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Protective effects of palmatine on substance P-induced pyroptosis via down-regulation of P2X4 receptor in neuron-like PC12 cells

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ABSTRACT: Palmatine has a wide range of medical applications due to its anti-inflammatory, antiviral, and neuroprotective effects. The P2X4 receptor is a potential therapeutic target for chronic neuropathic pain. This study aimed to investigate whether palmatine could prevent pyroptosis induced by substance P (SP) in rat pheochromocytoma-derived PC12 cells through P2X4 receptor. The PC12 cells were cultured with a medium containing SP in the presence or absence of palmatine. Subsequently, the cytotoxicity and protective effects of palmatine were assessed using CCK-8 assays. Expression of the P2X4 receptor in PC12 cells was determined by Western blot analysis, RT-PCR, and immunofluorescence staining. The analyses showed that P2X4 expression was up-regulated in PC12 cells cultured with SP and co-localized with brain-derived neurotrophic factor (BDNF). The enhanced P2X4 expression in PC12 cells may lead to a release of neuronal BDNF, which cannot protect the PC12 cells from SP-induced damage but promotes damage. Furthermore, our work indicated that SP-induced cell pyroptosis was via the up-regulation of NLRP3, caspase-1, TNF-α, IL-1β, IL-18, and intracellular free Ca^{2+} levels. These detrimental effects were ameliorated by treatment with either palmatine or P2X4 shRNA. These findings suggest that palmatine may be an effective drug candidate for the treatment of chronic pain since it can protect PC12 cells from SP-induced pyroptosis by inhibiting the expression of P2X4 receptor.

KEYWORDS: palmatine, substance P, PC12 cells, P2X4 receptor, pyroptosis

INTRODUCTION

Adenosine Triphosphate (ATP) plays an important role in chronic peripheral neuropathic pain (NPP) by activating purinergic receptors, including cation-permeable ion channels (P2X receptors) and G-protein-coupled receptors (P2Y receptors) [1]. P2X receptors contain 7 subtypes (P2X1-7), and the P2X4 receptor is closely related to the generation and transmission of pain signals [2]. Activation of P2X4 receptors can induce and aggravate pain by activating relevant nerve cells, increasing the transmission of sensory information, and sensitizing the central nervous system [3].

Brain-derived neurotrophic factor (BDNF) and its high-affinity receptor TrkB (tyrosine kinase receptor B) are important regulators of neuronal development, synaptic transmission, and synaptic plasticity and thereby are essential for neural maintenance and repair [4]. However, studies have shown that BDNF plays an important role in the development of NPP [5]. P2X4 receptors and BDNF/TrkB-related signaling pathways have been reported to be involved in abnormal trigeminal pain induced by repeated durable stimulation [6]. Pyroptosis, a form of programmed necrosis discovered in recent years, is characterized by cell lysis and release of cell contents, which is mediated by inflammatory caspase [7]. Pyroptosis is often aggravated in chronic pain such as diabetic peripheral neuropathy [8]. Previous experiments in our lab found that P2X4 receptor could induce pyroptosis in hippocampus of rats with chronic pain and depression comorbidity [9].

Palmatine, a natural isoquinoline alkaloid, has a wide range of medical applications due to its anti-inflammatory, antiviral, and neuroprotective effects [10]. Pre-laboratory studies showed that palmatine could alleviate trigeminal neuralgia (TN) by reducing BDNF and TrkB receptor expression in trigeminal ganglion of TN rats [11]. However, the molecular mechanism of pharmacological action of palmatine for the treatment of chronic pain remains to be elucidated. Substance P (SP) has long been considered an important effector of pain [12]. The rat pheochromocytoma cell line (PC12 cells) has been widely used as a cell model for research of neurological diseases [13]. The current study was designed to investigate the potential protective effects of palmatine on SP-induced neuronal cell damage in PC12 cells and elucidate the underlying neuroprotective mechanism.

MATERIALS AND METHODS

Cell culture and treatment

The PC12 cells used in the experiments were highly differentiated cell line from rat adrenal medullary pheochromocytoma (purchased from Procell Life Science & Technology Co., Ltd., China). PC12 cells were cultured in RPMI 1640 medium (Biological Industries, USA) supplemented with 10% fetal bovine serum (FBS; Biological Industries) and 1% penicillin/streptomycin. PC12 cells were incubated at 37 °C in a 95% humidified incubator with 5% CO_2 [13, 14]. The number of cell passages used for the experiments was less than 15.

In the cell injury assay, PC12 cells were randomly divided into 6 groups as follows: Control group (Ctrl), Control + palmatine group (Palmatine), Control + Substance P group (SP), SP + palmatine group, SP + P2X4 shRNA group, and SP + Scramble shRNA group. SP was obtained from Sigma (GLPBIO, USA), while palmatine was supplied by Chengdu DST Biological Technology (China). Both SP and palmatine were dissolved in phosphate-buffered saline (PBS; Beijing Fir Golden Bridge Biotechnology Co., Ltd, China). P2X4 was knocked down using short hairpin RNA (shRNA) using a TransIntro® EL transfection reagent system (TransGen, China). The sequences of P2X4 shRNAs are shown in the Table S1.

Cell viability assay

The viability and proliferation of PC12 cells were quantified using the CCK-8 (Cell Counting Kit-8) cell counting kit (GLPBIO) [15]. To examine the neuroprotective effect of palmatine on SP-induced cell injury, PC12 cells were treated with the indicated concentrations of SP in the presence of palmatine for 24 h, followed by CCK-8 assessment. For the assay, three independent experiments were performed.

Enzyme-linked immunosorbent assay

The production of inflammatory factors (IL-1β, IL-18, and TNF-α) in the supernatant of PC12 cells in each group was detected by ELISA kits (Shenke Experimental Technology Co., Ltd., China) [15].

Real-time quantitative polymerase chain reaction

Total RNA was extracted from PC12 cell cultures by using TransZol Up Plus RNA Kit (Beijing TransGen Biotech Co., Ltd., China) consisting of lysis buffer and washing buffers (Transzol up and CB9/WB9). Extracted RNA was converted to cDNA using EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (Beijing TransGen Biotech Co., Ltd.). RT-PCR was conducted using TB Green® Premix Ex Taq™ in an CFX96 Real-Time PCR Detection System (Bio-Rad, USA) [15].

Western blot analysis

PC12 cells were collected in EP tubes, and appropriate RIPA lysis solution was added according to the number of cells. The immunoreactive bands were visualized for the labeled proteins using enhanced chemiluminescence kit on the Bio-Rad system. Densitometry analysis was performed using Image-Pro Plus software [15].

Immunofluorescence

Immunofluorescence labeling was conducted to identify P2X4 and BDNF expression in PC12 cells. Images were obtained using a confocal laser scanning microscope (Leica, Germany), and immunofluorescence intensity was analyzed using Image-Pro Plus software.

Detection of intracellular free Ca^{2+} concentration

PC12 cells were uniformly seeded into 96-well plates for transfection and drug treatment for 24 h. The cells were washed 3 times with Hanks balanced salt solution (HBSS), and the 800-time diluted BB Cell Probe®F03 solution was added to cover the cells. Then, the cells were incubated at 37 °C for 30 min and washed twice with HBSS, resuspended in HBSS, and incubated for another 30 min at 37 °C. The detection of fluorescent calcium ion was recorded through ratio fluorometry at an excitation wavelength of 488 nm and an emission wavelength of 516 nm [16].

Statistical analysis

All results were expressed as the mean ± SD, and statistical analyses were performed using SPSS 21.0 software. Differences between treatment groups were analyzed by ANOVA, followed by Dunnett's post hoc test for multiple comparisons. p-values < 0.05 were statistically significant.

RESULTS

Effects of palmatine on viability and inflammatory factors of PC12 cells cultured with SP

The cell viability of PC12 cells treated with SP for 24 h was decreased in dose-dependent manner (0.1–1,000 nM) (Fig. 1A). Meanwhile, to avoid possible adverse effects of palmatine *per se* on PC12 cells, cells were incubated with 1–100 μM of palmatine for 24 h, followed by viability assay. The results showed that there was almost no cytotoxicity when palmatine at 50 μM or lower concentration was applied to PC12 cells (Fig. 1B). SP of 100 nM and palmatine at 50 μM were thus selected as the modeling concentrations.

SP significantly increased the generation of TNF-α mRNA, while pretreatment of PC12 with palmatine (1–100 μM) markedly inhibited SP-induced increase in TNF-α mRNA. No significant rise of SP-induced TNF-α mRNA production was seen when the concentration of palmatine treatment was 50 and 100 μM (Fig. 1C). Compared with the Ctrl group, the cell viability of SP

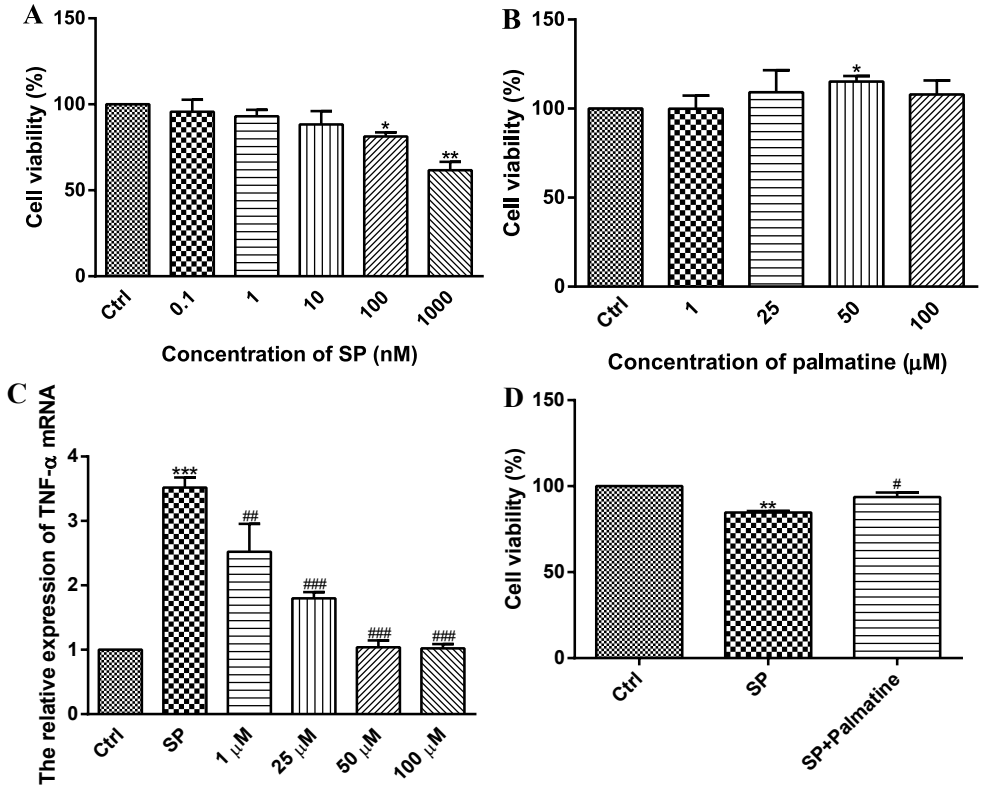

Fig. 1 Effects of palmatine on PC12 cells cultured with SP. Effects of (A) SP and (B) palmatine on the viability of PC12 cells. (C) Effects of varying concentrations of palmatine on the expression level of TNF-α mRNA in PC12 cells incubated with 100 nM SP. (D) Effect of 50 μM palmatine on the viability of PC12 cells cultured with 100 nM SP. The results are presented as the mean ± SD, $n = 6$ in each group. $^{*}p < 0.05$ vs. Ctrl group; $^{**}p < 0.01$ vs. Ctrl group; $^{***}p < 0.001$ vs. Ctrl group; $^{\#}p < 0.05$ vs. SP group; $^{\#\#}p < 0.01$ vs. SP group; and $^{\#\#\#}p < 0.001$ vs. SP group.

group (100 nM) was decreased, and the treatment with 50 μM palmatine could effectively protect the cells from SP-induced damage (Fig. 1D).

Screening of the optimal interference sequence of shRNA against P2X4 receptor

Real-time PCR was used to detect the relative expression of P2X4 mRNA in PC12 cells under various conditions (Control group, Scramble shRNA group, and P2X4 shRNA-1, -2, and -3 groups). The results showed that the interference efficiency of the second P2X4 shRNA sequence was the best (Fig. 2A).

Effects of palmatine on P2X4-BDNF signaling pathway in SP-induced PC12 cell injury model

Results from RT-PCR and Western blot analysis found that the mRNA and protein expression levels of P2X4, BDNF, and TrkB in the SP group were significantly higher than those in the Ctrl group ($p < 0.001$). After treatment with palmatine and P2X4 shRNA, the mRNA and protein expression levels of P2X4, BDNF, and TrkB in the SP + palmatine and SP + P2X4 shRNA groups were significantly decreased ($p < 0.001$). By contrast, there was no significant difference between the SP group and SP + Scramble shRNA groups (Figs. 2 and 3).

Furthermore, the expression levels of P2X4 and BDNF in PC12 cells were detected using an immunofluorescence labeling assay. The results showed that the expression levels of P2X4 and BDNF in the SP were significantly higher than those in the Ctrl group ($p < 0.001$). After administration of palmatine and P2X4 shRNA, P2X4 and BDNF co-expression levels in the SP + palmatine and SP + P2X4 shRNA groups were significantly reduced ($p < 0.001$); however, there was no significant difference in the co-expression levels of P2X4 and BDNF between SP + Scramble shRNA group and SP group ($p > 0.05$) (Fig. 4).

Moreover, we conducted an ELISA assay to determine TNF-α expression in PC12 cells. TNF-α expression levels in the SP and SP + Scramble shRNA groups were higher than those in the Ctrl group ($p < 0.01$). TNF-α expression levels in the palmatine and P2X4 shRNA treatment groups were significantly lower than those in the SP and SP + Scramble shRNA groups ($p < 0.05$) (Fig. 5F).

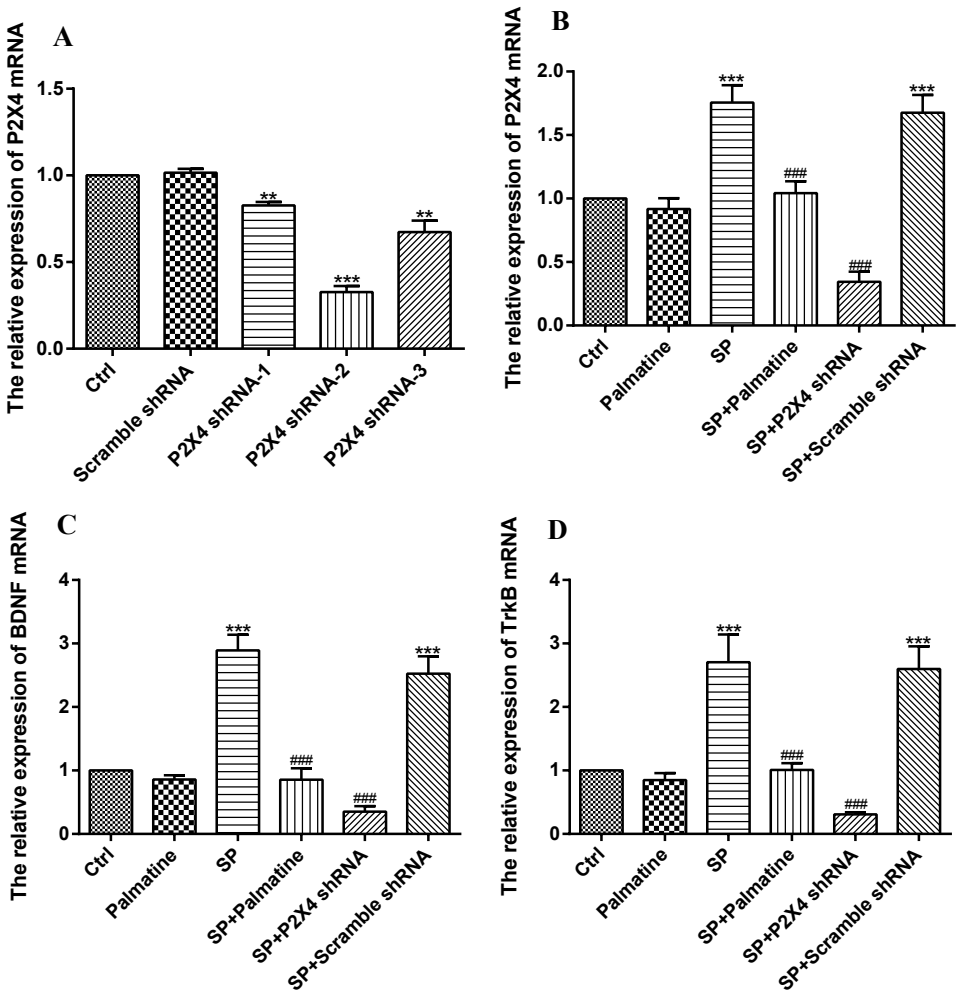

Fig. 2 Effects of palmatine, SP, and P2X4 shRNA treatments on P2X4, BDNF, and TrkB mRNA expression. (A) Effects of different P2X4 shRNA sequences on P2X4 mRNA in PC12 cells. Expression levels of mRNA of (B) P2X4, (C) BDNF, and (D) TrkB in PC12 cells of each treatment group. Data were expressed as mean ± SD, $n = 3$ in each group. ** $p < 0.01$ vs. Ctrl group; *** $p < 0.001$ vs. Ctrl group; and ### $p < 0.001$ vs. SP group.

The above results revealed that palmatine decreased the expression of P2X4, BDNF, TrkB, and TNF-α in SP-induced PC12 cells.

Effects of palmatine on NLRP3 / caspase-1 / pyroptosis signaling pathway in SP-induced PC12 cell pain model

NLRP3 inflammasome plays a key role in pyroptosis. The protein expression levels of pyroptosis related molecules (caspase-1, NLRP3, GSDMD, and ASC) were significantly elevated in the SP-induced pain model of PC12 cells, while the levels of the above proteins were reduced after the application of palmatine and P2X4 shRNA treatment (Fig. 5A–D). The increases of IL-1β and IL-18, the pyroptosis-associated proinflammatory cytokines induced by SP, were down-regulated by palmatine and P2X4 shRNA treatments (Fig. 5E,G). These results suggest that palmatine attenuates SP-induced pyroptosis, which may also involve P2X4 receptors.

Palmatine inhibiting SP-induced up-regulation of p38 MAPK phosphorylation and Ca^{2+} signal in PC12 cells

There was no significant difference in the total expression of p38 MAPK in PC12 cells among all groups ($p > 0.05$). However, the expression of p–p38 MAPK protein in SP and SP + Scramble shRNA groups was significantly higher than that in Ctrl group ($p < 0.001$). Furthermore, the expression levels of p–p38 MAPK protein in SP + palmatine and SP + P2X4 shRNA groups were decreased compared with SP group (Fig. 6A–C). These findings indicate that p–p38 MAPK is associated with

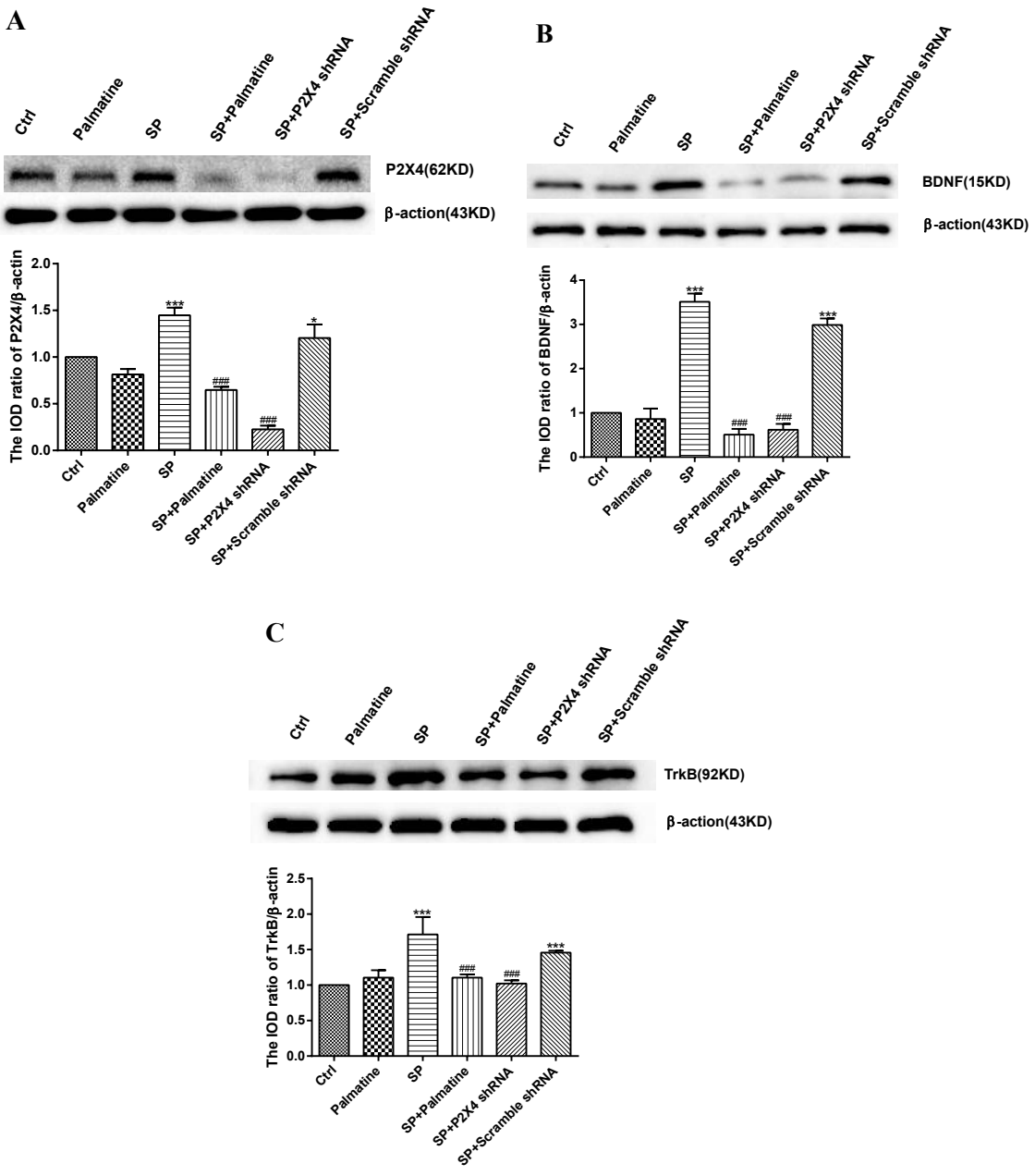

Fig. 3 Effects of palmatine, SP, and P2X4 shRNA treatments on P2X4, BDNF, and TrkB protein expression. Protein expression of (A) P2X4, (B) BDNF, and (C) TrkB in PC12 cells of each group detected by Western blot analysis. Data were expressed as mean ± SD, $n = 3$ in each group. * $p < 0.05$ vs. Ctrl group; *** $p < 0.001$ vs. Ctrl group; and $^{\#\#\#}$ $p < 0.001$ vs. SP group.

P2X4 receptor-mediated SP-induced damage in PC12 cells, which could be suppressed by palmatine.

The BBcellProbeTM F3 fluorescence probe was used to determine the palmatine effects on the Ca^{2+} signal in PC12 cells under the SP challenge. The results found an increase in Ca^{2+} signal in the SP and SP + Scramble shRNA groups compared with that in the Ctrl group, while the levels of Ca^{2+} signal were reduced after the application of palmatine and P2X4 shRNA treatment (Fig. 6D).

DISCUSSION

Activation of P2X4 receptor can induce and aggravate neuropathic pain by activating related cells such as microglia, increasing the transmission of sensory information, and sensitizing the central nervous sys-

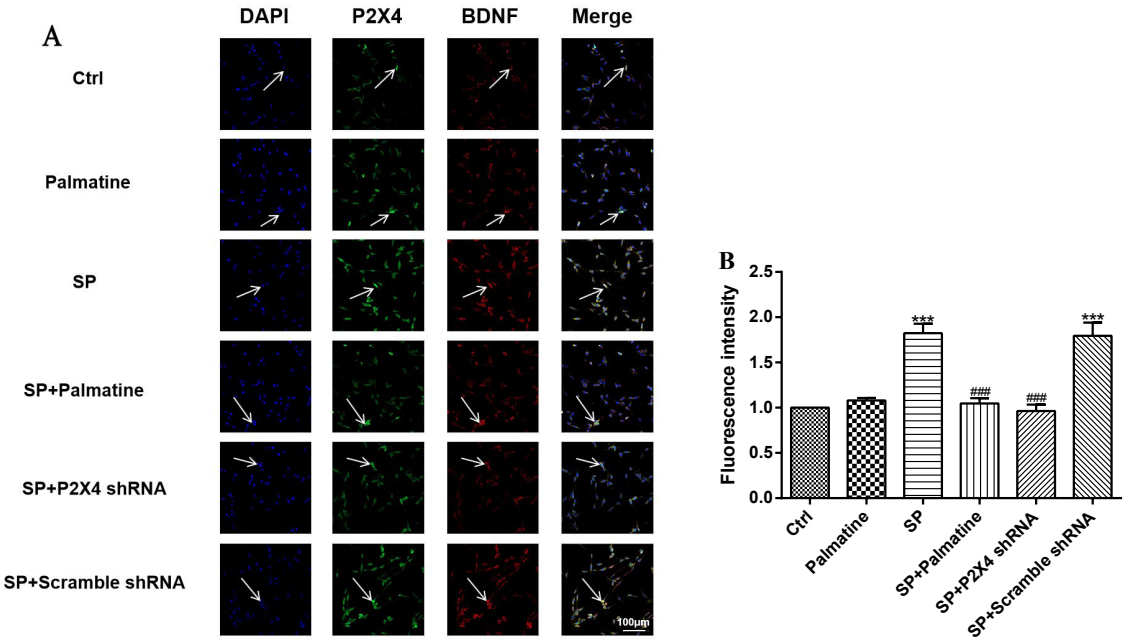

Fig. 4 The expression of P2X4 and BDNF in PC12 cells detected by immunofluorescence labeling assay. (A) The expression of P2X4 and BDNF shown in each group. The blue signal indicates nuclear staining with DAPI. The green signal represents P2X4 staining with FITC, and the red signal indicates BDNF staining with TRITC. The arrows indicate the immunostaining cells. (B) P2X4 and BDNF expression levels in the different groups of experiment. The fluorescence intensity of P2X4 and BDNF was increased in the SP group, whereas palmatine and P2X4 shRNA treatment decreased the expression level. Data were expressed as mean ± SD, $n = 3$ in each group. *** $p < 0.001$ vs. Ctrl group and $^{\#\#\#}$ $p < 0.001$ vs. SP group. Scale bar: 100 μm.

tem [16]. P2X4 receptors are also expressed in satellite glial cells, and overexpression of P2X4 receptors can significantly activate cellular pyroptosis in the stellate sympathetic ganglia and production of inflammatory factor levels in obese rats [17]. In addition, P2X4 expression was also found in neurons [18]. In neuropathic pain, however, it is unclear whether elevated expression of P2X4 through activation of neurons can directly promote pain transmission. In this study, results of RT-PCR and Western blot analysis showed that the expression of both mRNA and protein of P2X4 receptor was up-regulated in PC12 neuron-like cells cultured with SP. Meanwhile, the cell viability of PC12 cells treated with SP for 24 h was decreased in a concentration dependent manner. Importantly, the SP-induced damage of cell viability could be inhibited with the treatment by palmatine or P2X4 shRNA. These findings suggest that P2X4 receptors are involved in SP-induced damage in PC12 cells and palmatine can protect PC12 cells from such injury by down-regulating the expression of P2X4 receptors.

BDNF is a neurotrophic factor involved in the pathological development of a variety of neuronal diseases [19]. BDNF/TrkB has been shown to be associated with neuropathic pain (NPP) and is a potential therapeutic target for NPP [20]. In the central spinal cord, intrathecal injection of recombinant BDNF can directly induce persistent hyperalgesia, but reduction or inhibition of BDNF expression can reduce the degree of NPP [5, 21]. Thus, persistently elevated BDNF levels may be closely associated with pain transmission rather than nerve repair during NPP.

In neuropathic pain, P2X4 is believed to mediate the cellular release of BDNF, which triggers hyperalgesia [22]. In this study, the expressions of P2X4 and BDNF/TrkB were up-regulated in PC12 cells cultured with SP. The enhanced P2X4 expression in PC12 cells may lead to release of neuronal BDNF, which cannot protect the PC12 cells from SP-induced damage but promotes damage. Furthermore, application of P2X4 shRNA or palmatine significantly reduced elevation of mRNA, protein, and fluorescent staining of BDNF in SP-induced PC12 cells. Therefore, we speculate that P2X4 may be involved in the terminal release of BDNF from damaged neurons under inflammatory or neurological injury conditions. Additional studies have found that the high expression of P2X4 receptor in neurons can induce the increase of neuronal Ca^{2+} influx, up-regulate the expression level of BDNF, and promote inflammatory pain [23]. Consistent with previous findings, the SP-induced pain model in neuronal PC12 cells showed elevated P2X4 expression, increased intracellular Ca^{2+}

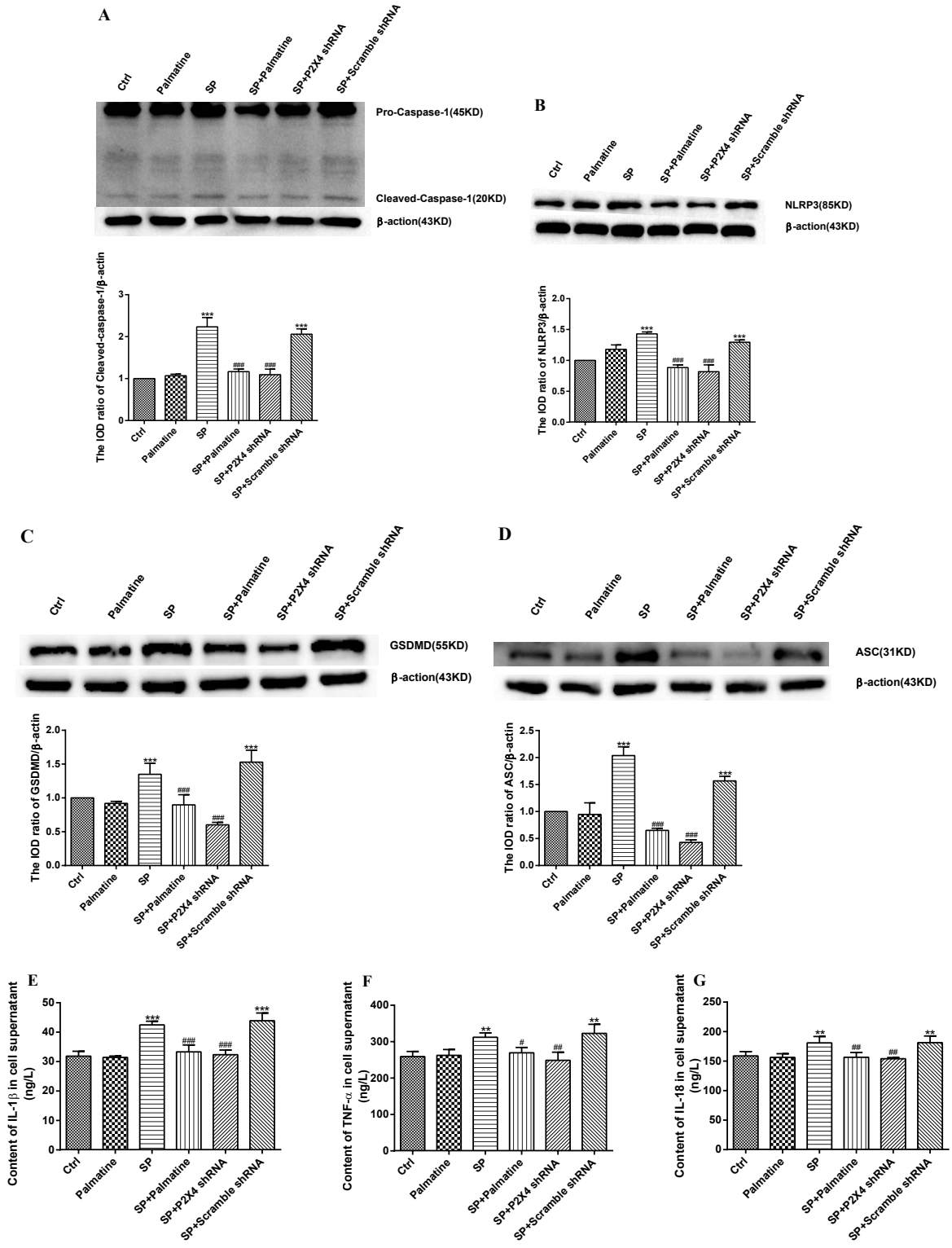

Fig. 5 Palmatine inhibiting pyroptosis of SP-induced PC12 cells. (A) Caspase-1, (B) NLRP3, (C) GSDMD, and (D) ASC protein expression levels in PC12 cells of each group detected using Western blot analysis. Concentrations of (E) IL-1β, (F) TNF-α, and (G) IL-18 in PC12 cells from each group determined by ELISA. Data were expressed as mean ± SD, $n = 3$ in each group. ** $p < 0.05$ vs. Ctrl group; *** $p < 0.001$ vs. Ctrl group; $^{\#}$ $p < 0.05$ vs. SP group; $^{\#\#}$ $p < 0.01$ vs. SP group; and $^{\#\#\#}$ $p < 0.001$ vs. SP group.

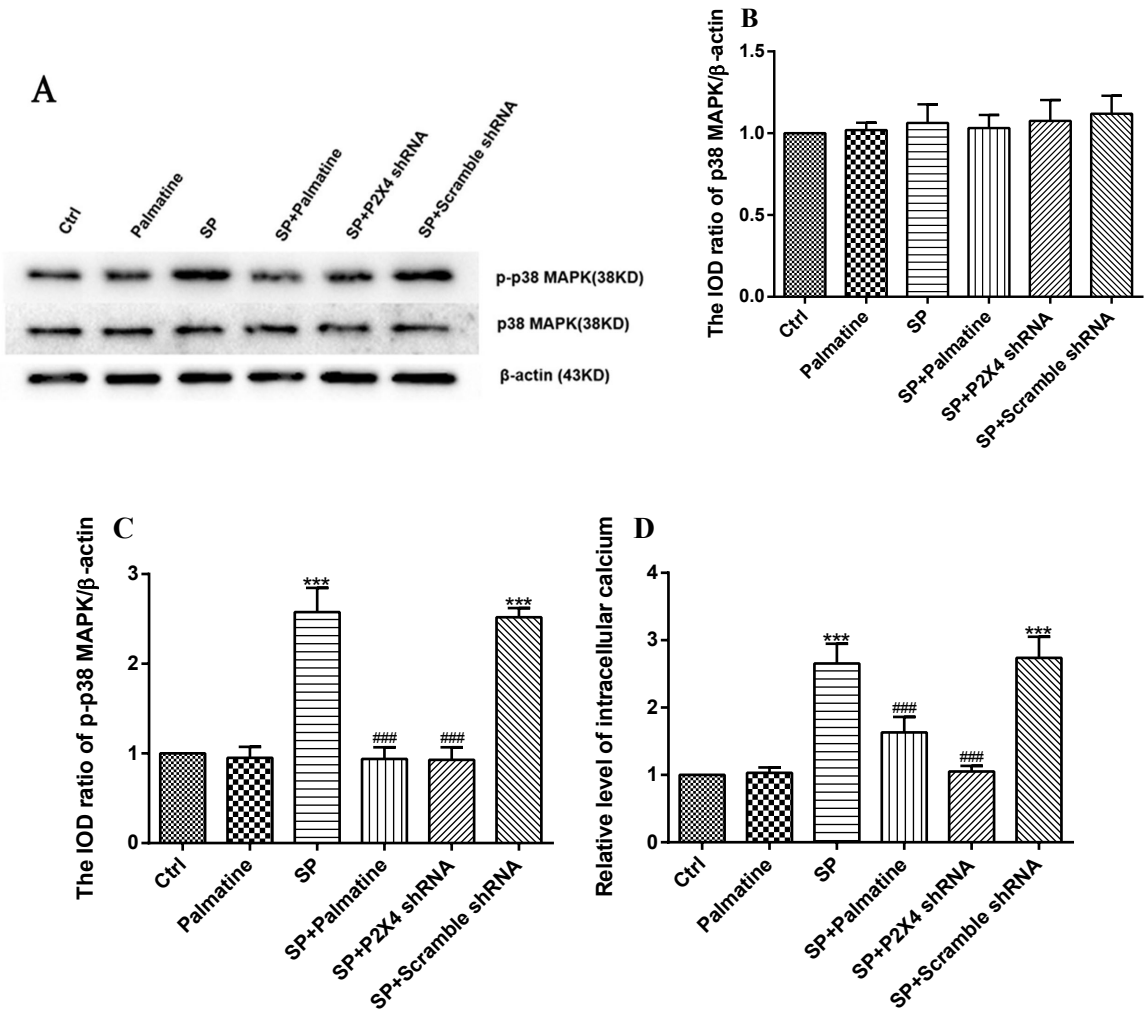

Fig. 6 Effects of palmatine on phosphorylation of (A–C) p38 MAPK and (D) Ca^{2+} signal in SP-induced PC12 cells. Data are expressed as mean ± SD, $n = 3$ in each group. *** $p < 0.001$ vs. Ctrl group and ### $p < 0.001$ vs. SP group.

level, and up-regulated production of inflammatory factors.

Palmatine has a wide range of pharmacological effects, which are closely related to its effects on the induction of programmed cell death. Study shows that palmatine selectively induces mitochondrial autophagy and restores mitochondrial function in an Alzheimer's disease mouse model, thereby improving cognitive function in mice [24]. It has also been reported that palmatine further alleviates LPS-induced depression-like behavior by improving apoptosis and oxidative stress [25]. However, the role of palmatine in relation to cellular pyroptosis has rarely been reported. This study also investigated the effect of palmatine on pyroptosis-implicated signaling events. The results showed that SP could induce pyroptosis of PC12 cells through activating NLRP3/caspase-1 signaling pathway since the expression levels of relevant proteins including NLRP3, caspase-1, GSDMD, and ASC were significantly increased in these cells. Such SP-evoked changes in the pyroptosis-related NLRP3/caspase-1 pathway could be effectively reversed by treatment with palmatine. It is well known that caspase-1 is activated by the NLRP3 inflammasome, resulting in the releases of proinflammatory factors IL-1β and IL-18 [26]. We found that SP could significantly raise the production of IL-1β and IL-18 in PC12 cells, which was also inhibited by treatment with palmatine. These results indicated that palmatine could alleviate pyroptosis of PC12 cells induced by SP. Therefore, it is hypothesized that palmatine can improve pyroptosis in neuropathic pain. Further experimental results showed that pyroptosis of PC12 cells induced by SP could be also inhibited by transfected P2X4 shRNA.

This supports the notion that palmatine may inhibit the activation of P2X4 receptor-NLRP3/caspase-1 signaling pathway in PC12 cell pain model, reducing the release of inflammatory cytokines (IL-1β and IL-18) and alleviating the pyroptosis injury caused by SP.

In this experiment, we established a cellular pain model by co-culturing PC12 cells with SP and investigated the effect and mechanism of palmatine on the expression of P2X4 receptors in the cellular pain model. However, to provide more comprehensive evidence for our conclusions and to demonstrate the direct effect of palmatine through P2X4 receptors, further experiments involving *in vitro* co-culture of primary neurons and glial cells as well as establishing an NPP animal model are necessary in future studies. These additional experiments will enable us to observe the precise molecular mechanism underlying palmatine interaction with P2X4 receptors from both cellular and animal perspectives, thereby enhancing our understanding of the pathomechanisms involved in chronic pain.

CONCLUSION

The results of this study demonstrate SP-induced upregulation of P2X4 receptor expression in PC12 cells and the release of neuronal BDNF, which may lead to cell damage during neuropathic pain and serve as a new potential therapeutic target in chronic pain. Palmatine protects PC12 cells from SP-induced injury by reversing the increased expression of P2X4 receptors. The underlying molecular mechanism may be related to the inhibition of BDNF/TrkB receptor and P38 MAPK phosphorylation, attenuation of calcium signaling, and blockade of NLRP3/caspase-1 mediated pyroptosis. Our work indicates that palmatine may be an effective drug candidate for the treatment of chronic pain.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2024.063.

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REFERENCES

1. Burnstock G (2007) Physiology and pathophysiology of purinergic neurotransmission. *Physiol Rev* **87**, 659–797.
2. Illes P, Müller CE, Jacobson KA, Grutter T, Nicke A, Fountain SJ, Kennedy C, Schmalzing G, et al (2021) Update of P2X receptor properties and their pharmacology: IUPHAR review 30. *Br J Pharmacol* **178**, 489–514.
3. Cheng R-d, Ren J-j, Zhang Y-y, Ye X-m (2014) P2X4 receptors expressed on microglial cells in post-ischemic inflammation of brain ischemic injury. *Neurochem Int* **67**, 9–13.
4. Zhou W, Xie Z, Li C, Xing Z, Xie S, Li M, Yao J (2021) Driving effect of BDNF in the spinal dorsal horn on neuropathic pain. *Neurosci Lett* **756**, 135965.
5. Vanelderen P, Rouwette T, Kozicz T, Roubos E, Van Zundert J, Heylen R, Vissers K (2010) The role of brain-derived neurotrophic factor in different animal models of neuropathic pain. *Eur J Pain* **14**, 471–479.
6. Liu C, Zhang Y, Liu Q, Jiang L, Li M, Wang S, Long T, He W, et al (2018) P2X4-receptor participates in EAAT3 regulation via BDNF-TrkB signaling in a model of trigeminal allodynia. *Mol Pain* **14**, 1744806918795930.
7. Jorgensen I, Miao EA (2015) Pyroptotic cell death defends against intracellular pathogens. *Immunol Rev* **265**, 130–142.
8. Cheng YC, Chu LW, Chen JY, Hsieh SL, Chang YC, Dai ZK, Wu BN (2020) Loganin attenuates high glucose-induced Schwann cells pyroptosis by inhibiting ROS generation and NLRP3 inflammasome activation. *Cells* **9**, 1948.
9. Yang RN, Yang JJ, Li ZJ, Su RC, Zou LF, Li L, Xu XM, Li GL, et al (2022) Pinocembrin inhibits P2X4 receptor-mediated pyroptosis in hippocampus to alleviate the behaviours of chronic pain and depression comorbidity in rats. *Mol Neurobiol* **59**, 7119–7133.
10. Long J, Song J, Zhong L, Liao Y, Liu L, Li X (2019) Palmatine: A review of its pharmacology, toxicity and pharmacokinetics. *Biochimie* **162**, 176–184.
11. Liu L, He L, Yin C, Huang R, Shen W, Ge H, Sun M, Li S, et al (2020) Effects of palmatine on BDNF/TrkB-mediated trigeminal neuralgia. *Sci Rep* **10**, 4998.
12. Chang CT, Jiang BY, Chen CC (2019) Ion channels involved in substance P-mediated nociception and antinociception. *Int J Mol Sci* **20**, 1596.
13. Lai MC, Liu WY, Liou SS, Liu IM (2020) The protective effects of moscatilin against methylglyoxal-induced neurotoxicity via the regulation of p38/JNK MAPK pathways in PC12 neuron-like cells. *Food Chem Toxicol* **140**, 111369.
14. Wang W, Huang L, Hu Y, Thomas ER, Li X (2020) Neuroprotective effects of notoginsenoside R1 by upregulating Trx-1 on acrylamide-induced neurotoxicity in PC12. *Hum Exp Toxicol* **39**, 797–807.
15. Ge H, Sun M, Wei X, Zhang M, Tu H, Hao Y, Chen R, Ye M, et al (2020) Protective effects of dihydromyricetin on primary hippocampal astrocytes from cytotoxicity induced by comorbid diabetic neuropathic pain and depression. *Purinergic Signal* **16**, 585–599.
16. Wang M, Cai X, Wang Y, Li S, Wang N, Sun R, Xing J, Liang S, et al (2020) Astragalin alleviates neuropathic pain by suppressing P2X4-mediated signaling in the dorsal root ganglia of rats. *Front Neurosci* **14**, 570831.
17. Zhang M, Wen Y, Liang P, Yang C, Tu H, Wei J, Du J, Zhan T, et al (2023) Imperatorin improves obesity-induced cardiac sympathetic nerve injury mediated by P2X4 receptor in stellate sympathetic ganglion. *Int J Mol Sci* **24**, 783.
18. Xu J, Bernstein AM, Wong A, Lu XH, Khoja S, Yang XW, Davies DL, Micevych P, et al (2016) P2X4 receptor reporter mice: Sparse brain expression and feeding-related

presynaptic facilitation in the arcuate nucleus. *J Neurosci* **36**, 8902–8920.
19. Zhao S, Wang F, Wang L, Xu Y, Lv L, Duan W, Bai R, Meng Z, et al (2022) Involvement of the BDNF-TrkB-KCC2 pathway in neuropathic pain after brachial plexus avulsion. *Brain Behav* **12**, e2464.
20. Wu Y, Shen Z, Xu H, Zhang K, Guo M, Wang F, Li J (2021) BDNF participates in chronic constriction injury-induced neuropathic pain via transcriptionally activating P2X(7) in primary sensory neurons. *Mol Neurobiol* **58**, 4226–4236.
21. Merighi A, Salio C, Ghirri A, Lossi L, Ferrini F, Betelli C, Bardoni R (2008) BDNF as a pain modulator. *Prog Neurobiol* **85**, 297–317.
22. Kohno K, Tsuda M (2021) Role of microglia and P2X4 receptors in chronic pain. *Pain Rep* **6**, e864.
23. Aby F, Whitestone S, Landry M, Ulmann L, Fossat P (2018) Inflammatory-induced spinal dorsal horn neurons hyperexcitability is mediated by P2X4 receptors. *Pain Rep* **3**, e660.
24. Lee D-Y, Lee K-M, Um J-H, Kim Y-Y, Kim D-H, Yun J (2023) The natural alkaloid palmatine selectively induces mitophagy and restores mitochondrial function in an Alzheimer's disease mouse model. *Int J Mol Sci* **24**, 16542.
25. Pei H, Zeng J, He Z, Zong Y, Zhao Y, Li J, Chen W, Du R (2023) Palmatine ameliorates LPS-induced HT-22 cells and mouse models of depression by regulating apoptosis and oxidative stress. *J Biochem Mol Toxicol* **37**, e23225.
26. Sun Q, Zhang R, Xue X, Wu Q, Yang D, Wang C, Yan B, Liang X (2021) Jinmaitong alleviates diabetic neuropathic pain through modulation of NLRP3 inflammasome and gasdermin D in dorsal root ganglia of diabetic rats. *Front Pharmacol* **12**, 679188.

Appendix A. Supplementary data

Table S1 The sequences of P2X4 shRNA.

	Sequence(5′–3′)
P2X4 shRNA-1	GATCCCGCCCTCTTGGTAAAGAACAATTG ATATCCGTTGTTCTTTACCAAGAGGGTTT TTTCCAAA
P2X4 shRNA-2	GATCCCGTCTACTGCATGAAGAAGAATTG ATATCCGTTCTTCTTCATGCAGTAGATTTT TTCCAAA
P2X4 shRNA-3	GATCCCGCCAACGCCGGCTTTCTTAAATT GATATCCGTTTAAGAAAGCCGGCGTTGG CTTTTTTCCAAA