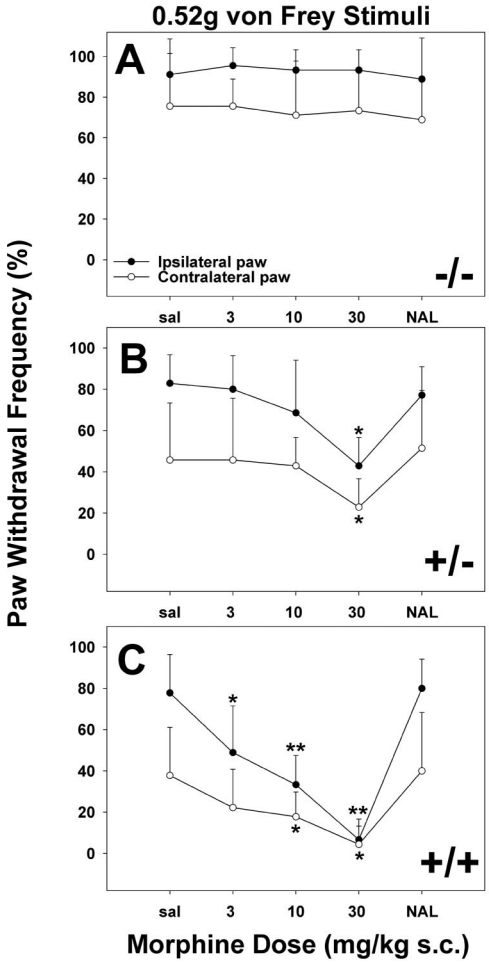

Fig. 6. Effects of systemic morphine administration on paw withdrawal frequency to a 0.52 g–force von Frey hair in (*A*) homozygous (−/−) and (*B*) heterozygous (+/−) μ-opioid receptor knockout and (*C*) wild-type (+/+) mice. Testings were performed immediately before and 20 min after each subcutaneous (s.c.) dose of morphine in mice 20 days after the spinal nerve injury. Morphine had no effect on the paw withdrawal responses in homozygous mice; in heterozygous mice, only the highest dose of morphine was effective in attenuating paw withdrawal responses. In wild-type mice, significant attenuation of responses was seen even at the lowest dose of morphine. The effects were naloxone reversible. Wild type, n = 9; homozygous, n = 9; heterozygous, n = 7. * $P < 0.05$, ** $P < 0.01$ compared with values after saline. *Error bars* = SDs; NAL = naloxone, 1 mg/kg subcutaneous; sal = saline.

heterozygous and wild-type mice were naloxone reversible (fig. 6).

Effects of Morphine on Paw Withdrawal Latencies to Radiant Heat

Systemic administration of morphine (3 and 10 mg/kg subcutaneous) or naloxone (1 mg/kg) did not have significant effects on the paw withdrawal latencies to radiant heat in homozygous and heterozygous μ-opioid knockout mice (fig. 7A). The effects of the 30-mg/kg dose of morphine on paw withdrawal latencies to heat stimuli could not be tested because this dose resulted in increased locomotor activity. In contrast to transient (approximately 1 s) stimulus durations with von Frey hairs and acetone, successful measurement of paw withdrawal latencies to heat required a stimulus duration of approximately 10 s. In the wild-type mice, morphine (10 mg/kg) attenuated the paw withdrawal latencies to radiant heat ipsilateral to the nerve injury (fig. 7A; $P < 0.05$) but had no significant effect on the contralateral paw response. The effects of morphine were reversed by naloxone (1 mg/kg subcutaneous).

Effects of Morphine on Paw Withdrawal Frequencies to Acetone

Systemic administration of morphine (3–30 mg/kg subcutaneous) or naloxone (1 mg/kg) did not have significant effects on the paw withdrawal frequencies to acetone in homozygous μ-opioid receptor knockout mice

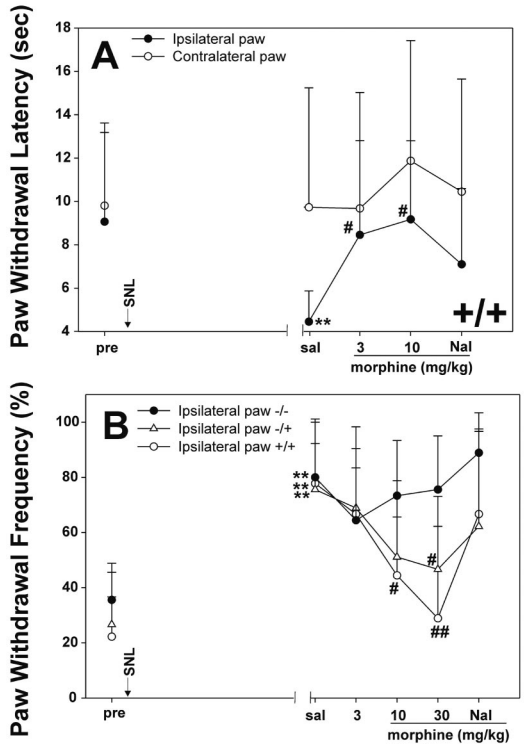

Fig. 7. (*A*) Effects of systemic morphine administration on paw withdrawal latencies to radiant heat in wild-type (+/+) mice. Morphine increased the paw withdrawal latencies to heat ipsilateral to the nerve injury at the 10-mg/kg subcutaneous dose; the effects were naloxone (1 mg/kg) reversible. Morphine, however, had no effects on heat hyperalgesia in homozygous and heterozygous μ-opioid receptor knockout mice (data not shown). n = 9. ** $P < 0.01$ compared with values after normal saline. (*B*) Effects of systemic morphine administration on paw withdrawal frequencies to acetone in homozygous and heterozygous μ-opioid receptor knockout and wild-type mice. Morphine had no effect on the response to acetone in homozygous mice; in contrast, morphine significantly decreased the withdrawal frequencies of the ipsilateral paw to acetone in heterozygous and wild-type mice. The effects were naloxone (1 mg/kg) reversible. n = 9 or 10. # $P < 0.05$, ## $P < 0.01$ compared with normal saline. ** $P < 0.01$ compared with pre-SNL values. *Error bars* = SDs; Nal = naloxone, 1 mg/kg subcutaneous; sal = saline; SNL = spinal nerve ligation and section.

(fig. 7B). In the heterozygous μ-opioid receptor knockout mice and wild-type mice, morphine dose-dependently attenuated the paw withdrawal frequencies ipsilateral to nerve injury, but the effect of morphine in heterozygous μ-opioid knockout mice was less than that in wild-type mice. The effects of morphine in the heterozygous μ-opioid receptor knockout mice and wild-type mice were reversed by naloxone (1 mg/kg subcutaneous).

Effects of U50488H on Mechanical Withdrawal Frequencies

Systemic administration of U50488H had significant effects on mechanical withdrawal frequencies in homozygous, heterozygous, and wild-type mice. In the homozygous mice, U50488H decreased the mechanical withdrawal frequencies in both paws (fig. 8). After U50488H administration, a significant difference persisted between ipsilateral and contralateral paw mechanical withdrawal responses in the homozygous mice. In the heterozygous and wild-type mice, administration of U50488H also attenuated mechanical withdrawal responses bilaterally and dose dependently (fig. 8). After 10 mg/kg subcutaneous U50488H in the wild-type and in the heterozygous mice, there was no significant difference in mechanical withdrawal responses between the nerve-injured and the contralateral side. The effects of U50488H could be reversed by naloxone (1 mg/kg subcutaneous; fig. 8).

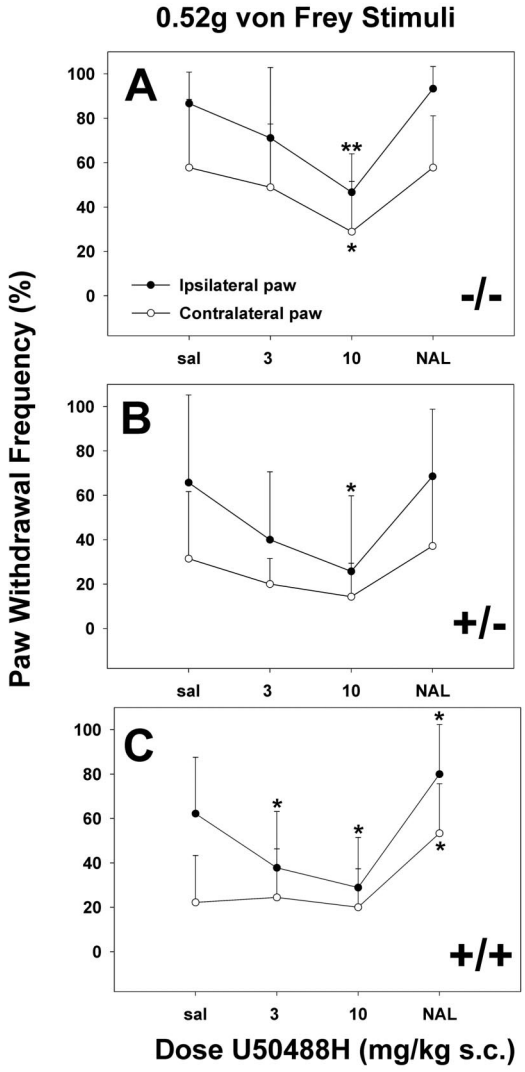

Fig. 8. Effects of systemic administration of U50488H on paw withdrawal frequency to a 0.52 g–force von Frey hair in (*A*) homozygous (−/−) and (*B*) heterozygous (+/−) μ-opioid receptor knockout and (*C*) wild-type (+/+) mice. Test protocols were similar to that described in figure 6. The κ-opioid agonist U50488H attenuated mechanical withdrawal frequencies dose dependently in all three genotypes; the effects were naloxone reversible. Wild type, n = 9; homozygous, n = 9; heterozygous, n = 7. * $P < 0.05$, ** $P < 0.01$ compared with values after saline. *Error bars* = SDs; NAL = naloxone, 1 mg/kg subcutaneous (s.c.); sal = saline.

Discussion

The primary observations in the current study are that (1) L5 spinal nerve injury–induced mechanical allodynia is enhanced bilaterally, *i.e.*, ipsilateral and contralateral paw, in μ-opioid receptor knockout mice; (2) L5 spinal nerve injury–induced hyperalgesia to heat and cooling stimuli is not affected by the absence of μ-opioid receptors; (3) systemic naloxone administration in wild-type mice after spinal nerve injury results in enhanced bilateral mechanical allodynia that mimics the neuropathic pain behavior observed in μ-opioid receptor knockout mice; (4) systemic morphine attenuates the nerve injury–induced increased responses to mechanical, heat, and cooling stimuli in control mice primarily by a μ-opioid receptor–mediated effect; and (5) systemic administration of a κ-opioid receptor agonist reduces neuropathic pain behavior, an effect that is independent of the μ-opioid receptor.

Studies in transgenic mice have been used in recent years to understand the role of different receptor mechanisms in the signaling of pain. However, it has been suggested that the use of gene knockout mice for studies of pain must be interpreted with caution because of the potential for confounding, compensatory factors.[28] Knocking out a gene might lead to compensatory changes in the expression of other genes or nonspecific changes affecting the behavior of the animal. The μ-opioid receptor knockout mice are a random mix of 129/sv and C57BL/6 alleles, which might also be a confounding factor because different strains have different sensitivities to noxious stimuli.[29] However, in the current study, mechanical and thermal withdrawal responses were similar before the nerve injury in wild-type and μ-opioid receptor knockout mice, which suggests that the general behavior and sensitivity to acute noxious stimuli were not affected by the deletion of the μ-opioid receptor

gene. Moreover, autoradiographic studies failed to show major quantitative differences in κ- or δ-opioid receptor binding in the brain of μ-opioid receptor mutant mice.[30] Finally, the observed effects in μ-opioid receptor knockout mice were mimicked by the opioid receptor antagonist naloxone in wild-type animals.

Our observations of an enhanced mechanical allodynia in the absence of μ-opioid receptors provide evidence to suggest that nerve injury induces the development of a tonic opioid receptor–mediated inhibitory mechanism—a potential compensatory neuronal response to attenuate the pain signals. The withdrawal frequencies to mechanical stimuli were similar in homozygous, heterozygous, and wild-type mice before the nerve injury. This finding is similar to our previous observations[15] that mechanical withdrawal responses are not different between μ-opioid receptor knockout and wild-type mice under normal conditions. Earlier reports with the use of opioid receptor antagonists in normal human subjects also indicated that tonic endogenous opioid receptor–mediated modulatory effects on nociception are minimal in the absence of tissue injury.[31,32] However, we observed that the mechanical allodynia resulting from spinal nerve injury was enhanced in the μ-opioid receptor knockout mice, and in wild-type mice after naloxone administration. These observations suggest that tonic μ-opioid receptor–mediated control mechanisms are induced by the nerve injury. A similar activation of the endogenous opioid system has been reported after peripheral inflammation and spinal cord injury.[23,24,33] In addition, dental postoperative pain has been reported to be enhanced after naloxone administration.[34] However, naloxone does not affect pain thresholds in normal subjects or in normal rats, indicating that activating the endogenous opiate system requires a long-lasting noxious stimulus.[31,34,35]

This endogenous μ-opioid receptor–mediated inhibition of neuropathic pain is not restricted to the side of nerve injury because knockout mice expressed also a robust contralateral mechanical allodynia. Our observations are consistent with previous studies showing a bilateral increase in μ-opioid receptor binding in spinal dorsal horn after a unilateral chronic constriction injury of the sciatic nerve.[36] Unilateral nerve injury in rats has also been shown to induce mechanical hyperalgesia in the contralateral side.[25,26,37] Contralateral changes are usually less marked and have a shorter time course than ipsilateral changes. Other chronic pain states, such as persistent inflammation, also alter the pharmacology and physiology of supraspinal neurons and lead to an increased potency of opioid agonist in suppressing nociceptive responses of the contralateral, uninflamed paw.[38] Our results indicate that contralateral pain behavior after nerve injury is under control of endogenous opiate mechanisms because contralateral allodynia to mechanical stimuli was evident in the control mice only after the administration of naloxone. This contralateral hyperalgesia seems also to be modality specific because contralateral changes were observed only with mechanical, not thermal, stimuli.

The site of modulatory actions of the μ-opioid system in neuropathic pain must be defined by additional studies. The bilateral effects of endogenous opioid mechanisms on mechanical allodynia suggest a central site of action. A recent report indicates that, compared with control animals, the efficacy of intrathecal morphine in attenuating pain behavior was decreased after spinal nerve ligation, whereas the effects of morphine administered in the periaqueductal gray region was increased.[39] Spinal nerve injury produces a discrete ipsilateral loss of μ-opioid receptors in laminae I and II of the spinal cord at the level of injury.[40] These observations suggest that supraspinal sites may be involved in the enhanced μ-opioid receptor–mediated pain modulation after nerve injury. Other studies in animal models of neuropathic pain, however, point to a possible peripheral opioid receptor–mediated modulation of pain.[9,41]

The mechanisms leading to contralateral increases in pain behavior are uncertain. Bilateral changes may result from crossing projections from primary afferents or intrinsic spinal neurons. It has been postulated that the mechanism linking the two sides of the body is *via* spinal interneurons that cross the midline rather than by peripheral mechanism (reviewed by Koltzenburg *et al.*,[42] 1999). Nerve injury may also activate descending pain modulating pathways from brain stem nuclei that have opioidergic neurons. Additional studies are needed to determine which of these potential mechanisms play a critical role in the development of tonic bilateral inhibition of pain after nerve injury.

The lack of a difference in hyperalgesia to thermal stimuli after nerve injury between the wild-type and the μ-opioid receptor knockout mice was an unexpected but interesting observation. These observations suggest that the mechanical allodynia and thermal hyperalgesia that develop after nerve injury have distinct mechanisms. Moreover, our findings indicate a differential effect of spinal nerve injury–induced endogenous μ-opioid receptor–mediated inhibition on mechanical allodynia *versus* thermal hyperalgesia. It has been postulated that central mechanisms are involved in mechanical allodynia, whereas peripheral mechanisms contribute to heat hyperalgesia.[43] Studies using resiniferatoxin to selectively abolish vanilloid receptor 1–sensitive C fibers indicate that whereas heat hyperalgesia is signaled by C fibers, the mechanical hyperalgesia may be independent of C fiber activity.[44] Recent observations that the *N*-methyl-D-aspartate receptor antagonist MK-801 dose-dependently ameliorated Freund's adjuvant–induced mechanical allodynia but did not significantly affect thermal hyperalgesia support the hypothesis that the mechanisms of mechanical and thermal hyperalgesia differ.[45]

Similar neurochemical distinctions in the mechanisms of hyperalgesia to mechanical and thermal stimuli after nerve injury have been postulated.[46] For example, the absence of neurokinin-1 receptors results in attenuation of mechanical but not thermal hyperalgesia after spinal nerve injury.[47] The intrathecal administration of the *N*-methyl-D-aspartate antagonist AP-5 reversed mechanical allodynia but was ineffective on thermal hyperalgesia in a rat model of spinal cord injury pain.[48] Nerve injury leads to a reduction in the potency and efficacy of intrathecal morphine in attenuating the response to noxious heat stimuli.[49]

In previous studies, neuropathic pain has been reported to be resistant to opioids. In various animal models of neuropathic pain, both increased and decreased effects of opioids have been shown.[7–11] Studies suggest that systemic and supraspinal administration of opioids are able to suppress enhanced responsiveness to mechanical stimuli after nerve injury, whereas spinal opioids are not.[50,51] However, a recent electrophysiologic study in spinal nerve–injured rats suggested that spinal morphine has enhanced potency on C-fiber and noxious mechanical and thermal stimuli–evoked spinal neuronal responses 7–10 days after nerve injury. However, this enhanced potency of morphine tended to decrease at 14–17 days after spinal nerve injury.[52] Results from the current study suggest that systemically administered morphine is able to attenuate neuropathic pain. These experimental observations are consistent with recent clinical reports from our group as well as from other investigators that opioids attenuate but do not abolish peripheral and central neuropathic pain.[4–6] Also, our results suggest that the antihyperalgesic effects of morphine on neuropathic pain are primarily mediated *via* μ-opioid receptors. This finding is consistent with previous studies using various acute nociceptive tests that show that the analgesic actions of morphine are mediated predominantly by μ-opioid receptors.[13–15]

In the current study, the κ-opioid agonist U50488H was effective in attenuating mechanical hyperalgesia dose dependently in μ-opioid receptor knockout and wild-type mice. This finding is in line with previous observations that the κ-opioid agonist GR89696 was effective in decreasing the enhanced responses to mechanical, heat, and cooling stimuli in a chronic constriction injury model of neuropathic pain.[53] Also, in a rat model of surgical pain, κ-opioid agonists blocked the mechanical and heat hyperalgesia.[54] Results from the current study also suggest that the κ-opioid receptor–mediated effects on neuropathic pain are not modulated by the μ-opioid receptor. This observation is consistent with previous studies showing that κ-opioid receptor–mediated analgesia is not affected in the μ-opioid receptor knockout mice in various acute pain tests.[15,16]

In conclusion, we have demonstrated that μ-opioid receptor contributes to the severity of mechanical but not thermal hypersensitivity in a spinal nerve injury model of neuropathic pain. The current study suggests that endogenous opiates acting *via* μ-opioid receptors exert a tonic inhibitory role in suppressing mechanical allodynia induced by nerve injury. We have also demonstrated that systemically administered morphine acting *via* μ-opioid receptors is effective in reversing mechanical allodynia in neuropathic pain, and we support the use of opioids for clinical neuropathic pain states. In addition, the κ-opioid agonist, U50488H, is also effective in attenuating mechanical allodynia, and this class of drugs may be worthy of clinical investigation.

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