Original Article

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Inhibition of morphine tolerance is mediated by painful stimuli *via* central mechanisms

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ABSTRACT: Tolerance to morphine analgesia following repeated administration disturbs the continuation of opioid therapy for severe pain. Emerging evidence suggests that the development of morphine tolerance may be antagonized by painful stimuli. To clarify the detailed mechanisms of these phenomena, we examined the effects of several pain stimuli on morphine-induced tolerance. Subcutaneous (*s.c.*) injection of morphine (10 mg/kg) produced an analgesic effect, which was evaluated by tail-pinch test. Morphine-induced analgesia was diminished by repeated administration of morphine (10 mg/kg, *s.c.*) once a day for 5 days, demonstrating the development of tolerance. Morphine analgesic tolerance was suppressed by nerve injury-induced neuropathic pain and formalin- or carrageenan-induced inflammatory pain. Tolerance to serum corticosterone elevation by morphine (10 mg/kg), which was evaluated by fluorometric assay, was also suppressed by formalin-induced inflammatory pain. Moreover, morphine analgesia induced by intracerebroventricular (10 nmol) or intrathecal (5 nmol) injection was diminished by repeated administration of morphine *s.c.*, and this was also suppressed by carrageenan-induced inflammatory pain. These results suggest that morphine tolerance is inhibited by several pain stimuli, including neuropathic and inflammatory pain, through central mechanisms.

Keywords: Opioid, analgesia, neuropathic pain, inflammatory pain

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1. Introduction

Opioid ligands including morphine are potent analgesics that are widely used for the treatment of severe pain, *e.g.*, cancer progression (*1,2*). In addition to other serious adverse effects such as respiratory depression and constipation, tolerance and dependence may also disturb the continuation of opioid therapy (*3*). Therefore, an understanding of the molecular mechanisms underlying opioid tolerance and dependence is required. Several hypotheses concerning opioid tolerance have been published. For instance, alterations of neural networks and glial activities after chronic exposure to opioids were revealed (*4-6*). These phenomena might be responsible for the opioid tolerance and dependence.

In contrast, emerging evidence suggests that the development of opioid tolerance may be antagonized by painful stimuli (*7,8*). Experimental inflammatory pain or surgical pain suppressed the morphine tolerance (*9,10*). Despite several lines of evidence, the effects of different types of pain on opioid tolerance have not been yet clarified. Moreover, detailed mechanisms underlying the inhibition of opioid tolerance by painful stimuli are unclear. Therefore, we examined the effects of several pain stimuli (*i.e.*, neuropathic and inflammatory pain) on morphine-induced tolerance, and investigated whether the central nervous system (CNS) is a key region relating to these phenomena.

2. Materials and Methods

2.1. *Animals*

Male ICR mice (15-25 g) were obtained from Japan SLC, Inc. (Osaka, Japan), and they were housed in an air-conditioned (23-24°C, 60-70% relative humidity) vivarium with a 12 h dark/light cycle (light on from 8:00 to 20:00). Water and food were available *ad libitum*. All experimental procedures were approved by the Animal Research Committee of Wakayama Medical University and complied with the ethical guidelines of the International Association for the Study of Pain (*11*).

2.2. *Morphine administration*

Morphine hydrochloride (Takeda, Osaka, Japan) was dissolved in physiological saline, and administered to mice by subcutaneous (*s.c.*, 0.1 mL/10 g), intracerebroventricular (*i.c.v.*, 5 μL), or intrathecal (*i.t.*, 5 μL) injection. The *i.c.v.* injections were administered into the left lateral ventricle, and the *i.t.* injections were administered into the region between spinal L5 and L6 segments as described in a previous report (*12,13*). For the *i.c.v.* and *i.t.* injections, Hamilton microsyringes fitted with 32-gauge or 26-gauge needles were used, respectively.

2.3. *Tail-pinch test*

The analgesic effects of morphine were evaluated by the tail-pinch test as described in our previous report (*14*). Briefly, the mouse tail root was pressed with a 6-mm-wide flattened clip, which was adjusted to about 500 g pressure. Nociceptive responses were indicated by the latency required for response to the pressure by biting the clip. To avoid tissue damage, a cut-off time of 15 sec was set. The clip was applied every 15 min following morphine administration over a 120 min period. The percentage of maximum possible effect (%MPE) was calculated using the following formula: %MPE = 100 × (measured latency – baseline latency)/(15 – baseline latency). The magnitude of analgesia was evaluated by the area under the curve (AUC) calculated from the time course of %MPE.

2.4. *Estimation of serum corticosterone (SCS) level*

Because the level of SCS due to circadian rhythm was most stable before noon each day, the blood was collected for SCS determination at 10:00-11:30. Mice were killed by decapitation and trunk blood was collected 1 h after morphine injection. The serum was separated by centrifugation and SCS level was measured by fluorometric assay according to the method of Zenkar and Bernstein (*15*).

2.5. *Pain models*

Three pain models were used in this study. For the neuropathic pain model, mice were anesthetized with sodium pentobarbital (70 mg/kg, *i.p.*) and the sciatic nerves of both hind limbs were exposed through a small incision. Then, 1/3 of the nerve thickness was tightly ligated with a silk suture (*16,17*). As a sham control, the incision site was closed without ligation. To induce acute inflammatory pain, mice were given 2% formalin or 1% λ-carrageenan into the intraplantar (*i.pl.*, 20 μL) surface of both hind paws (*18,19*). Formalin and carrageenan were dissolved in phosphate buffered saline (PBS, vehicle control). The *i.pl.* injection was performed using a Hamilton microsyringe fitted with a 30-gauge needle.

2.6. *Statistical analysis*

Data are presented as the mean ± S.E.M. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by Tukey multiple comparisons test, or two-way ANOVA followed by Bonferroni multiple comparisons test. Significance was established at $p < 0.05$.

3. Results

3.1. *Evaluation of morphine analgesia and its tolerance*

Subcutaneous injection of morphine (10 mg/kg) prolonged the withdrawal latency at 15 min after administration. The prolonged latency lasted for 75 min and was restored within 120 min, indicating the analgesic effect of morphine. Saline injected as a control had no effect on the pain threshold (Figure 1A). By repeated administration of morphine (10 mg/kg) once a day for 5 days, morphine-induced analgesia was gradually diminished, demonstrating the development of analgesic tolerance as shown in AUC (Figure 1B).

3.2. *Inhibition of morphine analgesic tolerance by nerve ligation-induced neuropathic pain*

To examine whether an analgesic tolerance to morphine is affected by neuropathic pain, mice were given partial sciatic nerve ligation (PSL) 1 day before the first morphine administration. Morphine (10 mg/kg) was administered once a day for 5 days, and morphine-induced analgesia was evaluated on days 1, 3 and 5. On day 1, the time-course of morphine analgesia in PSL-operated mice was similar to that in sham-operated mice (Figure 2A), indicating that morphine analgesia was not affected by neuropathic pain. In contrast, on day 5, the analgesic

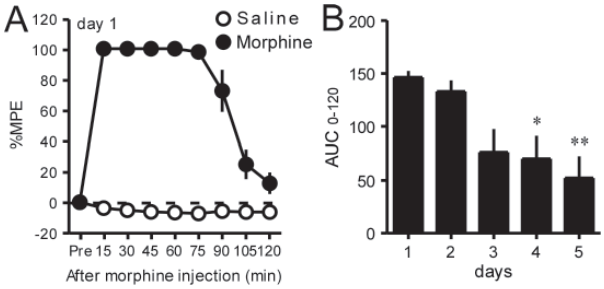

Figure 1. Evaluation of morphine analgesia and tolerance. **(A)** Time course of morphine analgesia on day 1. Morphine (10 mg/kg) or saline was subcutaneously (*s.c.*) administered, and the analgesic effects during a 120 min period after injection were evaluated by tail-pinch test. Analgesic effects are shown as time course of %MPE. **(B)** The development of tolerance to morphine analgesia, estimated by AUC. Morphine (10 mg/kg, *s.c.*) was repeatedly administered once a day for 5 days, and analgesic effect on each day is shown as AUC calculated from time course of %MPE. Data are presented as the mean ± S.E.M. of 6-8 mice. %MPE: the percentage of maximum possible effect; AUC: area under the curve. * $p < 0.05$, ** $p < 0.01$ *vs.* day 1.

effect in PSL-operated mice was greater than that in sham-operated mice (Figures 2B and 2C), indicating that the development of tolerance to morphine analgesia was significantly suppressed by PSL-induced neuropathic pain.

3.3. *Inhibition of morphine analgesic tolerance by formalin- or carrageenan-induced inflammatory pain*

We examined the effects of inflammatory pain on the development of analgesic tolerance to morphine. First, 2% formalin was *i.pl.* injected into mice 2 h before the first morphine administration. Repeated morphine administration and the evaluation of morphine tolerance were performed as shown in Figure 2. On day 1, there was no difference between morphine (10 mg/kg)-induced analgesia in formalin-treated mice and that in PBS-treated control mice, indicating that morphine analgesia was not affected by inflammatory pain (Figure 3A). In contrast, on day 5, the analgesic effect in FOR-treated mice was greater than that in PBS-treated mice (Figure 3B). As shown in AUC, the development of morphine tolerance was significantly suppressed by formalin-induced

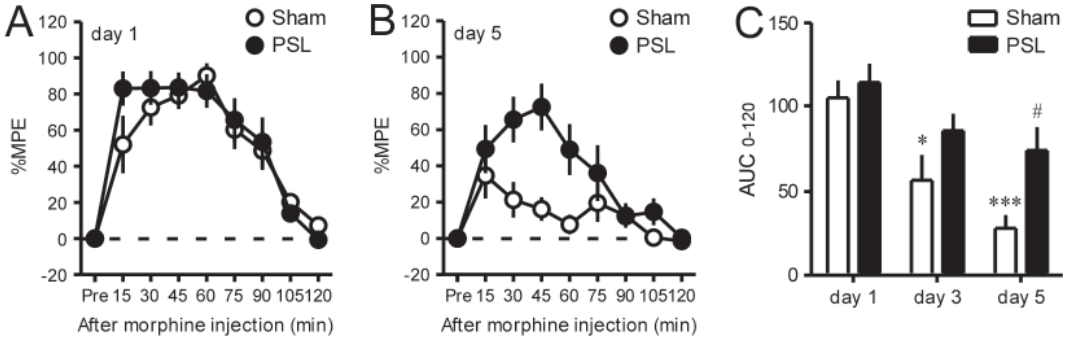

Figure 2. Inhibition of morphine analgesic tolerance by nerve ligation-induced neuropathic pain. Partial sciatic nerve ligation (PSL) was performed 1 day before morphine administration. As sham control, nerve was exposed. Morphine (10 mg/kg, *s.c.*) was administered once a day for 5 days, and morphine analgesia was evaluated for 120 min on days 1, 3, and 5. Analgesic effects of morphine on days 1 **(A)** and 5 **(B)** are shown as time course of %MPE and AUC **(C)**. Data are presented as the mean ± S.E.M. of 8-9 mice. %MPE: the percentage of maximum possible effect; AUC: area under the curve. * $p < 0.05$, *** $p < 0.001$ *vs.* day 1. # $p < 0.05$ *vs.* sham.

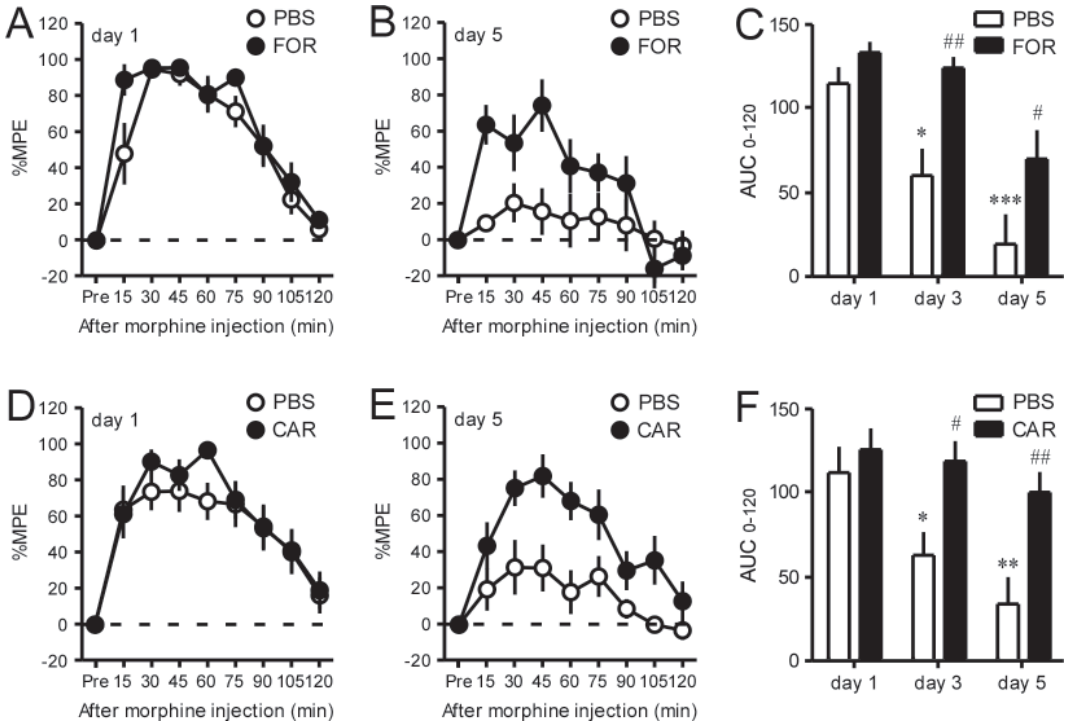

Figure 3. Inhibition of morphine analgesic tolerance by formalin- or carrageenan-induced inflammatory pain. 2% formalin (FOR; **A**-**C**) or 1% carrageenan (CAR; **D**-**F**) was *i.pl.* injected 2 h or 3 h before morphine administration on day 1, respectively. As vehicle control, PBS was injected. Morphine (10 mg/kg, *s.c.*) was administered once a day for 5 days, and morphine-induced analgesia was evaluated for 120 min on days 1, 3, and 5. Analgesic effects of morphine in FOR- or CAR-treated group on days 1 (**A**, **D**) and 5 (**B**, **E**) are shown as time course of %MPE (**A**, **B**, **D**, **E**), and AUC (**C**, **F**). Data are presented as the mean ± S.E.M. of 8-9 mice. %MPE: the percentage of maximum possible effect; AUC: area under the curve. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs*. day 1. # $p < 0.05$, ## $p < 0.01$ *vs*. PBS.

inflammatory pain on days 3 and 5 (Figure 3C). Second, as another inflammatory pain model, 1% carrageenan was *i.pl.* injected into mice 3 h before the first morphine administration. On day 1, there was no difference between morphine (10 mg/kg)-induced analgesia in carrageenan-treated mice and that in PBS-treated control mice, suggesting that carrageenan injection also had no effect on morphine-induced analgesia on day 1 (Figure 3D). On day 3 and 5, the analgesic effect in carrageenan-treated mice was greater than that in PBS-treated mice (Figures 3E and 3F).

3.4. *Effects of formalin-induced inflammatory pain on tolerance to SCS elevation by morphine*

Morphine produces not only analgesia but also SCS elevation. To confirm the inhibition of morphine analgesic tolerance by inflammatory pain, we examined the effect of formalin-induced inflammatory pain on tolerance to SCS elevation by repeated morphine administration. Morphine (10 mg/kg) was administered once a day for 5 days, and the SCS level was evaluated 1 h after morphine injection (10 mg/kg) on day 6. Basal SCS level was 6.8 ± 1.3 μg/dL (n = 5) in naive mice. After repeated saline injection for 5 days, SCS was markedly elevated by morphine. The morphine-induced SCS elevation was significantly suppressed after repeated morphine administration for 5 days, while tolerance was developed following *i.pl.* administration of PBS. The repeated morphine-induced suppression of SCS elevation was reversed by formalin *i.pl.*, indicating the inhibition of tolerance by formalin-induced inflammatory pain (Figure 4).

3.5. *Inhibition of tolerance to intracerebroventricular or intrathecal morphine analgesia by carrageenan-induced inflammatory pain*

Generally, it is believed that morphine develops analgesic tolerance through a central mechanism, although morphine acts on both the peripheral and central nervous systems. To investigate the key region, *i.e.*, supraspinal or spinal sites, of the inhibitory effects of painful stimuli on tolerance to morphine analgesia, we examined the effects of carrageenan-induced inflammatory pain on tolerance to *i.c.v.* or *i.t.* morphine. The analgesic tolerance to morphine was developed by the administration of morphine (10 mg/kg, *s.c.*) once a day for 3 days (on days 2, 3, and 4), and morphine *i.c.v.* or *i.t.*-induced analgesia was evaluated on days 1 and 5. On day 1, there was no effect of carrageenan *i.pl.* injection on the time course of morphine *i.c.v.* (10 nmol)-induced analgesia (Figure 5A). In PBS *i.pl.*-treated mice, the analgesic effect of *i.c.v.* morphine on day 5 was significantly diminished in comparison with that on day 1 by repeated morphine *s.c.* administration, which indicated the development of analgesic tolerance to *i.c.v.* morphine. However, on day 5, the analgesic effect of morphine *i.c.v.* in carrageenan *i.pl.*-treated mice was greater than that in PBS-treated mice (Figures 5B and 5C). On day 1, there was no effect of carrageenan *i.pl.* injection on morphine *i.t.* (5 nmol)-induced analgesia (Figure 5D). However, on day 5, the analgesic effect of morphine *i.t.* in carrageenan *i.pl*.-treated mice was greater than that in PBS-treated mice (Figures 5E and 5F). These results suggest that the development of tolerance to *i.c.v.* or *i.t.* morphine analgesia was significantly inhibited by carrageenan-induced inflammatory pain on day 5.

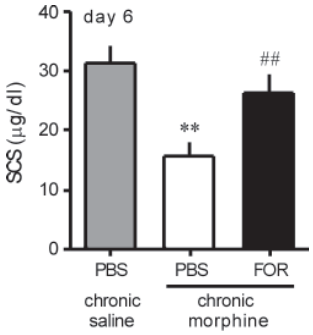

Figure 4. Inhibitory effect of intraplanter formalin on tolerance to morphine-induced corticosterone elevation. 2% formalin (FOR) was *i.pl.* injected 2 h before morphine administration on day 1. As vehicle control, PBS was injected. Morphine (10 mg/kg, *s.c.*) or saline was administered once a day for 5 days, and SCS elevation at 1 h after morphine (10 mg/kg, *s.c.*) administration was evaluated on day 6. Data are presented as the mean ± S.E.M. of 5-6 mice. ** $p < 0.01$ *vs.* chronic saline. $^{\#\#}$ $p < 0.01$ *vs.* PBS.

4. Discussion

It is generally considered that opioid tolerance may be antagonized under painful conditions in clinic (*1,8*). Here, we demonstrated that morphine-induced analgesia was gradually decreased by repeated morphine administration in mice. This indicated the development of tolerance to morphine analgesia, which is consistent with previous reports (*20,21*). Therefore, we examined the effect of neuropathic and inflammatory pain on the development of tolerance to morphine analgesia in mice. PSL-induced neuropathic pain (*i.e.*, allodynia and thermal hyperalgesia) is usually observed on the next day of PSL, and lasts for at least 14 days (*22*). After *i.pl.* injection of formalin or carrageenan, hyperalgesia and edema were observed, which are typical prognostics of inflammation, and these phenomena lasted for 7-14 days (*18,19*). On day 1, morphine analgesia evaluated by the time-course and AUC was not affected by both PSL-induced neuropathic pain and formalin- or carrageenan-induced inflammatory pain. We found that tolerance to morphine analgesia was inhibited by not only PSL-induced neuropathic pain, but also formalin- or carrageenan-induced inflammatory pain.

To confirm the inhibitory effects of pain stimuli on morphine tolerance, we focused on SCS elevation, which is one of characteristic effects of morphine. Normally, acute administration of morphine increases

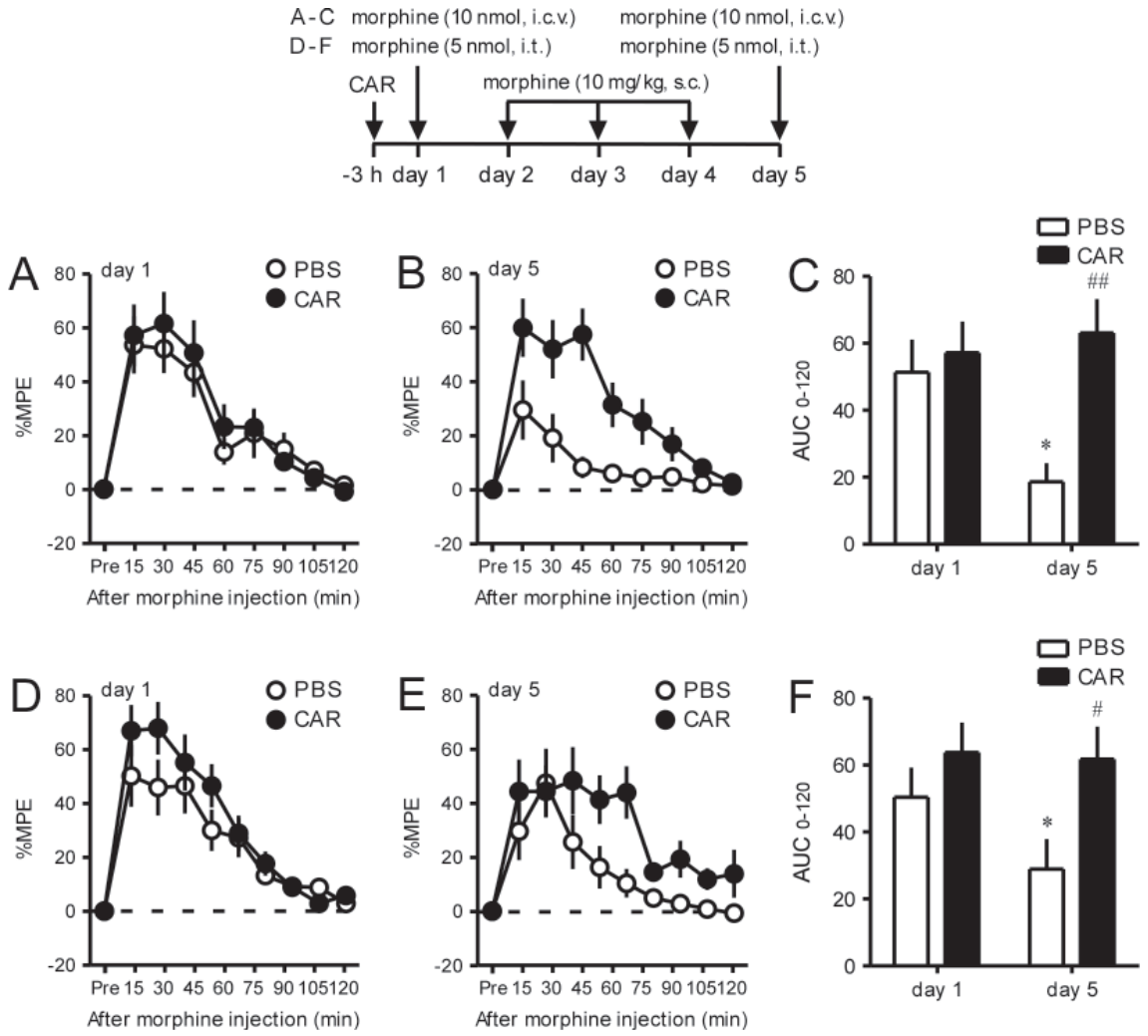

Figure 5. Inhibition of intracerebroventricular or intrathecal morphine tolerance by carrageenan-induced inflammatory pain. 1% carrageenan (CAR) was *i.pl.* injected 3 h before morphine administration on day 1. As vehicle control, PBS was injected. Morphine was administered *i.c.v.* (10 nmol, **A-C**) or *i.t.* (5 nmol, **D-F**) on days 1 and 5. For the development of tolerance, morphine (10 mg/kg) was *s.c.* administered on days 2, 3, and 4. Morphine-induced analgesia was evaluated for 120 min on days 1 and 5. Experimental schedules are indicated above. Analgesic effects of *i.c.v.* or *i.t.* morphine on days 1 (**A**, **D**) and 5 (**B**, **E**) are shown as time course of %MPE, respectively, and AUC (**C**, **F**) was calculated from time course of %MPE. Data are presented as the mean ± S.E.M. of 11-13 mice. *i.c.v.*: intracerebroventricular; *i.t.*: intrathecal; %MPE: the percentage of maximum possible effect; AUC: area under the curve. * $p < 0.05$ *vs*. day 1. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs*. PBS.

the SCS level in a dose-dependent manner. This effect is also diminished by repeated administration of morphine, indicating the development of tolerance (*23*). The tolerance to SCS elevation was also suppressed by formalin-induced inflammatory pain, strongly suggesting that painful stimuli might affect the mechanism of tolerance through not only an analgesic effect, but also an endocrine effect. It is well known that morphine acts on the hypothalamus to release corticotropin-releasing factor, which then releases adrenocorticotropic hormone (ACTH) from the pituitary. The released ACTH works on the adrenal cortex and results in the elevation of SCS (*24*). These observations indicate that tolerance to SCS elevation by morphine may be mediated by a supraspinal site, and are consistent with analgesic tolerance.

Several lines of evidence indicate the potential molecular mechanisms of opioid tolerance. It is well known that opioid receptors are 7-transmembrane G protein-coupled receptors that are classified in three types, μ, δ, and κ (*25*). All of the opioid receptors are associated with an intracellular Gαi protein, and are thought to produce analgesic effects through the activation of Gαi-related pathways (*26*). Notably, the μ-opioid receptor largely contributes to opioid analgesia, and desensitization of the μ-opioid receptor may be responsible for opioid tolerance (*27*). According to the cAMP hypothesis reported previously (*28*), opioid tolerance develops due to receptor internalization and subsequent desensitization at the single cell level (*29*). The relative activity versus endocytosis hypothesis was also suggested as a mechanism to explain the receptor internalization-dependent desensitization (*3,28*). Furthermore, the relationship between the activation of protein kinase C (PKC) and receptor desensitization was reported. Indeed, blockade of the

PKC pathway inhibited the development of morphine tolerance (*30*). Recently, growing evidence suggests that opioid tolerance is mediated by anti-opioidergic neural networks in the CNS, *i.e.*, the midbrain (*6*). Namely, anti-opioidergic neural networks in the CNS were enhanced by chronic morphine treatment, and cholecystokinin, neuropeptide, and glutamate, which are candidates of typical anti-opioid mediators, prevented the opioid effects (*31-33*). For example, concomitant treatment with MK801 (NMDA glutamatergic receptor antagonist) suppressed the development of tolerance to morphine analgesia. Furthermore, chronic morphine exposure activates microglia and astrocytes in the CNS (*5*). These cells express μ-opioid receptor and produce several inflammatory mediators following morphine treatment (*34*). For instance, the critical roles of interleukin (IL)-1β and IL-6 in morphine tolerance were identified (*21,35*). Taken together, anti-opioidergic neural networks and inflammation in the CNS largely participate in the opioid tolerance.

It was reported that analgesic effects were produced through not only the CNS but also the peripheral nervous system, *e.g.* primary afferents (*36*). To clarify a key region underlying inhibition of morphine tolerance by painful stimuli, we evaluated the effect of carrageenan-induced inflammatory pain on the analgesia and its tolerance by *i.c.v.*- or *i.t.*- administered morphine. In these experiments, analgesic tests after morphine *i.c.v.* or *i.t.* administration were performed on days 1, and 5. To avoid excess tissue damage by the manipulation of *i.c.v.* and *i.t.*, morphine was systemically (*s.c.*) administered on days 2, 3, and 4 for the development of tolerance. Tolerances to both *i.c.v.*- and *i.t.*-administered morphine were inhibited by carrageenan-induced inflammatory pain. These results indicate that interaction between opioid actions and pain stimuli in the CNS (midbrain and spinal cord) is crucial for the inhibition of opioid tolerance mediated by painful stimuli.

Although we could not determine the exact mechanisms underlying the inhibition of morphine tolerance by painful stimuli in this study, a possible mechanism is suggested. Previous reports showed that the inhibition of morphine tolerance and dependence by painful stimuli was partially mediated by activation of the endogenous κ opioid system (*10,37*). Therefore, this mechanism our observations in the midbrain and spinal cord presented here. However, further research into the mechanisms is still required.

In conclusion, morphine analgesic tolerance is inhibited by several pain stimuli, including neuropathic and inflammatory pain, through central mechanisms. The mechanistic details remain to be established, but these findings suggest that the clinical use of opioids should not be limited by its adverse tolerance, and we hope that these findings may lead to the development of more efficacious therapeutics for patients suffering from severe pain.

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