P2X7 Receptor of Microglia Mediates Neuropathic pain by Regulating Autophagy After Chronic-Constriction Injury

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Research

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Abstract

P2X7 receptor is a crucial receptor related to neuronal activation, neurosensitization, and pain transmission, and increasing evidences indicated that glial cells is thought to be a major contributor to the chronic neuropathic pain after nerve injury. In present study, we designed to investigate whether the P2X7R in glial plays a role in chronic neuropathic pain. We divided adult male Sprague Dawley rats were respectively into four groups:(1) vehicle group (Veh), (2) CCI (C group), (3) P2X7 inhibitor group (P group), (4) CCI+ P2X7R inhibition (CP group). Behavior test, real-time polymerase chain reaction, western blot, immunofluorescence staining, and transmission electron microscope were used to analyze the scientific hypothesis. The results of the experiment is that(i) P2X7R of microglia was downregulated by A-70003 after CCI. (ii) Downregulation of P2X7R on microglia is coincident with remission of NP after CCI. (iii) P2X7R of microglia participates in NP via regulating autophagy and apoptosis. In summary, our results support P2X7R inhibition can counteract the CCI-induced NP due to microglia activation via a modulation of autophagy and apoptosis in mPFC and spinal cord. This may provide an importantly neuroprotective mechanism for the improved NP and also help devising new therapeutic to improve chronic pain in patients.

Introduction

Neuropathic pain (NP) can develop after lesion or disease in the sensory system, which serves no protective function and affects 7%~8% of the population [1].Despite recent advances in the knowledge of nociceptive information processing in NP, which remains a vital major clinical and social problem [2], so it is still necessary to go further into the study of the pathophysiology of this condition to facilitate the development of new more effective drugs or, at least, to repurpose some that are currently in clinical use.

Recently, investigations have highlighted the role of glial cells in the development of chronic pain. Particularly, researches demonstrated that peripheral nerve injury can result in microglia and astrocyte activation of medial prefrontal cortex (mPFC) area in NP models [3, 4]. Activated microglia release various algesic substances that enhance pain transmission by neurons. Autophagy, as a process to remove and recycle aggregated proteins, is an important function for maintaining cellular homeostasis, regulated by the activation of astrocytes and has been participated in allodynia and hyperalgesia. Piao et al [5] reported that lack of the p62 autophagic protein plays a key role in the pathophysiology of NP. Autophagy inducers have been shown to protect neurons, findings indicated that NP activates autophagy [6], and increased glial autophagy activity exerted a protective effect in NP via inammasome inactivation [7]. However, how glial autophagy mediated NP has not been fully investigated.

In addition, P2X7 receptors are widely distributed in the central nervous system neurons, such as microglia and astrocytes. They are closely associated with pathologic pain, such as in the central nervous system, where they are expressed primarily in microglia cells and are thought to perform similar functions by inducing the release of inflammatory cytokines [8]. Early reports of the use of antagonists and knockout mice suggest that the microglia P2X7R is involved in chronic neuropathic and inflammatory
pain by releasing interleukin-1β (IL-1β)[9]. Previous studies have shown that P2X7R is upregulated in the spinal cord after nerve injury [10]. In addition, P2X7R seems to play an important role in NP [11]. For example, P2X7R-knockout mice fail to develop behavioral hypersensitivity after partial sciatic nerve ligation. Moreover, studies have shown that an autophagy inducer could result in long-lasting analgesia, inhibit IL-1β secretion [12], improves nerve myelination [13] and prevent pain chronification [14]. Takenouchi et al [15] proposed a mechanism for the P2X7R signaling pathway that may play an important role in activated microglial cells. However, whether P2X7R in glial cells contribute to NP through autophagy is unknown.

Therefore, the research hypothesis is that P2X7R in glials plays a role in chronic pain, and its descending pathway may be associated with autophagy. In the study, we established a chronic pain model for rats, and applied P2X7R inhibitor. After that, mPFC and spinal cord regions were selected and the relevant molecular biological level was studied to observe the indicators and demonstrate the hypothesis.

Materials And Methods

Animal Studies

The ethical approval for this experiment was approved by the Ethical Committee of the Animal Use and Care Committee of Zhongnan Hospital, and all animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals.

Animals

Five-Eight-week-old adult male Sprague Dawley rats wild-type mice with weight ranging from 200 to 300g were obtained from the experimental animal center of Wuhan University School of Medicine. Prior to the study, animals were housed and fed at a temperature of 23 ± 2°C and a humidity of 60–70%, with a 12-h light/dark cycle and free access to food and water. All experiments were conducted between 9 am and 5 pm. Mice were positioned on a heating pad to prevent hypothermia during experiments.

Chronic-Constriction Injury Model

The Chronic-Constriction Injury (CCI) model was established as previously described (Bennett and Xie, 1988). Briefly, the rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.), an incision was made on the lateral surface of the midthigh, the biceps femoris muscle was blunt-dissected, and the left sciatic nerve was exposed and loosely ligated with 4−0 silk at four sites with an interval of 1mm. For the sham group, all procedures were identical to those in the CCI group, except that there was no nerve ligation. Afterward, the muscle and skin incisions were closed separately. The wound was sutured with 3−0 silk.

P2X7R inhibition (A-740003 [N-(1-{[(Cyanoimino) (5-quinolinylamino) methyl]amino}-2,2-dimethylpropyl)-2-(3,4-dimethoxyphenyl) acetamide], a selectively P2X7 antagonist, Tocris, China) was injected to mPFC (bregma rostrocaudal 3.2 mm, mediolateral 0.7 mm, dorsoventral 3.5 mm) of rat by
bilateral stereotaxic (Techman, DW-2000, Chengdu, China). The rats were randomly divided into four groups according to the random number table: (1) vehicle group (Veh), which received vehicle (PBS, 0.5µl) (Aspen, AS1025, Wuhan, China) in the bilateral mPFC of the rats; (2) CCI group(C group), which was established CCI model; (3) P2X7 inhibitor group (P group), which received A-740003 (100M) in the bilateral mPFC of the rats at the 14th day for three consecutive days; (4) CCI + P2X7R inhibition (CP group), which received A-740003 (100M) in the bilateral mPFC of the rats at the 14th day of CCI for three consecutive days. A-740003 was freshly dissolved in DMSO.

**Behavioral test**

Pain-related behavioral tests were performed according to previous described methods. All animals were acclimated to the testing environment two days before baseline testing. Von Frey hairs were used to stimulate the plantar surface of the hind paw to evaluate paw-withdrawal threshold (PWT), and 50% PWT was determined using the up-down method. Briefly, a series of Von Frey hairs with ascending stiffness was used to stimulate the plantar surface of the hind paw, a positive response was recorded if the paw was suddenly withdrawn upon application of a filament as well as licking or biting of stimulated paw. Testing of a filament was done for five rounds per paw. The threshold filament size was set as the smaller filament resulted in three withdrawals out of five successive trials. The paw-withdrawal latency (PWL) was measured by the plantar test (7370, Ugo Basile, Comeria, Italy) and used the method of Hargreaves et al. Briefly, a radiant heat source beneath a glass floor was applied at the plantar surface of the hind paw. Each rat was tested three times with 5 min intervals between consecutive, alternating tests. The average of the three measurements was taken as the result per test.

**RT-PCR for P2X7R mRNA**

The area of mPFC in brain and spinal cord were collected and extracted using TRIzol reagent (Invitrogen™, USA). The concentration of RNA was detected using ultraviolet absorption spectrometry. The cDNA was synthesized using Entilink™ 1st Strand cDNA Synthesis Kit (ELK Biotechnology, EQ003). For quantitative real-time PCR reaction, EnTurbo™ SYBR Green PCR SuperMix® (ELK Biotechnology, EQ001) was used in StepOne™ Real-Time PCR detection system (Life Technologies, USA). The PCR was carried out at the following condition: pre-denaturing at 95°C for 3mins, followed by 40 cycles of annealing reaction at 58°C for 30s, and extension 72°C for 30s. The PCR primers for P2X7R are as follows: forward 5′-GAAAAGCGGACATTGATCAAAG-3′ and reverse 5′-CAAGTCAATACACACGCGTGGC′, respectively, and the product size was 147 bp. β-actin was used as references for mRNAs. Each sample was analyzed in triplicate.

**Western blot analysis**

Animals were anesthetized after behavioral tests at predefined time points, the lumbar spinal cords and the area of mPFC in brain were removed, and the collected tissues were frozen in liquid nitrogen and stored at −80°C. Tissue samples were homogenized in RIPA buffer containing protease and phosphatase inhibitor. Lysates were centrifuged at 12000 rpm for 15 min at 4°C, and protein concentration was determined by a bicinchoninic acid (BCA) Protein Assay Kit (Beyotime Institute of
Biotechnology, Shanghai, China). Samples with equal amount of protein were separated by 9%-12% PAGE (40 g total protein per lane) and transferred onto polyvinylidene difluoride membranes (Millipore, USA), followed by blockage with 5% skim milk for 2 h and incubation overnight with the following primary polyclonal antibodies: rabbit anti-P2X7R (1:250; Alomone, Israel), rabbit anti-LC3B (1:1000, Cell Signaling Technology, Beverly, MA, United States), rabbit polyclonal anti Beclin 1 (1:1000, MBL International Corporation, Nagoya, Japan), rabbit anti-Bcl-2 (1:1000; 2876, Cell Signaling Technology, USA), rabbit anti-Bax (1:1000; 2772, Cell Signaling Technology, USA), rabbit anti-cleaved-Caspase-3 (1:1000; 9661, Cell Signaling Technology, USA) . The membranes were washed and then incubated with secondary antibodies (anti-rabbit IgG at 1:10000, Aspen, USA). The immune complexes were identified using an enhanced chemiluminescence (ECL) detection system. β-actin (1:10000, Tdy Biotech, Beijing, China) and GAPDH (1:200; Santa Cruz Biotechnology) was blotted on the same membrane as a loading control.

Transmission electron microscopy study

After the brain and spinal cord tissues were fixed with paraformaldehyde, and removed, then fixation in 2.5% (w/v) glutaraldehyde overnight, the specimens were post-fixed in 2% (v/v) osmium tetroxide and blocked with 2% (v/v) uranyl acetate. Tissues were embedded in araldite after dehydration in a series of acetone washes. Semi-thin sections were cut and toluidine blue staining was performed for observation of localization. Finally, ultra-thin sections from at least three blocks per sample were cut and observed using a transmission electron microscopy (TEM).

Immunostaining and image analysis

Four mice in each group were humanely sacrificed at 1 h after the A740003 injection at the last time. The rats were deeply anaesthetized and transcardially perfused with 0.9% ice-cold saline solution followed by 4% paraformaldehyde (Aspen, Wuhan, China) at 4°C. The fixed brain and spinal cord were immersed in the same fixative overnight, and embedded in paraffin. The paraffin-embedded tissue arrays were performed in sections for immunostaining. For staining P2X7R-positive cells, sections of Spinal cord slice were incubated with primary antibody (rabbit anti-P2X7R, 1:50, APR008, Alomone, Israel) and then biotin-conjugated secondary antibody (goat anti-rabbit IgG, 1:50, Aspen, USA). For P2X7R-Iba1 (ionized calcium binding adaptor molecule 1) / P2X7R-GFAP (glial acidic fibrillary protein) co-labeling in brain, sections were incubated with a mix of primary antibodies (rabbit anti-P2X7R, 1:100, and mouse anti-Iba1 or mouse anti-GFAP, 1:100, Abcam, UK) and then secondary antibodies (goat anti-rabbit IgG at 1:100, goat anti-mouse IgG at 1:100, Aspen, USA, DAPI). For double immunofluorescence staining, the primary antibody for P62 (rabbit anti-P62, 1:200, Proteintech, United States) was incubated together with the mouse monoclonal antibody (anti Iba1 or anti GFAP, 1:100, Abcam, UK) and then incubated with secondary antibodies (goat anti-rabbit IgG at 1:100, goat anti-mouse IgG at 1:100, Aspen, USA). Images were acquired using a fluorescence microscope (Olympus, Tokyo, Japan) equipped with an imaging system.

Statistical analysis

GraphPad Prism version 8 software was used for statistical analysis. Because of the available number of animals and estimated statistical power, the sample size was 6/group for Behavioral test, 4/group for
PCR, western blotting, TEM and immunofluorescence analysis. Data were expressed as the mean ± standard error of the mean (SEM). All analyses were conducted using SPSS 20 statistical software. More than two groups were compared, statistical evaluation of the data was performed with one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. P < 0.05 was considered to be significant for all statistical comparison.

Results

Inhibition of P2X7R can increase thermal and mechanical pain thresholds in rats with chronic neuropathic pain

NP seriously affects the health and life quality of patients. However, the mechanism of NP has not been fully investigated and there is still lack of effective treatment for it. Previous study showed that P2X7 was involved in the pain transmission and the occurrence of NP in mice, in this study, we planned to explore whether P2X7R played a role in the process of chronic pain.

As shown in Fig. 1A, rats established by CCI model showed significantly lower pain threshold than the control rats from the third day after surgery, and there was no significant difference in heat and mechanical perception of rats given P2X7R inhibitor alone compared with the control group (thermal pain thresholds: Day 3: 13.20 ± 1.67 for group V, 13.55 ± 2.90 for group P, 8.23 ± 1.44 for group C, p < 0.001, 7.48 ± 1.22 for group CP, p < 0.001; mechanical pain thresholds: Day 3: 33.27 ± 4.22 for group V, 35.64 ± 4.76 for group P and 19.89 ± 5.46 for group C, p < 0.001, 21.79 ± 2.47 for CP group, p < 0.001) and continued until 14 days after surgery (thermal pain thresholds: Day 7: 14.32 ± 0.97 for group V, 15.48 ± 1.38 for group P, 5.87 ± 1.58 for group C, p < 0.001, 6.56 ± 0.18 for CP group, p < 0.001; Day 10: 15.24 ± 1.28 for group V, 14.74 ± 2.53 for group P, 5.76 ± 1.00 for group C, p < 0.001, 4.62 ± 1.37 for CP group, p < 0.001; Day 14: 13.71 ± 0.69 for group V, 13.99 ± 1.11 for group P, 3.84 ± 0.88 for group C, p < 0.001, 7.44 ± 1.29 for group CP, p < 0.001; Day 15: 14.23 ± 1.37 for group V, 14.33 ± 2.28 for group P, 3.60 ± 0.85 for group C, p < 0.001, 7.10 ± 1.10 for group CP, p = 0.001; Day 16: 14.14 ± 1.60 for group V, 14.52 ± 0.42 for group P, 3.79 ± 0.42 for group C, p < 0.001, 7.24 ± 0.77 for group CP, p < 0.001; mechanical pain thresholds: Day 7: 37.71 ± 2.52 for V group, 34.56 ± 4.75 for P group, 15.65 ± 6.19 for C group, p = 0.001, 16.97 ± 1.05 for CP group, p < 0.001; Day 10: 36.57 ± 1.50 for group V, 35.89 ± 3.64 for group P, 12.46 ± 2.03 for group C, p < 0.001, 11.75 ± 1.05 for CP group, p < 0.001; Day 14: 34.59 ± 2.58 for group V, 36.41 ± 6.37 for group P, 10.37 ± 3.36 for group C, p < 0.001, 16.00 ± 1.42 for CP group, p < 0.001; Day 15: 35.07 ± 3.00 for group V, 35.12 ± 5.82 for group P, 9.88 ± 1.38 for group C, p < 0.001, 15.85 ± 2.36 for CP group, p = 0.001; Day 16: 34.54 ± 2.33 for group V, 34.53 ± 3.15 for group P, 10.76 ± 2.88 for group C, p < 0.001, 15.95 ± 1.62 for CP group, p < 0.001). However, after treated with P2X7R inhibitor at the 14th days of CCI, CP group postoperatively suffered longer heat pain radiation than C group (p < 0.001), which verified that P2X7R can increase thermal and mechanical pain thresholds in chronic pain model rats.

Chronic pain was partially dependent on P2X7R in mPFC and spinal cord
Previous studies have shown that the mPFC, which integrates the sensory, emotional and attentional components of pain perception, is the focus of chronic pain. To explore whether P2X7R in the mPFC region is associated with chronic pain processes, we examined P2X7R levels using WB and PCR. As shown in Fig. 2, the content of P2X7R in the brain of chronic pain rats was significantly higher than that of control rats (0.04 ± 0.01 for group V, 0.04 ± 0.00 for group P, 0.19 ± 0.03 for group C, \( p < 0.001 \), 0.10 ± 0.01 for group CP, \( p < 0.001 \), Fig. 2B), and in the chronic pain group, P2X7R inhibitor treatment reduced the content of P2X7R in CP group (group C vs. CP, \( p < 0.001 \), Fig. 2B). PCR showed the same trend (0.93 ± 0.34 for group V, 0.77 ± 0.21 for group P, 4.89 ± 0.48 for group C, \( P < 0.001 \), 2.88 ± 0.71 for CP group, \( p < 0.001 \), group C VS CP, \( p < 0.001 \), Fig. 2C).

In addition, the spinal cord acts as the primary center of pain transmission and plays a role in chronic pain. As shown in Fig. 2, the trend of P2X7R content in the chronic pain spinal cord was consistent to that in the brain (western blot:0.25 ± 0.02 for group V, 0.27 ± 0.03 for group P, 0.47 ± 0.07 for group C, \( p < 0.001 \), 0.35 ± 0.01 for CP group, \( p < 0.001 \), Group C VS CP, \( P = 0.012 \), Fig. 2E; PCR:0.90 ± 0.55 for V group, 1.00 ± 0.31 for P group, 3.85 ± 0.23 for C group, < 0.001, 1.93 ± 0.47 for CP group, \( p < 0.001 \), group C VS CP, \( p < 0.001 \), Fig. 2F).

**P2X7 elimination decreases autophagy and apoptosis in rats of chronic pain**

Autophagy plays a key role in the progression of pain pathology, researches have demonstrated that the inhibition of autophagy can alleviate neuropathic pain behavior in animals. However, whether autophagy was regulated by P2X7R in chronic pain has not been fully investigated.

In Fig. 3A, the number of autophagosomes in chronic pain rats can be observed was significantly higher than that in control rats (mPFC: 7.97 ± 1.94 for group V, 8.79 ± 0.72 for P, 22.14 ± 3.01 for group C, \( p < 0.001 \), 14.03 ± 1.62 for CP group, \( p < 0.001 \), Fig. 3A; spinal cord: 3.59 ± 1.17 for group V, 3.97 ± 1.35 for group P, 16.93 ± 4.15 for group C, \( p < 0.001 \), 9.74 ± 2.40 for CP group, \( p < 0.001 \), Fig. 4A), and in rats of the chronic pain group, treatment with P2X7R inhibitors reduced the number of autophagosomes in the model rats (mPFC: Group C vs. CP, \( p = 0.001 \), Fig. 3B; spinal cord: Group C vs. CP, \( p = 0.017 \), Fig. 4B). Moreover, we measured the levels of Beclin 1, LC3\(^{II} \) / LC3\(^{II} \) in the mPFC and spinal cord at 14 days of model establishment (Fig. 3C,4C) using Western Blot. It was consistent with the trend of the number of autophagosomes (mPFC: Beclin 1: 0.25 ± 0.05 for V group, 0.27 ± 0.03 for P group, 0.82 ± 0.08 for C group, \( p < 0.001 \), 0.67 ± 0.04 for CP group, \( p < 0.001 \), group C vs. CP, \( P < 0.001 \), Fig. 3D; LC3\(^{II} \)/ LC3\(^{II} \): 0.14 ± 0.08 for V group, 0.20 ± 0.02 for P group, 0.42 ± 0.03 for C group, \( p < 0.001 \), 0.40 ± 0.05 for group CP, \( p < 0.001 \),C vs. CP group, \( P = 0.018 \), Fig. 3E; spinal cord: Beclin 1: 0.32 ± 0.05 for group V, 0.25 ± 0.07 for group P, 0.74 ± 0.10 for C group, \( p < 0.001 \), 0.48 ± 0.02 for group CP, \( P = 0.001 \),Group C vs. CP group, \( p = 0.002 \), Fig. 4D; LC3\(^{II} \)/ LC3\(^{II} \): 0.16 ± 0.04 for V group, 0.15 ± 0.03 for group P, 0.45 ± 0.02 for group C, \( p < 0.001 \), 0.26 ± 0.01 for group CP, \( p < 0.001 \), group C vs. CP, \( p < 0.001 \), Fig. 4E). It confirms that inhibition of P2X7R can reduce autophagy and apoptosis in chronic pain.
Next, we measured the levels of apoptotic indexes Bcl-2, Bax, and Cleaved caspase3 at 14 days after model establishment using Western Blot (Fig. 3F) in mPFC and spinal cord (Fig. 4F). It could be seen that the ratio of Bax/Bcl-2 in mPFC and spinal cord of chronic pain rats were significantly higher than those in the control group (mPFC: 0.27 ± 0.17 for group V, 6.72 ± 1.21 for group P, 0.23 ± 0.02 for group C, p < 0.001, 1.92 ± 0.63 for the CP group, p = 0.001, Fig. 3G; spinal cord: 0.39 ± 0.07 for group V, 7.00 ± 1.60 for group P, 0.38 ± 0.10 for group C, p < 0.001, 1.38 ± 0.34 for CP group, p < 0.001, Fig. 4G), and Bax/Bcl-2 level decreased after treatment with P2X7R inhibitors (mPFC: Group C vs. CP group P, p < 0.001, Fig. 3G; spinal cord: group C vs. CP, p < 0.001, Fig. 4G), the contents of Cleaved caspase3 were consistent to the trend (mPFC: Cleaved caspase3: 0.14 ± 0.09 for V group, 0.14 ± 0.02 for P group, 0.57 ± 0.07 for C group, p < 0.001, 0.36 ± 0.07 for CP group, p < 0.001, group C vs. CP, p < 0.001, Fig. 3H; spinal cord: Cleaved caspase3: 0.13 ± 0.04 for V group, 0.13 ± 0.02 for P group, 0.59 ± 0.07 for C group, p < 0.001, 0.29 ± 0.04 for CP group, p < 0.001, group C vs. CP, p < 0.001, Fig. 4H).

P2X7R in microglia of the mPFC and spinal cord contributed to chronic pain but not in astrocytes

In order to explore whether P2X7R in microglia plays important roles in chronic pain, double P2X7R and microglial immunostaining of mPFC and spinal cord were performed 14 days after the establishment of CCI (Fig. 5A, 6A). The number of microglia expressed by P2X7R+ -Iba1+ in chronic pain rats was significantly higher than that in control rats (39.18 ± 2.28 for group V, 38.14 ± 5.95 for group P, 79.37 ± 8.35 for group C, p < 0.001, 44.95 ± 1.64 for CP group, p = 0.001, Fig. 5B; 3.42 ± 0.28 for group V, 3.46 ± 0.11 for group P, 8.61 ± 0.81 for group C, P < 0.001, 6.96 ± 1.01 for CP group, p < 0.001, Fig. 6B), moreover, P2X7R inhibitor treatment resulted in a decrease in the number of P2X7R+ -Iba1+ microglia in the chronic pain group of rats (group C vs. CP, p < 0.001, Fig. 5B; group C vs. CP, p = 0.034, Fig. 6B), suggesting that P2X7R in microglia mediates neuropathic pain.

However, we did not identify any P2X7R-positive cells that were also positive for the astrocytic marker GFAP in the samples, there was no significant difference between groups. (Fig. 5C, 6C) The variation trend of P2X7R was consistent with other experimental results. We further applied A-740003, the antagonist of P2X7R, by microinjection injection, and found it significantly decreased the content of P2X7R in the chronic group. This may confirm that P2X7R expressed in microglia plays a role in chronic pain, but P2X7R expressed in astrocytes does not.

Microglia autophagy in mPFC and spinal cord contributed to chronic pain but not in astrocytes

The results of a double P62 and microglial immunostaining of mPFC and spinal cord (Fig. 7A, 8A) show that P62 was predominantly co-localized with microglia. The number of microglia expressed by P62+ - Iba1+ in chronic pain rats was significantly higher than that in control rats (9.36 ± 1.73 for group V, 9.47 ± 3.12 for group P, 41.63 ± 3.80 for group C, p < 0.001, 34.85 ± 4.21 for CP group, P < 0.001, Fig. 7B; 2.91 ± 1.19 for group V, 3.16 ± 0.38 for group P, 9.72 ± 1.64 for group C, p < 0.001, 6.06 ± 1.20 for CP group, p < 0.001, Fig. 8B), moreover, P2X7R inhibitor treatment resulted in a decrease in the
number of P62*-Iba1+ microglia in the chronic pain group of rats (group C vs. CP, \( p = 0.001 \), Fig. 7B; group C vs. CP, \( p < 0.001 \), Fig. 8B), suggesting that autophagy in microglia mediates neuropathic pain. However, P62-positive cells that were also positive for the astrocytic marker GFAP in the samples (Fig. 7C, 8C), which was consistent with the trend of P2X7R*-Iba1+ cells. This also confirm that P62 expressed in microglia plays a role in chronic pain, but P62 expressed in astrocytes does not.

**Discussion**

It should be noted that neuropathic pain may cause the damage of tissue, central nerve fibers and cell, increase the probability of various neurodegenerative dysfunctions, such as chronic pain, sleep disorders, even mild cognitive impairment [16]. However, the mechanisms of neuropathic pain is unknown. The multiple neural circuits involved and the cause-and-effect mechanisms of chronic pain itself require further investigation.

In our experiment, we established CCI, a widely used model of peripheral nerve injury. We have analyzed pain-related behaviors by PWT and PWL[16], consistent with previous studies[17], a stable hypersensitivity to pain after CCI establishment existed here, in addition, we have demonstrated that P2X7 inhibition rescues this CCI-treated phenotype, which verified that increased levels of P2X7 have participated in rats of CCI. Previous results have shown that P2X7R plays a role in chronic pain [18], and changes in mPFC may be mediated through P2X7R in others experiments [19]. The dependence of the negative association between CCI-induced pain related behaviors and NP on the presence of P2X7 observed in this study suggests that P2X7 may be important and could function as a regulator of NP. Studies with animal models have suggested that neuropathic pain points to the structure and function of mPFC, which integrates the sensory, emotional and attentional components of pain perception, furthermore, spinal cord stimulation is reversible therapy for treatment of severe, otherwise nonresponsive chronic pain [20]. However, except for a few studies reporting the NP appeared after alterations of glutamatergic transmission in mPFC and spinal cord [21], little is known about the alterations in them and how they are affected. So this study was designed to observe changes in mPFC and spinal cord.

Additionally, this improved CCI-induced neuropathic pain in P2X7 inhibition animals, whether owing to an autophagy mechanism in mPFC and spinal cord, requires further proceeding. In the present study, we found that CCI increased P2X7 mRNA and protein expression in the mPFC and spinal cord of rat, demonstrating P2X7 may be an important participant in neuropathic pain. Recent studies have also demonstrated that high expression of P2X7R can be detected in mPFC and spinal dorsal horn cells. P2X7R is closely related to neuronal activation, neurosensitization, pain transmission and neuroinflammation [22]. Moreover, autophagy also plays a key role in the pathological progression of pain. Studies have shown that the possible mechanism of the abnormal pain persisted after nerve injury is that the P2X7R mediated the sustained release of injury-related molecules, further promotes the formation of autophagy, when P2X7R impaired, the production of P2X7-triggered neuronal autophagy were attenuated [23].In this study, we found that the levels of LC3II, beclin and the number of autophagy body in mPFC were significantly higher after after CCI, and P2X7 inhibition showed an opposite condition.
These results suggest that the autophagy activated by CCI was through P2X7, the increased fluorescence under the electronic microscope also enhanced credibility [24]. Autophagy is a lysosomal degradation pathway and a homoeostatic cellular mechanism that is essential for survival, differentiation, development and homoeostasis. Meanwhile, recent investigations showed that autophagy was involved in neuropathic pain processing [25]. As a result of the dysfunction of autophagy, increased markers of autophagy (LC3 and Beclin) are observed in the early stages of CCI, which represents the accumulation of dysfunctional autophagosomes. It has also been shown that P2X7, as a positive regulator of the autophagic process, could apparently increase autophagy in glial. These demonstrated that the attenuated neuropathic pain after CCI in rat after P2X7 inhibition are partly due to reductions in autophagy.

On a mechanistic ground, the CCI-induced autophagy has been demonstrated to involved modulation of apoptosis. In previous study, the accumulation of autophagy also could induced the increase of apoptosis [26, 27]. Adult apoptosis is a process that by which a cell ceases to grow and results in the controlled death of the cell [28]. Apoptosis is regulated by two large families of proteins, the caspase family and the Bcl-2 family. The ratio of Bcl-2/Bax is an indicator of cell susceptibility to apoptosis, with a decreasing ratio indicating increase in apoptosis [29]. The caspases are a family of protease enzymes. They are mainly classified into initiators such as caspase-9 and effectors such as caspase-3. In order to know whether the decrease apoptosis may result from the change of the Bcl-2/Bax ratio and caspase-3, we quantified the level of them. As expected, the ratio of Bcl-2/Bax was reduced and caspase-3 was significantly increased after CCI. In addition, all of changes were less severe in P2X7 inhibition mice. A correlation has been shown between NP and decreased plasma levels of Bcl-2/Bax ratio [30]. Moreover, P2X7 activation and an increase in the autophagy can reduce adult apoptosis and produce the pain. It has been proven that P2X7R existed in neurons, and its over-expression can cause neuronal damage. It is also found, after tissue damage, that P2X7R was highly expressed in neurons, up-regulated the expression levels of caspase-3 and increased neuronal apoptosis [31]. Application of P2X7 inhibitor can inhibit neurons apoptosis and reduce tissue damage [32]. When P2X7 was activated by CCI in our study, it could increase cell membrane permeability, and the production of P2X7-triggered inflammatory factors and apoptosis were increased to activate microglia [32], which result in pain induced by tissue injury. These suggested that apoptosis activated by P2X7 was implicated in CCI-induced autophagy.

Numerous studies have predominantly demonstrated a critical role of glia in the development of pain hypersensitivity in neuropathic pain [33]. During the process of NP, microglial cells become hypertrophied and have increased IBA-1 expression; while astrocytes become hypertrophied with an increased expression of GFAP for instance. The activated glial cells have been reported was activate by P2X7R, and released other various cytokines to sustain plasticity changes in neurons and the eventual development of NP [34]. In our study, the microglial and astroglial were significantly activated after CCI, which were reversed in P2X7 inhibition rats. After peripheral nerve injury, microglia cells activate and change their morphology, and neuropathic pain is caused by neuropathic active factors and cytokines released. But consistently with some previous studies [35], our results exhibited that P2X7R is predominantly localized to microglia throughout the CNS after CCI establishment, but not localized to astrocytes. The activation
of microglial plays a key role in the development and transmission of pain. Huang et al. reported that P2X7R is expressed mostly in microglia, but not in astrocytes [36, 37] and P2X7R is upregulated along with microglia activation by the development of pain in rats [37], induce abnormal neuronal activity, enhance synaptic transmission of nociceptive neurons, and produce abnormal pain [38], and antagonism of P2X7R can significantly attenuated pain and reduced the upregulation of P2X7R as well as microglia activity in rats [39]. On the other hand, over-expression of P2X7R by studying animal models induced NP can activate microglial, increase the levels Tumor necrosis factor-alpha (TNF-α) and IL-1β, and result in hyperalgesia and allodynia [40] [41]. These demonstrated that P2X7R expressed in microglia plays a crucial role in the development of NP.

Microglial autophagy is suggested to be involved in modulating homeostasis in the central nervous system, when it was activated, it could directly enhance the excitability of pain-transmitting neurons [42], and increased autophagy activity exerted a protective effect in NP via inflammasome inactivation [7]. Given the same trend of glial seen with neuropathic pain in the spinal cord vs. the mPFC, it is not surprising that our data showed P2X7R activation in microglia appears to play an important role in pain regulation in both the spinal cord and brain, through autophagy.

There are some limitations to our study. First, P2X7R inhibitor may represent a limitation because the half time of drugs. Second, we did not observe the damage effects of P2X7R on CCI-induced NP using agonist and we didn’t verify our research through systematical injection. In addition, only the most basic measurements of the autapaghy and apoptosis have been researched, and the downstream mechanism has not been further investigated. Finally, mPFC and spinal cord were investigated, but we did not study which was in the upstream.

In this study, we found a pronounced increase of P2X7R expression in rats of CCI, which confirming a possible correlation with CCI-induced NP. Conversely, P2X7R inhibition can counteract the CCI-induced NP due to autophagy and apoptosis via a modulation of microglia in mPFC and spinal cord. This may provide an importantly neuroprotective mechanism for the improved NP and also help devising new therapeutic to improve chronic pain in patients.

**Abbreviations**

NP
Neuropathic pain; mPFC:medial prefrontal cortex; IL-1β:Interleukin-1β; CCI:Chronic-Constriction Injury; Veh:vehicle group; C group:CCI group; P group:P2X7 inhibitor group; CP group:CCI + P2X7R inhibition group; PWT:paw-withdrawal threshold; PWL:paw-withdrawal latency; BCA:bicinchoninic acid; ECL:enhanced chemiluminescence; TEM:transmission electron microscopy; IBA-1:ionized calcium binding adaptor molecule 1; GFAP:glial acidic fibrillary protein; SEM:standard error of the mean; ANOVA:one-way analysis of variance; TNF-α:Tumor necrosis factor-alpha

**Declarations**
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Authors’ contributions

QZ and YH performed most of the experiments and wrote the manuscript. CC and ZZ were responsible for the original design and providing key revisions, which are important for the content. YH completed behavioral Assays, data management and statistical analysis. TL,PF and JC participated in the experimental design and coordination. All authors contributed and approved the final version of the manuscript.

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Availability of data and materials

The data supporting the findings of this study are included within the article.

Ethics approval

All animal protocols were reviewed and approved by the Animal Ethics Committee of the Zhongnan Hospital and Research Center in Hubei, China, and all animal experiments complied with the ARRIVE guidelines and was carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Figures

Figure 1

Inhibition of P2X7R can increase thermal and mechanical pain thresholds in rats with chronic neuropathic pain. All groups of rats were tested in paw-withdrawal threshold (PWT) (a) and paw-withdrawal latency (PWL) (b) after surgery (n = 12/subgroup) within 3 consecutive days. The data were analyzed using two-way ANOVAs used Tukey's multiple comparisons test. Values are presented as the means ± SD. Significant differences are expressed as follows: * p < 0.05 vs. Veh group, # p < 0.05 vs. CP group for rats in CCI group. C = CCI, P= P2X7R Inhibition, CP= CCI&P2X7R Inhibition.
Figure 2

Chronic neuropathic pain was partially dependent on P2X7R in mPFC and spinal cord. The intensity of P2X7R in the mPFC(a,b,c) and spinal cord(d,e,f) of the rats were determined at 16 days after CCI surgery (n = 4/subgroup). a,d. Western blotting of P2X7 protein from rats in the mPFC and spinal cord, respectively. b,e. β-actin was used as a loading control and the graphs represent the mean ± SD in Western blot. c,f. β-actin was used as a loading control and the graphs represent the mean ± SD in PCR. The data were analyzed using two-way ANOVAs used Tukey’s multiple comparisons test. Values are presented as the means ± SD. Significant differences are expressed as follows: * p < 0.05 vs. Veh group, # p < 0.05 vs. CP group for rats in CCI group. C = CCI, P= P2X7R Inhibition, CP= CCI & P2X7R Inhibition.
Figure 3

P2X7 elimination relieved chronic neuropathic pain through decreasing autophagy and apoptosis in mPFC of rats. a. Representative images of autophagosomes under transmission microscope in mPFC region of rats. Scale bar = 500 nm. b. Quantitative analysis of the number of autophagosomes. c-e. Western blot analysis of autophagy-related proteins Beclin-1 and LC3, β-actin was used as an internal control. f-h. Western blot analysis of Bcl-2, Bax and cleaved-caspase-3 in vivo experiment, β-actin was used as an internal control (n = 4/subgroup). Values are presented as the means ± SD. Significant differences are expressed as follows: * p < 0.05 vs. Veh group, # p < 0.05 vs. CP group for rats in CCI group. C = CCI, P= P2X7R Inhibition, CP= CCI& P2X7R Inhibition.
Figure 4

P2X7 elimination relieved chronic neuropathic pain through decreasing autophagy and apoptosis in spinal cord of rats. a. Representative images of autophagosomes under transmission microscope in spinal cord of rats. Scale bar = 500 nm. b. Quantitative analysis of the number of autophagosomes. c-e. Western blot analysis of autophagy-related proteins Beclin-1 and LC3, β-actin was used as an internal control. f-h. Western blot analysis of Bcl-2, Bax and cleaved-caspase-3 in vivo experiment, β-actin was used as an internal control (n = 4/subgroup). Values are presented as the means ± SD. Significant differences are expressed as follows: * p < 0.05 vs. Veh group, # p < 0.05 vs. CP group for rats in CCI group. C = CCI, P= P2X7R Inhibition, CP= CCI & P2X7R Inhibition.
P2X7R in microglia of the brain contributed to chronic pain but not in astrocytes. P2X7R was predominantly co-localized with Iba1 (a microglial marker), but not with GFAP (astrocytic marker) in mPFC. 

- **Figure 5**

  a. Immunofluorescence staining of mPFC sections in microglial cells from rats. P2X7R (green), Iba1 (red).  
  b. Quantitative analysis of P2X7R+ and Iba1+ positive cells.  
  c. Immunofluorescence staining of mPFC sections in microglial cells from rats. P2X7R (green), GFAP (red).  
  d. Quantitative analysis of P2X7R+ and GFAP+ positive cells. Original magnification: × 100; Scale bar = 50 μm. Values are presented...
as the means ± SD. Significant differences are expressed as follows: * p < 0.05 vs. Veh group, # p < 0.05 vs. CP group for rats in CCI group. C = CCI, P= P2X7R Inhibition, CP= CCI& P2X7R Inhibition.

Figure 6

P2X7R in microglia of the spinal cord contributed to chronic pain but not in astrocytes P2X7R was predominantly co-localized with Iba1 (a microglial marker), but not with GFAP (astrocytic marker) in spinal cord. a. Immunofluorescence staining of spinal cord sections in microglial cells from rats. P2X7R (green), Iba1 (red). b. Quantitative analysis of P2X7R+ and Iba1+ positive cells. c. Immunofluorescence
staining of spinal cord sections in microglial cells from rats. P2X7R (green), GFAP (red). d. Quantitative analysis of P2X7R+ and GFAP+ positive cells. Original magnification: × 100; Scale bar = 50 μm. Values are presented as the means ± SD. Significant differences are expressed as follows: * p < 0.05 vs. Veh group, # p < 0.05 vs. CP group for rats in CCI group. C = CCI, P= P2X7R Inhibition, CP= CCI& P2X7R Inhibition.

Figure 7
Microglia autophagy in mPFC contributed to chronic pain but not in astrocytes. P62 was predominantly co-localized with Iba1 (a microglial marker), but not with GFAP (astrocytic marker) in mPFC. a. Immunofluorescence staining of mPFC sections in microglial cells from rats. P62 (green), Iba1 (red). b. Quantitative analysis of P62+ and Iba1+ positive cells. c. Immunofluorescence staining of mPFC sections in microglial cells from rats. P62 (green), GFAP (red). d. Quantitative analysis of P62+ and GFAP+ positive cells. Original magnification: × 100; Scale bar = 50 μm . Values are presented as the means ± SD. Significant differences are expressed as follows: * p < 0.05 vs. Veh group, # p < 0.05 vs. CP group for rats in CCI group. C = CCI, P= P2X7R Inhibition, CP= CCI& P2X7R Inhibition.

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