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ORIGINAL ARTICLE

Microglial activation involved in morphine tolerance is not mediated by toll-like receptor 4

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Abstract
Purpose Morphine is a powerful analgesic but its effect is often diminished owing to the development of tolerance. It has been suggested that morphine activates microglia through its action on the toll-like receptor 4 (TLR4) in the spinal cord, leading to suppression of the morphine effect. However, it has not been examined whether the development of morphine tolerance is affected by the deletion and mutation of the *TLR4* gene.
Methods Mice were treated with morphine (60 mg/kg) or vehicle once daily for five consecutive days to induce morphine tolerance, which was assessed by the tail-flick test before and after the treatment period. The effect of the microglial inhibitor minocycline, and the effect of *TLR4* mutation (C3H/HeJ mouse) and deletion (*TLR4*-knockout mouse) on the development of morphine tolerance were tested. The expression of the microglial activation marker, CD11b, in the spinal cords of *TLR4*-knockout and wild-type mice after morphine treatment for 5 days was assessed by reverse-transcription polymerase chain reaction.
Results Minocycline attenuated the development of morphine tolerance in mice. Mutation or deletion of the *TLR4* gene did not significantly affect the development of morphine tolerance. CD11b mRNA expression was increased after morphine treatment both in *TLR4*-knockout and wild-type mice.
Conclusion Microglial activation caused by a mechanism independent of TLR4 is involved in the development of morphine tolerance. Further studies are necessary to clarify the cellular mechanisms of morphine-induced microglial activation.

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Introduction

Opioids are the most powerful analgesic drugs used for acute and chronic pain management [1]. However, their clinical utility is often limited by the development of analgesic tolerance, in which dose increases are needed without change of the disease state. The mechanism of opioid tolerance has been extensively studied, but has not been completely clarified. Neurons have been the main targets of studies of the development of opioid tolerance, and it has been shown that a number of protein kinases, ion channels, second messenger-synthesizing enzymes, cytoskeletal proteins, and neurotrophic factors are involved in the functional modulation of neurons in opioid tolerance [2]. Recently, it has been suggested that the activation of glial cells, including astrocytes and microglia, at the level of the spinal cord plays an important role in the development of opioid tolerance [3–6]. It has been reported that morphine induces microglial activation through its action on the toll-like receptor 4 (TLR4) expressed in microglia, leading to the release of cytokines, including interleukin 1b, and suppression of the analgesic effect of morphine [7]; these features suggest that TLR4 may be involved in the microglial activation that occurs in the development of opioid tolerance, but it is not clear whether TLR4 is involved in the development of opioid tolerance itself.

In this investigation, we aimed to clarify whether TLR4 activation was involved in the development of opioid tolerance in vivo. For this purpose, we used the mouse strain C3H/HeJ, which is known to have a missense mutation of the *TLR4* gene and to produce non-functional TLR4-protein [8], and *TLR4*-knockout mice [9].

Materials and methods

Animals

Male adult mice, aged 6–8 weeks, of the following strains were used in the experiments described below: C57BL/6, C3H/HeJ, and C3H/HeN, obtained from Japan SLC (Shizuoka, Japan); and *TLR4*$^{-/-}$ mice (*TLR4*-knockout) on a C57BL/6 background, obtained from Oriental Bioservice (Kyoto, Japan). All mice were housed in an air-conditioned room (24 ± 2 °C, 50 % relative humidity) with the lights on from 7 a.m. to 9 p.m. Food and water were available ad libitum. The experiments were performed after at least 1 week of acclimatization. All tests were performed between 9 a.m. and 3 p.m. The study was approved by the Animal Experiment Committee of Kyoto University. All efforts were made to minimize animal suffering and the number of animals used.

Morphine treatment

Morphine hydrochloride (Takeda, Osaka, Japan) and minocycline hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in physiologic saline. Mice received morphine (60 mg/kg) or saline subcutaneously once daily for five consecutive days to induce chronic morphine tolerance. This dose of morphine is similar to that used to induce tolerance in mice in a previous study [10]. To assess the effect of microglial activation on the development of morphine tolerance, C57BL/6 mice were intraperitoneally administered with minocycline (50 mg/kg) or saline 30 min before morphine treatment for 5 consecutive days. Morphine tolerance was assessed by the tail-flick test on the day before and the day after completion of the morphine treatment. The mice were divided into 4 groups that were subjected to different combinations of pretreatment and treatment: vehicle/vehicle, minocycline/vehicle, vehicle/morphine, and minocycline/morphine.

To investigate whether TLR4 is involved in morphine tolerance, we compared C3H/HeJ, a mouse strain producing non-functional TLR4, and the wild-type (WT) strain C3H/HeN, as well as comparing the strain *TLR4*-knockout on a C57BL/6 background and the WT strain C57BL/6. The tail-flick test was performed on the day before the morphine treatment began, on the third day of the treatment, and on the day after the treatment was completed.

Tail-flick test

The pain threshold to a thermal stimulus was assessed by the tail-flick latency evoked by a noxious hot stimulus, as determined with a tail-flick analgesic meter (Tail Flick Analgesia Meter; Muromachi Kikai, Kyoto, Japan). Tail-flick latencies (TFLs) were determined from the mean of two consecutive latencies. The level of heat intensity was preset so that the baseline TFL ranged between 2.7 and 3.3 s. To prevent tissue damage, the cut-off time was 6 s. After the measurement of baseline TFL, morphine 4 mg/kg was injected subcutaneously to each mouse. After 30 min, the post-treatment TFL was determined. The maximum possible effect (%MPE) was calculated as follows: $\%\text{MPE} = (\text{post-treatment TFL} - \text{baseline TFL})/(\text{cut-off time} - \text{baseline TFL}) \times 100$.

RNA isolation and real-time polymerase chain reaction (PCR)

Mice were sacrificed by carbon dioxide treatment and cervical dislocation. The lumbar part of the spinal cord (approximately the L4/L5 level) was flushed out with phosphate-buffered saline, using a 5-ml syringe and an 18-gauge needle, and collected into ice-cold tubes and stored at −80 °C until the time of analysis.

Total RNA was isolated from the stored spinal cord tissues, using a Takara FastPure RNA Kit (Takara Biochemicals, Shiga, Japan) following a protocol provided by the manufacturer. RNA was purified using RNeasy (Qiagen, Valencia, CA, USA) and treated with DNase. UV spectrophotometry was used to assess purity and concentration. First-strand complementary DNA synthesis and real-time PCR were performed using the QuantiTect SYBR green PCR kit (Qiagen) and an Applied Biosystems 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA) following a protocol provided by the manufacturer. PCR primers were purchased from Qiagen. The relative change in expression of each target mRNA relative to 18S rRNA was calculated.

Statistics

The behavioral data are presented as means ± SEM. GraphPad Prism (version 5 for Mac; San Diego, CA, USA) software was used for all statistical analyses. Two-way analysis of variance (ANOVA) with Bonferroni post-hoc tests were used to determine the statistical significance of differences between behavioral measures. Unpaired *t*-tests were used to determine statistical significance in the mRNA tests. For all analyses, $p < 0.05$ was considered significant.

Results

Minocycline suppresses the development of morphine tolerance

First, the effect of minocycline, an inhibitor of microglia, on the development of morphine tolerance was examined.

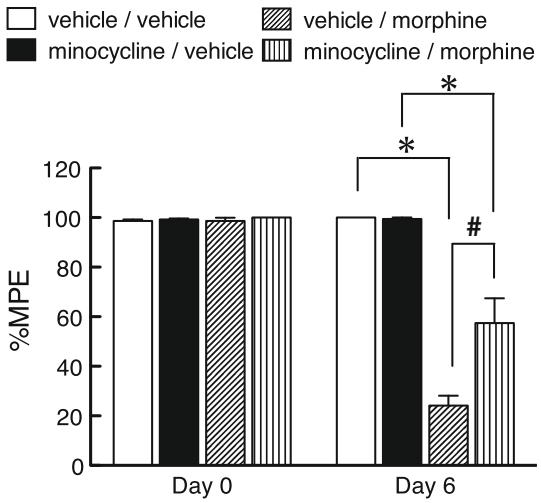

Fig. 1 Effect of minocycline pretreatment on the development of morphine tolerance. Four groups of mice (vehicle/vehicle, minocycline/vehicle, vehicle/morphine, and minocycline/morphine) were subjected to different combinations of pretreatment with vehicle or minocycline and treatment with vehicle or morphine (60 mg/kg) for 5 consecutive days. On the day before (day 0) and the day after (day 6) the treatment period, the tail-flick test was performed and the percentage of the maximum possible effect (*%MPE*) was calculated. Data are shown as means ± SEM ($n = 6$). $^{*}p < 0.001$, $^{\#}p < 0.01$

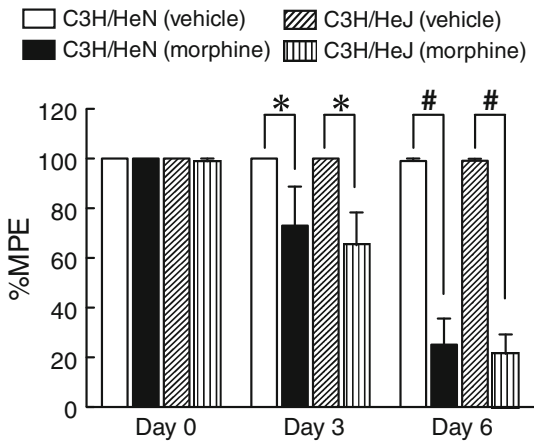

Fig. 2 Morphine tolerance induced in mouse strain C3H/HeJ (*TLR4* missense mutation) and C3H/HeN (wild-type). Mice received vehicle or morphine (60 mg/kg) injection for 5 consecutive days. On day 0 (1 day before the treatment period), day 3, and day 6 (1 day after the treatment period), the tail-flick test was performed and the percentage of the maximum possible effect (%MPE) was calculated. Data are shown as means ± SEM ($n = 6$). $^{*}p < 0.05$, $^{\#}p < 0.01$

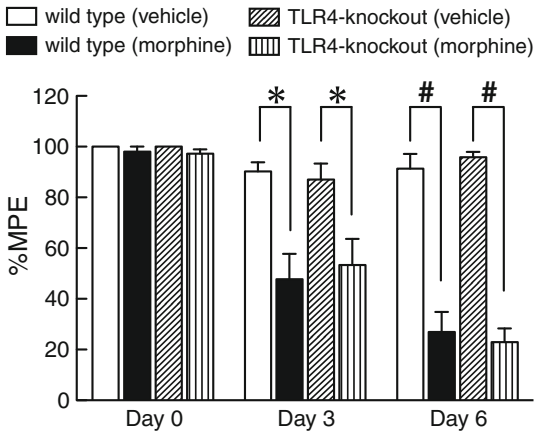

Fig. 3 Morphine tolerance induced in wild-type and *TLR4*-knockout mice. Mice received vehicle or morphine (60 mg/kg) injection for 5 consecutive days. On day 0 (1 day before the treatment period), day 3, and day 6 (1 day after the treatment period), the tail-flick test was performed and the percentage of the maximum possible effect (%MPE) was calculated. Values are shown as means ± SEM ($n = 6$). $^{*}p < 0.05$, $^{\#}p < 0.01$

Administration of minocycline for 5 days without morphine did not affect the %MPE (98.6 ± 0.6 –100 ± 0 %), indicating that minocycline itself does not significantly affect the analgesic effect of morphine. Repeated injections of morphine with prior administration of saline significantly reduced the analgesic effect of morphine, assessed as %MPE [98.6 ± 1.4–24.2 ± 4.0 % ($n = 6$)] (Fig. 1), indicating the development of tolerance to morphine. In the mice administered with minocycline 30 min prior to morphine injection, the analgesic effect of morphine was also reduced after repeated injections of morphine [100 ± 0–57.4 ± 10.1 % ($n = 6$)], but this effect was significantly smaller compared with that in mice without minocycline pretreatment. These results indicate that minocycline pretreatment significantly suppresses the development of morphine tolerance, suggesting the involvement of microglia activation in the development of morphine tolerance.

Involvement of TLR4 in the development of morphine tolerance

To explore the possibility that morphine induces TLR4 activation leading to microglial activation and morphine tolerance, we examined whether the development of morphine tolerance in the mice was affected by *TLR4* mutation. The *TLR4*-mutated mouse strain C3H/HeJ and the WT strain C3H/HeN developed a similar degree of morphine tolerance (Fig. 2). Furthermore, *TLR4*-knockout and WT mice showed similar degree of morphine tolerance (Fig. 3). These results indicate that the development of morphine tolerance is not affected by the presence of functional *TLR4*.

Microglia are activated by morphine in *TLR4*-knockout mice

To assess microglial activation by morphine, the mRNA level of the microglial activation marker CD11b was examined [11]. Figure 4 reveals that CD11b mRNA was significantly increased after repeated injections of

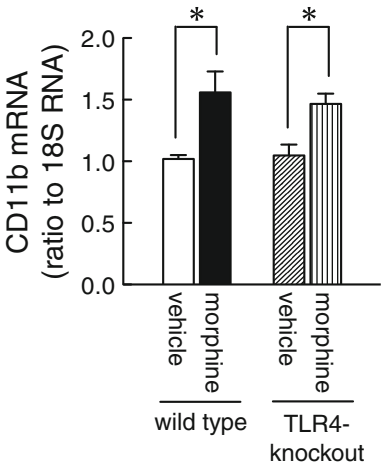

Fig. 4 Effect of morphine treatment on CD11b mRNA expression in the mouse spinal cord. Wild-type and *TLR4*-knockout mice were injected with vehicle or morphine (60 mg/kg) subcutaneously once daily for five consecutive days, and then RNA was prepared from the spinal cord to be analyzed by real-time polymerase chain reaction (PCR). The amount of CD11b mRNA compared to that of 18S RNA is expressed as the mean ± SEM ($n = 6$) of the ratio to that in vehicle-treated mice. *$p < 0.01$

morphine (once daily for 5 consecutive days) both in *TLR4*-knockout and WT mice. These results suggest that microglia are activated by morphine through a mechanism which does not involve TLR4.

Discussion

We showed that morphine tolerance was attenuated by minocycline pretreatment and that CD11b mRNA expression was increased by morphine treatment, suggesting that microglial activation is induced by repeated administration of morphine and that this activation is involved in the development of morphine tolerance. Furthermore, it was shown that a missense mutation or genetic deletion of the *TLR4* gene in mice affected neither the development of morphine tolerance nor the morphine-induced increase in CD11b mRNA expression, suggesting that TLR4 is not involved in morphine-induced microglial activation.

It has been reported that glial cells, including astrocytes and microglia, are involved in the development of morphine tolerance. Propentofylline, which depresses the activation of microglia and astrocytes, was shown to attenuate morphine tolerance, withdrawal-induced hyperalgesia, and associated spinal inflammatory immune responses in rats, suggesting that spinal glia and proinflammatory cytokines contribute to the mechanism of morphine tolerance and associated abnormal pain sensitivity [6]. Narita et al. [5] reported an increase in the level of glial fibrillary acidic protein (GFAP)-positive astrocytes in the spinal cord after chronic administration of morphine, and suggested that astrocytes may play a role in the development of opioid tolerance. The involvement of microglia was originally suggested by the suppression of morphine tolerance by minocycline, which inhibits the activation of microglia [3, 4]. Our results demonstrating the suppression of morphine tolerance by minocycline and activation of microglia by repeated injection of morphine are consistent with previous reports.

The mechanism of morphine-induced microglial activation has yet to be clarified. Hutchinson et al. [7] have hypothesized that TLR4 activation is involved in morphine-induced microglial activation, which suggests that TLR4 may be involved in morphine tolerance. However, they did not investigate whether morphine tolerance was affected in *TLR4*-knockout mice. Our results demonstrated that *TLR4*-knockout, *TLR4*-mutated, and WT mice showed a similar degree of morphine tolerance. We cannot exclude the possibility that a greater extent of morphine tolerance could have been induced in *TLR4*-knockout mice than in WT mice if morphine had been administered daily for 5 consecutive days at a dose less than 25 mg/kg, because the analgesic potency of morphine is threefold higher in *TLR4*-knockout mice than in WT mice [7]. However, we speculate that the dose of morphine used to induce tolerance in the present study, 60 mg/kg, which produced nearly maximum analgesic effects both in *TLR4*-knockout mice and WT mice, would induce a similar extent of tolerance. Thus, our results suggest that microglial activation is at least partially involved in the development of morphine tolerance, but is not induced by TLR4 activation. Zhang et al. [12] reported that in mice deficient in TLR2, morphine-induced microglial activation and the main symptoms of morphine withdrawal were attenuated compared with these features in WT mice. It has also been reported that P2X4 and P2X7 receptors are involved in morphine-induced microglial activation [13, 14]. Although microglia have been shown to express opioid receptors [15], the involvement of opioid receptors in morphine-induced microglial activation via TLRs is controversial.

In conclusion, our findings suggest that microglial activation in the development of morphine tolerance is not mediated by TLR4. Further investigations are necessary to clarify the precise mechanism of opioid tolerance.

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