Brief Communications

Involvement of Spinal Microglial P2X$_7$ Receptor in Generation of Tolerance to Morphine Analgesia in Rats

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Morphine loses analgesic potency after repeated administration. The underlying mechanism is not fully understood. Glia are thought to be involved in morphine tolerance, and P2X$_7$ purinergic receptor (P2X$_7$R) has been implicated in neuron–glia communication and chronic pain. The present study demonstrated that P2X$_7$R immunoreactivity was colocalized with the microglial marker OX42, but not the astrocytic marker GFAP, in the spinal cord. The protein level of spinal P2X$_7$R was upregulated after chronic exposure to morphine. Intrathecal administration of Brilliant Blue G (BBG), a selective P2X$_7$R inhibitor, significantly attenuated the loss of morphine analgesic potency, P2X$_7$R upregulation, and microglial activation. Furthermore, RNA interference targeting the spinal P2X$_7$R exhibited a similar tolerance-attenuating effect. Once morphine analgesic tolerance is established, it was no longer affected by intrathecal BBG. Together, our results suggest that spinal P2X$_7$R is involved in the induction but not maintenance of morphine tolerance.

Introduction

Morphine is a highly potent analgesic for pain management. However, its analgesic potency fades rapidly during repetitive administration, so that progressively higher doses are required to achieve comparable analgesic levels. Considerable progress has been made concerning mechanisms underlying morphine tolerance; however, a clear understanding is still lacking regarding its complexity.

Lines of evidence have demonstrated that multiple factors are known to be involved in morphine tolerance, including desensitization of opioid receptors (Martini and Whistler, 2007) and functional changes in glutamate receptors (Mao et al., 1995; Mayer et al., 1999) and transporters (Mao et al., 2002; Tai et al., 2007). Noticeably, studies by us (Song and Zhao, 2001) and others suggest that spinal glia may contribute to the development of morphine tolerance (Watkins et al., 2005).

Various signaling molecules are expressed in glia (Watkins et al., 2007). Among them, P2X$_7$ receptor (P2X$_7$R) is an ATP-gated nonselective cation channel (Sperlágh et al., 2006). In the spinal cord, P2X$_7$R is predominately present in microglia (Collo et al., 1997; Yu et al., 2008). There is mounting evidence that P2X$_7$R plays an important role in the production of proinflammatory cytokines such as interleukin-1 β (IL-1β) (Takenouchi et al., 2009) and tumor necrosis factor-α (TNFα) (Suzuki et al., 2004). Furthermore, P2X$_7$R is involved in the induction of neuropathic

pain (Honore et al., 2006; Broom et al., 2008). Together, these lines of evidence point to a potential role for P2X$_7$R in the development of morphine tolerance.

In the present study, we explored the effects of P2X$_7$R on morphine tolerance using behavioral, immunohistochemical, pharmacological, and gene interfering methods. Changes in spinal P2X$_7$R expression were detected during long-term morphine administration. Moreover, morphine tolerance was prevented with either blockade of spinal P2X$_7$R by antagonist or targeting RNA interference.

Materials and Methods

Animals. Adult male Sprague Dawley rats initially weighing 180–200 g (The Animal Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) were used. Animals were housed under a 12 h light/dark cycle at a room temperature of 22 $\pm$ 1°C with food and water available *ad libitum*. All experiments were performed with the approval of the Shanghai Animal Care and Use Committee and followed the policies issued by the International Association for the Study of Pain on the use of laboratory animals.

Drugs. Morphine hydrochloride was purchased from Shengyang First Pharmaceutical Factory. Brilliant Blue G (BBG), a noncompetitive selective antagonist of P2X$_7$R, was purchased from Sigma. Both drugs were diluted with saline to attain the final concentration.

Induction of morphine tolerance. Morphine was given subcutaneously twice daily with 12 h intervals, from day 1 to at most day 9 at 10 mg/kg body weight, to establish systemic analgesic tolerance. To evaluate the development of morphine tolerance, morphine antinociception to mechanical and thermal stimuli were assessed 30 min after an acute dose (5 mg/kg) of morphine given intraperitoneally, and the analgesic effects before and after a defined period of tolerance induction were compared.

Behavioral tests. Modified von Frey test was used to evaluate morphine analgesic effect to mechanical stimuli. A series of seven von Frey filaments (Stoelting) were applied on the plantar surface of a hindpaw in ascending order (2, 4, 6, 8, 10, 15, and 26 g). Each filament was applied five times with each time lasted for 2 s and a 30 s interval between applications. A positive response to a filament was when at least three of
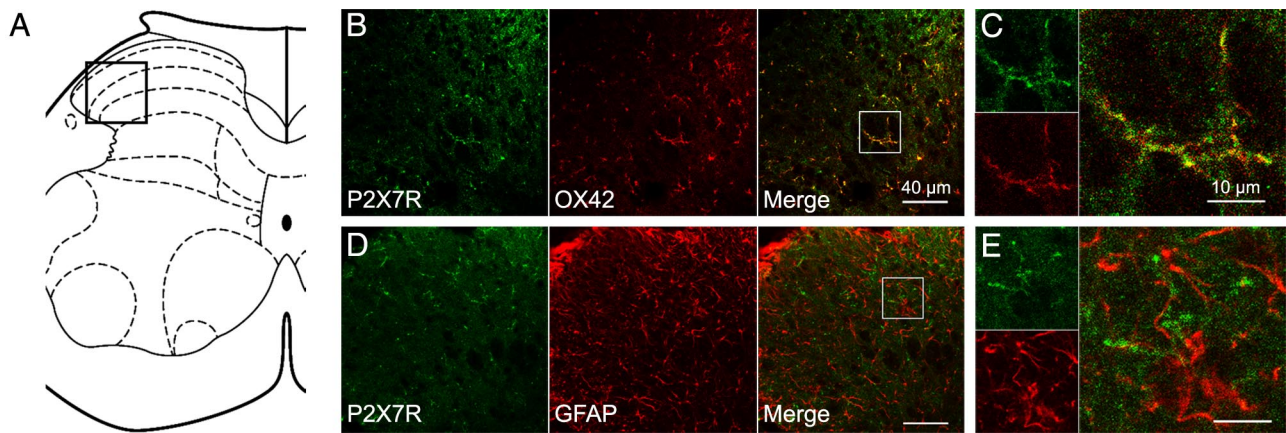
Figure 1. Double immunostaining of P2X₇R and cell-specific markers in morphine-tolerated rats. **A**, Schematic diagram of the spinal cord. Black open square (**A**) marks the corresponding scope of confocal images (**B**, **D**) on the spinal cord section. White open squares (**B**, **D**) mark the corresponding scope of amplified images (**C**, **E**) on the confocal images. **B**, **C**, Double immunostaining of P2X₇R and the microglial marker OX42. **D**, **E**, Double immunostaining of P2X₇R and the astrocytic marker GFAP. Scale bars: **B**, **D**, 40 μm; **C**, **E**, 10 μm.
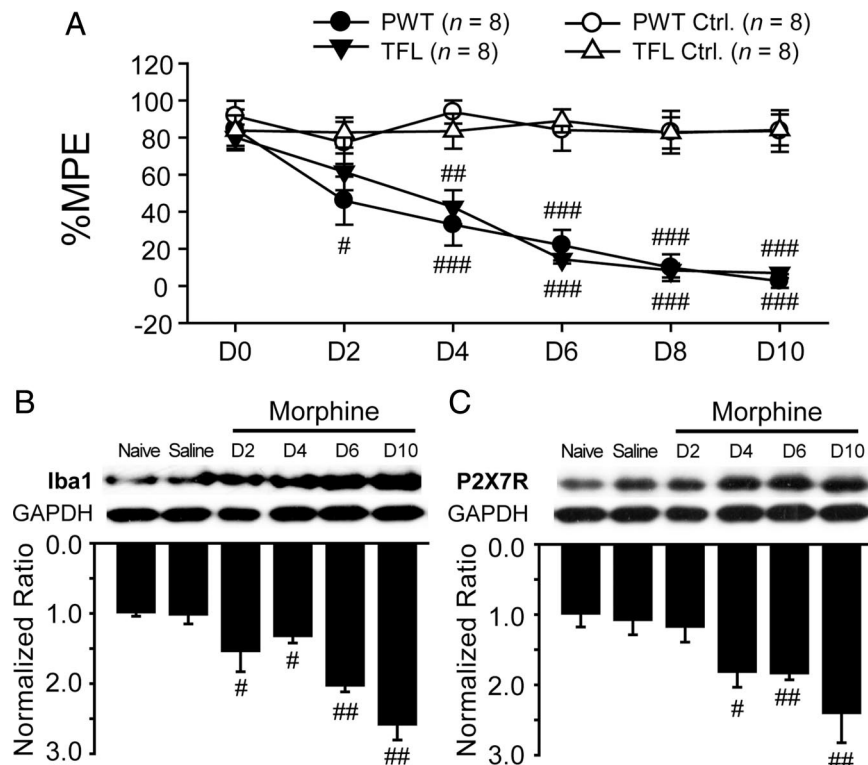

Figure 2. Upregulation of spinal expression of microglia and P2X₇R in morphine-tolerant rats. Nine days of subcutaneous morphine (10 mg/kg, twice daily) were given to induce analgesic tolerance. In behavioral tests (**A**), analgesic efficacy of acute morphine (5 mg/kg, i.p.) was decreased significantly during repeated administration (#$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, compared with the corresponding saline group). Western bolt analysis showed that the protein levels of microglial marker Iba1 (**B**) and P2X₇R (**C**) were upregulated significantly by chronic morphine treatment (**B**, **C**; #$p < 0.05$, ##$p < 0.01$, compared with saline group; $n = 4$). The spinal tissue samples of saline group were collected on day 10. PWT, Paw-withdrawal threshold; TFL, tail-flick latency.

the five applications evoked flinching. Paw-withdrawal threshold was defined as the lowest filament bending force that elicited positive responses. Hargreaves' test and tail-flick test were used to evaluate morphine analgesic effect to thermal stimuli and were performed as described previously (Sun et al., 2008; Wang et al., 2009).

Small interfering RNAs. Small interfering RNA (siRNA) targeting the rat P2X₇R mRNA containing four pooled SMARTselected duplexes or a nontargeting control siRNA (Thermo Scientific Dharmacon). The sense sequences of the four duplexes were as follows: (1) GUACAGUGGCU-UCAAGUAU; (2) GGAUGGACCCACAAAGUAA; (3) UUACAGAG-

GUGGCAGUUCA; (4) GAACGAUGUCUU-UCAGUAU. siGENOME RISC-Free Control siRNA was used as nontargeting control. Branched polyethylenimine (PEI) was purchased from Sigma, and siRNA–PEI complex (1.8 μl of 10 mM PEI/μg RNA) was prepared according to a published method (Tan et al., 2005).

Drug delivery. To minimize inflammatory responses from intrathecal surgery, drugs or siRNA–PEI complexes were delivered into the spinal space via lumbar puncture with a 30 gauge needle between L5 and L6 vertebrae as described previously (Xu et al., 2006). A brisk tail twitch was considered as an indicator of the accuracy of intrathecal injection.

Western blotting. Animals were anesthetized by overdose urethane (SCR Co.), and the spinal cord tissue (L4–L6) was rapidly removed. Collected tissue samples were homogenized in a lysis buffer containing a mixture of protease inhibitors (Roche) and PMSF (Sigma). Protein samples were separated on SDS-PAGE (5–12% gels; Bio-Rad) and transferred onto polyvinylidene fluoride membranes (Millipore Corporation). The membranes were blocked and then incubated overnight at 4°C with a primary antibody [rabbit anti-P2X₇R, 1:2000 (Alomone Labs); rabbit anti-Iba1, 1:5000 (Wako); rabbit anti-p-p38, 1:500 (Cell Signaling Technology; rabbit anti-p38, 1:1000 (Cell Signaling Technology)]. The membranes were then incubated with the goat anti-rabbit HRP-conjugated secondary antibody (1:1000; Santa Cruz Biotechnology) for 2 h at room temperature before the blots were visualized in ECL solution (Pierce) and exposed to x-ray films. The developed x-ray films were scanned for data analysis.

Immunohistochemistry. Animals were anesthetized with overdose urethane and perfused transcardially. Lumbar spinal cord samples (L4–L6) were removed, postfixed for 4 h at 4°C, and immersed in 10–30% gradient sucrose in 0.01 M phosphate buffer. Transverse spinal sections (35 μm) were cut in a cryostat. Sections were blocked and then incubated with primary antibodies [rabbit anti-P2X₇R, 1:400 (Alomone Labs); mouse anti-OX42, 1:100 (AbD Serotec); or mouse anti-GFAP, 1:1000 (Cell Signal Technology)] overnight at 4°C. The corresponding secondary antibodies were applied for 90 min at 4°C. Immunofluorescent sections were observed with a Leica flu-
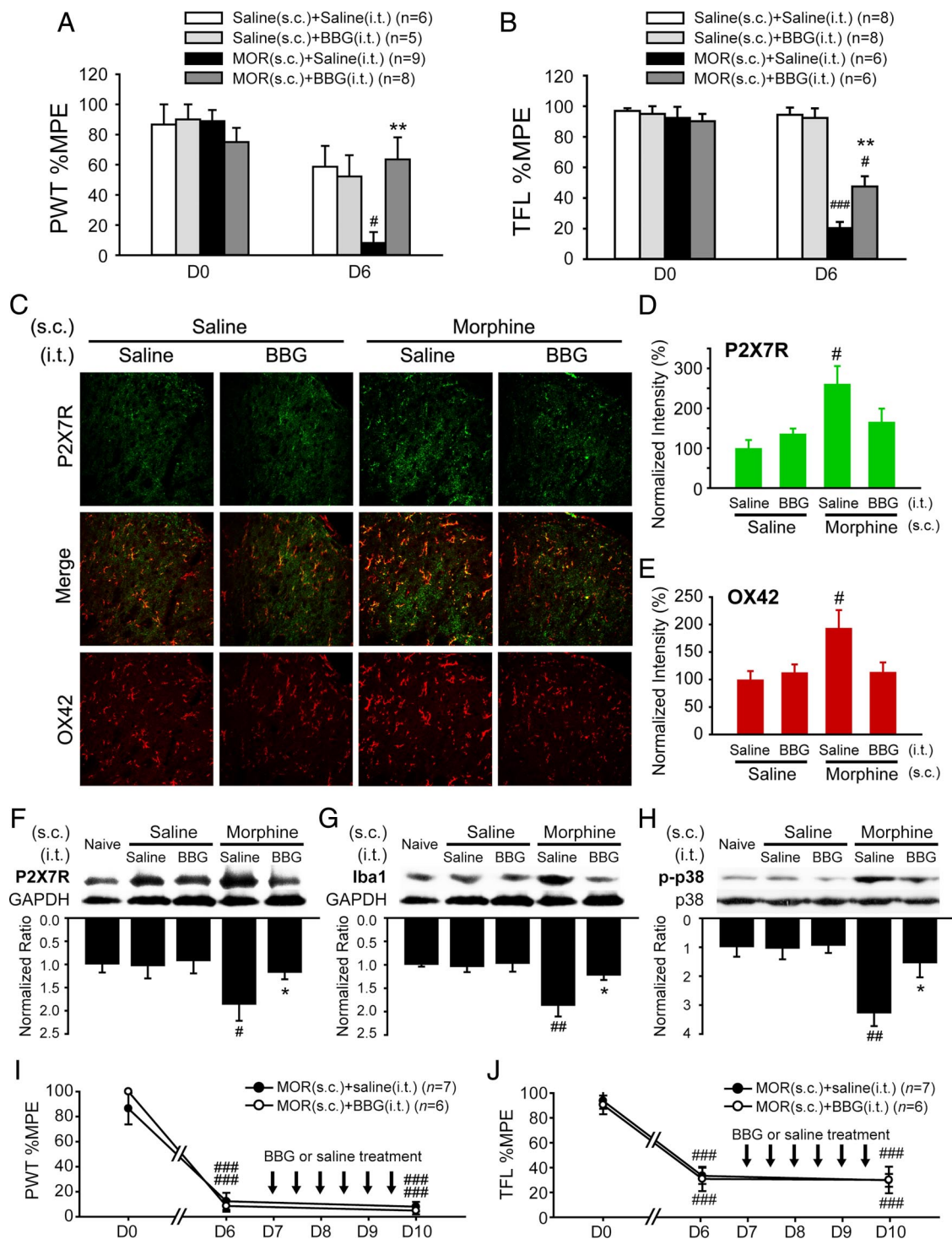
Figure 3. Effects of P2X₇R antagonist on the induction and maintenance of morphine analgesic tolerance. Chronic morphine (MOR)-induced analgesic tolerance was attenuated in von Frey test (**A**) and tail-flick test (**B**) by intrathecal BBG treatment ($^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, compared with Saline + Saline; $^{**}p < 0.01$, compared with MOR + Saline). Immunohistochemical analysis (**C**) showed that intrathecal BBG treatment abolished the immunoreactive upregulation of P2X₇R (**D**) and the microglial marker OX42 (**E**) induced by chronic morphine ($^{\#}p < 0.05$, compared with Saline + Saline). Western blot analysis indicated that the protein levels of P2X₇R (**F**), the microglial marker Iba1 (**G**), and phosphorylated p38 MAPK (**H**) with chronic morphine were also inhibited by BBG treatment ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with Saline + Saline; $^{*}p < 0.05$, compared with MOR + Saline; $n = 3$). Five days of subcutaneous morphine (10 mg/kg, twice daily) produced analgesic tolerance to acute morphine challenge (5 mg/kg, i.p.) in von Frey test (**I**) and tail-flick test (**J**). After 3 d of BBG treatment (10 μM, 20 μl, i.t., 30 min before subcutaneous morphine, twice daily) did not affect the onset of morphine tolerance. Data represent means ± SEM ($^{\#\#\#}p < 0.001$, compared with day 0).

orescence microscope, and images were captured with a CCD spot camera for data analysis.

Data analysis. Data were expressed as means ± SEM. For behavioral tests, morphine analgesic effects were converted to the percentage of maximal possible effect (%MPE) using the following formula: %MPE =

[(test − baseline)/(cutoff − baseline)] × 100. Student's *t* test was used to analyze the siRNA data. One-way repeated-measures ANOVA was used to analyze morphine tolerance time course. Two-way ANOVA was used to analyze the effect of BBG on the maintenance of morphine tolerance. For image data from Western blotting and immunohistochemistry, the
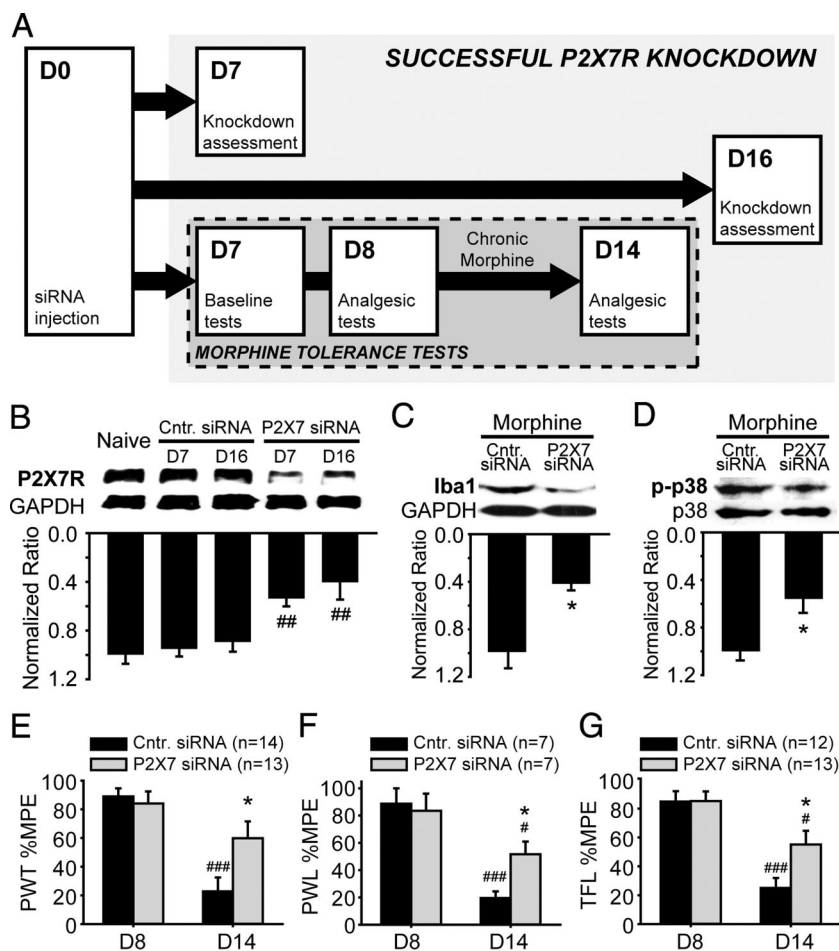
Figure 4. Intrathecal P2X$_7$ siRNA attenuated the induction of morphine analgesic tolerance. **A**, Schematic showing the experimental schedule. P2X$_7$ siRNA and control siRNA were injected intrathecally on day 0. Acute morphine analgesic tests were performed on day 8 and day 14, respectively. Chronic morphine consisted of subcutaneous morphine injection (twice daily) from day 9 to day 13. Western blot analysis (**B**) showed that intrathecal P2X$_7$ siRNA significantly reduced the protein level of P2X$_7$R in the spinal cord on day 7 and day 16 after siRNA injection ($^{##}p < 0.01$, compared with Naive). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. The protein levels of Iba1 (**C**) and phosphorylated p38 MAPK (**D**) in the spinal cord were significantly lower in the P2X$_7$ siRNA groups after chronic morphine exposure ($^{*}p < 0.05$, compared with control siRNA group; $n = 4$). P2X$_7$ siRNA-treated rats exhibited a significantly higher morphine analgesic potency in von Frey test (**E**), Hargreaves' test (**F**), and tail-flick test (**G**) after chronic morphine treatment ($^{#}p < 0.05$, $^{###}p < 0.001$, compared with day 8; $^{*}p < 0.05$, compared with control siRNA group). PWL, Paw-withdrawal latency.

intensity of immunoreactive bands or sections were measured using NIH ImageJ. Differences in intensity were compared using Student's t test.

Results

Colocalization of P2X$_7$R and OX42 in the spinal cord
In the spinal dorsal horn of saline treated rats, P2X$_7$R-immunoreactivity (IR) was predominantly colocalized with the microglial marker OX42 but not the astrocytic marker GFAP (data not shown). To investigate whether there are changes in distribution after chronic morphine treatment, we also examined P2X$_7$R expression in morphine-tolerant rats (Fig. 1). After 5 d of morphine treatment (10 mg/kg, s.c., twice daily), P2X$_7$R-IR remained exclusively colocalized with OX42 (Fig. 1 B,C) but not GFAP (Fig. 1 D,E).

Upregulation of spinal microglial expression and P2X$_7$Rs in morphine-tolerated rats
Chronic morphine treatment (10 mg/kg, s.c., twice daily) resulted in a significant loss of analgesic potency of acute morphine (5 mg/kg, i.p.) in both mechanical and thermal tests (Fig. 2A). We then investigated possible changes in both the expression of microglia marker

Iba1 and P2X$_7$R in morphine-tolerant rats. Parallel to behavioral changes, the protein levels of spinal microglia marker Iba1 were upregulated over time (Fig. 2B). Likewise, upregulation of P2X$_7$R in the dorsal horn was observed in morphine-tolerant rats starting at day 4 (Fig. 2C), suggesting an involvement of the spinal P2X$_7$R in the development of morphine tolerance.

P2X$_7$R antagonist prevented morphine tolerance and suppressed P2X$_7$R upregulation and microglial activation
The effects of BBG, a selective P2X$_7$R antagonist, on morphine tolerance were examined using the von Frey test and tail-flick test. The baseline levels of pain threshold were not altered by repeated intrathecal administration of BBG (10 μM, 20 μl, twice daily for 5 d) in either test compared with the intrathecal saline group (20 μl, twice daily). Chronic morphine treatment (10 mg/kg, s.c., twice daily) after intrathecal injection of saline (20 μl, 30 min before subcutaneous morphine application) produced significant tolerance to acute morphine challenge (5 mg/kg, i.p.) on day 6. In contrast, intrathecal BBG (10 μM, 20 μl, 30 min before subcutaneous morphine application) significantly retained morphine antinociceptive effects to both mechanical and thermal stimuli (Fig. 3A,B), indicating a critical role for the spinal P2X$_7$R in the development of morphine tolerance. Parallel to the behavioral changes, immunohistochemical data showed that BBG treatment inhibited the P2X$_7$R upregulation and the microglial activation induced by chronic morphine on day 6 without altering their baseline levels (Fig. 3C–E). These results were confirmed by the Western blot experiments (Fig. 3F,G). Similar results were also observed in the spinal expression of phosphorylated p38 mitogen-activated protein kinase (MAPK) (Fig. 3H). Given that chronic morphine can activate microglial p38 MAPK in the spinal cord (Cui et al., 2006), our data suggest that BBG treatment attenuated morphine analgesic tolerance at least partly by inhibiting the activation of spinal microglia.

We also examined whether the spinal P2X$_7$R is involved in the maintenance of morphine tolerance. After morphine tolerance was established on day 6, BBG (10 μM, 20 μl, twice daily) was applied via lumbar puncture 30 min before regular subcutaneous morphine (10 mg/kg, twice daily) from day 7 to day 9. BBG failed to restore morphine antinociceptive efficacy compared with the saline control group in behavioral tests on day 10 (Fig. 3I,J), implying that the spinal P2X$_7$R contributes to the induction, but not the maintenance, of morphine tolerance.

Spinal siRNAs targeting P2X$_7$R inhibited both induction of morphine tolerance and activation of spinal microglia
To confirm the pharmacological results, an RNA interference approach was used. Figure 4A illustrates the test schedule. Briefly,

a single injection (17 μl) of siRNA targeting P2X$_7$R mRNA was applied intrathecally. The control group received a single intra-thecal injection (17 μl) of nontargeting control siRNA. Expressions of the spinal P2X$_7$R on day 7 and day 16 post siRNA injection were assessed to determine the knockdown efficiency. At these two time points, P2X$_7$R protein levels were significantly lower in P2X$_7$ siRNA injected rats than those from control rats (Fig. 4B), indicating a substantial and stable P2X$_7$R knockdown. Significant differences in behavioral baseline levels were not found between day 0 and day 7 within each group or between these two groups at day 0 or day 7 (data not shown). Therefore, we performed the morphine tolerance tests from day 8 to day 14. After morphine challenge (5 mg/kg, i.p.) analgesic tests on day 8, morphine (10 mg/kg, s.c., twice daily) was given from day 9 to day 13 to induce tolerance. As shown in Figure 4E–G, analgesic effect of acute morphine showed no difference between the groups on day 8. Although significant reductions in morphine antinociception were observed in Hargreaves' test (Fig. 4F) and tail-flick test (Fig. 4G) on day 14, it was still significantly higher in the P2X$_7$ siRNA group compared with the control group. In addition, P2X$_7$ siRNA injection also inhibited the upregulation of Iba1 (Fig. 4C) and phosphorylated p38 MAPK (Fig. 4D) by chronic morphine.

Discussion

Much progress has been made toward illustrating the mechanisms underlying morphine tolerance with studies prevailingly focused on neuronal involvement. In the present study, we examined microglial involvement and obtained two major findings. First, P2X$_7$R was upregulated in the spinal cord along with microglia marker Iba1 by chronic morphine treatment. Second, antagonism of the spinal P2X$_7$R by either the selective antagonist BBG or targeting siRNA not only attenuated morphine tolerance but also reduced the upregulation of the spinal P2X$_7$R, Iba1, and phosphorylated p38 MAPK induced by chronic morphine. Our results highlight an intimate association between the spinal microglia-expressed P2X$_7$R and the development of morphine tolerance.

Our previous study showed that functional interruption of astrocyte by glial inhibitor fluorocitrate reduced GFAP expression and blocked morphine tolerance, suggesting a possible involvement of astrocytic action (Song and Zhao, 2001). This has been confirmed and extended by other investigators, showing that several pain-facilitatory cytokines are released from activated glia (Raghavendra et al., 2002, 2004). It is suggested that pain-facilitatory cytokines counteract morphine analgesia, thus contributing to the morphine tolerance (Watkins et al., 2005). Expression and function of astrocytic P2X$_7$R were described in *in vitro* studies (Duan et al., 2003; Narcisse et al., 2005). However, such expression of P2X$_7$R was not detected in spinal astrocytes by us or others (Yu et al., 2008). Therefore, astrocyte contribution to morphine analgesic tolerance is unlikely to be directly mediated by P2X$_7$R. Given that neuronal P2X$_7$R was mostly observed in spinal motoneurons (Wang et al., 2004), it seems less likely that neuronal P2X$_7$R is associated with morphine tolerance.

In the present study, neither pharmacological antagonism nor siRNA inhibition of spinal P2X$_7$R produced a significant change in baseline response to nociceptive stimuli or acute morphine analgesia, suggesting that P2X$_7$R is not involved in physiological pain. It has been reported that P2X$_7$R is activated only by high concentration ($>$100 μM) of its endogenous ligand ATP (Roberts et al., 2006). It is, therefore, conceivable that P2X$_7$R is inactive in physiological condition because of the insufficient ATP concen-

tration in the spinal cord. Given that morphine application increased glutamate concentration in the CSF in morphine-tolerant rats (Wen et al., 2004), it might be that elevation of glutamate results in excess ATP release from spinal glia in an AMPA receptor-mediated calcium-dependent manner (Queiroz et al., 1999; Liu et al., 2006). This may create an ATP-rich local extracellular environment that could contribute to the activation of spinal P2X$_7$R. Additionally, when activated, P2X$_7$R can directly mediate both ATP and glutamate release (Ye et al., 2003; Suadicani et al., 2006). This positive feedback may also contribute to the sustained activation of P2X$_7$R in morphine tolerance.

Our findings provided evidence of microglial P2X$_7$R being a crucial molecule in the development of morphine tolerance. It has been reported that p38 MAPK, a transducer of various extracellular stimuli, regulates the release of inflammatory factors (Saklatvala, 2004) and is involved in the development of morphine analgesic tolerance associated with microglia (Cui et al., 2006, 2008). Therefore, we examined the effect of p38 MAPK on P2X$_7$R-mediated morphine tolerance. In our experiments, both pharmacological antagonism and siRNA inhibition of spinal P2X$_7$R suppressed the activation of spinal microglia and p38 MAPK, suggesting that p38 MAPK is a downstream effector of P2X$_7$R activation in morphine tolerance, which is consistent with previous studies (Pfeiffer et al., 2004; Papp et al., 2007). Therefore, the involvement of spinal P2X$_7$R in morphine tolerance is achieved at least partially through activation of microglia via the p38 MAPK signaling pathway. Previous studies have provided clues for interaction between P2X$_7$R and other signal molecules. P2X$_7$R has been shown to regulate the production and release of IL-1β and TNFα (Suzuki et al., 2004; Takenouchi et al., 2009). These two proinflammatory factors are potent pain mediators, and they are implicated in morphine tolerance (Shavit et al., 2005; Mika, 2008). Additionally, NMDA receptor is critically involved in the induction and maintenance of morphine tolerance (Mayer et al., 1999). P2X$_7$R-mediated glutamate release may serve as an important component to initiate NMDA receptor activation. Furthermore, glutamate transporter is crucial to morphine antinociceptive effect. Chronic morphine downregulated glutamate transporter, and an activator of glutamate transporter attenuated morphine analgesic tolerance (Tai et al., 2007). Recent evidence suggested that activation of P2X$_7$R decreased glutamate transport efficiency (Morioka et al., 2008). It is highly possible that P2X$_7$R also contributes to morphine analgesic tolerance by disrupting the balance between glutamate release and uptake.

In summary, we found that P2X$_7$R is predominantly expressed in microglia in the spinal cord. Activation of microglia-expressed P2X$_7$R is involved in the generation of morphine tolerance. These findings highlight the possibility of a new clinical strategy to prevent morphine tolerance.

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