Over-expression of P2X7 receptors in spinal glial cells contributes to the development of chronic postsurgical pain induced by skin/muscle incision and retraction (SMIR) in rats

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Many patients suffer from chronic postsurgical pain (CPSP) following surgery, and the underlying mechanisms are poorly understood. In the present work, with use of the skin/muscle incision and retraction (SMIR) model, the role of P2X7 receptors (P2X7Rs) in spinal glial cells in the development of CPSP was evaluated. Consistent with previous reports, we found that SMIR decreased the ipsilateral 50% paw withdrawal threshold (PWT), lasting for at least 2 weeks. No injury was done to L3 dorsal root ganglia (DRG) neurons and no axonal or Schwann cell damage at the retraction site in the saphenous nerve was observed 7 days after SMIR. The results of immunofluorescence showed that both microglia and astrocytes were activated in the spinal dorsal horn following SMIR. In addition, both P2X7Rs and tumor necrosis factor-alpha (TNF-α) were up-regulated following SMIR. Double immunofluorescence staining revealed that the up-regulated P2X7R immunoreactivity was mainly located in microglia, and to a lesser extent in astrocytes, but not in neurons. Intrathecal delivery of specific P2X7R antagonist BBG (10 μM in 10 μl volume) or A438079 (10 μM in 10 μl volume), started 30 min before the surgery and once daily thereafter for 7 days, prevented the mechanical allodynia. Intrathecal injection of BBG inhibited the activation of microglia and astrocytes, and the up-regulation of TNF-α induced by SMIR. These data suggest that P2X7Rs in the spinal dorsal horn might mediate the development of CPSP via activation of glial cells and up-regulation of TNF-α.

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subtypes (P2X1–P2X7) (North, 2002). It has been shown that peripheral nerve injury activates P2X7Rs in the spinal dorsal horn and that P2X7R antagonist A-740033 dose-dependently reduces neuropathic pain induced by spinal nerve ligation (Honore et al., 2006). P2X7R gene knockout mice also show reduced mechanical allodynia after nerve injury as compared with matched wild-type mice (Chessell et al., 2005). These above studies suggest that P2X7Rs are critical for neuropathic pain induced by nerve injury. However, up to now, almost all studies demonstrating the roles and mechanisms of P2X7Rs in neuropathic pain are based on the animal models with nerve injury (McGaraughty et al., 2007; Honore et al., 2006) and the role of P2X7Rs in SMIR-induced chronic postsurgical pain has been reported to be without nerve injury remains unknown.

To evaluate the role of P2X7Rs in SMIR-induced chronic postsurgical pain, in the present study we first evaluated whether SMIR induces injury of dorsal root ganglia neurons and the saphenous nerve, then we investigated whether SMIR activates microglia and astrocytes, and up-regulates P2X7Rs and TNF-α in the L3/4 spinal dorsal horn. Whether P2X7R antagonist BBG could block the development of postsurgical pain induced by SMIR and whether P2X7Rs contribute to the activation of glial cells and the up-regulation of TNF-α following SMIR were also investigated.

Materials and methods

Animals and reagents

Male Sprague–Dawley (SD) rats weighing 150–250 g were housed under a 12-h light/dark cycle with free access to food and water at a constant room temperature of 25 °C. Experimental procedures were approved by the local animal care committee and were carried out in accordance with the guidelines of the National Institutes of Health on animal care and the ethical guidelines for investigation of experimental pain in conscious animals (Zimmerman, 1983).

P2X7R antagonist Brilliant Blue C (BBG) was purchased from Sigma (USA), dissolved in sterile normal saline, prepared as stock solutions and diluted to the required concentration with artificial cerebrospinal fluid (ACSF). P2X7 antagonist A438079 hydrochloride (Tocris, MO, USA) was dissolved in dimethyl sulfoxide (DMSO), stored as a stock solution of 0.1 M at −20 °C, and diluted to the required concentration before use.

Drug administration

All drugs were prepared just before experiments. 4% chloral hydrate was used for anesthesia, and aseptic technique was adopted to minimize infection to the greatest extent during the operation. Rats were implanted with a PE-10 intrathecal catheter (BD, USA) in the lumbar enlargement (close to the L3–4 segments) for intrathecal drug administration. The catheter placement was verified by observing transient hind-paw paralysis induced by intrathecal injection of lidocaine (2%, 7 μl) daily thereafter for 7 days. Animals that failed to show any paralysis were excluded from the experiments. The selective P2X7R antagonist BBG (10 μM, 10 μl), A438079 (10 μM, 10 μl), saline (vehicle control for BBG), and DMSO (vehicle control for A438079) were administered intrathecally 30 min before SMIR surgery and once daily thereafter for 7 days.

Skin/muscle incision and retraction (SMIR) surgery

The SMIR surgery was carried out according to the previous described procedures (Flatters, 2008). Briefly, under anesthesia with chloral hydrate (0.4 g/kg, i.p.), a 1.5–2 cm incision was made in the skin of the medial thigh approximately 4 mm medial to the saphenous vein, to expose the muscle of the thigh. An incision (7–10 mm long) was then made in the superficial muscle layer of the thigh, to allow the insertion of a micro dissecting retractor (Biomedical Research Instruments Inc., USA). The skin and superficial muscle of the thigh were retracted by 2 cm for 1 h. Animals were covered with an absorbent bench underpad to minimize heat loss and to prevent dehydration of the surgical site. Rats with skin incision but without retraction served as sham-SMIR controls. Following recovery from anesthesia, all animals could move around normally and freely to reach food and water.

Behavioral tests

Animals were habituated and basal pain sensitivity was tested before drug administration or surgery. Mechanical sensitivity was assessed with the up–down method described previously (Chaplan et al., 1994), using a set of von Frey hairs with logarithmically incremental stiffness from 0.41 g to 15.41 g (0.41, 0.70, 1.20, 2.04, 3.63, 5.50, 8.51, 15.14 g). The 2.04 g stimulus, in the middle of the series, was applied first. In the event of paw withdrawal absence, the next stronger stimulus was chosen. On the contrary, a weaker stimulus was applied. Each stimulus consisted of a 6- to 8-s application of the von Frey hair to the sciatic innervation area of the hindpaws with a 5-min interval between stimuli. The quick withdrawal or licking of the paw in response to the stimulus was considered as a positive response. Three persons performed the behavioral tests and only one of them knew the design of the study.

Electron microscopy

Saphenous nerve segments of 4 mm length at the SMIR surgery site were fixed with 2.5% glutaraldehyde in the 0.1 M phosphate buffer (pH 7.4) overnight at 4 °C. The fixed nerve was dehydrated in a graded alcohol series, and embedded in Epon812. Ultrathin sections (80 nm thick) were cut with an ultramicrotome (Leica, EMUCS, Germany), and then stained with uranyl acetate and lead citrate for 30 and 5 min, respectively. Myelin thickness and axon diameter were measured in photographs taken under an electron microscope (Tecnai 12 Spirit Twin; USA).

Western blot

Dorsal quadrants of the L3/4 spinal cord were dissected out from each animal and the spinal meninges were carefully removed. The ipsilateral side were separated and put into liquid nitrogen immediately, followed by homogenization in 15 mmol/l Tris buffer, pH 7.6 (250 mmol/l sucrose, 1 mM MgCl2, 1 mM DTT, 2.5 mM EDTA, 1 mM EGTA, 50 mM NaF, 10 μg/ml leupeptin, 1.25 μg/ml pepstatin, 2.5 μg/ml Aprotin, 2 mM sodium pyrophosphate, 0.1 mM NaVO4, 0.5 mM PMSF, and protease inhibitor cocktail). The samples were centrifuged at 14,500 ×g for 20 min at 4 °C to isolate supernatant containing protein samples. Protein samples were separated by gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane. The blots were probed with β-actin antibody (1:500; Boster, China), and detected as described above. The band intensities on the same membrane were determined by the ratio of the protein signal to the β-actin signal. These ratios were normalized to the control values.

Immunohistochemistry

Rats were perfused through the ascending aorta with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2–7.4, 4 °C.
The L3/4 spinal cord segments were removed and post-fixed in the same fixative for 3 h and then replaced with 30% of sucrose overnight. Transverse free-floating spinal sections (25 µm) were cut in a cryostat (Leica CM1900) and processed for immunostaining with immunofluorescence according to the method described previously (Xu et al., 2006). All of the cryostat sections were blocked with 3% donkey serum in 0.3% Triton X-100 for 1 h at room temperature and incubated overnight at 4 °C with rabbit anti-P2X7R (1:1000; Millipore, USA), rabbit anti-ATF-3 polyclonal antibody (1:200; Santa Cruz), mouse monoclonal glial fibrillary acidic protein (GFAP) (astrocyte marker, 1:300; Chemicon, Temecula, CA), and mouse Iba1 antibody (microglia marker, 1:800; abcam, USA). The sections were then incubated for 1 h at room temperature with FITC-conjugated secondary antibody (1:400; Jackson ImmunoResearch, USA). For double immunofluorescence staining, the spinal sections were incubated with a mixture of rabbit P2X7R antibody (1:200; Millipore, USA) and mouse neuronal specific nuclear protein (NeuN) (neuronal marker, 1:200; Chemicon, Temecula, CA), mouse GFAP, or mouse Iba1 antibody overnight at 4 °C. All of the above sections were treated by a mixture of FITC and Cy3-conjugated secondary antibody for 1 h at room temperature. To verify the specificity of P2X7R antibody used in the present study, negative controls were performed with only secondary antibody (FITC or Cy3-conjugated secondary antibody) but no primary antibody was added. The stained sections were examined with an Olympus IX71 (Olympus Optical, Tokyo, Japan) fluorescence microscope and images were captured with a CCD spot camera.

Quantification and statistics

For quantification of the immunofluorescence staining, the area of Iba1-immunoreactivity (IR), GFAP-IR or P2X7R-IR per section was measured using a Leica Qwin V3 digital image processing system (Germany). A density threshold was set above background level firstly to identify positively stained structure. The area occupied by these structures was measured as positive area. In each rat, every fifth section was picked from a series of consecutive sections; and four to six sections at each time point were selected randomly. An average percentage of area of Iba1-IR, GFAP-IR or P2X7R-IR relative to the total area of the sections was obtained for each animal across the different tissue sections, and was normalized to the control values. Six rats were included for each group for quantification of immunohistochemistry results.

All analysis was done in a blinded fashion with the same criterion. All data were expressed as means ± SEM. The data of behavioral tests were analyzed using repeated measures two-way ANOVA with time and treatment as main effects. For the data of immunofluorescence, differences in changes of values over time were tested using one-way ANOVA followed by individual post hoc comparisons (Tukey post hoc tests). The relative densities of Western blots between different groups were compared using ANOVA with the least significant difference test (LSD-t). Statistical tests were taken with SPSS 10.0 (SPSS Inc., USA). A difference was accepted as significant if the P value is less than 0.05.

Results

SMIR induced mechanical allodynia in the ipsilateral side without nerve injury

Consistent with the previous study (Flatters, 2008), we found that SMIR induced a significant decrease in 50% paw withdrawal threshold (50% PWT) in the ipsilateral side, starting on day 1, reaching a peak on day 7 and lasting until day 22 (P < 0.05) after surgery (Fig. 1). No significant changes in 50% PWT in the contralateral side were detected.

To determine whether SMIR could damage the DRG neurons and validate this model, expression of activating transcription factor (ATF-3), which is described as an adaptive response gene whose activity is rapidly up-regulated in response to injury, in L3 DRG neurons was examined. The results showed that 7 days after SMIR surgery, at the maximum of evoked pain syndrome, majority of L3 DRG cells were ATF3-negative (Fig. 2A), whereas 7 days following complete saphenous nerve transaction (SANT), a significant increase in ATF3 immunoreactivity, which was located in the nuclei was observed (Fig. 2B). These data indicated that very little or no damage was done to the DRG neurons following SMIR, which are in good agreement with previous results (Flatters, 2008), indicating that the SMIR model was copied.

To determine whether SMIR could damage the saphenous nerve, Flatters has observed the nerve with light microscopy and found that SMIR does not cause demyelination or edema in the saphenous nerve at the surgical site, proximally or distally to it. In the present study, we further examined the nerve at the retraction site 7 days after SMIR or sham operated under an electron microscope (EM). We found that there was no difference in the myelin thickness and axon diameter between the SMIR group (Figs. 2C, E, and F) and sham group (Figs. 2D, E and F). Moreover, morphology of the axons of myelinated A-fibers (indicated by the black arrow) and unmyelinated C-fibers (indicated by the white arrow) was generally normal 7 days after SMIR. These results suggest that SMIR may induce neither demyelination nor axonal degeneration in the saphenous nerve. These data, together with the results of ATF3 staining of L3 DRG, suggest that the mechanical allodynia induced by SMIR might not be a result of a direct injury of the sensory systems.

Microglial cells and astrocytes were activated in L3/4 spinal dorsal horn following SMIR

As shown in Fig. 3, compared with the sham group, microglial cells and astrocytes were activated after SMIR, manifesting as over-expression of Iba1 or GFAP. In the sections through the L3/4 spinal dorsal horn from the sham rats (7 days after the operation, Figs. 3A and E), only a few specific signals for Iba1 or GFAP could be detected. However, Iba1-IR and GFAP-IR increased significantly 3 days after SMIR (Figs. 3J, P < 0.01), increased further on day 7 (Figs. 3B, F, P < 0.001), remained at a significant high level on day 12 (Figs. 3I, P < 0.01), and returned to baseline on day 32 (Figs. 3C, G). No significant changes of Iba1 and GFAP expression were observed in the contralateral side of the spinal dorsal horn (Figs. 3D, H, I and J).
P2X7Rs were up-regulated in L3/4 spinal dorsal horn following SMIR

Western blot showed that compared with the sham group, P2X7R expression in the ipsilateral L3/4 spinal dorsal horn increased on day 1 ($P < 0.05$), peaked on day 7 ($P < 0.01$), sustained to day 22 ($P < 0.001$), and returned to baseline on day 32 after SMIR (Fig. 4A). Similar changes of P2X7Rs were also observed in the spinal dorsal horn by immunohistochemistry (Figs. 4C–F). No significant changes of P2X7R expression were observed in the contralateral side of the spinal dorsal horn (Fig. 4F). No signals were detected in the negative control groups, in which only the secondary antibody but not the primary antibody was added to the spinal sections (Fig. 4B). Double immunofluorescence staining showed that P2X7Rs were mainly co-localized with Iba1 (Fig. 5A), and to a lesser extent with GFAP (Fig. 5B), but not with NeuN (Fig. 5C) (a marker for neuron).

SMIR up-regulates TNF-α in the L3/4 spinal dorsal horn

To determine whether TNF-α contributes to SMIR-induced CPSP, the expression of TNF-α, a leading pro-inflammatory cytokine, was measured in the tissue of the L3/4 spinal dorsal horn. Western blot analysis revealed that the expression of TNF-α in the ipsilateral spinal dorsal horn was markedly increased after SMIR (Fig. 6). A significant increase of TNF-α was detected on day 1 ($P < 0.001$), peaked on day 7 ($P < 0.001$), remained at a significant high level for at least 22 days, and then returned to baseline on day 32 ($P > 0.05$) after SMIR surgery.

P2X7R antagonist BBG inhibits mechanical allodynia, the activation of glial cells and the up-regulation of TNF-α in spinal dorsal horn induced by SMIR

Having demonstrated that SMIR activated microglia and astrocytes and up-regulated P2X7Rs and TNF-α, we next evaluated the role of P2X7Rs in SMIR-induced mechanical allodynia and whether P2X7Rs contributed to the activation of glial cells and TNF-α up-regulation. To do so, BBG, a P2X7R antagonist, was injected intrathecally, started 30 min before surgery and once daily thereafter for 7 days. The results showed that 50% PWT in BBG-treated rats was significantly higher than those treated with saline, and was not different from those in sham rats (Fig. 7A), indicating that mechanical allodynia was completely prevented by antagonism of P2X7Rs. Intrathecal injection of BBG had no effects on the PWTs of sham operated rats (Fig. 7A). To test whether the effect of BBG is specific, another P2X7R antagonist, A438079, was tested with the same experimental procedure. We found that A438079, injected intrathecally daily for 7 days, was also able to prevent the mechanical allodynia induced by SMIR (Fig. 7B).
Immunofluorescence showed that the expression of Iba1 and GFAP in the ipsilateral spinal dorsal horn was significantly lower in the BBG-treated group (Figs. 8C, F and G) than that in saline-treated rats at 7 days after SMIR (Figs. 8B, E and G, \( P < 0.001 \) compared with the saline-treated group). Western blot showed that the expression of TNF-\( \alpha \) in the ipsilateral spinal dorsal horn was also significantly lower in the BBG-treated group than that in the saline-treated rats at 7 days after SMIR (Fig. 8H, \( P < 0.01 \) compared with the saline-treated group).

**Fig. 3.** SMIR induced the activation of microglia and astrocyte in the ipsilateral spinal dorsal horn. A–C: Representative immunohistochemistry staining experiments show the time course of Iba1 expression in the ipsilateral L3/4 spinal dorsal horn 1, 7, and 22 days after SMIR and 7 days after sham operation. D: Representative immunohistochemistry staining experiments show the expression of Iba1 in bilateral spinal dorsal horn 7 days after SMIR. E–G: Representative immunohistochemistry staining experiments show the time course of GFAP expression in the ipsilateral L3/4 spinal dorsal horn 1, 7, and 22 days after SMIR and 7 days after sham operation. H: Representative immunohistochemistry staining experiments show the expression of GFAP in bilateral spinal dorsal horn 7 days after SMIR. I–J: The histogram shows the summary data of Iba1 or GFAP positive area at different time points following SMIR (\( n = 6 \) group), \( *P < 0.05, **P < 0.01, ***P < 0.001 \) compared with sham group. Scale bars (E–G, I–J) = 100 \( \mu \)m. Scale bars (D and H) = 400 \( \mu \)m.

**Fig. 4.** SMIR induced the up-regulation of P2X7Rs in the ipsilateral spinal dorsal horn. A: The bands show the expression of the P2X7Rs and \( \beta \)-actin in the ipsilateral L3/4 spinal dorsal horn after SMIR and the histogram shows the quantification of P2X7Rs normalized by \( \beta \)-actin (\( n = 6 \)). \( *P < 0.05, **P < 0.01, ***P < 0.001 \) compared with sham group. B: Representative Western blot experiments show the results of negative controls (\( n = 6 \)). C–E: Representative immunohistochemistry experiments show the expression of P2X7Rs in the ipsilateral L3/4 spinal dorsal horn 7 (D), and 22 days (E) after SMIR and 7 days after sham operation (C). F: Representative immunohistochemistry experiments show the expression of P2X7Rs in bilateral spinal dorsal horn 7 days after SMIR (\( n = 6 \)). Scale bars (B–E) = 100 \( \mu \)m. Scale bars (F) = 400 \( \mu \)m.
Discussion

Prolonged tissue retraction that is necessary for various common surgeries, such as thoracotomy and inguinal hernia repair, often causes persistent postoperative pain. Consistent with previous work (Flatters, 2008), in the present study, we found that SMIR induced mechanical allodynia in the ipsilateral side, which was not associated with injury of the peripheral nerve and L3 DRG neurons. We further investigated whether P2X7Rs in the L3/4 spinal dorsal horn are involved in the development of SMIR-induced chronic postsurgical pain and found that SMIR activated spinal microglia and astrocytes exhibited P2X7R and TNF-α up-regulation in the L3/4 spinal dorsal horn of SMIR rats. In addition, double immunofluorescence staining revealed that P2X7R immunoreactivity was mainly located in microglia, and to a lesser extent in astrocytes, but not in neurons. Importantly, we found that intrathecal injection of P2X7R antagonist attenuated mechanical hypersensitivity, the activation of glial cells and the up-regulation of TNF-α induced by SMIR. These results suggest that P2X7R activation in the spinal dorsal horn might contribute to the development of CPSP via glia activation and TNF-α up-regulation.

The genesis of pathological pain without nerve injury

It is well-known that nerve injury which produced by trauma, chemicals, diabetes and tumor induce chronic neuropathic pain (Zimmermann, 2001). However, there is also abundant evidence showing that some physical and chemical changes in the peripheral or central nervous system directly induce behavioral signs of neuropathic pain in the absence of nerve injury. For example, peri-sciatic application of TNF-α (Wei et al., 2007) or IL-1β (Wei et al., 2012) dose-dependently induces mechanical allodynia in rats without nerve injury. Direct activation of spinal glial cells by a single systemic injection of lipopolysaccharides also induces mechanical hyperalgesia in rats (Guo and Schluesener, 2006). Zymosan (yeast cell walls) acutely (within 3 h) injected around the sciatic nerve of rats produces mechanical allodynia (Chacur et al., 2001). The proinflammatory cytokine high mobility group-1 also dose-dependently creates unilateral and bilateral hindpaw mechanical allodynia (Wang et al., 1999). Consistent with the previous study (Flatters, 2008), we found that SMIR induced chronic postsurgical pain but did not damage the peripheral nerve and the DRG neurons, as revealed by electron microscopy and ATP3 staining. Therefore, mechanical allodynia induced by this surgery might not be a result of a direct injury of the saphenous nerve trunk and its afferents ascending to the spinal cord.

The role of P2X7Rs in the development of CPSP following SMIR

It has been well established that activation of P2X7Rs in the spinal dorsal horn contributes to the chronic pain produced by peripheral nerve injury. Genetic deletion of P2X7Rs abolishes chronic inflammatory and neuropathic pain completely, while normal nociceptive processing is preserved (Chesell et al., 2005). A specific P2X7R antagonist A-74003 dose-dependently reduces neuropathic pain in rats (Honore et al., 2006). P2X7Rs and downstream signaling pathways are also involved in LTP of C-fiber evoked field potentials in the spinal dorsal horn (Chu et al., 2010). In the present work, we found that SMIR without nerve injury up-regulated P2X7Rs in the L3/4 spinal dorsal horn and that the P2X7R antagonist BBG or A438079 dramatically blocked mechanical allodynia induced by SMIR, indicating that P2X7Rs are involved in the development of SMIR-induced CPSP.

The only known physiological activator of P2X7Rs is ATP (Ferrari et al., 2006). Under normal conditions, extracellular ATP is present in only low concentrations. Extracellular ATP concentrations increase significantly under inflammatory conditions in vivo (Lazarowski et al., 2000) and in response to tissue trauma (e.g., ischemia/hypoxia) (Nieber et al., 1999). Prolonged retraction of skeletal muscles during SMIR may render the vessels semi-permeable and the soft tissue anoxic, which may induce a large amount of ATP release from primary afferent terminals into the spinal dorsal horn to activate P2X7Rs. P2X7R activation causes a massive upset of cytoplasmic ion homeostasis, as P2X7Rs mediate the influx of Ca2+ and Na+ ions, and the efflux of K+ from cells (Khakh and North, 2006). P2X7R activation also results in activation of phospholipases A2 and D (PLA2, PLD), as well as phosphorylation of tyrosine (P-Tyr) and activation of mitogen-activated protein kinase (MAPK) pathway proteins (MEK, ERK 1/2); the latter can then influence the activity of transcription factors such as nuclear factor-κB (NF-κB) and activator

![Fig. 5. Cell types that express P2X7Rs 7 days after SMIR. Double immunofluorescence staining shows that P2X7Rs were mainly co-localized with Iba1 (C), and to a lesser extent with GFAP (B), but not with NeuN (A) (n = 6). Scale bar = 50 μm.](image)

![Fig. 6. SMIR induced the up-regulation of TNF-α in the ipsilateral spinal dorsal horn. The bands show the expression of TNF-α and β-actin in the ipsilateral L3/4 spinal dorsal horn at different time periods after SMIR or sham-operation. The histogram shows the quantification of TNF-α normalized by β-actin (n = 6). *P < 0.05, **P < 0.01, ***P < 0.001 compared with sham group.](image)
protein-1 (AP-1), which up-regulate the expression of proinflammatory genes, such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) (Skaper et al., 2010). As PLA2, PLD, tyrosine phosphorylation and the MAPK pathway have been well demonstrated to participate in the development of chronic pain (Ma et al., 2010; Wei et al., 2012; Xu et al., 2006; Zhuang et al., 2005), activation of P2X7Rs may induce CPSP via these signaling pathways.

A previous study has reported that p38 MAPK, which is suggested to be a marker of microglial activation (Ji and Suter, 2007), is activated in spinal microglia following SMIR (Huang et al., 2011). In the present

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**Fig. 7.** Effects of intrathecal injection of BBG or A438079 on SMIR surgery-induced mechanical allodynia. A: The time course of changes in 50% PWT in different groups, as indicated (n = 5/group). *P < 0.05, **P < 0.01, ***P < 0.001 versus sham group. #P < 0.05, ##P < 0.01, ###P < 0.001 versus saline or DMSO treated SMIR group.

**Fig. 8.** Effects of intrathecal injection of BBG on SMIR surgery-induced activation of microglia and astrocytes, and TNF-α up regulation in the ipsilateral spinal dorsal horn. A–C: Representative immunohistochemistry experiments show the expression of Iba1 in the ipsilateral L3/4 spinal dorsal horn in different groups 7 days after SMIR or sham operation. D–F: Representative immunohistochemistry experiments show the expression of GFAP in the ipsilateral L3/4 spinal dorsal horn in different groups 7 days after SMIR or sham operation. G: The histogram shows the summary data of Iba1 or GFAP positive area in different groups 7 days after SMIR or sham operation (n = 6/group). ***P < 0.001 versus sham group. ###P < 0.001 versus saline-treated SMIR group. H: The western blot bands show the expression of TNF-α and β-actin in the ipsilateral L3/4 spinal dorsal horn in different groups. The histogram shows the quantification of TNF-α normalized by β-actin (n = 5/group). *P < 0.05, **P < 0.01, ***P < 0.001 versus sham group. #P < 0.05, ##P < 0.01, ###P < 0.001 versus saline-treated SMIR group.
study, we found that P2X7Rs were also located mainly in microglia, to a lesser extent in astrocytes in the spinal dorsal horn after SMIR. This is also in line with previous studies that apart from microglia, P2X7Rs are expressed in astrocytes of the cerebral cortex (Oliveira et al., 2011) and of the spinal dorsal horn (Aoyama et al., 2011). P2X7Rs play a pivotal role in the cross-talk between microglia and neurons (Sperlagh et al., 2006). Thus, following SMIR, P2X7Rs in glial cells in the spinal dorsal horn may be activated to cooperate with neurons to induce chronic pain.

P2X7Rs in the spinal dorsal horn contribute to the activation of microglia and astrocytes, and the up-regulation of TNF-α following SMIR

Brief oxygen deprivation in the rat causes a marked decline of brain intracellular ATP levels with a concomitant efflux of ATP into the extracellular space (Volanté et al., 2003), which can act as a chemoattractant for microglia (Honda et al., 2001). The over-expression of P2X7Rs is sufficient to activate microglia in primary hippocampal cultures (Monif et al., 2009). Stimulation of P2X7Rs by ATP evoked the mRNA expression and release of proinflammatory cytokines IL-6, TNF-α but not in P2X7 (-/-) cells (Shieh et al., 2014). Adjuvant induced increases in IL-1β, IL-6, IL-10, and macrophage chemoattractant protein-1 were reduced in these P2X7 (-/-) knockout animals (Chessell et al., 2005). The above studies suggest that signaling via P2X7Rs may allow glial cells to sense and respond to ATP in the extracellular environment, and regulate cytokine release. In the present study, we found that P2X7Rs were up-regulated 1 day after SMIR, while microglia and astrocytes were activated 2 days later (3 days after SMIR). These results raise a possibility that P2X7R may contribute to the activation of glial cells after SMIR. To test this hypothesis, we observed whether antagonism of P2X7Rs affects the activation of glial cells and TNF-α release. The results showed that intrathecal injection of the P2X7 antagonist BBG for 7 days (started 30 min before the surgery and once daily thereafter for 7 days), prevents the activation of microglia and astrocytes, and the up-regulation of TNF-α in the L3/4 spinal dorsal horn following SMIR. Our previous study has reported that inhibition of TNF-α synthesis before L5 VRT surgery was able to block the up-regulation of IL-6 (Wei et al., 2013), indicating that if the up-regulation of TNF-α was prevented, IL-6 might not be up-regulated as well. Thus, P2X7Rs may contribute to the activation of glial cells and the release of cytokines including TNF-α following SMIR, which may make a critical contribution to pathologically enhanced pain processing in the spinal dorsal horn. Thus, antagonism of P2X7Rs might be a good strategy for prevention of CPSP produced by prolonged muscle retraction during common surgery.

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