Specific down-regulation of spinal μ-opioid receptor and reduced analgesic effects of morphine in mice with postherpetic pain

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ABSTRACT

The analgesic effects of opioid agonists and the expression of μ- and κ-opioid receptors were compared between mice with herpetic pain and those with postherpetic pain induced by herpetic virus inoculation. Morphine inhibited herpetic pain more effectively than postherpetic pain. Intrathecal injection reduced the analgesic effects of morphine on postherpetic pain, but intracerebroventricular injection did not. The κ-opioid receptor agonist nalfurafine suppressed herpetic and postherpetic pain to similar degrees. μ-Opioid receptor-like immunoreactivities in the lumbar dorsal horn were markedly decreased at the postherpetic, but not herpetic, stage of pain. In the dorsal root ganglia, the expression of μ-opioid receptor mRNA was significantly decreased in mice with postherpetic pain, whereas the κ-opioid receptor mRNA level was not altered. These results suggest that specific down-regulation of the μ-opioid receptor in the primary sensory neurons is responsible for the reduced analgesic action of morphine on postherpetic pain. The κ-opioid receptor may be a useful target for the analgesic treatment of postherpetic neuralgia.

1. INTRODUCTION

Peripheral nerve injury often results in persistent symptoms including spontaneous pain, tactile pain (allodynia) and hyperalgesia. Neuropathic pain is difficult to treat and is considered resistant to conventional analgesics, especially opioids such as morphine. Several studies have tried to explain the spinal mechanisms of reduced effect of morphine on neuropathic pain, including the reduced expression of the μ-opioid receptor (Porreca et al., 1998), enhanced release of dynorphin A (Nichols et al., 1997) and cholecystokinin (Nichols et al., 1996), and increased phosphorylation of the μ-opioid receptor (Narita et al., 2004). However, the mechanisms underlying the reduction of morphine efficacy are complex and not fully understood.

Postherpetic neuralgia, characterized as spontaneous pain and allodynia that persist long after the healing of herpes zoster, is also difficult to treat with opioids (Dworkin and Portenoy, 1996; Watson, 1996). The pathophysiological mechanisms of postherpetic neuralgia are not well understood. We previously established mouse models of herpetic pain and postherpetic pain using herpes simplex virus type-1 (HSV-1) (Takasaki et al., 2000b, 2002). When mice are given transdermal HSV-1 inoculation on the hind paw, they show herpes zoster-like skin lesions throughout the inoculated dermatome, as well as allodynia, hyperalgesia, and spontaneous pain (Takasaki et al., 2000b). These symptoms become apparent from day 5 after inoculation, a day when the viruses actively proliferate in the sensory ganglion (Takasaki et

al., 2000a). The skin lesions heal up by day 15 after inoculation. Pain-related responses subside in about half of mice by day 20, but the responses remain in the rest long after the lesions heal, suggesting the development of postherpetic pain (Takasaki et al., 2002; Kuraishi et al., 2004).

To clarify the cause of the ineffectiveness of opioids against postherpetic neuralgia, this study compared the analgesic effects of morphine between mice models of herpetic and postherpetic pain and identified the expression of the μ-opioid receptor to confirm the behavioral results. We recently found that the κ-opioid receptor agonist nalfurafine (TRK-820) effectively suppresses herpetic pain (Takasaki et al., 2004). Therefore, we also compared the efficacy of nalfurafine between herpetic and postherpetic pain and examined the alteration of κ-opioid receptor in the herpetic and postherpetic pain conditions.

2. MATERIALS AND METHODS

2.1. Animals

Female BALB/c mice (six weeks old at the start of experiments; Japan SLC, Shizuoka, Japan) were used. They were housed six per cage under controlled temperature (22 ± 1ºC) and humidity ($55 \pm 10\%$). The room was lighted from 07:00 to 19:00 h and during medication. Food and water were freely available. In this study, a total of 182 mice were used (naive, $n = 12$; herpetic pain, $n = 84$; postherpetic pain+, $n = 73$; postherpetic pain−, $n = 13$) for behavioral and biochemical studies. Experiments were conducted with the approval of the Animal Care Committee at University of Toyama, and according to the guidelines for investigations of experimental pain in animals published by the International Association for the Study of Pain (Zimmermann, 1983).

2.2. HSV-1 infection

The mice were inoculated with HSV-1 as described previously (Takasaki et al., 2000b). In brief, HSV-1 (7401H strain; 1×10^6 plaque-forming units in 10 µl) was inoculated on the shin skin of the right hind paw (5×5 mm) after scarification with 27-gauge needles. The contralateral hind paw was without inoculation. To prevent motor paralysis and death, mice were given oral acyclovir (10 mg/kg) (GlaxoSmithKline, Tokyo, Japan) five times (09:00, 12:00, 15:00, 18:00 and 21:00 h) daily from day 5 to 11 after inoculation.

2.3. Assessment of pain-related responses

Pain-related responses of the hind paw were assessed using von Frey filaments, as described previously (Takasaki et al., 2000b). After at least 15-min acclimation period, von Frey filaments with bending force of 0.17 or 1.20 g were pressed perpendicularly against the plantar skin of the hind paw and held for 3–5 s with it slightly bent. The responses to these

stimuli were ranked as follows: 0, no response; 1, withdrawing the hind paw from von Frey filament; 2, immediate flinching or licking of the hind paw. The stimulation of the same intensity applied six times to each hind paw at intervals of several seconds and response score was calculated as the mean value of six trials.

Allodynia and hyperalgesia were determined as described (Kuraishi et al., 2004). All naive mice tested do not respond to von Frey filament of 0.17-g strength, but it is occasionally difficult to distinguish pain-related responses from walking behaviors. On day 6 after inoculation, most mice show 0.32 or higher response scores (Kuraishi et al., 2004). Therefore, mice that showed 0.5 or higher response scores were considered to have allodynia. When stimulated with von Frey filament of 1.20-g strength, naive mice showed 0.5 or lower response scores and inoculated mice showed 1.0 or higher response scores on day 6 after inoculation (Kuraishi et al., 2004). Thus, mice that showed 1.17 or higher response scores were considered to have hyperalgesia. Mice that showed allodynia or hyperalgesia were considered to have herpetic or postherpetic pain, and they were used in the behavioral study.

Analgesic effect was calculated as follows:

$$\text{Analgesic effect (\%)} = \frac{\text{RS (inoculated, before drug)} - \text{RS (inoculated, after drug)}}{\text{RS (inoculated, before drug)} - \text{RS (contralateral, before drug)}} \times 100$$

where RS is response score.

2.4. Intrathecal and Intracerebroventricular injections

Intrathecal injection was done by the method of Hylden and Wilcox (1980). In brief, mice were given it in a volume of 5 µl by a lumbar puncture using a 25-µl Hamilton microsyringe with a 30-gauge needle. The animals were not anaesthetized during the intrathecal injection.

Intracerebroventricular injection was performed by the method of Haley and McCormick (1957). In brief, mice were anaesthetized with diethyl ether and a 27-gauge needle attached to a microsyringe was inserted into the lateral ventricle. The injection volume was 5 µl.

2.5. Agents

Morphine hydrochloride (Sankyo, Tokyo, Japan) was dissolved in physiological saline and was injected subcutaneously, intrathecally, and intracerebroventricularly. The κ-opioid receptor agonist nalfurafine hydrochloride (Toray Industries Inc., Kamakura, Japan) was dissolved in distilled water and was administered per os. Effects of these opioid agonists on herpetic and postherpetic pain were tested in a blinded manner on day 6 and day 30–35, respectively, after HSV-1 inoculation.

2.6. Fluorescence immunohistochemistry

Mice were deeply anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal) and perfusion-fixed with 4% paraformaldehyde. Then the spinal cords were removed and

post-fixed in 4% paraformaldehyde for 4 h. They were permeated with 30% sucrose for 16 h, and were frozen in an embedding compound (OTC compound, Sakura Finetechnical, Tokyo, Japan) on dry ice. Frozen tissues were sectioned at 16 μm in thickness with a cryostat and sections were mounted on glass slides. The sections were fixed with 4% paraformaldehyde for 30 min and washed three times in phosphate-buffered saline. They were incubated in phosphate-buffered saline containing 1.5% fetal bovine serum at room temperature for 30 min and then with guinea pig antiserum against the μ-opioid receptor (1:100, Chemicon International Inc., Temecula, CA, USA) overnight at 4°C in a humid chamber. After incubation with the primary antibodies, they were washed with phosphate-buffered saline, and incubated with biotin-labeled goat anti-guinea pig IgG (1:100) (Vector Laboratories Inc., Burlingame, CA, USA) for 1 h at room temperature. They were then rinsed with phosphate-buffered saline, and were incubated with fluorescein isothiocyanate-labeled avidin (1:1,000) (Vector Laboratories Inc.). The slides were coverslipped with glycerol-phosphate-buffered saline containing 2.5% triethylenediamine. The sections were observed with a confocal laser-scanning microscope (Zeiss LSM 510, Carl Zeiss Co. Ltd., Jena, Germany). To quantify the μ-opioid receptor-like immunoreactivities, fluorescence intensity in a region of the spinal dorsal horn (see Fig. 3A) was measured with NIH image.

2.7. Reverse transcription-polymerase chain reaction

Semi-quantitative reverse transcription-polymerase chain reaction was employed to measure the levels of μ- and κ-opioid receptor mRNAs. After decapitation under diethyl ether anesthesia, the dorsal horn of lumbar cord and the dorsal root ganglia at the L4 and L5 levels were rapidly removed. Total RNA was isolated using Trizol reagent (Invitrogen Co., Carlsbad, CA, USA) and first strand cDNA was synthesized from total RNA using oligo $(dT)_{16}$ primer and reverse transcriptase. The first strand cDNA was amplified with using the following primers (Hokkaido System Science Co. Ltd., Sapporo, Japan): 5'- CCC AAC TTC CTC CAC AAT CGA A -3' (sense) and 5'- AAA TCA CAT ACC ACA GAC TAT TG -3' (antisense) for μ-opioid receptor, 5'- TTT ATC CTG GTG GAG GCT CTG GGA -3' (sense) and 5'- CTC ATG GAA GCA GGA TCC TGA ACT -3' (antisense) for κ-opioid receptor, 5'- CAA AGG TCA TCC ATG ACA AC -3' (sense) and 5'- TTA CTC CTT GGA GGC CAT GT -3' (antisense) for glyceraldehyde-3-phosphate dehydrogenase. Reaction products were separated on a 1.5% agarose gel and stained with ethidium bromide. To determine the expression levels, the density of the bands was measured with a densitometer (DensitoGraph, Atto, Tokyo, Japan).

2.8. Statistical analysis

The means of data are presented together with standard error of the means. Significant difference between groups was analyzed with Dunnett's multiple comparisons. $P < 0.05$ was considered significant.

3. RESULTS

3.1. Effects of opioid agonist on herpetic and postherpetic pain

The effects of systemic administration of morphine and nalfurafine on herpetic and postherpetic pain were tested.   A subcutaneous injection of morphine inhibited herpetic and postherpetic pain (allodynia and hyperalgesia) dose-dependently (Fig. 1A, B). The effects were significantly smaller on postherpetic pain than on herpetic pain. An oral administration of nalfurafine suppressed herpetic and postherpetic pain dose-dependently (Fig. 1C, D). The effects were not significantly different between herpetic and postherpetic pain.

The effects of intrathecal and intracerebroventricular injection of morphine on herpetic and postherpetic pain were tested. Intrathecal injection of morphine dose-dependently suppressed both herpetic and postherpetic pain (Fig. 2A, B). The effects were significantly smaller on postherpetic pain than on herpetic pain. Intracerebroventricular injection of morphine dose dependently inhibited herpetic and postherpetic pains (Fig. 2C, D). The effects were not significantly different between herpetic and postherpetic pain.

3.2. μ-Opioid receptor-like immunoreactivity in the dorsal horn

μ-Opioid receptor-like immunoreactivity was localized mainly in the superficial dorsal horn of the lumbar cord in the naive mouse (Fig. 3A). The distribution of μ-opioid receptor-like immunoreactivity on the inoculated side of the herpetic pain mice was similar to that of the naive mouse (Fig. 3B). μ-Opioid receptor-like immunoreactivity was markedly decreased on the inoculated side in the lumbar dorsal horn of the postherpetic pain mice as compared to the naive mice (Fig. 3C). The decrease in μ-opioid receptor-like immunoreactivity was also found in mice without postherpetic pain (Fig. 3D). The μ-opioid receptor-like immunoreactivities in the lumbar dorsal horn on the contralateral (uninoculated) side were not altered (data not shown). Semi-quantitative analysis showed that μ-opioid receptor-like immunoreactivities were significantly decreased in mice with postherpetic pain ($66.0 \pm 12.7\%$ of naive group) and in those without postherpetic pain ($68.7 \pm 10.3\%$ of naive group) (Fig. 3E).

3.3. Opioid receptor mRNAs in the dorsal root ganglia and dorsal horn

μ-Opioid and κ-opioid receptor mRNAs in the dorsal root ganglia and dorsal horn were determined by reverse-transcription polymerase chain reaction (Fig. 4A). Although the expression level of the μ-opioid receptor mRNA in the dorsal root ganglia was not altered in herpetic pain mice, it was markedly decreased in the dorsal root ganglia on the inoculated side of postherpetic pain mice, without changes on the contralateral side (Fig. 4B). The expression level of μ-opioid receptor mRNA in the lumbar dorsal horn did not differ between herpetic

and postherpetic pain mice (Fig. 4C). The expression levels of κ-opioid receptor mRNA in the dorsal root ganglia and lumbar dorsal horn were not altered in herpetic pain and postherpetic pain mice (Fig. 4D, E). The expression levels of these mRNAs in the dorsal root ganglia and the lumbar dorsal horn on the uninoculated side were not altered (data not shown).

4. DISCUSSION

Clinically, it has been reported that morphine is less effective against the pain of postherpetic neuralgia after intravenous or epidural injection (Eide et al., 1994; Watt et al., 1996). Reduced analgesic effects of morphine have also been reported in animals with neuropathic pain induced by peripheral nerve injury or with streptozotocin-induced allodynia (Lee et al., 1994; Field et al., 1999; Rashid et al., 2004; Yaksh et al., 1995). Consistent with these reports, the analgesic effects of systemic injection of morphine were markedly decreased in mice with postherpetic pain; systemic morphine (5 mg/kg) almost completely ameliorated herpetic pain, but inhibited postherpetic pain by only about 50% (Fig. 1A, B).

Systemically administered morphine may produce analgesic effects through spinal and supraspinal actions. In the present study, to reveal the regions responsible for the reduced efficacy of systemic morphine, the effects of intrathecal and intracerebroventricular injection of morphine were compared between postherpetic and herpetic pain. A marked decrease in the analgesic effects of morphine on postherpetic pain was observed after intrathecal injection (Fig. 2A, B). On the other hand, the analgesic effects of intracerebroventricular morphine did not differ between postherpetic and herpetic pain (Fig. 2C, D), suggesting that the analgesic action of morphine was not affected at the supraspinal level. Taken together, these results suggest the reduction in the inhibitory effects of systemic morphine on postherpetic pain is at least partly due to its reduced action at the spinal level. A relatively high systemic dose (5 mg/kg) of morphine inhibits postherpetic pain (Takasaki et al., 2002; present experiment). We did not test higher doses of morphine, as they would have increased locomotor activity and made it difficult to assess pain-related responses. But it is conceivable that higher doses of morphine substantially inhibit postherpetic pain, because the supraspinal inhibitory effect did not differ between postherpetic and herpetic pain.

To clarify the mechanisms underlying the reduction in the inhibitory effects of morphine on postherpetic pain, we determined the expression of μ-opioid receptor by reverse-transcription polymerase chain reaction and an immunohistochemical method. Interestingly, μ-opioid receptor-like immunoreactivities were markedly decreased in the spinal dorsal horn on the inoculated side in mice with postherpetic pain, whereas they were not altered in mice with herpetic pain (Fig. 3). These results support the idea that the reduction in inhibitory effects of systemic and intrathecal morphine on postherpetic pain is

attributed to the reduced distribution of μ-opioid receptors in the spinal dorsal horn. In mice with postherpetic pain, μ-opioid receptor mRNA expression was markedly decreased in the affected dorsal root ganglia but not in the lumbar dorsal horn (Fig. 4). Thus, the reduction of μ-opioid receptor-like immunoreactivities in the spinal cord is probably due to the down-regulation of the μ-opioid receptor in the primary sensory neurons rather than in the dorsal horn neurons. The idea that μ-opioid receptors in the spinal dorsal horn are synthesized mainly in the dorsal root ganglia is consistent with other reports, in which dorsal rhizotomy and spinal nerve axotomy substantially reduce μ-opioid receptor-like immunoreactivity in the spinal dorsal horn (Arvidsson et al., 1995; deGroot et al., 1997).

The decrease in μ-opioid receptor mRNA in the dorsal root ganglia and the decrease in μ-opioid receptor-like immunoreactivities in the spinal dorsal horn were observed not only in postherpetic pain mice but also in mice without postherpetic pain. There was no difference between mice with and those without postherpetic pain in the extent to which μ-opioid receptor mRNA and immunoreactivities were reduced. Therefore, the down-regulation of μ-opioid receptor in the dorsal horn may not be the cause of postherpetic pain.

The expression levels of κ-opioid receptor mRNA in the dorsal root ganglia and dorsal horn were not altered in the herpetic and postherpetic pain phases. Therefore, we tested the effects of nalfurafine, a selective and potent agonist of κ-opioid receptor (Endoh et al., 1999; Nagase et al., 1998), on herpetic and postherpetic pain. It has been reported that nalfurafine produces potent antinociception in the mouse acetic acid writhing test (Nagase et al., 1998) and, in healthy and inflamed rats, in the paw pressure test through κ-opioid receptors (Endoh et al., 2000). Consistent with our previous data (Takasaki et al., 2004), nalfurafine strongly alleviated herpetic pain (Fig. 2C, D). Nalfurafine also exerted potent inhibitory effects on postherpetic pain (Fig. 2C, D). Unlike morphine, the suppressive efficacy of nalfurafine was similar between postherpetic and herpetic pain. These results strongly support the idea that expression levels of opioid receptor in the primary afferents are responsible for the analgesic effects of its agonists on postherpetic pain.

HSV-1 is a neurotropic virus and becomes latent in the nervous system after infection. HSV-1 actively proliferates in the dorsal root ganglia during a short period of the herpetic pain phase (Takasaki et al., 2000b). HSV-1 DNA is detectable in the ipsilateral dorsal root ganglia of mice with or without postherpetic pain (Takasaki et al., 2002), whereas viral antigens are not detected in the postherpetic pain phase (unpublished data), suggesting latent infection of HSV-1 in the dorsal root ganglia at the postherpetic pain phase. The down-regulation of the μ-opioid receptor was observed at the postherpetic, but not the herpetic, pain phase. Therefore, herpes viral activation itself may not be a direct cause of the down-regulation of μ-opioid receptors. In our preliminary experiments, primary afferent degeneration was observed at the postherpetic, but not the herpetic, pain phase. Peripheral axotomy produces down-regulation of μ-opioid receptors in the dorsal root ganglia (Zhang et

al., 1998). Thus, nerve injury may be a cause of the down-regulation of μ-opioid receptors. Unlike the expression of μ-opioid receptors, that of κ-opioid receptors was not altered at the postherpetic or herpetic pain phase. In contrast to μ-opioid receptors, κ-opioid receptors are up-regulated in some dorsal root ganglion neurons and are not affected in the others after peripheral axotomy (Sung et al., 2000). The specific down-regulation of μ-opioid receptors after HSV-1 infection may be due at least partly to a difference in expression regulation between κ- and μ-opioid receptors.

In summary, decreased μ-opioid receptor-like immunoreactivity in the spinal dorsal horn was observed in mice with postherpetic pain. This decrease is likely due to reduced synthesis of μ-opioid receptor mRNA in the primary sensory neurons and may contribute, at least in part, to the reduced effects of morphine to alleviate postherpetic pain. On the other hand, κ-opioid receptor expression was not affected by HSV-1 infection, and the κ-opioid receptor agonist nalfurafine exerts potent inhibitory effects on herpetic and postherpetic pain. κ-Opioid receptor agonists may be useful analgesics in the treatment of postherpetic neuralgia.

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Fig. 1.   Effects of morphine and the κ-receptor agonist TRK-820 on herpetic and postherpetic pain in mice. Morphine (A, B) and TRK-820 (C, D) were administered subcutaneously (s.c.) and per os (p.o.), respectively. The effects on herpetic (open circles) and postherpetic pain (closed circles) were tested on day 6 and 30–35 after viral inoculation, respectively. The data presented are means and standard error of the means (morphine, $n =$ 5–6; TRK-820, $n = 6$). *$P < 0.05$ when compared with the analgesic effects of the same doses of agent on herpetic pain.
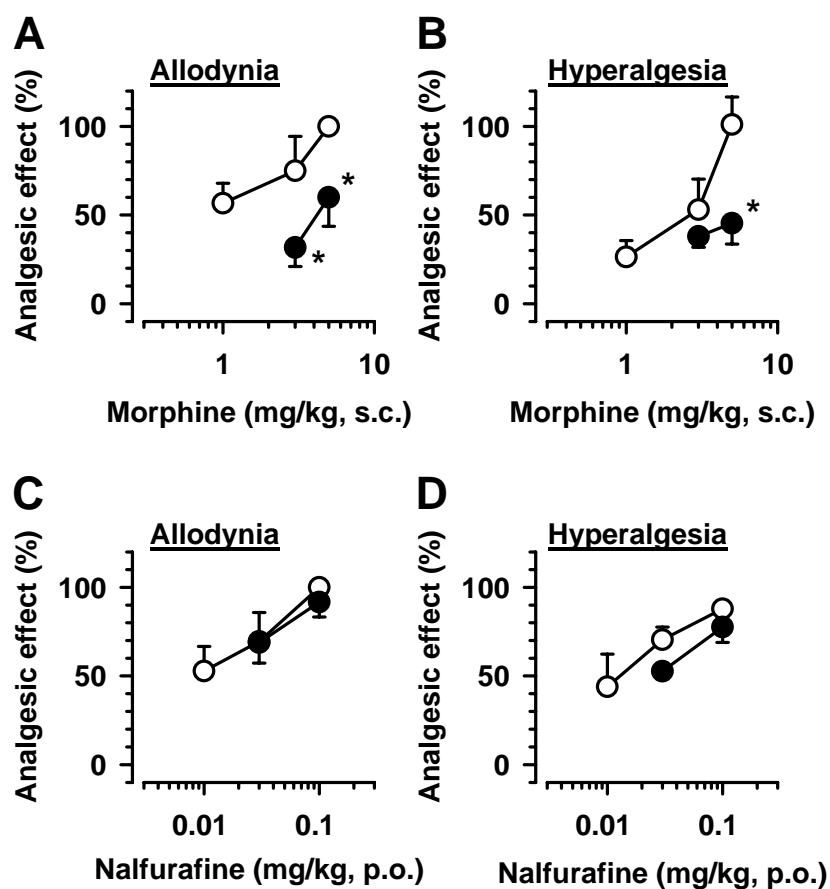
Fig. 2.   Effects of central injection of morphine on herpetic and postherpetic pain in mice. Mice were given intrathecal (i.t.; A, B) or intracerebroventricular (i.c.v.; C, D) injection of morphine. The effects on herpetic (open circles) and postherpetic pain (closed circles) were tested on day 6 and 30–35 after viral inoculation, respectively. The data presented are means and standard error of the means ($n = 6$–7). *$P < 0.05$ when compared with the analgesic effects of the same doses of agent on herpetic pain.
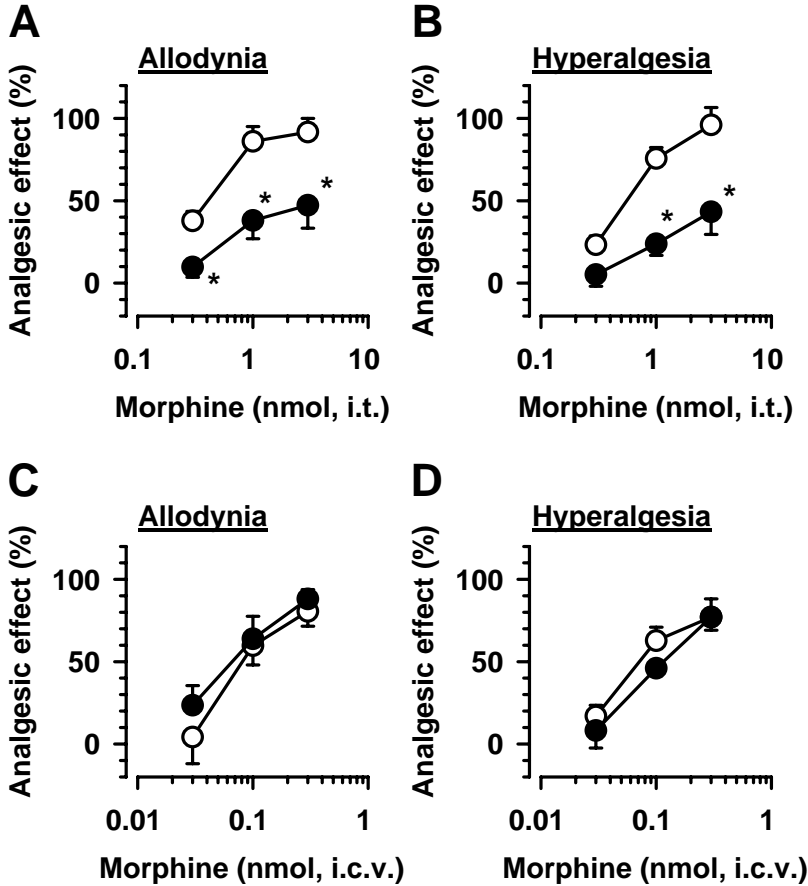
Fig. 3. μ-Opioid receptor-like immunoreactivities in the spinal dorsal horn of mice. (A) Naive mouse, (B) mouse with herpetic pain (day 6), (C) mouse with postherpetic pain (day 35), and (D) mouse without postherpetic pain (day 35). Scale bar = 200 μm. (E) The density of μ-opioid receptor-like immunoreactivities in the spinal dorsal horn were normalized to those of naive mice. The data presented are means and standard error of the means (naive, n = 6; herpetic pain, n = 6; postherpetic pain+, n = 6; postherpetic pain−, n = 5). *$P < 0.05$ vs. naive group.
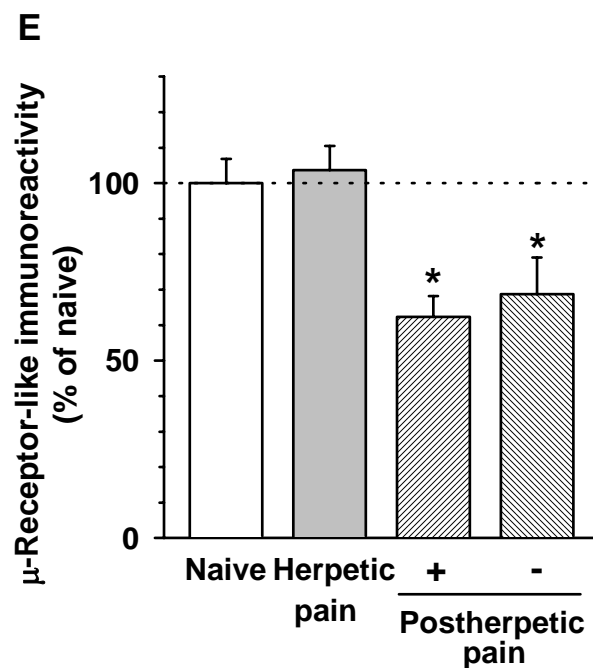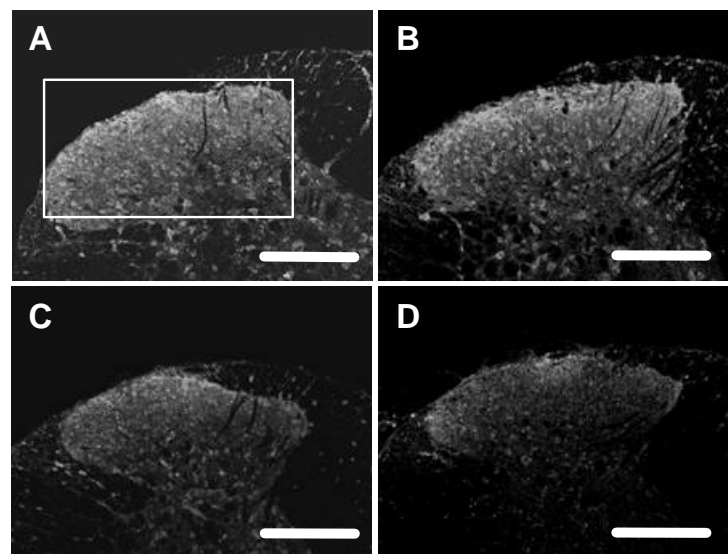
Fig. 4.   Expression of μ-opioid receptor (MOR) and κ-opioid receptor (KOR) mRNAs in the dorsal root ganglia (DRG) and spinal dorsal horn (SDH). (A) Typical examples of the expression of MOR and KOR mRNAs. (B–E) The expression levels of MOR and KOR mRNAs normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA were compared with those of naive mice. The data presented are means and standard error of the means (naive, $n = 6$; herpetic pain (HP, day 6), $n = 6$; postherpetic pain (PP)+ (day 35), $n = 8$; PP− (day 35), $n = 8$). *$P < 0.05$ vs. naive group.