Involvement of microglial P2X7 receptors and downstream signaling pathways in long-term potentiation of spinal nociceptive responses

Yu-Xia Chu [a,b], Yan Zhang [a], Yu-Qiu Zhang [a], Zhi-Qi Zhao [a,*]

[a] Institute of Neurobiology, Institutes of Brain Science and State Key Laboratory of Medical Neurobiology, Fudan University, Shanghai 200032, China
[b] College of Medicine, Jiaxing University, Jiaxing, Zhejiang Province 314001, China

ABSTRACT

Tetanic stimulation of the sciatic nerve (TSS) produces long-term potentiation (LTP) of C-fiber-evoked field potentials in the spinal cord. This potentiation is considered to be a substrate for long-lasting sensitization in the spinal pain pathway. Because microglia have previously been shown to regulate the induction of spinal LTP, we hypothesize that P2X7 receptors (P2X7R), which are predominantly expressed in microglia and participate in the communication between microglia and neurons, may play a role in this induction. This study investigated the potential roles of P2X7Rs in spinal LTP and persistent pain induced by TSS in rats. OxATP or BBG, a P2X7R antagonist, prevented the induction of spinal LTP both *in vivo* and in spinal cord slices *in vitro* and alleviated mechanical allodynia. Down-regulation of P2X7Rs with P2X7-siRNA blocked the induction of spinal LTP and inhibited mechanical allodynia. Double immunofluorescence showed colocalization of P2X7Rs with the microglial marker OX-42, but not with the astrocytic marker GFAP or the neuronal marker NeuN. Intrathecal injection of BBG suppressed the up-regulation of microglial P2X7Rs and increased expression of Fos in the spinal superficial dorsal horn. Further, pre-administration of BBG inhibited increased expression of the microglial marker Iba-1, phosphorylated p38 (*p*-p38), interleukin 1β (IL-1β) and GluR1 following TSS. Pre-administration of the IL-1 receptor antagonist (IL-1ra) blocked both the induction of spinal LTP and the up-regulation of GluR1. These results suggest that microglial P2X7Rs and its downstream signaling pathways play a pivotal role in the induction of spinal LTP and persistent pain induced by TSS.

© 2010 Elsevier Inc. All rights reserved.

1. Introduction

Tetanic stimulation of the sciatic nerve (TSS) has previously been shown to produce long-lasting hyperalgesia and allodynia in rats (Ying et al., 2006; Zhang et al., 2005). Electrophysiological studies have also shown that long-term potentiation (LTP) of C-fiber/lamina I neuron synapses in the spinal cord is induced by TSS (Sandkuhler, 2007). Induction of this LTP is blocked by antagonists of spinal N-methyl-D-aspartic acid (NMDA) and NK-1 receptors, both of which are know to function in the spinal pain pathways (Liu and Sandkuhler, 1995, 1997; Sandkuhler and Liu, 1998; Rygh et al., 1999). It is therefore plausible that spinal LTP is the cellular mechanism underlying central sensitization of nociceptive signals (Ji et al., 2003; Sandkuhler, 2007).

Increasing evidence suggests that microglia play an important role in the modulation of neuronal plasticity and the induction of neuropathic pain (Watkins et al., 2007, 2009; Watkins and Maier, 2002). In our previous studies, we showed that disruption of microglial and astrocytic function blocked both tetanic stimula-tion-induced spinal LTP and pain behaviors (Ma and Zhao, 2002; Ying et al., 2006). More recently, other investigators showed that activation of Src-family kinases (SFKs) in microglia in spinal dorsal horn controls the direction of TSS-induced LTP and that the cytokine tumor necrosis factor α (TNF-α) is involved in LTP induction (Zhong et al., 2010). Cytokines that are primarily released from microglia, including interleukin 1β (IL-1β) and TNF-α, have also been shown to modulate the induction of LTP in the hippocampus by increasing the surface expression and phosphorylation of alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Cunningham et al., 1996; Beattie et al., 2002; Ikeda et al., 2007; Pedersen et al., 2009). Although the contribution of microglia to the spinal LTP has been confirmed, the exact mechanisms are uncertain.

P2X7 receptors (P2X7R) are abundantly expressed in microglia and have been proposed to play a pivotal role in the cross-talk between microglia and neurons (Sperlagh et al., 2006; Yu et al., 2008). Activation of P2X7Rs by ATP or BzATP significantly blocked the uptake of glutamate into microglia (Morioka et al., 2008). Also, disruption of P2X7Rs by oxidized ATP (oxATP), a selective P2X7R antagonist, prevents the release of proinflammatory cytokines IL-1β and TNF-α (Clark et al., 2010; Hide et al., 2000; Hughes et al.,

* Corresponding author. Fax: +86 21 54237647.
  E-mail address: zqzhao@fudan.edu.cn (Z.-Q. Zhao).

2007; Labrousse et al., 2009). Activation of P2X7Rs increases phosphorylation of p38 mitogen activated protein kinase (MAPK), which functions upstream in the IL-1β signaling pathway (Solle et al., 2001; Donnelly-Roberts et al., 2004; Ferrari et al., 2006; Sperlagh et al., 2007). Moreover, deletion of the P2X7R gene abolishes chronic inflammatory and neuropathic pain in P2X7−/− mice (Chessell et al., 2005). Furthermore, blockade of P2X7Rs by a P2X7R antagonist reduces nerve injury-induced spontaneous firing of spinal neurons and pain behaviors (McGaraughty et al., 2007). Taken together, these results suggest that microglial P2X7Rs may regulate tetanic stimulation-induced spinal LTP through a similar signaling pathway.

In this study, we demonstrate that microglial P2X7Rs and the downstream IL-1β signaling pathway play pivotal roles in the induction of spinal LTP and behavioral persistent pain induced by TSS.

2. Materials and methods

2.1. Animals and reagents

Adult male Sprague–Dawley rats (180–350 g) (Animal Center, Chinese Academy of Sciences, Shanghai) were housed in single plastic cages in a light (12:12 light/dark cycle) and temperature (23 ± 2 °C) controlled room with standard rodent chow and water available ad libitum. The treatment of the animals conformed to the guidelines of the protocols of the International Association for the Study of Pain concerning the use of laboratory animals.

OxATP and Brilliant Blue G (BBG), P2X7R antagonists, were purchased from Sigma. The rat recombinant interleukin-1 receptor antagonist (IL-1ra), an IL-1β antibody, was purchased from R&D Systems. All of the drugs were dissolved in sterile normal saline. Bicuculline methiodide and strychnine were purchased from sigma. Both of the drugs were prepared as stock solutions and diluted to the required concentration with artificial cerebrospinal fluid (ACSF).

2.2. Lumbar puncture

Under intraperitoneal (i.p.) anesthesia with Chloral Hydrate (0.3 g/kg), oxATP (300 μM, 20 μl), BBG (20 μM, 20 μl) or saline was initially loaded into a 0.25 ml vitric syringe connecting with a number 4 pinhead. The pinhead penetrated the skin and entered between lumbar vertebrae L4 and L5 as described previously (Xu et al., 2006). An instaneous and fast tail-flick was considered as a sign of reaching subarachnoid space in the spinal lumbar enlargement. Drugs or saline were injected at a rate of 5 μl/min for 4 min 0.5 h before tetanic stimulation and then the needle was withdrawn.

As to the RNA interference experiments, under brief isoflurane anesthesia (2% in oxygen), P2X7-siRNA (20 μl) or control-siRNA (20 μl) was administered in the same manner. Rats showed full recovery from anesthesia within 10 min after the acute injection. No abnormal motor behavior was observed after any injection. The lumbar puncture was performed 7 days before tetanic stimulation and the down-regulation of P2X7Rs was confirmed by Western blots.

2.3. siRNAs

siRNA targeting rat P2X7R mRNA containing four pooled SMART selected duplexes or a non-targeting control-siRNA (Thermal scientific Dharmacon) was used in this study. The sense sequences of the four duplexes were as follows: (1) GUACAGUGGCUUCAAGU AU; (2) GGAUGGACCCACAAAGUAA; (3) UUACAGAGGUGGCAGUU

CA; (4) GAACGAUGUCUUUCAGUAU. SiGENOME RISC-Free control-siRNA was used as non-targeting control. Branched polyethyleneimine (PEI) was purchased from Sigma and siRNA-PEI complex (1.8 μl of 10 mM/μg RNA) was prepared according to previous description (Tan et al., 2005).

2.4. Electrophysiological recording of spinal LTP

The procedures were similar to the previous study (Ma and Zhao, 2001). Briefly, adult male Sprague–Dawley rats (250–350 g) were initially anesthetized with urethane (1.5 g/kg, i.p.) for surgery. The right external jugular vein, carotid artery and the trachea were cannulated for drug injection, monitoring blood pressure and artificial ventilation, respectively. A laminectomy was performed at vertebrae T13-L1 to expose the lumbar enlargement of the spinal cord. An intrathecal catheter (PE-10) filled with sterile saline (approximately 4 μl) was inserted into the intervertebral gap between the L4 and L5 and extended to subarachnoid space of the spinal lumbar enlargement for drug injection. The flowing out of clear cerebrospinal fluid from the catheter was considered as a successful insertion and then the outer end was plugged. OxATP (300 μM, 10 μl), BBG (20 μM, 10 μl), IL-1ra (3.5 μg/ml, 20 μl) or saline was initially loaded into a Hamilton syringe and separated from 10 μl saline solution by an air bubble. During testing, the intrathecal catheter was connected to the pre-filled Hamilton syringe and drugs or saline was injected 0.5 h before tetanic stimulation. The surgery of intrathecal implanting catheters was not performed in the rats subjected to intrathecal injection of P2X7-siRNA or control-siRNA 7 days before electrophysiological recording. The skin of left thigh just above the sciatic nerve was incised and muscle and fascial layers were bluntly separated to expose the sciatic nerve. The left sciatic nerve was separated carefully with adjacent fascia and muscles by a glass seeker and spared for stimulation using bipolar silver electrodes. The spinal column was firmly suspended by vertebral clamps rostral and caudal to the exposed spinal cord. The skin around the incision was slightly slinged and fixed on a metal circle to form a skin gutter filled with warm paraffin oil. Animals were artificially ventilated (Capstar-100, IITC Life Science, USA) and body temperature (37.5–38 °C) was maintained by means of a feedback controlled heating blanket. Throughout the experiment, the electrocardiogram, end-tidal CO_2 and rectal temperature were monitored and controlled within the physiological condition.

The field potentials were recorded in the ipsilateral dorsal horn at L4–L5 segments, 300–500 μm from the surface of the spinal cord with glass microelectrodes (impedance 3–6 MΩ). The low-pass filter was adjusted to 100 Hz. A single rectangular pulse (0.5 ms, 20–30 V), enough to excite C afferent fibers, was applied to the sciatic nerve at 1-min intervals as a test stimulus. Stable responses for more than 40 min served as baseline control. A conditioning tetanic stimulation (0.5 ms, 100 Hz, 40 V, 10 trains of 2-s duration at 10-s intervals) was delivered to the sciatic nerve to induce spinal LTP. Four consecutive C-fiber-evoked field potentials were averaged, stored, and analyzed by a data processing system (SMUP-E, Shanghai Medical College, Fudan University, China).

2.5. Tetanic stimulation of the sciatic nerve

Under Chloral Hydrate (0.3 g/kg, i.p.) anesthesia and sterilizing, the left sciatic nerve was carefully exposed at mid-thigh level and separated from neighboring tissues. The hind paws, pelvis and tail were fastened on the surgical board with tape. A pair of silver hooks was placed under the sciatic nerve for stimulation with the identical parameters used in electrophysiological recording. The exposed nerve was covered with mineral oil (37 °C). In the sham group, the exposed left sciatic nerve was placed on the stim-

ulation electrode without electrical stimulation. After surgery, the animals were put back into the cages and allowed to recover for 2 days before behavioral test.

2.6. Preparation of spinal cord slices

Young Sprague–Dawley rats (postnatal days 14–21) were deeply anesthetized with diethyl ether, and for LTP recording, about 1 ml lidocaine (5 ml:0.1 g) was injected to both sides of lumbar vertebrae (L4–L5). Laminectomy was performed from mid-thoracic to low lumbar levels and the cord was quickly removed to cold modified ACSF: (in mM) NaCl, 80; KCl, 2.5; NaH_2PO_4, 1.25; $CaCl_2$, 0.5; $MgCl_2$, 3.5; $NaHCO_3$, 25; sucrose, 75; ascorbate, 1.3; sodium pyruvate, 3.0; oxygenated with 95% O_2 and 5% CO_2; pH 7.4; measured osmolarity 310.5 mOsm. Transverse 500 μm slices, with attached dorsal roots (8–12 mm) were obtained. Slices were then incubated for at least 1 h at 32 °C in a solution that consisted of: (in mM) NaCl, 125; KCl, 2.5; $CaCl_2$, 2; $MgCl_2$, 1, NaH_2PO_4, 1.25; $NaHCO_3$, 26; D-glucose, 25; ascorbate, 1.3; sodium pyruvate, 3.0; oxygenated with 95% O_2 and 5% CO_2, pH 7.4; measured osmolarity 324.5 mOsm. The slice was then transferred into a recording chamber and perfused with oxygenated recording solution at a rate of 5 ml/min prior to electrophysiological recording at room temperature (RT).

2.7. Field potential recording from spinal cord slices

Field potential recording from the superficial spinal dorsal horn was performed with glass microelectrodes (impedance 2–5 MΩ) filled with (in mM) NaCl 135, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5 (pH adjusted to 7.2 with NaOH). A bipolar tungsten electrode was used to stimulate Lissauer's tract (LT) (0.1 ms, 0.7–1 mA). Low-pass filter was set to 1 kHz, amplification 500× (Axopatch 200B, Axon Instruments). To minimize current spread to the dorsal roots and the recording sites, the electrode was placed at the most ventrolateral border of LT (Terman et al., 2001). Recording of field excitory postsynaptic potentials (fEPSPs) was made in the presence of bicuculline methiodide (10 μM) and strychnine (1 μM) to block tonic inhibitory action of $GABA_A$ and glycine and data are normalized (to mean values of 10 min before high-frequency stimulation (HFS)) mean peak fEPSPs amplitudes.

2.8. von Frey test for mechanical allodynia

The baseline response and allodynia to mechanical stimulation were measured on hind paws by von Frey filaments with bending force ranging from 2 to 26 g before tetanic stimulation and on days 3, 5, and 7 after surgery, respectively. Each rat was placed in a chamber (20 × 10 × 20 cm) with a customized platform made of 3 mm thick plexiglas containing 1.5 mm diameter holes in a 5 mm grid of perpendicular rows throughout the entire area. Each rat was allowed to acclimate for about 30 min. After acclimation, a series of von Frey filament stimuli was delivered in an ascending order of forces to the central region of the plantar surface of the hind paws. A particular hair was applied until buckling of the filament occurred. This was maintained for approximately 2 s. A withdrawal response was considered valid only if the hind paw was completely removed from the platform, not just flinched after a single application. A trial consisted of application of von Frey filament to the hind paw five times at 15 s intervals, and only when the hind paw withdrew from a particular filament at least three times out of the five applications, the value of the filament in grams was considered to be the 'paw withdrawal threshold' (PWT) to mechanical stimuli. Otherwise, the next larger filament in the series was tested until the rat withdrew the hind paw at least three times. Stimulated and sham-operated hind paws were

tested in the same manner. As above, the investigator was blind to the group being tested.

2.9. Fos immunohistochemistry staining

Under urethane (1.5 g/kg, i.p.) anesthesia, 20 μl saline or BBG (20 μM) was intrathecally injected by lumbar puncture. The LTP-inducing electrical stimulation (500 ms, 100 Hz, 40 V, 10 trains of 2 s duration at 10 s intervals) of the left sciatic nerve was performed 0.5 h after intrathecal administration. In the sham group, the exposed left sciatic nerve was placed on the stimulation electrode without electrical stimulation. The rats were perfused transcardially with 250–300 ml of 4% cold paraformaldehyde in 0.1 M PB (pH 7.4) following 100–150 ml 0.9% warm saline 2 h after tetanic stimulation. In sham group, the rats were perfused 2 h after sham surgery. The spinal cord was then removed, kept in the same fixative for 2–4 h at 4 °C and immersed in 10% and 20% sucrose solution step by step until sinking to the bottom, followed by 30% sucrose solution overnight. Coronal cryostat sections of 35 μm thick of L4–L6 spinal cord were cut on a cryostat and collected free-floating for the following studies.

Sections were immersed in 0.01 M PBS containing 3% hydrogen peroxide for 15 min and blocked with 10% goat serum for 2 h at RT. Then the sections were incubated for 48 h at 4 °C in the rabbit anti-Fos primary antibody (1:10,000; Calbiochem) diluted in 0.01 M PBS containing 3% Triton X-100. After washing in 0.01 M PBS, the sections were incubated 2 h at 4 °C in biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories) and then in avidin–biotin-peroxidase complex (ABC; 1:200; Vector Laboratories) for 2 h at 4 °C. Staining was developed in 0.05% diaminobenzidine with 0.01% H_2O_2. The sections were mounted on slides, dried, dehydrated, cleared and coverslipped. The sections were examined under bright-field microscopy (Olympus BX51, Japan) at 10× objective magnification and images were captured with a CCD spot camera (Diagnostic Instruments, USA). Camera aperture, magnification, light power and exposure time were fixed for all images. The quantification of Fos-immunoreactivity (Fos-IR) neurons was performed with the aid of Image J (version 1.38; NIH, USA). The defined range including layers I–IV of the dorsal spinal cord was selected and the value of the area was calculated by Image J. The total number of Fos-IR neurons in this range was counted and divided by the value of the selected area to get the mean density of Fos-IR neurons. All the counts were from consecutive sections (n = 5–6 sections per rat; n = 4 rats per group) and averaged to produce a mean. All results were expressed as mean ± SEM.

2.10. Immunofluorescence staining

Sham and treated rats were anesthetized with urethane (1.5 g/kg, i.p.) on days 1, 3 and 7 after tetanic stimulation and then perfused through the ascending aorta with warm saline, followed by 4% cold paraformaldehyde, pH 7.2–7.4. After perfusion, the L4–L6 spinal cord segments were removed and postfixed in the same fixative for 2–4 h. Then the tissue was immersed in 10% and 20% sucrose solution step by step until sinking to the bottom, followed by 30% sucrose solution overnight. Finally, the L4–L6 segments of the lumber spinal cord was transected into 35 μm in a cryostat and six non-adjacent sections were randomly selected for immunostaining. All of the sections were blocked with 10% donkey serum in 0.3% Triton X-100 for 2 h at RT and incubated at 4 °C with rabbit anti-ionized calcium binding adaptor protein, Iba-1 (a microglial marker, 1:2000; Wako) overnight or P2X7R (1:150; Alomone Labs) over two nights. Then the sections were incubated for 2 h at 4 °C with RRX-conjugated donkey anti-rabbit IgG (1:200; Jackson). To verify the specificity of P2X7R antibody used in the immunostaining, negative controls were performed in which only secondary

antibody (RRX-conjugated donkey anti-rabbit IgG) but no primary antibody was added ($n = 2$).

All sections were observed with a fluorescence microscope (Leica DMRXA, Germany) and images were captured using a computerized image analysis system (Leica Qwin 500, Germany). Because the morphology of microglia is complex and immunoreactive staining includes both cell bodies and their processes, cell counts may not sufficiently quantify activation. Thus, the optical density of immunoreactive staining for Iba-1 was measured. The relative density of images was determined by subtracting the background density in each image. These corrected density values of six sections were averaged to provide a mean density for each animal ($n = 4$).

For double immunofluorescence, spinal sections were incubated with a mixture of rabbit anti-P2X7R (1:150; Alomone Labs) or phosphorylated p38 (p-p38) (1:500; Cell Signaling) and mouse anti-OX-42 (a microglial marker, 1:500; Serotec) over two nights at 4 °C, glial fibrillary acidic protein (GFAP, an astrocytic marker, 1:2000; Sigma) or neuronal nuclei (NeuN, a neuronal marker, 1:2000; Chemicon) overnight at 4 °C, followed by a mixture of RRX- and FITC-conjugated secondary antibodies for 2 h at 4 °C.

Sections were examined with confocal microscopy system (Leica TCS SP2, Germany) with 488 nm (green) and 543 nm (red) laser lines. The overlay of green signal and red signal resulted in yellow signal, if both red and green signals expressed in the same place. If the expression of P2X7Rs (red) in microglia (green) increased, the area of merged signal (yellow) should be larger. In addition, individual microglial cells were occasionally difficult to distinguish, therefore we compared the area of yellow signal but not the number of colocalization cells to reflect the colocalization intensity. For the quantitative analysis of the colocalization of P2X7Rs and the microglial marker OX-42 in different treatment groups, at least 6 sections of L4-L6 segments of spinal cord ($n = 4$/group) were randomly selected. For each experiment, images were processed simultaneously and collected using identical acquisition parameters and analyzed using ImagePro Plus (version 6.0; Media Cybernetics, USA). Quantitative analyses were done in a blinded manner.

2.11. Western blots

Under urethane (1.5 g/kg, i.p.) anesthesia on days 1, 3 and 7 after tetanic stimulation, the animals were rapidly sacrificed by decapitation ($n = 3$ or 5/group). The L4-L6 lumbar spinal cord was rapidly removed, immediately frozen in liquid nitrogen and stored at –70 °C until use. The segments were homogenized in an SDS sample buffer that contained a mixture of proteinase and phosphatase inhibitors. The protein concentrations were determined and Western blots were conducted. Aliquots of sample (10 μl) including 8 μl protein and 2 μl 5× sample buffer were loaded, subjected to 12% SDS–PAGE, and electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore Immobilon-p Transfer Membrane) using Mini-Protean 3 electrophoresis system and Mini Trans-Blot electrophoretic transfer system (Bio-Tanon, Shanghai, China). Blots were blocked with 5% non-fat milk in Tris-buffered saline (pH 7.5) containing 0.1% Tween 20 for 2 h, and then incubated overnight at 4 °C with rabbit anti-P2X7 (1:1000; Alomone Labs), p-p38 (1:500; Cell Signaling), p38 (1:500; Cell Signaling), IL-1β (1:1000; Peprotech) or GluR1 (1:500; Chemicon), respectively. After washing, the blots were incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:1000; Pierce) for 2 h at 4 °C and then developed in ECL solution (Pierce) for about 1 min, and finally exposed onto films. The membranes were stripped with stripping buffer (Beyotime, China) and reblotted with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:10,000; Cell Signaling). For the quantification of Western signals, the densities of specific P2X7R, p-p38,

p38, IL-1β and GluR1 bands were measured with a computer-assisted imaging analysis system (Photoshop) and were normalized against the corresponding GAPDH level as a control for sample loading.

2.12. Statistical analysis

In each experiment, areas of four consecutive field potentials recorded at 1 min intervals were averaged. The mean areas before drug or saline application served as baseline. The area of C-fiber-evoked field potentials was expressed as percentage of baseline. The summary data from different animals in the same group were expressed as mean ± SEM. Statistical tests were carried out with SPSS (version 13.0, SPSS Inc., USA) or SigmaStat (version 3.5, Systat Software Inc., USA). The values (% of baseline) showed in the results was obtained at 1 h after TSS or immediately before TSS. The effects of electrical stimulation or drugs, as well as the application of siRNA, on C-fiber-evoked field potentials or PWTs were analyzed by one way repeated measure ANOVA followed by Tukey post hoc test when compared within the group and by two way repeated measures ANOVA when compared between groups. In immunohistochemistry experiments, the effects of tetanic stimulation with or without pre-injection of the P2X7R antagonist BBG on the expression of the microglial marker Iba-1 were analyzed by one way ANOVA followed by LSD post hoc test. The expression of Fos and microglial P2X7Rs in sham and test groups was analyzed using independent samples t-test. In Western blot experiments, the expression of P2X7Rs, p-p38, IL-1β and GluR1 in sham and saline or BBG injection groups was analyzed by one way ANOVA followed by LSD post hoc test. The effects of P2X7-siRNA on the down-regulation of P2X7Rs and the effects of IL-1ra on the expression of GluR1 protein were analyzed using independent samples t-test.

3. Results

3.1. Blockade of the induction of spinal LTP by P2X7R antagonists and P2X7-siRNA

In consistent with our previous studies (Ma and Zhao, 2001; Ying et al., 2006; Zhang et al., 2005), the C-fiber-evoked field potentials by electrical stimulation (0.5 ms, 20–30 V) were recorded at the depth of 300–500 μm from the surface of the spinal cord. Four consecutive C-fiber-evoked field potentials were averaged as control. The baseline of C-fiber-evoked field potentials was stable during the recording periods. Tetanic stimulation of the sciatic nerve (0.5 ms, 100 Hz, 40 V, 10 trains of 2-s duration at 10-s intervals) augmented the average area of C-fiber-evoked field potentials in all rats tested (Fig. 1A; 188.61 ± 13.64% of baseline control, $F(34, 136) = 30.44$, $p < 0.001$ vs. baseline, $n = 5$), which lasted throughout recording periods, indicating occurrence of spinal LTP.

To investigate the roles of P2X7Rs in the induction of spinal LTP, the effects of oxATP or BBG, a P2X7R antagonist, on the spinal LTP were tested. OxATP (300 μM, 10 μl) and BBG (20 μM, 10 μl) were intrathecally injected 30 min before tetanic stimulation, respectively. Both oxATP and BBG completely blocked the induction of LTP (Fig. 1B; 94.53 ± 6.83% of baseline control, $F(34, 136) = 1.15$, $p > 0.05$ vs. baseline, $n = 5$; 78.33 ± 7.05% of baseline control, $F(34, 136) = 4.05$, $p > 0.05$ vs. baseline, $n = 5$, respectively) without affecting the baseline responses (Fig. 1B; 104.63 ± 4.53% of baseline control, $n = 5$; 97.43 ± 3.82% of baseline control, $n = 5$, respectively), which suggests that P2X7Rs are implicated in the induction of LTP of C-fiber-evoked field potentials in the spinal dorsal horn.
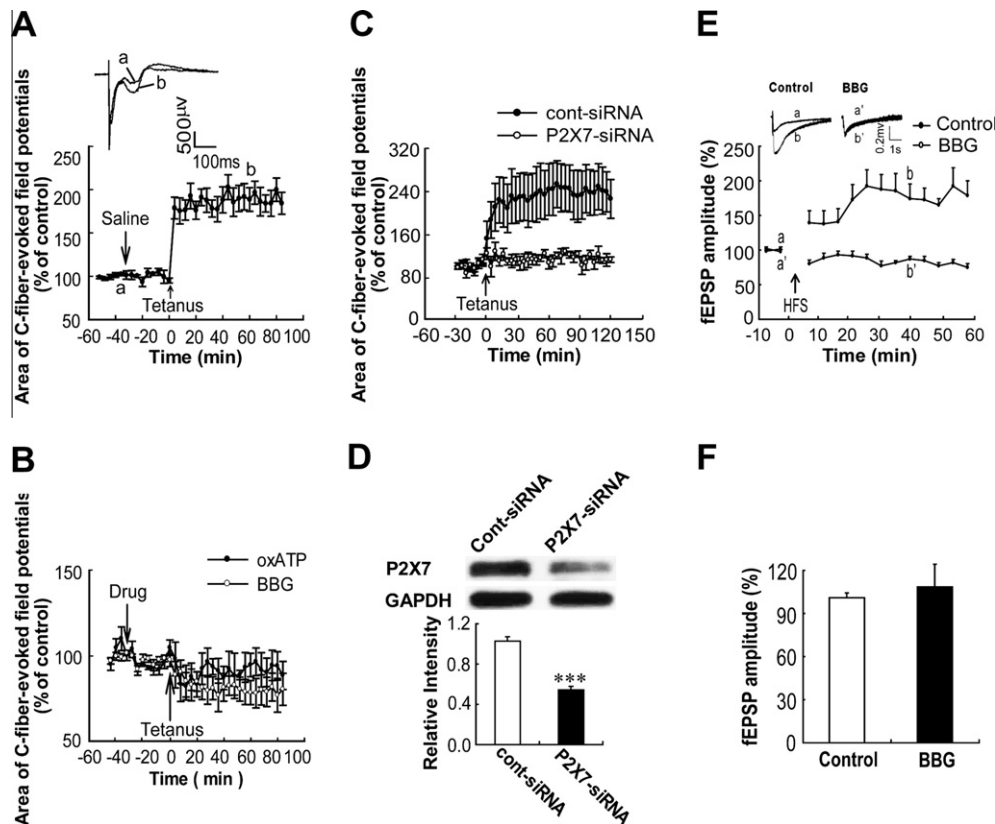
Fig. 1. Blockade of tetanic stimulation-induced spinal LTP of C-fiber-evoked field potentials by P2X7 receptor antagonists and P2X7-siRNA in both *in vivo* and *in vitro* preparations. (A) Tetanic stimulation-induced LTP of C-fiber-evoked field potentials ($n = 5$, $p < 0.001$). (B) OxATP (300 μM, 10 μl, i.t.) and BBG (20 μM, 10 μl, i.t.), P2X7 antagonists, completely blocked the induction of spinal LTP ($n = 5$/group, $p > 0.05$). (C) Pretreatment with P2X7-siRNA (20 μl) 7 days before tetanic stimulation prevented the induction of spinal LTP ($n = 5$/group, $p > 0.05$). (D) Compared with control-siRNA (cont-siRNA) group, the expression of P2X7Rs significantly decreased on day 7 after intrathecal injection of P2X7-siRNA (***$p < 0.001$). (E) 2 μM BBG applied 1 h before conditioning high-frequency stimulation (HFS) blocked the induction of LTP ($p > 0.05$) (control $n = 6$; BBG $n = 7$). n: the number of slices. (F) The baseline response of C-fiber-evoked field potentials induced by test stimulation was not affected by the application of BBG ($p > 0.05$) ($n = 5$/group).

To further confirm the results from P2X7R antagonists, the RNA interference experiments targeted to P2X7Rs was performed on the spinal LTP test. Intrathecal administration of a non-targeting control-siRNA (20 μl) 7 days before tetanic stimulation failed to alter the spinal LTP (Fig. 1C; 243.08 ± 48.63% of baseline control, $F(37, 148) = 7.86$, $p < 0.001$ vs. baseline, $n = 5$). In contrast, intrathecal application of P2X7-siRNA (20 μl) 7 days before tetanic stimulation obviously blocked the induction of LTP (Fig. 1C; 105.63 ± 11.64% of baseline control, $F(37, 148) = 0.95$, $p > 0.05$ vs. baseline, $n = 5$). The Western blots showed that P2X7Rs were significantly down-regulated by pre-application of P2X7-siRNA, compared with control-siRNA group (Fig. 1D; P2X7R: control-siRNA 1.03 ± 0.04; P2X7-siRNA 0.54 ± 0.04, $t(4) = 8.445$, $p < 0.001$). These results provide a further evidence for a vital role of P2X7Rs in the induction of LTP of C-fiber-evoked field potentials induced by tetanic stimulation in the spinal dorsal horn.

We examined the effects of BBG on LTP of C-fiber-evoked field potentials in the spinal cord slices *in vitro*. The C-fiber-evoked field potentials in the dorsal horn were induced by stimulating the LT via a bipolar tungsten electrode. After perfusion with bicuculline methiodide (10 μM) and strychnine (1 μM) for 1 h, the LT was stimulated once every 5 min at a current strength sufficient to excite C-fibers (0.1 ms, 0.7–1 mA). The fEPSPs recorded 10 min before tetanic HFS (100 Hz for 1 s, three times at 10-s intervals) served as control. The fEPSPs were enhanced after tetanic HFS in all recorded cases (Fig. 1E; 174.3 ± 21% of baseline control, $F(13, 65) = 4.37$, $p < 0.001$ vs. baseline, $n = 6$). However, perfusion with 2 μM BBG 1 h before conditioning HFS of LT completely blocked the LTP of

fEPSPs (Fig. 1E; 89.8 ± 6.56% of baseline control, $F(13,78) = 1.73$, $p > 0.05$ vs. baseline, $n = 7$) without changing the baseline responses (Fig. 1F; 108.67 ± 15.72% of baseline control, $p > 0.05$ vs. baseline, $n = 5$). It is reasonable to conclude that P2X7Rs are involved in the induction of LTP of fEPSPs.

3.2. Partial blockade of mechanical allodynia induced by tetanic stimulation and spinal Fos expression by P2X7R antagonists and P2X7-siRNA

As the observation in the previous behavioral studies (Ying et al., 2006), the identical tetanic stimulation used in the electrophysiological test produced a sustained decrease in bilateral PWTs to mechanical stimulation (Ipsilateral: $F(3, 59) = 36.06$, $p < 0.001$; Contralateral: $F(3, 59) = 21.56$, $p < 0.001$). As shown in Fig. 2A and B, the ipsi- and contra-lateral PWTs to von Frey filament stimulation rapidly fell from 16.2 ± 1.85 g to 3.87 ± 0.5 g ($p < 0.01$) and from 14.07 ± 1.81 g to 4.67 ± 0.64 g ($p < 0.05$), respectively, on day 7 in saline group, without changes in sham group (Ipsilateral: $F(3, 27) = 0.09$, $p > 0.05$; Contralateral: $F(3, 27) = 0.28$, $p > 0.05$).

The pre-injection of P2X7R antagonists oxATP (300 μM, 20 μl) and BBG (20 μM, 20 μl) 30 min before tetanic stimulation partially reversed the decrease of PWTs following tetanic stimulation (oxATP: $F(1, 76) = 14.92$, $p < 0.001$; BBG: $F(1, 80) = 11.59$, $p < 0.001$). The ipsilateral PWTs dropped from 17 ± 3 g to 8.67 ± 1 g (Fig. 2A; $p < 0.05$) and from 14.14 ± 1.7 g to 7.14 ± 0.86 g (Fig. 2A; $p < 0.01$), respectively, at the time point of day 7 after tetanic stimulation. However, the values of PWTs were significantly higher than that
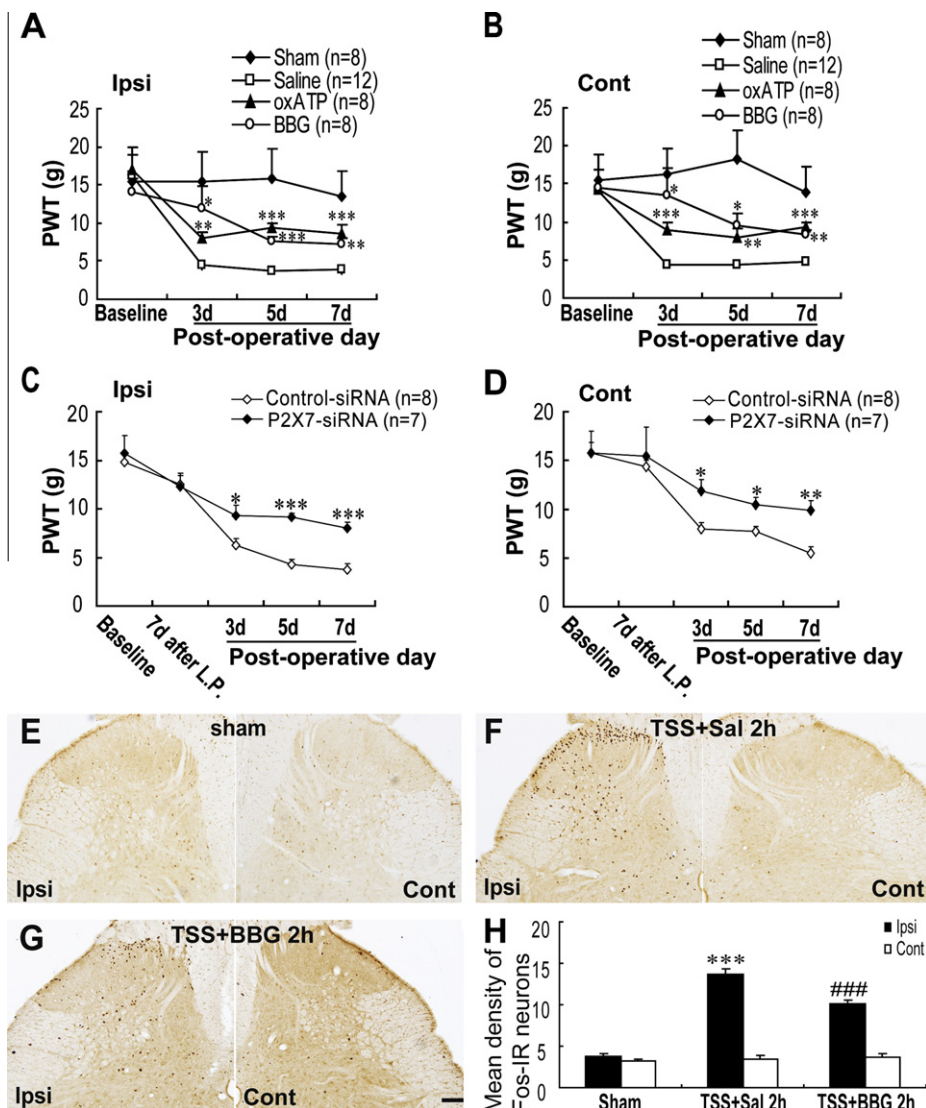
Fig. 2. Alleviation of bilateral mechanical allodynia and Fos expression following tetanic stimulation by P2X7 antagonists and P2X7-siRNA. (A and B) show the effects of oxATP and BBG on nociceptive behaviors on ipsilateral (Ipsi) and contralateral (Cont) sides of tetanic stimulation, respectively. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. saline groups. (C and D) indicate the effects of P2X7-siRNA or control-siRNA (cont-siRNA) injected by lumbar puncture (L.P.) on mechanical allodynia on ipsilateral and contralateral sides of tetanic stimulation, respectively. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. cont-siRNA group. (E–G) Photomicrographs show the staining of Fos-immunoreactivity (Fos-IR) neurons in sham (E), saline (Sal, F) and BBG (G) group 2 h following tetanic stimulation of the sciatic nerve (TSS) of both ipsilateral and contralateral sides. (H) The histogram suggested that BBG inhibited the increased expression of Fos-IR in the spinal dorsal horn neurons 2 h after tetanic stimulation of the sciatic nerve ($n = 4$/group). Scale bar, 100 μm (E–G). $***p < 0.001$ vs. sham group, $###p < 0.001$ vs. saline group.

in saline group (Fig. 2A; 8.67 ± 1, $p < 0.001$ and 7.14 ± 0.86, $p < 0.01$ vs. 3.87 ± 0.5, respectively). A similar result was observed in the contralateral hind paws (Fig. 2B; oxATP: $F(1, 76) = 12.23$, $p < 0.001$; BBG: $F(1, 80) = 18.61$, $p < 0.001$). The results indicated that tetanic stimulation-induced persistent pain was partially alleviated by P2X7R antagonists.

To further confirm the pharmacological results, P2X7-siRNA was applied instead of P2X7R antagonists. PWTs to mechanical stimulation markedly decreased following TSS (Ipsilateral: $F(4, 39) = 39.315$, $p < 0.001$; Contralateral: $F(4, 39) = 38.41$, $p < 0.001$; $n = 8$) from day 3 to at least day 7 in the control-siRNA group. As shown in Fig. 2C and D, in control-siRNA group, the ipsilateral and contra-lateral PWTs to von Frey filament stimuli rapidly fell from 14.81 ± 1.07 g to 3.75 ± 0.59 g ($p < 0.001$) and from 15.75 ± 1.11 g to 5.5 ± 0.63 g ($p < 0.001$) on day 7, respectively. In the P2X7-siRNA group, P2X7-siRNA (20 μl) was administrated by lumbar puncture 7 days before tetanus. The values of ipsilateral PWTs were unchanged on day 7 after P2X7-siRNA injection compared with base-

line (12.29 ± 1.38 vs. 15.71 ± 1.87, $p > 0.05$). The ipsilateral PWTs dropped significantly after tetanic stimulation (Fig. 2C; $F(4, 34) = 6.89$, $p < 0.001$, $n = 7$), indicating that allodynia was still induced. However, the values of PWTs were significantly higher than control-P2X7 group (Fig. 2C; $F(1, 65) = 16.77$, $p < 0.001$), which indicated that the mechanical allodynia was partially alleviated by down-regulation of P2X7Rs. The mechanical allodynia induced by tetanic stimulation in the contralateral hind paw was also partially inhibited by pre-application of P2X7-siRNA (Fig. 2D; $F(1, 65) = 8.12$, $p < 0.01$). Therefore, the data provided another strong evidence for the involvement of P2X7Rs in mechanical allodynia.

Fos is usually used as a molecular marker of pain-sensitive neurons activation in response to noxious stimulation (Harris, 1998). TSS induced an obvious increase of Fos-IR neurons mainly in the ipsilateral superficial dorsal horn 2 h after stimulation (Fig. 2E, F and H; from 3.73 ± 0.35 to 13.63 ± 0.67, $p < 0.001$), but not on the contralateral side (Fig. 2E, F and H; 3.46 ± 0.43 vs. 3.19 ± 0.28, $p > 0.05$). To investigate the effects of P2X7R antagonist on spinal

Fos expression, BBG (20 μM, 20 μl) was injected onto surface of the spinal dorsal horn by lumbar puncture 30 min before tetanic stimulation. Pre-administration of BBG significantly reduced the density of Fos expression (Fig. 2G and H; 10.16 ± 0.38, $p < 0.001$ vs. saline group) in the spinal dorsal horn (laminae I to IV), suggesting involvement of P2X7Rs in the sensitization of spinal nociceptive neurons induced by tetanic stimulation.

3.3. Expression of P2X7Rs in microglia

3.3.1. Colocalization of a microglial marker and P2X7Rs

Positive staining of P2X7Rs was shown in Fig. 3B. In contrast, no signals were detected in the negative control groups, in which only secondary antibody but no primary antibody was added to tissues (Fig. 3A). To identify the cell type that expresses P2X7Rs, double immunostaining of P2X7Rs with OX-42 (a microglial marker), GFAP (a astrocytic marker) and NeuN (a neuronal marker) was detected. P2X7Rs were colocalized with OX-42 (Fig. 3E–J), but not with GFAP (Fig. 3C) and NeuN (Fig. 3D).

3.3.2. Tetanic stimulation-induced activation of microglia

It has been reported that the activation of microglia was observed in the process of many pathological pain (Cao and Zhang, 2008; Clark et al., 2007). In this work, tetanic stimulation-induced activation of microglia was examined. As shown in Fig. 4, microglia were obviously activated following tetanic stimulation ($F(3, 59) = 8.04$, $p < 0.001$, $n = 4$/group), exhibiting the characteristics of swollen cell bodies and retracted processes (Fig. 4F), compared with the sham group (Fig. 4E). The microglial activation started from day 3 after TSS (Fig. 4C and G; 15.88 ± 0.6 vs. sham 12.66 ± 1.06, $p < 0.05$) and lasted at least to day 7 (Fig. 4D and G; 18.8 ± 1.58, $p < 0.01$ vs. sham). The staining of contralateral microglia remained unchanged (Fig. 4A–D and G; $F(3, 57) = 0.97$, $p > 0.05$, $n = 4$/group).

3.3.3. Blockade of up-regulation of spinal P2X7Rs and activation of microglia by the P2X7R antagonist BBG

Tetanic stimulation induced a significant increase of P2X7R expression (Fig. 5A and B; 6.89 ± 1.46 vs. 1 ± 0.28 of sham, $p < 0.01$) in microglia on day 7 after surgery. Intrathecal administration of BBG (20 μM, 20 μl) 30 min before tetanic stimulation inhibited the increased expression of P2X7Rs in microglia (Fig. 5A and B; 3.03 ± 0.5 vs. 6.89 ± 1.46, $p < 0.05$) and the staining intensity of ipsilateral microglia (Fig. 5C and D; 14.4 ± 1.07 vs. saline group 18.8 ± 1.58, $p < 0.05$) on day 7 after tetanic stimulation.

The Western blots showed that tetanic stimulation induced a significant increase in P2X7R levels in the lumbar spinal cord on days 1, 3 and 7 and pre-injection of P2X7R antagonist BBG suppressed this increase ($F(6, 67) = 6.72$, $p < 0.001$, $n = 3$/group). As shown in Fig. 5E and F, the expression level of P2X7Rs significantly increased from day 1 after tetanic stimulation (0.95 ± 0.03, $p < 0.01$ vs. 0.85 ± 0.01 of sham group) and maintained to at least day 7 (1.01 ± 0.03, $p < 0.001$ vs. 0.85 ± 0.01 of sham group). Pre-administration of BBG (20 μM, 20 μl) significantly inhibited the increase of P2X7Rs protein levels on day 1 (Fig. 5E and F; 0.74 ± 0.05 vs. 0.95 ± 0.03 of saline, $p < 0.001$), day 3 (Fig. 5E and F; 0.76 ± 0.05 vs. 0.95 ± 0.04 of saline, $p < 0.01$) and day 7 (Fig. 5E and F; 0.86 ± 0.05 vs. 1.01 ± 0.03 of saline, $p < 0.05$) following tetanic stimulation. These results further indicated a specific role of P2X7Rs in the induction of spinal LTP.

3.4. Signaling pathway analysis

3.4.1. Increase in levels of p-p38, IL-1β and GluR1 following tetanic stimulation and antagonism by BBG

Because microglial P2X7Rs could modulate the phosphorylation of p38 MAPK, which is closely associated with pathological pain (Sperlagh et al., 2007; Tsuda et al., 2004), we investigated the effects of the P2X7R antagonist BBG on the expression level of p-p38 MAPK. The expression level of p-p38 protein was significantly upregulated and was inhibited by pre-application of the P2X7R antagonist BBG ($F(6, 36) = 8.12$, $p < 0.001$, $n = 3$/group). Compared with the sham group, the level of p-p38 protein expression was significantly increased on days 1, 3 and 7 (Fig. 6A and B; 0.99 ± 0.01, $p < 0.05$; 1.13 ± 0.02, $p < 0.001$ and 1.12 ± 0.06, $p < 0.01$ vs. sham 0.9 ± 0.03, respectively) following tetanic stimulation. The increase in levels of p-p38 was blocked by pre-injection of BBG (20 μM, 20 μl, i.t.) on day 1 (Fig. 6A and B; 0.57 ± 0.03, $p < 0.001$), day 3 (Fig. 6A and B; 0.81 ± 0.1, $p < 0.05$) and day 7 (Fig. 6A and B; 0.79 ± 0.08, $p < 0.01$). To identify the cell type that expresses p-p38, spinal sections were double immunostained with p-p38 and OX-42, GFAP or NeuN on day 7 after tetanus in saline group. As
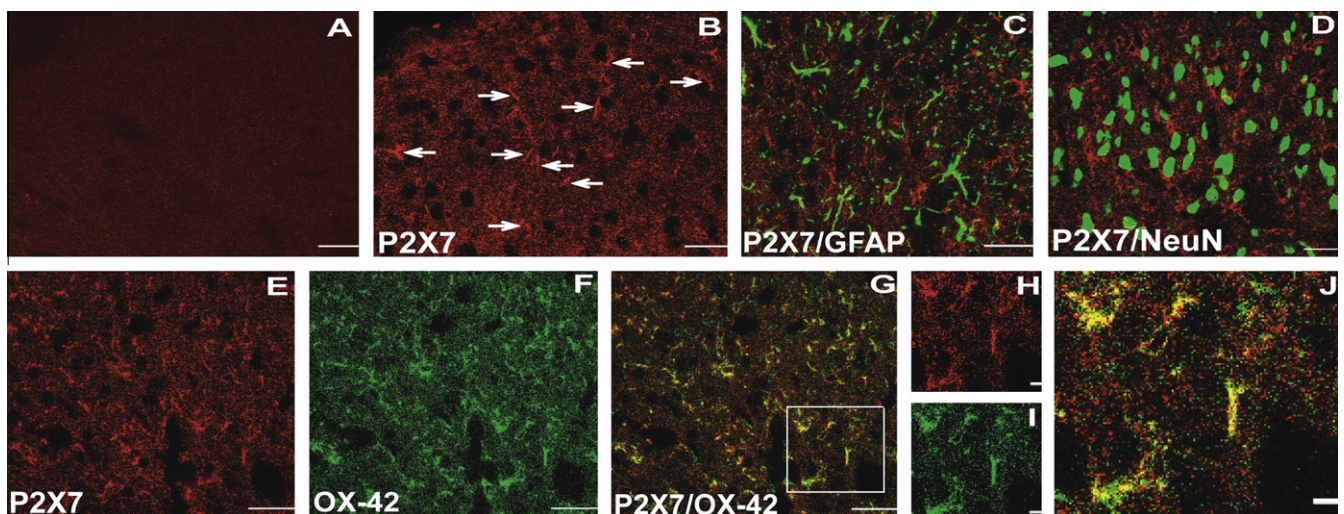

Fig. 3. The localization of P2X7Rs in the spinal dorsal horn. (A) No signals were detected in negative control groups, in which only secondary antibody but no primary antibody was added to tissues. (B) Arrows indicate the positive staining of P2X7Rs. (C and D) show no colocalization of P2X7Rs with the astrocytic marker GFAP and the neuronal marker NeuN, respectively. (E–G) Double immunofluorescence in the superficial spinal dorsal horn (I–IV layers) shows that P2X7Rs are colocalized with the microglial marker OX-42. White open square (in G) marks the corresponding scope of amplified images (H–J) on the confocal images. Scale bars: (A–G) 25 μm; (H–J) 5 μm.

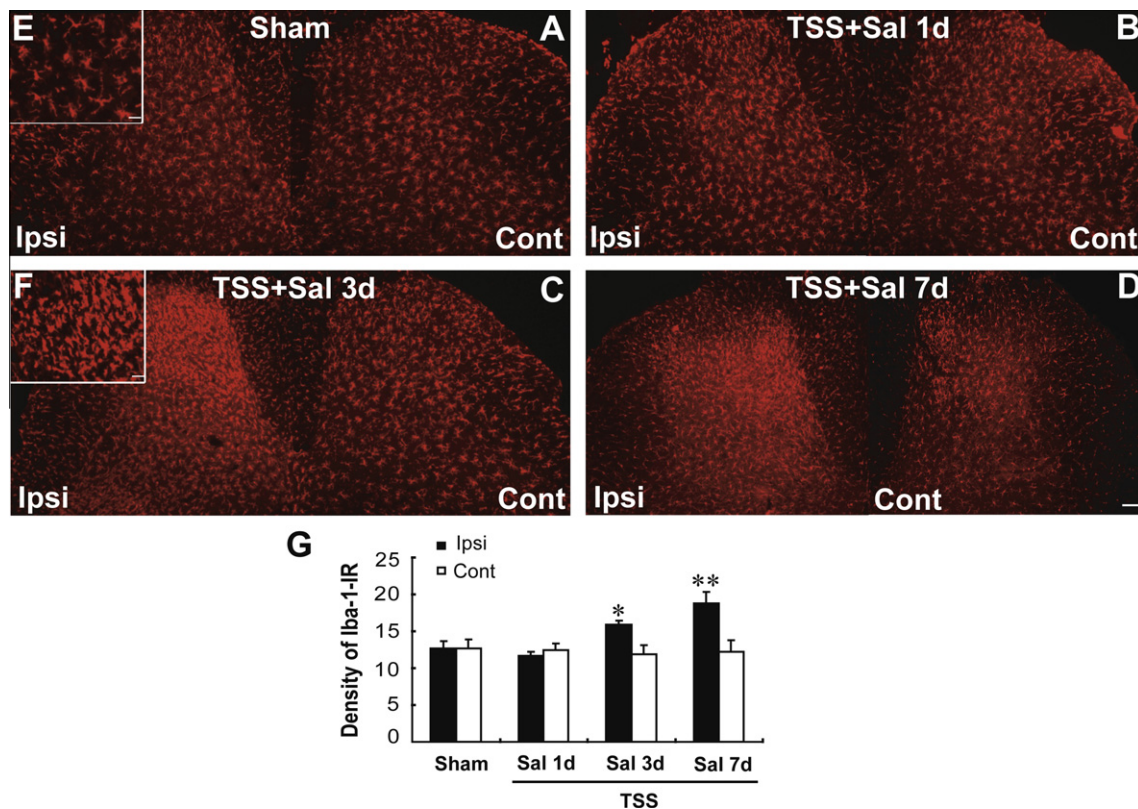**Fig. 4.** The activation of microglia induced by tetanic stimulation of the sciatic nerve. (A–D) Immunohistochemistry indicates a significant increase of Iba-1-immunoreactivity (Iba-1-IR) from day 3 to day 7 in the ipsilateral (Ipsi) but not the contralateral (Cont) lumbar cord following tetanic stimulation of the sciatic nerve (TSS). (E and F) show rest microglia with ramified morphology and activated microglia with swollen cell bodies and retracted processes at a higher magnification in sham and saline (Sal) injection groups of tetanic stimulation, respectively. Scale bars: (A–D) 100 μm; (E and F) 25 μm. (G) The histogram represents the statistic results of Iba-1 expression on bilateral sides of tetanic stimulation. $^*p < 0.05$, $^{**}p > 0.01$ vs. sham control ($n = 4$/group).

shown in Fig. 6C, p-p38 was almost completely colocalized with OX-42, but not with GFAP and NeuN. Pre-application of the P2X7R antagonist BBG inhibited the increased phosphorylation of p38 MAPK in microglia, suggesting that p38 MAPK in microglia functions as downstream of P2X7Rs.

IL-1β is one of most important proinflammatory mediators released from glia and is thought to function as the downstream of p38 MAPK. The expression level of the 28 kDa partially processed IL-1β significantly increased following tetanic stimulation and pre-injection of the P2X7R antagonist BBG suppressed this increase ($F(6, 25) = 7.99$, $p < 0.001$, $n = 3$ or 5/group). The 31 kDa precursor band and the 17 kDa mature band were too weak to be detected. Western blot analysis showed that the expression level of IL-1β (28 kDa) was dramatically increased on day 1 following tetanic stimulation ($1.01 ± 0.03$, $p < 0.05$ vs. sham $0.8 ± 0.03$), peaked on day 3 ($1.07 ± 0.07$, $p < 0.05$ vs. sham) and maintained to at least day 7 ($1.05 ± 0.07$, $p < 0.05$ vs. sham). Pre-administration of the P2X7R antagonist BBG (20 μM, 20 μl) 30 min before tetanic stimulation substantially inhibited the increased expression of IL-1β (28 kDa) (Fig. 7A and B; $0.79 ± 0.02$, $p < 0.05$ vs. saline group on day 1; $0.81 ± 0.04$, $p < 0.05$ vs. saline group on day 3; $0.7 ± 0.08$, $p < 0.05$ vs. saline group on day 7, respectively). These results indicated that IL-1β maybe underlied the antinociceptive actions of P2X7R antagonists.

Compelling evidence has demonstrated that the insertion and translocation to the membrane of GluR1, a subunit of AMPA receptor, is essential in the induction of hippocampical LTP (Lisman and Raghavachari, 2006; Sanderson et al., 2008). We examined the expression of GluR1 in the induction of spinal LTP. The expression level of GluR1 significantly increased following tetanic stimulation

and pre-injection of the P2X7R antagonist BBG inhibited this increase ($F(6, 46) = 12.82$, $p < 0.001$, $n = 3$/group). Compared with sham group, the expression level of GluR1 protein dramatically increased following tetanic stimulation from day 1 ($0.37 ± 0.05$, $p < 0.05$ vs. sham $0.2 ± 0.001$) to day 7 ($0.25 ± 0.02$, $p < 0.05$ vs. sham) and the peak was on day 3 ($0.46 ± 0.03$, $p < 0.001$ vs. sham). Pre-administration of the P2X7R antagonist BBG (20 μM, 20 μl) 30 min before tetanic stimulation substantially inhibited the increase in GluR1 protein level (Fig. 8A and B; $0.2 ± 0.03$, $p < 0.05$ on day 1; $0.3 ± 0.03$, $p < 0.01$ on day 3; $0.16 ± 0.02$, $p < 0.01$ on day 7, respectively).

3.4.2. IL-1ra blocking the increase in GluR1 protein level and the induction of spinal LTP

Because tetanic stimulation induced the increase in IL-1β expression, we examined the effects of IL-1ra, an IL-1β antibody, on the induction of spinal LTP. IL-1ra (3.5 μg/ml, 20 μl) was intrathecally injected 30 min before tetanic stimulation. Spinal LTP was completely blocked (Fig. 9A; $108.42 ± 4.79\%$ of baseline control, $F(55, 220) = 1.09$, $p > 0.05$ vs. baseline, $n = 5$) without affecting the baseline C fiber-evoked responses (Fig. 9A; $99.56 ± 1.71\%$ of baseline control), suggesting that IL-1β functions as the downstream of P2X7Rs and contributes greatly to the induction of spinal LTP of C-fiber-evoked field potentials.

The effects of IL-1ra on the expression level of GluR1 protein were also detected. Compared with the sham group ($0.2 ± 0.001$), the expression level of GluR1 significantly increased and reached the peak on day 3 following tetanic stimulation ($0.46 ± 0.03$, $p < 0.001$). Pre-administration of IL-1ra (3.5 μg/ml, 20 μl) 30 min before tetanic stimulation completely reversed the increase in
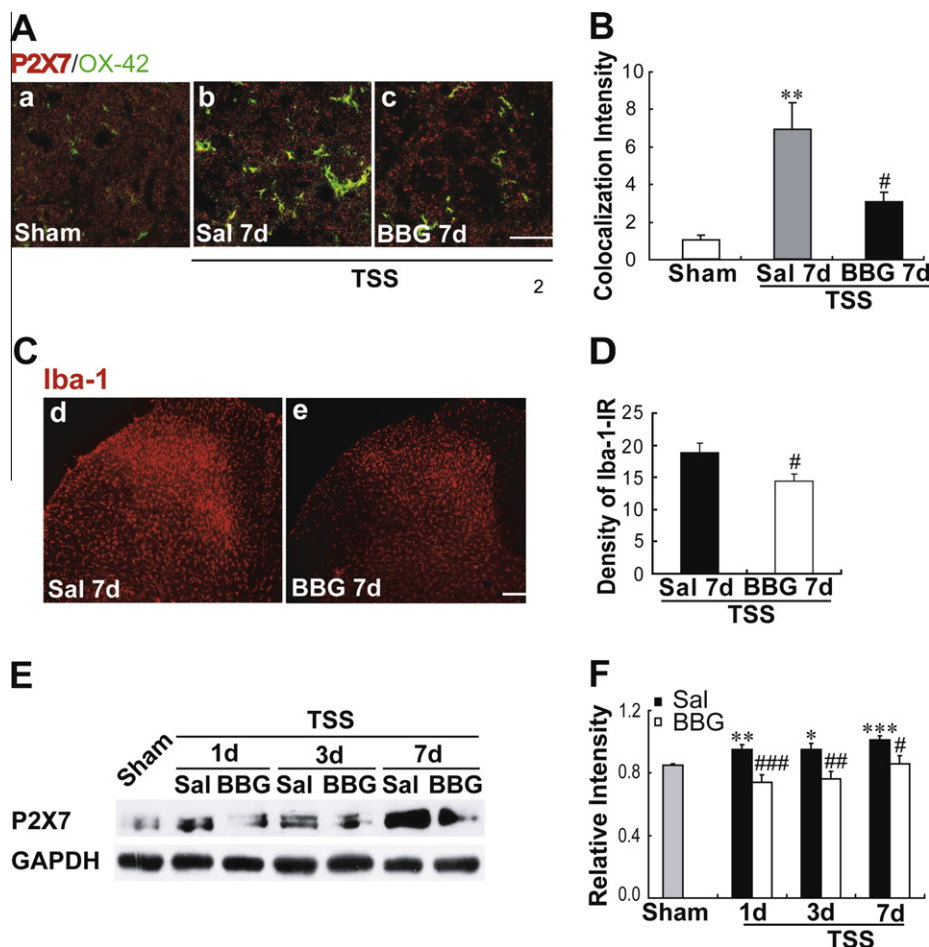
Fig. 5. Inhibition of microglial activation and the increased expression of P2X7Rs following tetanic stimulation by the P2X7R antagonist BBG. (A) Double immunohisto-chemistry shows the expression of P2X7Rs in the microglia of the superficial spinal dorsal horn (I–IV layers) in sham and tetanic stimulation of the sciatic nerve (TSS) groups received pre-injection of saline (Sal) or BBG, respectively. Scale bar, 25 μm. (B) The bar graph indicated that significant increase of P2X7R expression in microglia on day 7 following tetanic stimulation was significantly inhibited by pretreatment with BBG (20 μM, 20 μl, i.t.). $**p < 0.01$ vs. sham group, $\#p < 0.05$ vs. saline group ($n = 4$/group). (C) Representative immunofluorescence staining of the microglial marker Iba-1 on day 7 following tetanic stimulation in the saline and BBG injection groups. Scale bar, 100 μm. (D) The histogram shows that the increase of Iba-1-immunoreactivity (Iba-1-IR) induced by tetanic stimulation was partially inhibited by BBG. $\#p < 0.05$ vs. saline injection group ($n = 4$/group). (E) Representative Western blots showing the expression of P2X7Rs and GAPDH at different time points in sham or tetanus groups. (F) Histogram shows that intrathecal injection of BBG (20 μM, 20 μl) 30 min before TSS inhibits the increased expression of P2X7Rs. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. sham group; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ vs. saline group in the same day after tetanic stimulation ($n = 3$/group).

levels of GluR1 protein (Fig. 9B and C; 0.17 ± 0.01, $t(7) = 5.691$, $p < 0.001$), suggesting that glia-derived IL-1β may promote the expression of GluR1 contributing to the induction of spinal LTP.

4. Discussion

The main findings of this study are: (1) Activation of spinal P2X7Rs by tetanic stimulation of the sciatic nerve is intimately linked with the induction of spinal LTP of C-fiber-evoked field potentials and long-lasting behavioral pain hypersensitization; (2) P2X7Rs are expressed in spinal cord microglia; (3) Disruption of P2X7R signaling by antagonists or introduction of siRNA into spinal microglia blocked the spinal LTP and alleviated pain hypersensitization; and (4) IL-1β signaling pathway regulates tetanic stimulation-induced plasticity of spinal pain-sensitive neurons. The relationship between the cell and molecular actors of the spinal cord involved in TSS and spinal LTP is summarized in Fig. 10.

Tetanic stimulation-induced LTP of C-fiber-evoked field potentials in the spinal cord has been proposed to be the substrate for central sensitization in the spinal pain pathway (Liu and Sandkuh-ler, 1998; Lozier and Kendig, 1995; Ma and Zhao, 2001). Increased expression of Fos in superficial dorsal horn neurons is closely asso-

ciated with the long-term intracellular plastic changes of persistent pain provoked by noxious stimulation (Harris, 1998). The observations that tetanic stimulation induced Fos expression in superficial dorsal horn neurons and induced long-lasting pain-associated behavioral responses provide further support for a role of spinal LTP in central pain sensitization. The exact mechanisms underlying the induction of spinal LTP, however, are still not fully understood.

There is compelling evidence that ATP acts as an activity-dependent signaling molecule in synaptic transmission of peripheral and spinal nociception (Fields and Stevens, 2000) and in communication between neurons and glia by activating multiple P2X receptors in glia (Chizh and Illes, 2001; Haydon, 2001). ATP released in the spinal cord originates from several sources, including primary afferent terminals, interneurons and glial cells (Nakatsuka and Gu, 2001; Queiroz et al., 1997; Sawynok et al., 1993). P2X7Rs are expressed predominantly in immune cells (Sim et al., 2004) and function as a non-selective cationic channel (Chessell et al., 2005; Honore et al., 2006; McGaraughty et al., 2007). An early study showed that ATP is released from presynaptic nerve terminals when high-frequency stimulation induces hippocampal LTP, suggesting a role for ATP in the modulation of synaptic efficiency
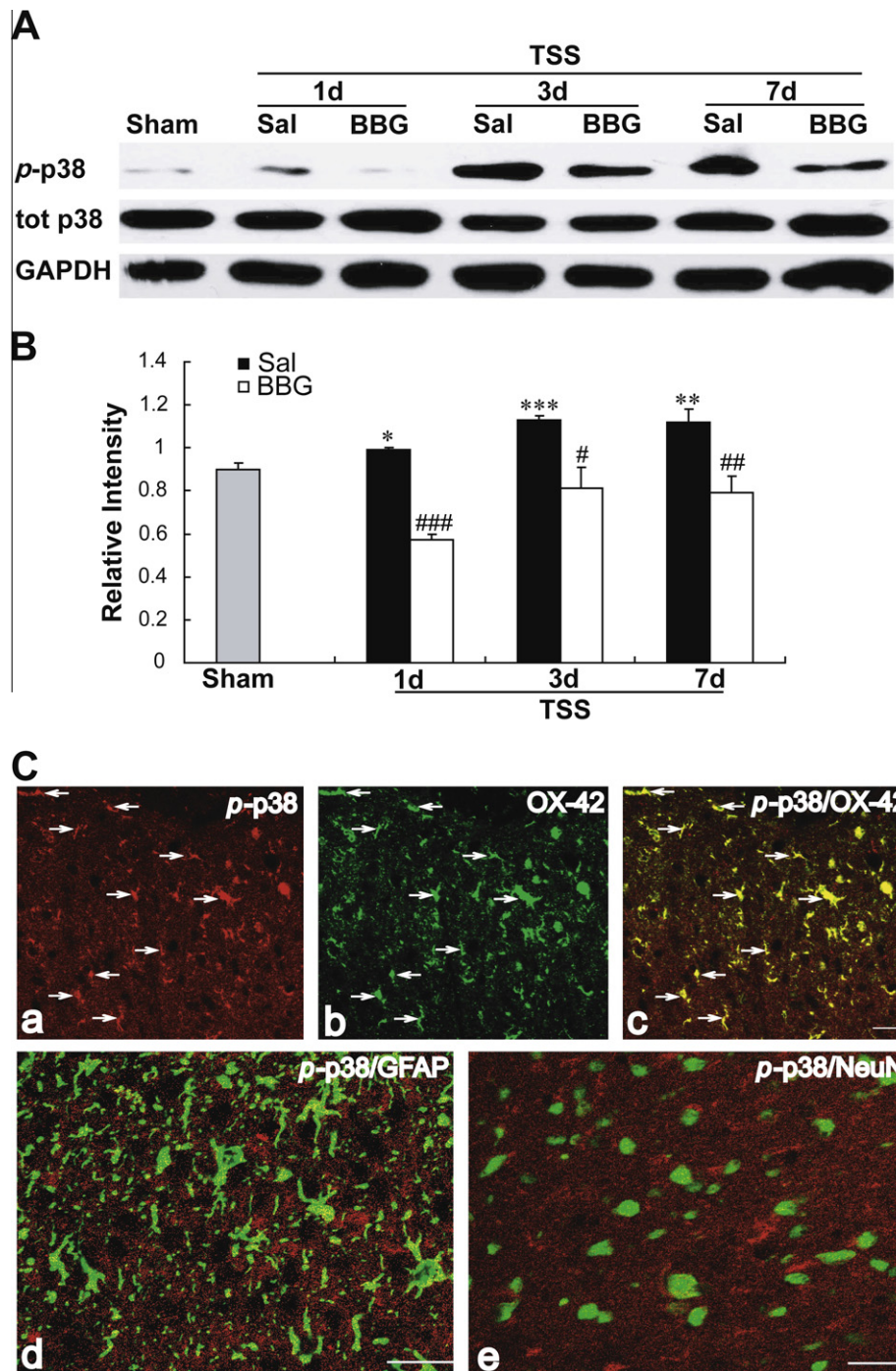
Fig. 6. Activation of microglial p38 in the spinal dorsal horn induced by tetanic stimulation and inhibitory action of the P2X7R antagonist BBG. (A) Representative Western blots for phosphorylated p38 (p-p38), total p38 (tot p38) and GAPDH in the ipsilateral dorsal horn at different time points in sham or tetanic stimulation of the sciatic nerve (TSS) groups. (B) Western blot data indicate that the increase in p-p38 MAPK protein level in saline (Sal) injection group occurs on days 1, 3 and 7 and is blocked by pre-administration of BBG (20 μM, 20 μl, i.t.). Data were normalized against GAPDH protein expression in each sample. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. sham group; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ vs. saline group in the same day ($n = 3$/group). (C) Double immunostaining suggests that p-p38 (red; a) is almost completely colocalized (yellow, c, arrow) with OX-42 (a microglial marker; green; b), but not with GFAP (an astrocytic marker; d) and NeuN (a neuronal marker; e). Scale bars: 25 μm (a–c), 10 μm (d–e). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Wieraszko et al., 1989). It is reasonable to speculate that tetanic stimulation of the sciatic nerve induces considerable release of ATP in the spinal cord and, in turn, activates P2X7Rs in microglia. Our observations that P2X7R antagonists and P2X7-siRNA prevent tetanic stimulation-induced spinal LTP and reduce mechanical allodynia strongly suggest that microglial P2X7Rs are required for plastic changes in pain-sensitive neurons in the spinal dorsal horn. The difference between the complete blockade of spinal

LTP and partial inhibition of mechanical allodynia may be attributed to the different physiological conditions under which the experiments were carried out: under anesthesia for the electrophysiological recording and awake for the behavioral tests.

In agreement with our previous study (Ying et al., 2006), tetanic stimulation induced bilateral mechanical allodynia, also called mirror-image pain. The mechanisms underlying the development of mirror-image pain have not yet been explored. Peripheral circu-
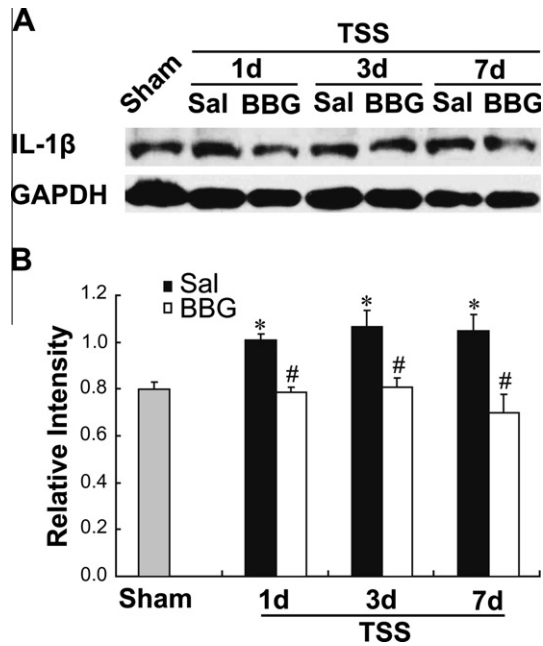
Fig. 7. Blockade of increased expression of IL-1β induced by tetanic stimulation by P2X7 antagonist BBG. (A) Representative Western blot bands of IL-1β using GAPDH as loading control. (B) Histogram shows the statistic results of Western blot analysis. The expression of IL-1β significantly increased on days 1, 3 and 7 in saline (Sal) injection group following tetanic stimulation of the sciatic nerve (TSS) compared with sham group. Intrathecal injection of BBG (20 μM, 20 μl) 30 min before tetanic stimulation significantly inhibited the increased expression of IL-1β at all time points. $*p < 0.05$ vs. sham group; $^{\#}p < 0.05$ vs. saline group of the same day after tetanic stimulation ($n = 3$ or 5/group).
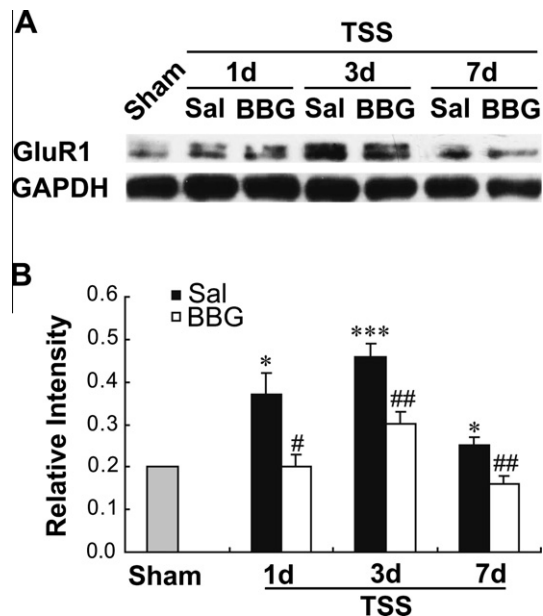

Fig. 8. Inhibition of the increased expression of GluR1 induced by tetanic stimulation by the P2X7 antagonist BBG. (A) Representative Western blots showing relative levels of GluR1 after surgery using GAPDH as loading control. (B) Histogram shows the statistic results of Western blots experiments. The expression level of GluR1 significantly increased on days 1, 3 and 7 in saline (Sal) group after tetanic stimulation of the sciatic nerve (TSS) compared with sham group. Intrathecal injection of BBG (20 μM, 20 μl) 30 min before tetanic stimulation inhibited the increased expression of GluR1 at all time points. $*p < 0.05$, $***p < 0.001$ vs. sham group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. saline injection group of the same day after tetanic stimulation ($n = 3$/group).
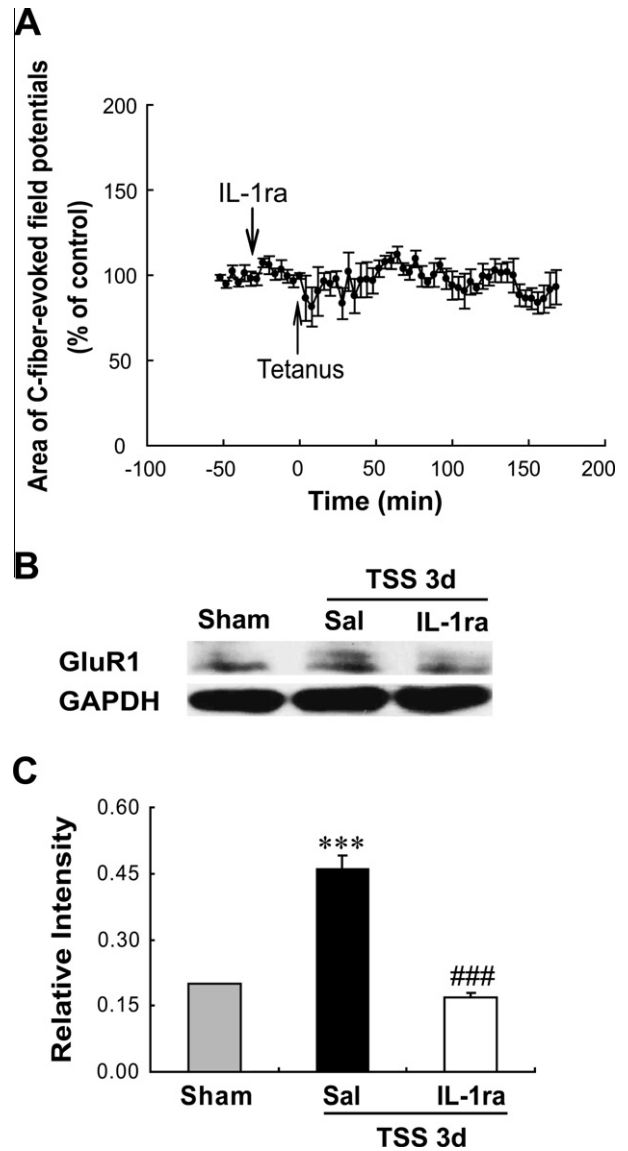

Fig. 9. Blockade of tetanic stimulation-induced LTP of C-fiber-evoked field potentials and the increase in levels of GluR1 protein by the IL-1β antibody IL-1ra. (A) IL-1ra (3.5 μg/ml, 20 μl, i.t.) completely blocked the induction of spinal LTP induced by tetanic stimulation of the sciatic nerve (TSS) ($n = 5$/group). The mean response recorded before the administration of IL-1ra served as control. Mean area of C-fiber-evoked field potentials (± SEM) was expressed as percentage of control and plotted versus time. (B) Representative Western blots showing relative levels of GluR1 after surgery using GAPDH as loading control. (C) Histogram shows the peaked expression of GluR1 on day 3 after tetanus in saline (Sal) group is significantly inhibited by intrathecal injection of IL-1ra (3.5 μg/ml, 20 μl) 30 min before tetanic stimulation. $***p < 0.001$ vs. sham group; $^{\#\#\#}p < 0.01$ vs. saline group ($n = 3$/group).

lating factors, transmedian sprouting, overmapping central terminals of afferents or dendrites of motoneurons, as well as commissural interneuron in the spinal cord and brainstem, have been proposed to mediate unilateral nerve injury-induced bilateral mirror effects (Koltzenburg et al., 1999). In addition, a role in mirror-image pain for diffusible proinflammatory cytokines released from spinal glia has also been proposed (Milligan et al., 2003). Our present and previous findings support roles of glial function in mirror-image pain, since bilateral allodynia could be reduced by blocking microglial P2X7Rs with the antagonist BBG and astroglial functions with fluorocitrate (Ying et al., 2006). It is well known that glia connect closely with each other via gap junctions to form a functional syncytium that allow the propagation of calcium waves from cell
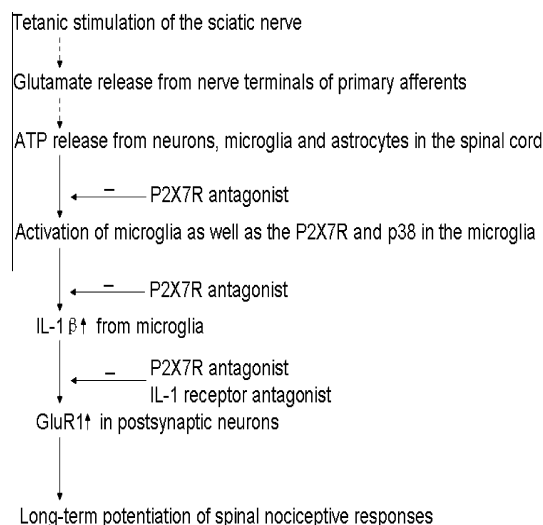
Tetanic stimulation of the sciatic nerve

Glutamate release from nerve terminals of primary afferents

ATP release from neurons, microglia and astrocytes in the spinal cord

— P2X7R antagonist

Activation of microglia as well as the P2X7R and p38 in the microglia

— P2X7R antagonist

IL-1β↑ from microglia

— P2X7R antagonist
IL-1 receptor antagonist

GluR1↑ in postsynaptic neurons

Long-term potentiation of spinal nociceptive responses

Fig. 10. Schematic illustration of mechanisms by which P2X7Rs in activated microglia are involved in the long-term potentiation of nociceptive responses induced by tetanic stimulation of the sciatic nerve. The tetanic stimulation promotes the release of glutamate from nerve terminals of primary afferents and, in turn, evokes ATP release from neurons, microglia and astrocytes in the spinal cord. ATP induces the activation of the ipsilateral microglia, as well as the increase in levels of P2X7Rs protein and p38 MAPK phosphorylation in the microglia by binding to P2X7Rs. P2X7R activation upon ATP leads to the increased expression of IL-1β. Il-1β acts on the postsynaptic neuronal membrane to increase the expression and phosphorylation of the GluR1 subunit, thus synaptic strength is finally strengthened. The microglial P2X7Rs act as a key factor in forming a net hyperexcitability in the spinal dorsal horn pain network and are responsible for long-term nociceptive responses. Dashed arrows indicate plausible events.

to cell (Suadicani et al., 2006). Thus, glia on contralateral side might be activated by the excited glia in the stimulated side to promote the induction of mirror-image pain through the release of glia-derived proinflammatory cytokines and nitric oxide. Although we think that it is likely that the same signaling pathways mediate the induction of mirror-image pain, a resolution of this question must await further studies.

In this work, we found that the increase in Fos-IR neurons is restricted to the ipsilateral side of tetanic stimulation, a result consistent with previous reports (Hunter et al., 1995; Ji et al., 1994). It has been proposed that spinal Fos-IR neurons are driven monosynaptically by small-diameter, presumed nociceptive primary afferents from the sciatic nerve (Presley et al., 1990). In addition, Fos is generally detected 2 h after noxious stimulation and this activation is thought to reflect activation of pain-sensitive neurons in acute pain conditions. Contralateral expression of spinal Fos is usually observed following prolonged noxious stimulation (Harris, 1998). Glia may be involved in the process.

Several studies have shown that P2X7Rs are expressed in both microglia and astrocytes (Collo et al., 1997; Duan et al., 2003; Hide et al., 2000; Lee et al., 2008). It was reported, however, that astrocytes express only P2X7R mRNA, but not protein (Deng and Fyffe, 2004). This observation was confirmed in our double-immunofluorescence-staining experiments, which showed that P2X7Rs are expressed in microglia, but are not detected in astrocytes and neurons. Numerous studies demonstrating that both P2X7R mRNA and protein are expressed in spinal microglia support these observations (Bernardino et al., 2008; Brough et al., 2002; Di Virgilio et al., 1999; Melani et al., 2006; Morioka et al., 2008). Although the expression of P2X7Rs in neurons has been reported (Atkinson et al., 2004) and the involvement of P2X7Rs in modulating neuronal function proposed (Bennett, 2007; Deuchars et al., 2001), this interpretation is complicated by the presence of 'P2X7R-like' protein staining in P2X7R gene-knockout mice (Sim et al., 2004). The expression of P2X7Rs in neurons, therefore, still needs to be con-

firmed. This study demonstrates that the P2X7R antagonist BBG inhibits the up-regulation of microglial P2X7Rs following tetanic stimulation. This result is consistent with the previous report that oxATP blocks LPS stimulation-induced increase in expression of microglial P2X7Rs (Choi et al., 2007) and further supports a role of microglial P2X7Rs in nociception. Taken together, our data strongly argues for a pivotal role for microglial P2X7Rs in the induction of spinal LTP, although possible presynaptic effects of neuronal P2X7Rs can not be absolutely excluded.

P2X7Rs regulate many aspects of microglia, including the activation of specific signaling pathways that are closely associated with allodynia (Monif et al., 2009; Sperlagh and Illes, 2007). Several lines of evidence suggest that activation of p38 MAPK in microglia plays an important role in the induction of pathological pain. For example, inhibition of the p38 MAPK signaling pathway significantly alleviates nerve injury-induced mechanical allodynia (Crown et al., 2008). In addition, the p38 inhibitor SB203580 suppresses the induction of LTP in synapses in the cingulate following presynaptic stimulation in the presence of postsynaptic depolarization (Toyoda et al., 2007). It is uncertain, however, that whether the p38 MAPK signaling pathway is involved in the induction of spinal LTP.

It has been reported that activation of the p38 MAPK signaling pathway mediates the induction of metabotropic glutamate receptor-dependent long-term depression (LTD) of CA3-CA1 synapses (Bolshakov et al., 2000). Moreover, expression of p38 MAPK decreases in LTP of hippocampal CA1 neurons (Sanes and Lichtman, 1999). All of these results imply a negative role for p38 signaling in synaptic plasticity in the hippocampus. In contrast to above results, activation of p38 MAPK increases central sensitization of the spinal cord neurons (Ji et al., 2003; Sanes and Lichtman, 1999). This study shows that the P2X7R antagonist BBG inhibits the increase in levels of p-p38 protein following tetanic stimulation, a result consistent with the report that activation of P2X7Rs by extracellular ATP increases the phosphorylation of p38 MAPK (Donnelly-Roberts et al., 2004; Sperlagh et al., 2007). As noted above, glial cell p38 MAPK is a key regulator of the production and release of IL-1β and TNF-α (Ji and Suter, 2007; Kumar et al., 2003). It is likely, therefore, that p38 MAPK has a positive role in the induction of spinal LTP through direct or indirect pathways.

P2X7Rs activated by extracellular ATP specifically promotes the maturation and release of the proinflammatory cytokine IL-1β (Ferrari et al., 2006; Simi et al., 2007; Solle et al., 2001). In this study, intrathecal injection of a P2X7R antagonist was found to inhibit the increase in levels of IL-1β following tetanic stimulation. Previous studies on the involvement of IL-1β in CA1 hippocampal LTP support our observations (Cunningham et al., 1996; Schneider et al., 1998). The release of IL-1β in the spinal cord is increased under different chronic pain conditions and plays an important role in chronic pain generation (Samad et al., 2001; Sweitzer et al., 2001). Intrathecal injection of IL-1β enhances pain and blockade of IL-1β signaling pathway attenuates chronic pain (DeLeo and Yezierski, 2001; Watkins et al., 2001). In addition to enhancing the frequency and amplitude of spontaneous EPSCs (sEPSCs) and reducing the frequency and amplitude of spontaneous IPSCs (sIPSCs), IL-1β induces phosphorylation of cAMP response element-binding protein (CREB) (Kawasaki et al., 2008). CREB phosphorylation has previously been shown to be essential for the maintenance of long-term neural plasticity in dorsal horn neurons (Ji et al., 2003). Taken together, the inhibition of IL-1β action may account for the blockade of LTP of C-fiber/lamina I neuron synapses and persistent pain after antagonism of P2X7Rs.

It is commonly accepted that insertion and phosphorylation of GluR1, an important subunit of AMPA receptor, is required for the increase in synaptic strength during CA1 hippocampal LTP (Lisman and Raghavachari, 2006; Sanderson et al., 2008). Both LTP and

LTD are abolished in mice with knockin mutations in the phosphorylation sites Ser831 and Ser845 of the GluR1 subunit. (Lee et al., 2003). In addition, it has been reported that exposure to exogenous IL-1β reduces both the surface expression and Ser831 phosphorylation of the GluR1 subunit in CA1 hippocampal LTP (Lai et al., 2006). In this work, we found that expression of GluR1 protein dramatically increases following tetanic stimulation and intrathecal injection of either the P2X7R antagonist BBG or the IL-1β antibody IL-1ra inhibits this increase. This result strongly suggests the involvement of GluR1, functions as the downstream of P2X7R and IL-1β, in the induction of spinal LTP.

Taken together, we speculate that tetanic stimulation of the sciatic nerve triggers the release of glutamate from primary afferent terminals in the spinal cord and, in turn, evokes ATP release from several sources, including spinal neurons, microglia and astrocytes. Activation of microglial P2X7Rs by this ATP induces the release of proinflammatory cytokine IL-1β from spinal microglia, which, in turn, acts on the postsynaptic neuronal membrane to increase the expression and phosphorylation of the GluR1 subunit. The net effect of these signaling events is to strengthen synaptic efficacy of spinal nociceptive transmission.

Acknowledgments

References

Atkinson, L., Batten, T.F., Moores, T.S., Varoqui, H., Erickson, J.D., Deuchars, J., 2004. Differential co-localisation of the P2X7 receptor subunit with vesicular glutamate transporters VGLUT1 and VGLUT2 in rat CNS. Neuroscience 123, 761–768.

Beattie, E.C., Stellwagen, D., Morishita, W., Bresnahan, J.C., Ha, B.K., Von Zastrow, M., Beattie, M.S., Malenka, R.C., 2002. Control of synaptic strength by glial TNF alpha. Science 295, 2282–2285.

Bennett, M.R., 2007. Synaptic P2X7 receptor regenerative-loop hypothesis for depression. Aust. N. Z. J. Psychiat. 41, 563–571.

Bernardino, L., Balosso, S., Ravizza, T., Marchi, N., Ku, G., Randle, J.C., Malva, J.O., Vezzani, A., 2008. Inflammatory events in hippocampal slice cultures prime neuronal susceptibility to excitotoxic injury: a crucial role of P2X (7) receptor-mediated IL-1 beta release. J. Neurochem. 106, 271–280.

Bolshakov, V.Y., Carboni, L., Cobb, M.H., Siegelbaum, S.A., Belardetti, F., 2000. Dual MAP kinase pathways mediate opposing forms of long-term plasticity at CA3-CA1 synapses. Nat. Neurosci. 3, 1107–1112.

Brough, D., Le Feuvre, R.A., Iwakura, Y., Rothwell, N.J., 2002. Purinergic (P2X7) receptor activation of microglia induces cell death via an interleukin-1-independent mechanism. Mol. Cell. Neurosci. 19, 272–280.

Cao, H., Zhang, Y.Q., 2008. Spinal glial activation contributes to pathological pain states. Neurosci. Biobehav. Rev. 32, 972–983.

Chessell, I.P., Hatcher, J.P., Bountra, C., Michel, A.D., Hughes, J.P., Green, P., Egerton, J., Murfin, M., Richardson, J., Peck, W.L., Grahames, C.B.A., Casula, M.A., Yiangou, Y., Birch, R., Anand, P., Buell, G.N., 2005. Disruption of the P2X (7) purinoceptor gene abolishes chronic inflammatory and neuropathic pain. Pain 114, 386–396.

Chizh, B.A., Illes, P., 2001. P2X receptors and nociception. Pharmacol. Rev. 53, 553–568.

Choi, H.B., Ryu, J.K., Kim, S.U., McLarnon, J.G., 2007. Modulation of the purinergic P2X7 receptor attenuates lipopolysaccharide-mediated microglial activation and neuronal damage in inflamed brain. J. Neurosci. 27, 4957–4968.

Clark, A.K., Gentry, C., Bradbury, E.J., McMahon, S.B., Malcangio, M., 2007. Role of spinal microglia in rat models of peripheral nerve injury and inflammation. Eur. J. Pain 11, 223–230.

Clark, A.K., Staniland, A.A., Marchand, F., Kaan, T.K., McMahon, S.B., Malcangio, M., 2010. P2X7-dependent release of interleukin-1beta and nociception in the spinal cord following lipopolysaccharide. J. Neurosci. 30, 573–582.

Collo, G., Neidhart, S., Kawashima, E., Kosco-Vilbois, M., North, R.A., Buell, G., 1997. Tissue distribution of the P2X7 receptor. Neuropharmacology 36, 1277–1283.

Crown, E.D., Gwak, Y.S., Ye, Z., Johnson, K.M., Hulsebosch, C.E., 2008. Activation of p38 MAP kinase is involved in central neuropathic pain following spinal cord injury. Exp. Neurol. 213, 257–267.

Cunningham, A.J., Murray, C.A., O'Neill, L.A., Lynch, M.A., O'Connor, J.J., 1996. Interleukin-1 beta (IL-1 beta) and tumour necrosis factor (TNF) inhibit long-term potentiation in the rat dentate gyrus in vitro. Neurosci. Lett. 203, 17–20.

DeLeo, J.A., Yezierski, R.P., 2001. The role of neuroinflammation and neuroimmune activation in persistent pain. Pain 90, 1–6.

Deng, Z., Fyffe, R.E., 2004. Expression of P2X7 receptor immunoreactivity in distinct subsets of synaptic terminals in the ventral horn of rat lumbar spinal cord. Brain Res. 1020, 53–61.

Deuchars, S.A., Atkinson, L., Brooke, R.E., Musa, H., Milligan, C.J., Batten, T.F., Buckley, N.J., Parson, S.H., Deuchars, J., 2001. Neuronal P2X7 receptors are targeted to presynaptic terminals in the central and peripheral nervous systems. J. Neurosci. 21, 7143–7152.

Di Virgilio, F., Sanz, J.M., Chiozzi, P., Falzoni, S., 1999. The P2Z/P2X7 receptor of microglial cells: a novel immunomodulatory receptor. Prog. Brain Res. 120, 355–368.

Donnelly-Roberts, D.L., Namovic, M.T., Faltynek, C.R., Jarvis, M.F., 2004. Mitogen-activated protein kinase and caspase signaling pathways are required for P2X7 receptor (P2X7R)-induced pore formation in human THP-1 cells. J. Pharmacol. Exp. Ther. 308, 1053–1061.

Duan, S., Anderson, C.M., Keung, E.C., Chen, Y., Chen, Y., Swanson, R.A., 2003. P2X7 receptor-mediated release of excitatory amino acids from astrocytes. J. Neurosci. 23, 1320–1328.

Ferrari, D., Pizzirani, C., Adinolfi, E., Lemoli, R.M., Curti, A., Idzko, M., Panther, E., Di Virgilio, F., 2006. The P2X7 receptor: a key player in IL-1 processing and release. J. Immunol. 176, 3877–3883.

Fields, R.D., Stevens, B., 2000. ATP: an extracellular signaling molecule between neurons and glia. Trends Neurosci. 23, 625–633.

Harris, J.A., 1998. Using c-fos as a neural marker of pain. Brain Res. Bull. 45, 1–8.

Haydon, P.G., 2001. GLIA: listening and talking to the synapse. Nat. Rev. Neurosci. 2, 185–193.

Hide, I., Tanaka, M., Inoue, A., Nakajima, K., Kohsaka, S., Inoue, K., Nakata, Y., 2000. Extracellular ATP triggers tumor necrosis factor-alpha release from rat microglia. J. Neurochem. 75, 965–972.

Honore, P., Donnelly-Roberts, D., Namovic, M.T., Hsieh, G., Zhu, C.Z., Mikusa, J.P., Hernandez, G., Zhong, C.M., Gauvin, D.M., Chandran, P., Harris, R., Medrano, A.P., Carroll, W., Marsh, K., Sullivan, J.P., Faltynek, C.R., Jarvis, M.F., 2006. A-740003 [N-(1-{[(cyanoimino)(5-quinolinylamino)methyl]amino}-2,2-dimethylpropyl)-2-(3,4-dimethoxyphenyl)acetamide], a novel and selective P2X (7) receptor antagonist, dose-dependently reduces neuropathic pain in the rat. J. Pharmacol. Exp. Ther. 319, 1376–1385.

Hughes, J.P., Hatcher, J.P., Chessell, I.P., 2007. The role of P2X (7) in pain and inflammation. Purinergic Signal. 3, 163–169.

Hunter, J.C., Woodburn, V.L., Durieux, C., Pettersson, E.K., Poat, J.A., Hughes, J., 1995. C-fos antisense oligodeoxynucleotide increases formalin-induced nociception and regulates preprodynorphin expression. Neuroscience 65, 485–492.

Ikeda, H., Tsuda, M., Inoue, K., Murase, K., 2007. Long-term potentiation of neuronal excitation by neuron–glia interactions in the rat spinal dorsal horn. Eur. J. Neurosci. 25, 1297–1306.

Ji, R.R., Kohno, T., Moore, K.A., Woolf, C.J., 2003. Central sensitization and LTP: do pain and memory share similar mechanisms? Trends Neurosci. 26, 696–705.

Ji, R.R., Suter, M.R., 2007. P38 MAPK, microglial signaling, and neuropathic pain. Mol. Pain 3, 33.

Ji, R.R., Zhang, X., Wiesenfeld-Hallin, Z., Hokfelt, T., 1994. Expression of neuropeptide Y and neuropeptide Y (Y1) receptor mRNA in rat spinal cord and dorsal root ganglia following peripheral tissue inflammation. J. Neurosci. 14, 6423–6434.

Kawasaki, Y., Zhang, L., Cheng, J.K., Ji, R.R., 2008. Cytokine mechanisms of central sensitization: distinct and overlapping role of interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha in regulating synaptic and neuronal activity in the superficial spinal cord. J. Neurosci. 28, 5189–5194.

Koltzenburg, M., Wall, P.D., McMahon, S.B., 1999. Does the right side know what the left is doing? Trends Neurosci. 22, 122–127.

Kumar, S., Boehm, J., Lee, J.C., 2003. P38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. Nat. Rev. Drug Discov. 2, 717–726.

Labrousse, V.F., Costes, L., Aubert, A., Darnaudery, M., Ferreira, G., Amedee, T., Laye, S., 2009. Impaired interleukin-1beta and c-Fos expression in the hippocampus is associated with a spatial memory deficit in P2X (7) receptor-deficient mice. PLoS One 4, e6006.

Lai, A.Y., Swayze, R.D., El-Husseini, A., Song, C., 2006. Interleukin-1 beta modulates AMPA receptor expression and phosphorylation in hippocampal neurons. J. Neuroimmunol. 175, 97–106.

Lee, H.K., Takamiya, K., Han, J.S., Man, H., Kim, C.H., Rumbaugh, G., Yu, S., Ding, L., He, C., Petralia, R.S., Wenthold, R.J., Gallagher, M., Huganir, R.L., 2003. Phosphorylation of the AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory. Cell 112, 631–643.

Lee, M., Lee, S.J., Choi, H.J., Jung, Y.W., Frokiaer, J., Nielsen, S., Kwon, T.H., 2008. Regulation of AQP4 protein expression in rat brain astrocytes: role of P2X7 receptor activation. Brain Res. 1195, 1–11.

Lisman, J., Raghavachari, S., 2006. A unified model of the presynaptic and postsynaptic changes during LTP at CA1 synapses. Sci. STKE 2006, re11.

Liu, X.G., Sandkuhler, J., 1995. Long-term potentiation of C-fiber-evoked potentials in the rat spinal dorsal horn is prevented by spinal N-methyl-D-aspartic acid receptor blockage. Neurosci. Lett. 191, 43–46.

Liu, X.G., Sandkuhler, J., 1997. Characterization of long-term potentiation of C-fiber-evoked potentials in spinal dorsal horn of adult rat: essential role of NK1 and NK2 receptors. J. Neurophysiol. 78, 1973–1982.

Liu, X.G., Sandkuhler, J., 1998. Activation of spinal N-methyl-D-aspartate or neurokinin receptors induces long-term potentiation of spinal C-fibre-evoked potentials. Neuroscience 86, 1209–1216.

Lozier, A.P., Kendig, J.J., 1995. Long-term potentiation in an isolated peripheral nerve-spinal cord preparation. J. Neurophysiol. 74, 1001–1009.

Ma, J.Y., Zhao, Z.Q., 2001. The effects of Zn2+ on long-term potentiation of C fiber-evoked potentials in the rat spinal dorsal horn. Brain Res. Bull. 56, 575–579.

Ma, J.Y., Zhao, Z.Q., 2002. The involvement of glia in long-term plasticity in the spinal dorsal horn of the rat. Neuroreport 13, 1781–1784.

McGaraughty, S., Chu, K.L., Namovic, M.T., Donnelly-Roberts, D.L., Harris, R.R., Zhang, X.F., Shieh, C.C., Wismer, C.T., Zhu, C.Z., Gauvin, D.M., Fabiyi, A.C., Honore, P., Gregg, R.J., Kort, M.E., Nelson, D.W., Carroll, W.A., Marsh, K., Faltynek, C.R., Jarvis, M.F., 2007. P2X7-related modulation of pathological nociception in rats. Neuroscience 146, 1817–1828.

Melani, A., Amadio, S., Gianfriddo, M., Vannucchi, M.G., Volonte, C., Bernardi, G., Pedata, F., Sancesario, G., 2006. P2X (7) receptor modulation on microglial cells and reduction of brain infarct caused by middle cerebral artery occlusion in rat. J. Cereb. Blood Flow Metab. 26, 974–982.

Milligan, E.D., Twining, C., Chacur, M., Biedenkapp, J., O'Connor, K., Poole, S., Tracey, K., Martin, D., Maier, S.F., Watkins, L.R., 2003. Spinal glia and proinflammatory cytokines mediate mirror-image neuropathic pain in rats. J. Neurosci. 23, 1026–1040.

Monif, M., Reid, C.A., Powell, K.L., Smart, M.L., Williams, D.A., 2009. The P2X7 receptor drives microglial activation and proliferation: a trophic role for P2X7R pore. J. Neurosci. 29, 3781–3791.

Morioka, N., Abdin, M.J., Kitayama, T., Morita, K., Nakata, Y., Dohi, T., 2008. P2X (7) receptor stimulation in primary cultures of rat spinal microglia induces down-regulation of the activity for glutamate transport. Glia 56, 528–538.

Nakatsuka, T., Gu, J.G., 2001. ATP P2X receptor-mediated enhancement of glutamate release and evoked EPSCs in dorsal horn neurons of the rat spinal cord. J. Neurosci. 21, 6522–6531.

Pedersen, L.M., Jacobsen, L.M., Mollerup, S., Gjerstad, J., 2009. Spinal cord long-term potentiation (LTP) is associated with increased dorsal horn gene expression of IL-1beta, GDNF and iNOS. Eur. J. Pain 14, 255–260.

Presley, R.W., Menetrey, D., Levine, J.D., Basbaum, A.I., 1990. Systemic morphine suppresses noxious stimulus-evoked Fos protein-like immunoreactivity in the rat spinal cord. J. Neurosci. 10, 323–335.

Queiroz, G., Gebicke-Haerter, P.J., Schobert, A., Starke, K., von Kugelgen, I., 1997. Release of ATP from cultured rat astrocytes elicited by glutamate receptor activation. Neuroscience 78, 1203–1208.

Rygh, L.J., Svendsen, F., Hole, K., Tjolsen, A., 1999. Natural noxious stimulation can induce long-term increase of spinal nociceptive responses. Pain 82, 305–310.

Samad, T.A., Moore, K.A., Sapirstein, A., Billet, S., Allchorne, A., Poole, S., Bonventre, J.V., Woolf, C.J., 2001. Interleukin-1beta-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. Nature 410, 471–475.

Sanderson, D.J., Good, M.A., Seeburg, P.H., Sprengel, R., Rawlins, J.N., Bannerman, D.M., 2008. The role of the GluR-A (GluR1) AMPA receptor subunit in learning and memory. Prog. Brain Res. 169, 159–178.

Sandkuhler, J., 2007. Understanding LTP in pain pathways. Mol. Pain. 3, 9.

Sandkuhler, J., Liu, X.G., 1998. Induction of long-term potentiation at spinal synapses by noxious stimulation or nerve injury. Eur. J. Neurosci. 10, 2476–2480.

Sanes, J.R., Lichtman, J.W., 1999. Can molecules explain long-term potentiation? Nat. Neurosci. 2, 597–604.

Sawynok, J., Downie, J.W., Reid, A.R., Cahill, C.M., White, T.D., 1993. ATP release from dorsal spinal cord synaptosomes: characterization and neuronal origin. Brain Res. 610, 32–38.

Schneider, H., Pitossi, F., Balschun, D., Wagner, A., del Rey, A., Besedovsky, H.O., 1998. A neuromodulatory role of interleukin-1beta in the hippocampus. Proc. Natl. Acad. Sci. USA 95, 7778–7783.

Sim, J.A., Young, M.T., Sung, H.Y., North, R.A., Surprenant, A., 2004. Reanalysis of P2X7 receptor expression in rodent brain. J. Neurosci. 24, 6307–6314.

Simi, A., Lerouet, D., Pinteaux, E., Brough, D., 2007. Mechanisms of regulation for interleukin-1beta in neurodegenerative disease. Neuropharmacology 52, 1563–1569.

Solle, M., Labasi, J., Perregaux, D.G., Stam, E., Petrushova, N., Koller, B.H., Griffiths, R.J., Gabel, C.A., 2001. Altered cytokine production in mice lacking P2X (7) receptors. J. Biol. Chem. 276, 125–132.

Sperlagh, B., Illes, P., 2007. Purinergic modulation of microglial cell activation. Purinergic Signal. 3, 117–127.

Sperlagh, B., Papp, L., Vizi, E.S., 2007. P2X7 receptor mediated phosphorylation of p38MAP kinase in the hippocampus. J. Neurochem. 102, 289.

Sperlagh, B., Vizi, E.S., Wirkner, K., Illes, P., 2006. P2X (7) receptors in the nervous system. Prog. Neurobiol. 78, 327–346.

Suadicani, S.O., Brosnan, C.F., Scemes, E., 2006. P2X7 receptors mediate ATP release and amplification of astrocytic intercellular Ca2+ signaling. J. Neurosci. 26, 1378–1385.

Sweitzer, S., Martin, D., DeLeo, J.A., 2001. Intrathecal interleukin-1 receptor antagonist in combination with soluble tumor necrosis factor receptor exhibits an anti-allodynic action in a rat model of neuropathic pain. Neuroscience 103, 529–539.

Tan, P.H., Yang, L.C., Shih, H.C., Lan, K.C., Cheng, J.T., 2005. Gene knockdown with intrathecal siRNA of NMDA receptor NR2B subunit reduces formalin-induced nociception in the rat. Gene Ther. 12, 59–66.

Terman, G.W., Eastman, C.L., Chavkin, C., 2001. Mu opiates inhibit long-term potentiation induction in the spinal cord slice. J. Neurophysiol. 85, 485–494.

Toyoda, H., Zhao, M.G., Xu, H., Wu, L.J., Ren, M., Zhuo, M., 2007. Requirement of extracellular signal-regulated kinase/mitogen-activated protein kinase for long-term potentiation in adult mouse anterior cingulate cortex. Mol. Pain 3, 36.

Tsuda, M., Mizokoshi, A., Shigemoto-Mogami, Y., Koizumi, S., Inoue, K., 2004. Activation of p38 mitogen-activated protein kinase in spinal hyperactive microglia contributes to pain hypersensitivity following peripheral nerve injury. Glia 45, 89–95.

Watkins, L.R., Hutchinson, M.R., Ledeboer, A., Wieseler-Frank, J., Milligan, E.D., Maier, S.F., 2007. Norman Cousins Lecture. Glia as the "bad guys": implications for improving clinical pain control and the clinical utility of opioids. Brain Behav. Immun. 21, 131–146.

Watkins, L.R., Hutchinson, M.R., Rice, K.C., Maier, S.F., 2009. The "toll" of opioid-induced glial activation: improving the clinical efficacy of opioids by targeting glia. Trends Pharmacol. Sci. 30, 581–591.

Watkins, L.R., Maier, S.F., 2002. Beyond neurons: evidence that immune and glial cells contribute to pathological pain states. Physiol. Rev. 82, 981–1011.

Watkins, L.R., Milligan, E.D., Maier, S.F., 2001. Glial activation: a driving force for pathological pain. Trends Neurosci. 24, 450–455.

Wieraszko, A., Goldsmith, G., Seyfried, T.N., 1989. Stimulation-dependent release of adenosine triphosphate from hippocampal slices. Brain Res. 485, 244–250.

Xu, J.J., Walla, B.C., Diaz, M.F., Fuller, G.N., Gutstein, H.B., 2006. Intermittent lumbar puncture in rats: a novel method for the experimental study of opioid tolerance. Anesth. Analg. 103, 714–720.

Ying, B., Lu, N., Zhang, Y.Q., Zhao, Z.Q., 2006. Involvement of spinal glia in tetanically sciatic stimulation-induced bilateral mechanical allodynia in rats. Biochem. Biophys. Res. Commun. 340, 1264–1272.

Yu, Y., Ugawa, S., Ueda, T., Ishida, Y., Inoue, K., Nyunt, A.K., Umemura, A., Mase, M., Yamada, A., Shimada, S., 2008. Cellular localization of P2X7 receptor mRNA in the rat brain. Brain Res. 1194, 45–55.

Zhang, X.C., Zhang, Y.Q., Zhao, Z.Q., 2005. Involvement of nitric oxide in long-term potentiation of spinal nociceptive responses in rats. Neuroreport 16, 1197–1201.

Zhong, Y., Zhou, L.J., Ren, W.J., Xin, W.J., Li, Y.Y., Zhang, T., Liu, X.G., 2010. The direction of synaptic plasticity mediated by C-fibers in spinal dorsal horn is decided by Src-family kinases in microglia: The role of tumor necrosis factor-alpha. Brain Behav. Immun. doi:10.1016/j.bbi.2010.01.007.