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Research report

Spinal $P2X_7$ receptor mediates microglia activation-induced neuropathic pain in the sciatic nerve injury rat model

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ABSTRACT

$P2X_7$ receptor is an important member of ATP-sensitive ionotropic P2X receptors family, which includes seven receptor subtypes ($P2X_1$–$P2X_7$). Recent evidence indicates that $P2X_7R$ participates in the onset and persistence of neuropathic pain. In tetanic stimulation of the sciatic nerve model, $P2X_7R$ was involved in the activation of microglia, but whether this happens in other neuropathic pain models remains unclear. In this study we used immunohistochemistry and Western blot to explore the relationship of $P2X_7R$ expression with microglia activation, and with mechanical allodynia and thermal hypersensitivity in the chronic constriction of the sciatic nerve (CCI) rat model. The results show that following nerve ligature, mechanical allodynia and thermal hypersensitivity were developed within 3 days (d), peaked at 14 d and persisted for 21 d on the injured side. $P2X_7R$ levels in the ipsilateral L4–6 spinal cord were increased markedly after injury and the highest levels were observed on day 14, significant difference was observed at I–IV layers of the dorsal horn. The change in $P2X_7R$ levels in the spinal cord was consistent with the development of mechanical allodynia and thermal hypersensitivity. Intrathecal administration of the $P2X_7R$ antagonist Brilliant Blue G (BBG) reversed CCI-induced mechanical allodynia and thermal hypersensitivity. Double-labeled immunofluorescence showed that $P2X_7R$ expression were restricted to microglia, spinal microglia were activated after nerve injury, which was inhibited by BBG. These results indicated that spinal $P2X_7R$ mediate microglia activation, this process may play an important role in development of mechanical allodynia and thermal hypersensitivity in CCI model.

1. Introduction

Neuropathic pain is a common and severely disabling state, affecting millions of people worldwide [1]. It typically develops when peripheral nerves are damaged due to surgery, bone compression in cancer, diabetes or infection [2,3]. Following nerve injury, there are many molecular and cellular changes of the peripheral and central nervous systems, among which ATP and its purine receptors have been widely studied.

It is well-known that adenosine 5′-triphosphate (ATP) can serve as an important chemical gliotransmitter that mediates a broad range of physiological and pathological processes in the nervous system [4]. Accumulated evidence indicates that ATP and its purine receptors are involved in the regulation of neuropathic pain [5,6]. P2 purinoceptors were divided into two different categories: the ATP-gated ionotropic P2X family and the G protein-coupled (metabotropic) P2Y receptors [7]. These two types of P2 purinoceptors are widely distributed in the sensory nervous system and exhibit various effects both at neuronal and glial cells [8–10]. In the spinal cord, much emphasis has been focused on the potential role of P2X receptors in sensory processing in the dorsal horn, because the sensory neurons for pain, touch, and temperature are located in this area. However, the exact role of these receptors in the neuropathic pain remains unclear.

The $P2X_7R$ is a unique member of the P2X receptor family, because it is activated only by high concentrations of ATP (>100 μM) and its prolonged exposure to ATP has been shown to form a much larger pore than any other P2X channel [11]. $P2X_7R$ is primarily expressed in microglia and peripheral macrophages, a recent study has demonstrated that $P2X_7R$ is required for the activation and proliferation of microglia, suggesting that it regulates immune function and inflammatory responses [12]. Studies have shown that activation of the $P2X_7R$ can promote the release of some cytokines such as interleukin-1β (IL-1β), tumor necrosis

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factor-α (TNF-α) and some superoxide products, all of which play important roles in the generation or maintenance of pain. Chessell et al. found that hypersensitivity is completely absent to both mechanical and thermal stimuli in $P2X_7$ gene-ablated (−/−) mice, whilst normal nociceptive processing is preserved [13]. Development of new potent and selective $P2X_7R$ antagonists also indicated a role for $P2X_7Rs$ in the onset and persistence of certain types of chronic pain [14–16]. Although these studies have revealed a role for $P2X_7Rs$ in the development of neuropathic pain, where alteration of $P2X_7R$ pathway is involved in the pathology of neuropathic pain is unclear.

A recent study by Chu et al. showed that in the dorsal horn of spinal cord, microglial $P2X_7$ receptors signaling pathway was involved in the induction of long-term potentiation in response to spinal nociceptive stimuli [17]; however, how about the role that microglial $P2X_7R$ plays in the chronic constriction injury (CCI) of the sciatic nerve remains to be elucidated. In order to address this question, in this study we observed the expression of $P2X_7R$ in spinal cord dorsal horn in neuropathic pain induced by CCI, and explored the relationship of expression of $P2X_7R$ with tactile allodynia and thermal hypersensitivity after peripheral nerve injury. The selective $P2X_7R$ antagonist, Brilliant Blue G (BBG), was used to indicate that activation of $P2X_7Rs$ in spinal microglia participates in the pathogenesis of CCI-induced neuropathic pain.

2. Materials and methods

All experiments were performed in strict accordance with the ethical guidelines of the International Association for the Study of Pain [18], and also approved by the Ethical Committee for Animal Research of Third Military Medical University.

2.1. Animal model of chronic constriction of the sciatic nerve (CCI)

Adult Sprague-Dawley(SD) rats (180–220 g) were housed under a 12-h light/dark cycle and free access to food and water at a constant room temperature of 25 °C. Rats were subjected to CCI as previously described [19]. The right sciatic nerve (just proximal to the trifurcation into the sural, peroneal and tibial nerve branches) of rats were tied with 4 × 4-0 chronic sutures under 4% chloral hydrate (Shanghai Xingya Medical Company; 10 ml/kg, i.p.). The sutures were not tied too tightly so as to barely constrict blood flow. Rats with right sciatic nerve exposed without ligature served as sham-CCI controls, and normal rats as naïve controls. Test animals were maintained for 3, 7, 14 or 21 days (d).

2.2. Drug administration

All drugs were prepared just before experiments. Under 4% chloral hydrate (10 ml/kg, i.p.), rats were implanted with PE-10 intrathecal catheter (BD, USA) in the lumbar enlargement (close to L4–5 segments) for intrathecal drug administration. After 7 d recovery, the catheter placement was verified by observing transient hindpaw paralysis induced by intrathecal injection of lidocaine (2%, 5 μl). Animals that failed to show any paralysis were excluded in the experiments. After peripheral nerve injury, rats were administered intrathecally with the selective $P2X_7R$ antagonist BBG (10 μM, 10 μl, Sigma, USA;) or normal saline (10 μl as a vehicle control) once a day from day 0 (right after nerve injury) to day 14.

2.3. Mechanical and thermal sensitivity measurement

Mechanical allodynia was assessed using von Frey hairs (vFh) with bending forces at a range of 0.3–20.3 g. Test was initiated with the 4.10 g vFh, the middle of the filament series. The filament was applied to the ventral surface of each hind paw for 4–6 s. A positive paw withdrawal response was recorded if the animal briskly lifted the hindpaw. The 50% paw-withdrawal mechanical threshold (PWMT) was determined by the up-down method [20]. Thermal sensitivity measurement was carried out using Hargreaves paw withdrawal, which measures the withdrawal latency from a radiant heat source directing at the proximal half of the plantar surface of each hindpaw. The paw-withdrawal thermal latency (PWTL) was recorded as the threshold of thermal sensitivity. Each hindpaw was tested five times at 5-min intervals.

2.4. Western blot analysis

Lumbar spinal cords were dissected out from each animal and the spinal meninges were carefully removed. The ipsilateral sides of L4–6 dorsal spinal cord were used in the following experiments. Protein was extracted by T-PER tissue protein extraction reagent (Pierce, USA) and separated on 10% SDS polyacrylamide gels, electrophoretically transferred onto polyvinylidene fluoride membranes, incubated with the blocking buffer (TBST with 5% fat free dry milk) for 6 h and probed with rabbit anti-$P2X_7$ (1:1000, Millipore, USA) overnight at 4 °C. The membrane sheet was then incubated with a goat anti-rabbit antibody (1:1000) for 2 h and a HRP-labeled streptavidin reagent (1:1000) for 1 h at 37 °C, and visualized with the 3,30-diaminobenzidene (DAB) for 5 min. The immunoreactive density was analyzed by Quantity One.

2.5. Immunohistochemistry

To evaluate the time course of $P2X_7R$ expression in dorsal horn of lumbar spinal cords after neuropathic pain, tissues were taken at days 0, 3, 7, 14 or 21 after ligation. Rats were deeply anesthetized with chloral hydrate (350 mg/kg, i.p.) and perfused intracardially with 4 °C pre-cooled 4% paraformaldehyde prepared in PBS. Spinal cords were quickly removed and fixed in the same fixative overnight at 4 °C. After dehydration, L4–6 segment of the frozen spinal cords were sectioned into slices at 16 μm in thickness. Endogenous peroxidase was inhibited with 3% H_2O_2 for 15 min. Slices were incubated with 10% normal goat serum to block nonspecific binding of immunoglobulins and then incubated with a rabbit $P2X_7R$ antibody (1:200; Millipore, USA) or a specific microglia marker mouse OX42 antibody (1:100, Millipore, USA), at 37 °C for 1 h then 4 °C overnight. After PBS wash, sections were incubated with a donkey anti-rabbit antibody (1:300) or a goat anti-mouse antibody (1:200) for 1 h at 37 °C. Subsequently sections were incubated with a horseradish peroxidase streptavidin (1:200) and visualized with a nickel-intensified DAB chromogen for 5 min as previous report [21]. Immunohistochemistry images were obtained with a microscope (Leica, Wetzlar, Germany). Five randomly selected spinal sections were chosen for each marker for each animal, and density (mean) of each section was analyzed with Image Pro Plus 6.0.

2.6. Double-labeled immunofluoresence

In double labeling experiments, sections were pre-treated as described above. Then they were incubated with 10% normal goat serum for 30 min at room temperature. Subsequently, sections were incubated with the rabbit polyclonal antibody against $P2X_7R$ (1:200; Millipore, USA), 37 °C for 1 h then 4 °C overnight. Antibody binding to tissue sections was visualized with a cy3 labeling goat anti-rabbit antibody (1:300) for 1.5 h at 37 °C. Then they were incubated with 10% normal goat serum again, incubated with different monoclonal second primary antibody (OX42: 1:100; Millipore, USA; Glial fibrillary acidic protein (GFAP): 1:200; Sigma, USA; Microtubule-associated protein 2 (MAP2): 1:200; Abcam, UK) at 37 °C for 1 h then 4 °C overnight. Consecutively, visualization was achieved by adding FITC labeling goat anti-mouse antibody for 1.5 h at 37 °C. Finally, all the sections were counterstained with 4′,6-diamidino-2-phenylindole DAPI (Sigma, USA) and examined with a laser scanning confocal microscope (Leica DMI 6000, Wetzlar, Germany).

2.7. Statistical analysis

The optical density of $P2X_7$ protein in Western blotting was determined by the ratio of the protein signals to β-actin signals. These ratios were normalized to the control values. The mean optical density of P2X7 and OX42 of immunohistochemistry was determined sensitivity (mean) of P2X7 and OX42 in dorsal horn in immunohistochemistry was normalized to the control values. All data are presented as means ± SEM. Statistical analysis was performed with One way ANOVA followed by Tukey post hoc test, $P < 0.05$ was considered to be statistical significant.

3. Results

3.1. CCI-induced mechanical allodynia was attenuated by the selective $P2X_7R$ antagonist BBG

CCI model is a well-established and widely-used preclinical model of neuropathic pain. In this study we revealed profound differences mechanical sensitivity between groups after nerve ligation (Fig. 1). Mechanical allodynia was recorded as a significant reduction of the withdrawal threshold in ipsilesional hindpaw of rats after ligation when compared to that of the naïve controls and the sham-operation group ($P < 0.001$). Naïve controls and the sham-CCI group were relatively stable and had no significant difference in the withdrawal threshold during the whole time course examined. A time-dependent pattern of mechanical allodynia was observed in ipsilesional hindpaw of CCI rats, significant decrease of withdraw threshold was seen at day 3 ($P < 0.001$ compared with naïve control) and then maintained until day 21. To investigate the role of $P2X_7R$ in neuropathic pain, we intrathecally administered BBG, a selective antagonist for $P2X_7R$, to rats once daily for 14 d, through a catheter

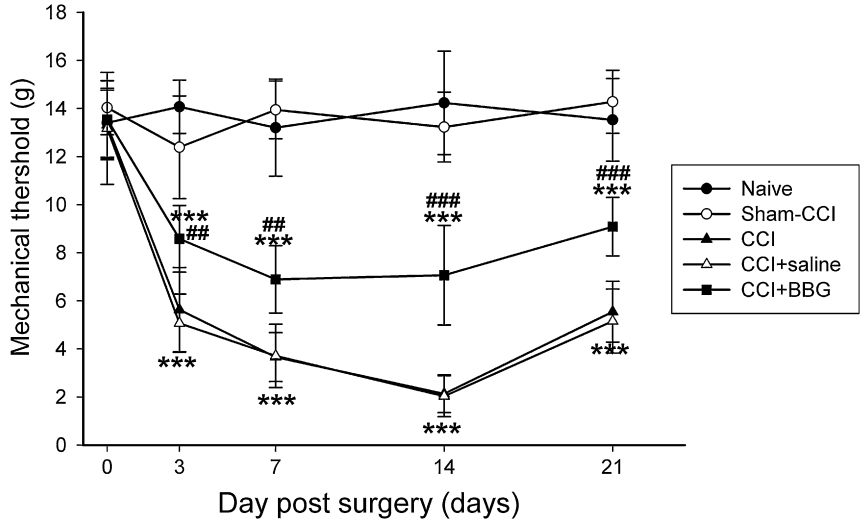

Fig. 1. Intrathecal injections of BBG attenuated CCI-induced mechanical allodynia. Following ligation, mechanical allodynia was developed at 3 d, which was significantly different from the naïve control and sham-CCI rat up to 21 d. After BBG injections, PWMT of CCI rats was higher than that of the saline treated rats and untreated rats, significant differences were observed from 3 d to 21 d. ($n = 6$; $^{*}P < 0.05$,$^{**}P < 0.01$,$^{***}P < 0.001$ compared to naïve control; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ compared to CCI).

whose tip was positioned near the L4/5 spinal cord. In the vehicle (saline)-treated rats, the PWMT was decreased after CCI, which were not significantly different compared to untreated CCI rats. In contrast, repeated intrathecal administration of BBG markedly suppressed this decrease in PWMT after nerve injury, which has shown significant after nerve ligation from 3 d to 21 d compared to saline-treated rats and untreated CCI rats (Fig. 1). However, intrathecal administration of BBG could not completely inhibit mechanical allodynia in neuropathic pain, which has also shown significant differences when compared to the naïve control.

3.2. CCI-induced thermal hypersensitivity was partial inhibited by the selective $P2X_7R$ antagonist BBG

We also detected the effect of BBG on thermal hypersensitivity induced by CCI (Fig. 2). The result has shown that thermal sensitivity displayed changes similar to that of the mechanical allodynia following nerve ligation. Analysis of thermal sensitivity in ipsilesional hindpaw of CCI rats revealed development of significant hyperalgesia from day 3 to 21 post-surgery, and peaked at day 14 ($P < 0.001$ compared with naïve control). Naïve controls and the sham-CCI group also had no significant difference in the thermal sensitivity during the whole time course examined. Intrathecal administration of BBG also reversed the decrease of PWTL induced by CCI (Fig. 2). Significant differences were shown from 3 d to 21 d when compared to the saline-treated rats and untreated CCI rats, and also were detected when compared to the naïve control after intrathecal administration of BBG. The results have suggested that BBG could partial inhibit CCI-induced thermal hypersensitivity.

3.3. Up-regulation of $P2X_7R$ in the dorsal horn after peripheral nerve injury

To explore the relationship $P2X_7R$ expression with CCI-induced neuropathic pain, we used western blot and immunochemistry to examined the level of $P2X_7R$ protein in dorsal spinal cord of CCI rats. Western blot analysis revealed that the expression of P2X7R

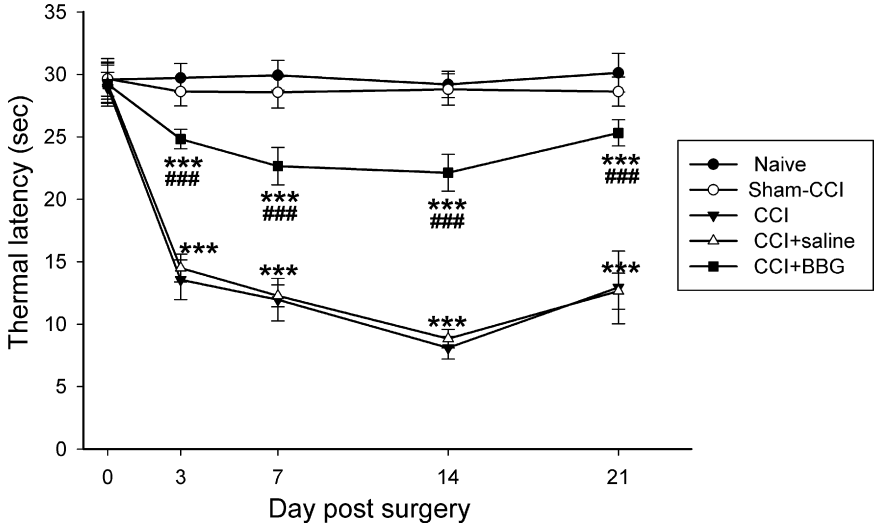

Fig. 2. Intrathecal injections of BBG reversed CCI-induced thermal hypersensitivity. Following ligation, rats developed significant thermal hypersensitivity by 3 d, which was maintained until day 21. Naïve control and sham-CCI rats completely failed to produce thermal hypersensitivity. After BBG intrathecal injections, thermal hypersensitivity induced by CCI was reversed. ($n = 6$; $^{*}P < 0.05$, $^{**}P < 0.01$,$^{***}P < 0.001$ compared to the naïve control; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ compared to the CCI animals).

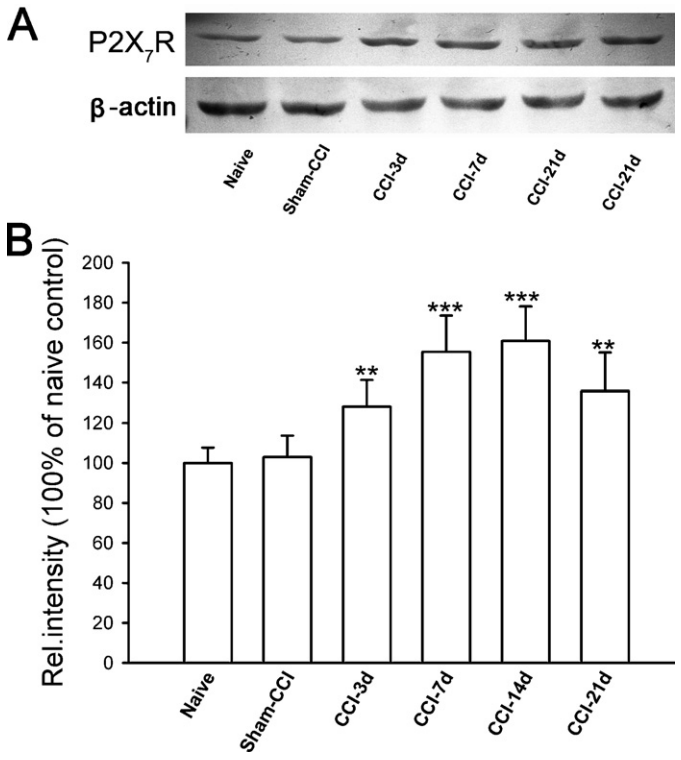

Fig. 3. Western blot analysis of $P2X_7R$ protein from the spinal cord ipsilateral to the nerve ligation. A: Western blot showed the up-regulation of $P2X_7$ receptor in the ipsilateral dorsal spinal cord in the CCI rats. B: Bar graph showing the relative intensity in the level of $P2X_7R$ expression in ipsilateral L4–6 spinal cord compared with the mean expression level of $P2X_7R$ in naïve animals. ($n = 7$; $^*P < 0.05$,$^{**}P < 0.01$,$^{***}P < 0.001$ compared to naïve control).

in the ipsilateral spinal cord was markedly increased after nerve injury (Fig. 3). A significant increase was observed from day 3 after injury; $P2X_7R$ protein levels peaked on day 14. We also performed immunohistochemistry and observed an up-regulation of $P2X_7R$ in the ipsilateral dorsal horn of spinal cord 3 d after nerve injury (Fig. 4), the results showed that $P2X_7R$ immunoreactivity (IR) was increased throughout the ipsilateral dorsal horn of L4–6, specially at layer I–IV, which is one of the major areas for nociceptive signaling processing. Up-regulation of $P2X_7R$ expression was significantly higher in ipsilateral dorsal horn of spinal cord after injury, peaked at 14 d and persisted for at least 21 d, but was occasionally observed in naïve control and sham-CCI groups.

3.4. Expression of $P2X_7R$ on microglia, but not neurons or astrocytes, in dorsal horn of spinal cord

There have been a number of studies exploring the distribution of $P2X_7$ receptors in the nervous system, our data clearly demonstrated that in the ipsilesional of rats post-surgery, the $P2X_7R^+$ cells exhibited a hypertrophic morphology, characterized by enlarged, darkened soma and shorter, thicker and less branched processes, which are similar to morphological features of activated microglia. To identify the type of cells expressing $P2X_7R$ in spinal cord after nerve injury, on day 14 we performed double immunofluorescence labeling for $P2X_7R$ and for cell-type-specific markers: Microtubule-associated protein 2 for neuron, glial fibrillary acidic protein (GFAP) for astrocyte and OX42 for microglia. We found that cells showing $P2X_7R$ immunofluorescence were not double-labeled with MAP2 or GFAP (Fig. 5). Instead, almost all $P2X_7R$-positive cells were double-labeled with OX42 (Fig. 5). Based on these results, we concluded that, in the dorsal horn after nerve injury, the $P2X_7R$ expression is highly restricted to microglia.

3.5. $P2X_7R$ mediated microglia activation in CCI animals

Double-labeled immunofluorescence experiment indicates that P2X7R express highly and exclusively in microglia, but is it associated with microglial activation, contributing to detrimental and/or protective functions in neuropathic pain? We detected OX42 expression in lumbar spinal cord following nerve ligation and activation of microglia by blocking $P2X_7R$. We have found that a tendency of change in expression was similar between $P2X_7R$ and OX42. Immunohistochemical results showed that OX42 protein expression up-regulates post-surgery, and was significantly higher in the ipsilesional dorsal horn of spinal cord after nerve injury (Fig. 6). Positive cells exhibited reactive microglia morphology and surround the dorsal horn following nerve ligation. OX42 expression was decreased significantly compare with saline treated rats, when we blocked $P2X_7R$ activation in L4–6 spinal cord using the selective $P2X_7R$ antagonist BBG and when we examined microglia activation in dorsal horn of spinal cord. The data also showed that in BBG treated 14 d rats, distribution of microglia was not as intensive as in saline treated 14 d rats or CCI rat, and less microglia

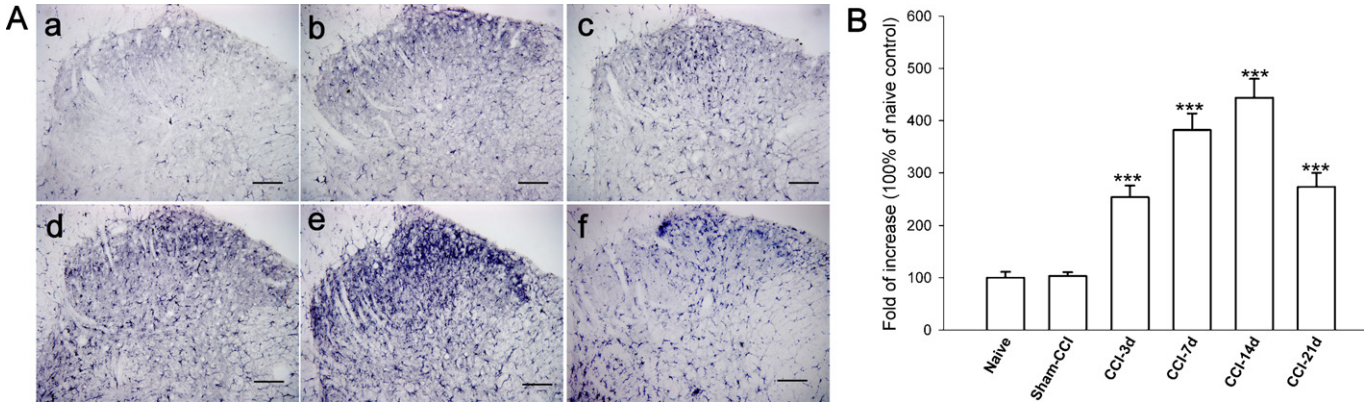

Fig. 4. Up-regulation of $P2X_7R$ expression levels in the spinal dorsal horn after the sciatic nerve ligation. A: $P2X_7R$ immunohistochemical results of the L4–6 dorsal spinal cord ($n = 6$, bar = 100 μm). The time course of change in $P2X_7R$ protein is similar to that of paw withdrawal threshold (bottom panel). a: Naïve control, b: Sham-CCI group, c: Day 3 after operation, d: Day 7 after operation, e: Day 14 after operation, f: Day 21 after operation. B: Mean optical density of $P2X_7R$ after peripheral nerve injury by immunohistochemistry. Bar graph shows the average fold increase in the level of $P2X_7R$ expression in dorsal horn of spinal cord compared with the mean expression level of $P2X_7R$ in naïve animals ($n = 6$; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to naïve control).

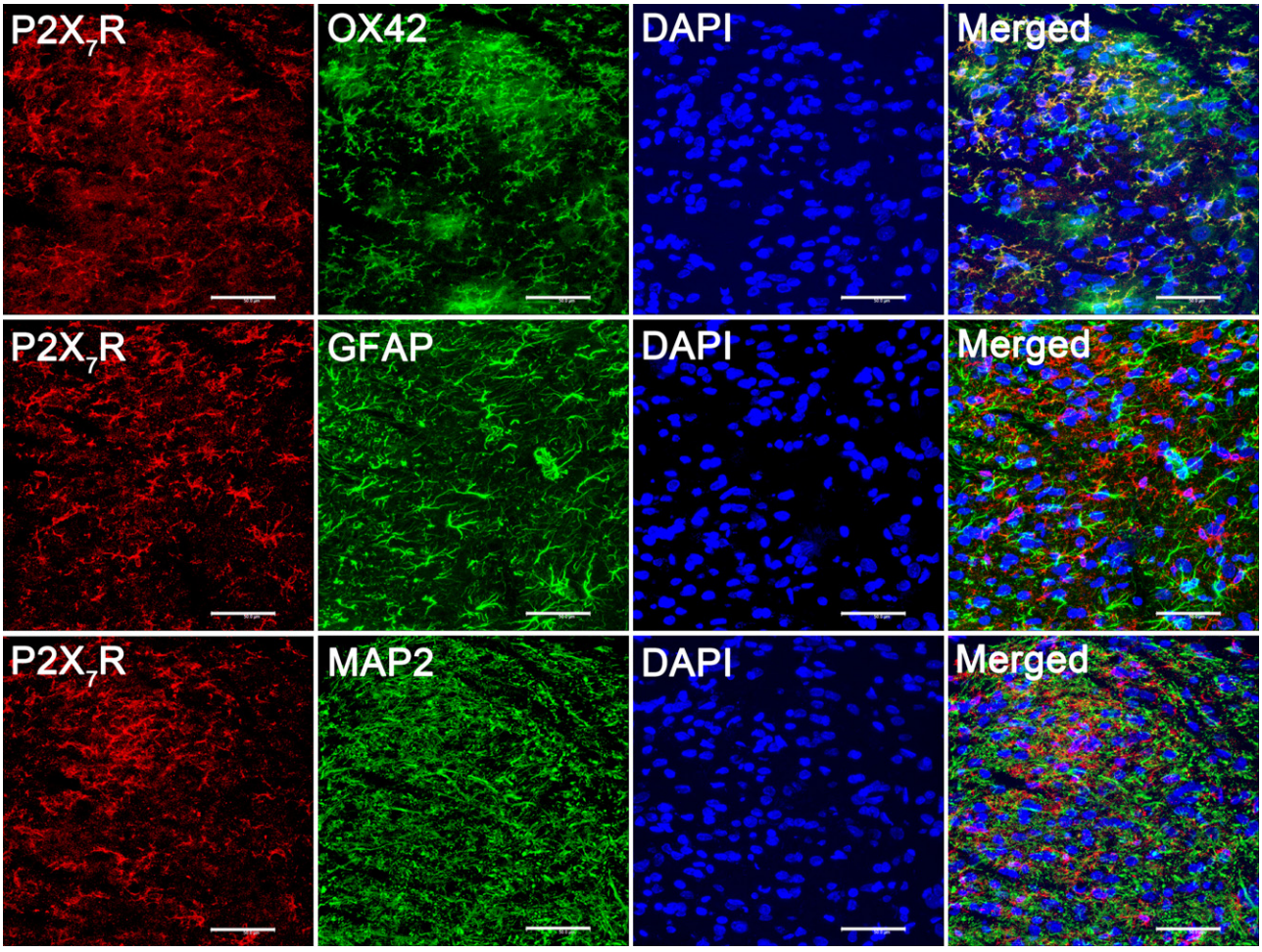

Fig. 5. $P2X_7R$ expression in microglia, but not neurons or astrocytes, in dorsal horn of spinal cord. All experiments were performed with L4–6 spinal cord sections, 14 d after nerve ligation (bar = 50 μm). Double immunofluorescence labeling for $P2X_7R$ and for cell-type-specific markers: Microtubule-associated protein 2 (MAP2) for neuron, glial fibrillary acidic protein (GFAP) for astrocyte and OX42 for microglia.

exhibited hypertrophic morphology, although still more so than in naïve controls.

4. Discussion

In the present study, the CCI model was used to investigate neuropathic pain, and we observed expression of $P2X_7R$ in dorsal horn of L4–L6 spinal cord after nerve injury. Our data from Western blot and immunohistochemistry showed that $P2X_7R$ expression was increased in the ipsilateral spinal cord after nerve injury, and the time course of the change in $P2X_7R$ levels in the dorsal horn of spinal cord and the difference in $P2X_7R$ levels matched the emergence of mechanical allodynia and thermal hypersensitivity. We also demonstrated that nerve injury-induced mechanical allodynia and thermal hypersensitivity was reversed by intrathecal administration of BBG. These data indicate that $P2X_7R$ in spinal cord may play important roles in CCI-induced neuropathic pain. We further noticed that $P2X_7R$ expression in the dorsal horn was highly restricted to microglia, and the numbers of microglia were also increased in the ipsilateral spinal cord of CCI rats, and possessed the short, thick processes that are characteristic of activated microglia, but the number of reactive microglia was decreased and the distribution of microglia was less intensive after intrathecal administration of a selective $P2X_7$ antagonist BBG. Thus, we suggest that $P2X_7R$ mediated microglia activation to maintain neuropathic pain.

ATP has been recognized as a key neurotransmitter in the development and maintenance of chronic neuropathic pain [22,23]. There are many studies indicate that $P2X_7R$ was linked to responses associated to spinal cord damage and inflammation being primary mediators of pain sensation [13–16]. $P2X_7(-/-)$ mice do not develop either mechanical allodynia or thermal hyperalgesia in a partial nerve ligation model [13]. Functional $P2X_7Rs$ have been demonstrated in peripheral glial cells in rat dorsal root ganglion, and this may play a role in peripheral sensory transduction of pain perception [24]. However, there is little histological evidence to show the role of $P2X_7R$ in dorsal horn of spinal cord after neuropathic pain.

In order to explore spinal $P2X_7R$ relationship with neuropathic pain, the CCI model was used in this study, which shows many of the pathophysiological properties of chronic neuropathic pain in human subjects [19]. Symptoms of CCI rats are similar to those seen with neuropathic pain induced by cancer compression, metabolic disorders and toxins. In CCI model, we detected that $P2X_7R$ expression was increased in the dorsal horn of spinal cord, and difference in $P2X_7R$ levels matched the time-course and the degree of allodynia and hyperalgesia against mechanical and thermal stimuli. The observations suggest that spinal $P2X_7R$ may play a role in CCI-induced neuropathic pain.

Recently, some new potent and selective $P2X_7R$ antagonists were developed, such as oxidized ATP (oxATP), A-740003 and A-438079. Administration of those selective $P2X_7R$ antagonist, can

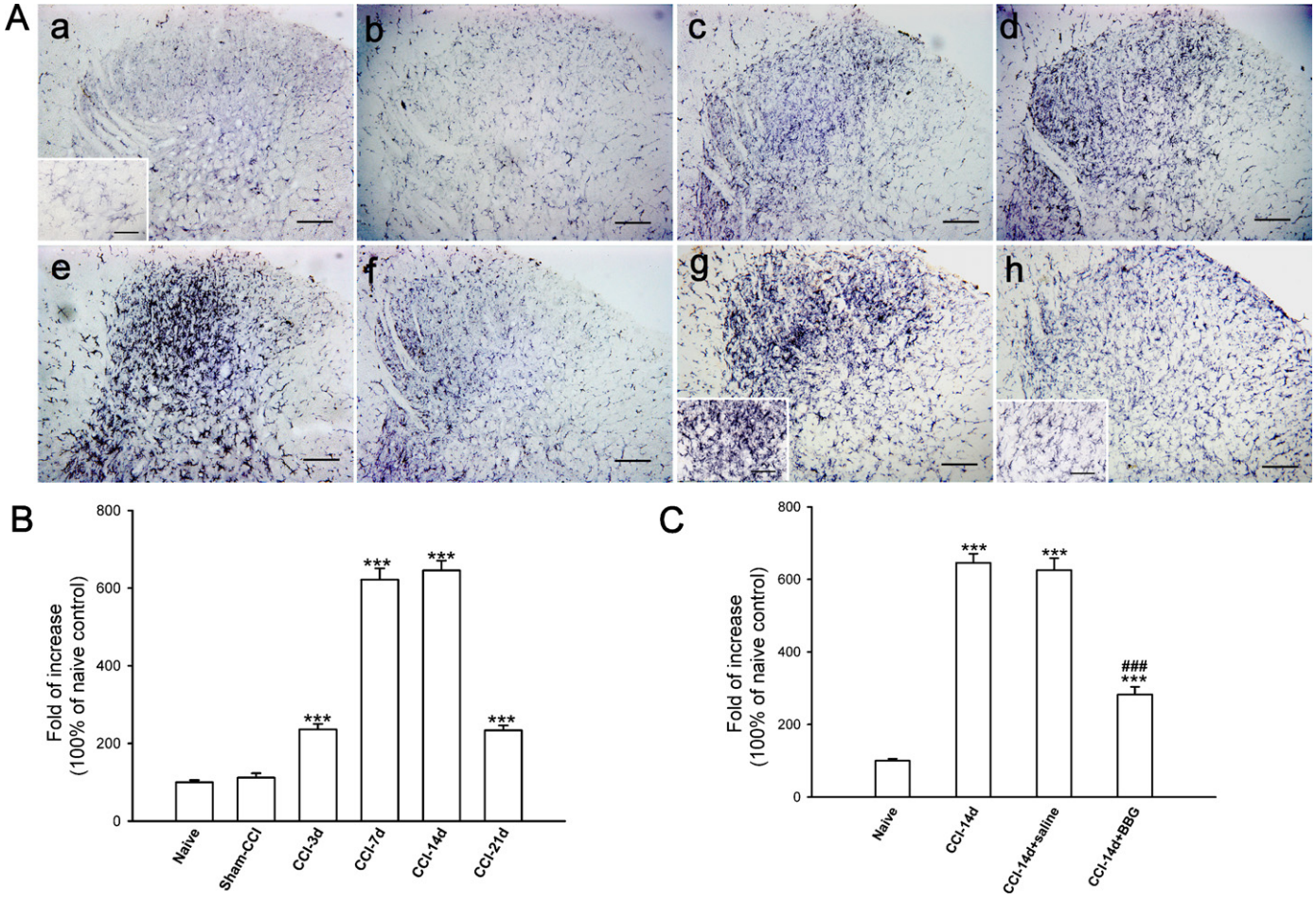

Fig. 6. OX42 expression was decreased by blocking $P2X_7$ receptor in CCI rats. A: Immunohistochemical observation of OX42 labeled microglia in the L5 dorsal spinal cord (bar = 100 μm). The time course of change in microglia was similar to that of the paw withdrawal threshold (bottom panel). Intrathecal injection of BBG reduced the up-regulation of OX42 induced by nerve injury. a: Naïve control, b: Sham-CCI, c: Day 3 after operation, d: Day 7 after operation, e: Day 14 after operation, f: Day 21 after operation. g: Intrathecal injection of saline after 14 d in CCI rats, h: Intrathecal injection of BBG after 14 d in CCI rats. Higher magnification images of OX42 expression show the change of microglia morphology under different treatments. (bar: 25 μm). B: Mean optical density analysis of OX42 expression after peripheral nerve injury by immunohistochemistry. C: Mean density analysis of OX42 expression after intrathecal injection of drug after 14 d in CCI rats by immunohistochemistry. Bar graph shows the average fold increase in the level of OX42 expression in dorsal horn of spinal cord compared with the mean expression level of OX42 in naïve animals. ($n = 6$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the naïve control, $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ compared to CCI intrathecal injections of BBG or intrathecal injections of saline compare to the untreated CCI rats)

reduce both allodynia and hyperalgesia in several animal models of chronic pain [13–16]. In our study, we used BBG as an antagonist to block spinal $P2X_7R$ in CCI-induced pain model. BBG is a derivative of a commonly used blue food color (FD&C blue No. 1), which crosses the blood-brain barrier, and is a selective $P2X_7R$ antagonist. The low toxicity [25] and high selectivity of BBG [26] made this compound an ideal candidate for blocking the potential adverse effect of spinal $P2X_7R$ activation in neuropathic pain. Results of this study showed that BBG can reversed mechanical allodynia in CCI model, which is concordant with an earlier report in tetanic stimulation of the sciatic nerve (TSS) model. Moreover, BBG also can reduce CCI-induced thermal hypersensitivity. The effect of BBG was observed from 3 d to 21 d, which indicated a role of $P2X_7$ in the onset and persistence of CCI-induced neuropathic pain.

Previous studies indicated that $P2X_7$ receptors were expressed in several types of cell in the CNS [27–30], but in the spinal cord, $P2X_7R$ expression is remains in debate. Although several studies have demonstrated that $P2X_7R$ mRNA and protein were detected in dorsal neurons and ventral motoneurons of spinal cord ventral horn [31,32], Some studies have shown that staining of a "$P2X_7$-like" protein in neurons and astrocytes is not eliminated by $P2X_7$ gene-knockout [33], so whether $P2X_7R$ is actually expressed in neurons needs to be further investigation. In addition to neurons, $P2X_7R$ mRNA can also be detected in astrocytes [27]. However, most of the reports on $P2X_7R$ in spinal astrocytes come from culture preparations that could alter the expression profile [34], Immunohistochemistry failed to detected astrocytic signal for this receptor in the spinal cord [32]. In this study, double-labeled immunofluorescence experiment show that dorsal spinal $P2X_7$ receptors expression is highly strict in microglia, which is consistent with some studies that P2X7R mRNA and protein are expressed in microglia [17,35–37]. Chu et al. have shown that there are no colocalization of $P2X_7Rs$ with neuron-specific nuclear protein (NeuN) in the dorsal horn of spinal cord [17]. To exclude $P2X_7$ expression in nerve terminals, we used MAP2 to label Neuron, which mainly localizes to the somato-dendritic compartments in neurons. Colocalization of $P2X_7R$ with MAP2 or GFAP also was not observed in the dorsal horn of spinal cord in CCI rats, which suggested that there was no $P2X_7$ expression in dorsal horn neurons or astrocytes.

Microglia are activated in response to a diversity of stimuli, ranging from peripheral inflammation to CNS injury, which is necessary and sufficient to induce neuropathic pain. There is abundant evidence that microglia are activated in the dorsal horn in a wide variety of nerve injury models [38,39]. Our current data have also shown that OX42 expression was increased in CCI model.

The signal(s) for activating microglia in the models of neuropathic pain is unclear, but it has been proven that microglia are exquisitely responsive to extracellular ATP, which is released by cellular damage, nearby astrocytes or neurons from either synaptic or non-synaptic regions [40,41]. $P2X_7R$ is highly expressed in microglia, and many studies have shown that $P2X_7R$ immunoreactivity is increased in activated microglia of many models of diseases, such as Alzheimer's disease, multiple or amyotrophic later sclerosis and focal cerebral ischemia [42–45], suggesting that microglial $P2X_7R$ might be a general mediator of stress during pathological states.

Activated microglia are characterized by a specific morphology, proliferation, increased expression of cell surface markers and receptors, and changes in function, such as migration to areas of damage, phagocytosis, and production/release of pro-inflammatory cytokines [46]. Spinal microglia proliferation occurs following peripheral nerve injury and this cell proliferation is correlated with the neuropathic pain [47]. It was reported that overexpression alone of $P2X_7R$is sufficient to drive the activation and proliferation of microglia and chronic blockade of the $P2X_7$ receptor by antagonists (oxATP, KN62 and BBG), or treatment with the ATP-hydrolase apyrase, severely decreases microglia proliferation. Down-regulation of $P2X_7R$ expression by small RNA interference (SiRNA) also decreases microglia proliferation [12]. Our current data also demonstrates that blocking $P2X_7R$ expression on microglia is consistent with microglia proliferation and activation in dorsal horn of spinal cord after CCI, which suggests $P2X_7R$ expression may be necessary for microglia activation and proliferation, which is associated with neuron–glial signaling in neuropathic pain. Thus, blocking of microglial $P2X_7R$ in spinal cord might represent a therapeutic strategy for treating neuropathic pain.

5. Conclusions

Our results indicate that$P2X_7R$ expression was increased in the ipsilateral spinal cord after sciatic nerve ligation and was highly restricted to microglia, and CCI-induced mechanical allodynia and thermal hypersensitivity was reversed by intrathecal administration of a selective $P2X_7$ antagonist BBG. In addition, up-regulation of OX42 expression was also observed in the ipsilateral spinal cord of CCI rats, but the number of reactive microglia was decreased and distribution of microglia was less intensive after intrathecal administration of BBG. These results suggest that $P2X_7R$ may contribute to microglia activation-induced neuropathic pain in the CCI model.

Competing interests

Authors declare that they have no competing interests.

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