# P2X4R/p38 signaling pathway in the spinal cord contribute to hyperalgesia in diabetic neuropathic pain

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**Research Article**

**Keywords:** diabetic neuropathic pain, spinal cord, microglia, P2X4 receptor

**Posted Date:** May 6th, 2022

**DOI:** https://doi.org/10.21203/rs.3.rs-1613857/v1



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# Abstract

Management of diabetic neuropathic pain (DNP), a prevalent, refractory diabetic complication, remains a challenge. Previous research evidences have implicated microglia P2X4 receptor (P2X4R) in occurrence and development of DNP. To date, however, the specific mechanism of microglia P2X4R action in DNP needs to be further explored. We elucidated the role and underlying mechanism of microglia P2X4R in DNP. The DNP rat model was established by injection of streptozotocin (STZ). DNP rats developed thermal hyperalgesia from day 14. Western blot and immunofluorescence analyse revealed that microglia, P2X4R, p-p38 mitogen-activated protein kinase (p-p38 MAPK), and downstream targets brain-derived neurotrophic factor (BDNF), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) were upregulated in the spinal cord. In addition, a P2X4R antagonist 5-BDBD and a microglia inhibitor minocycline (mino) not only alleviated DNP, but also suppressed P2X4R, microglia, p-p38 MAPK, BDNF, TNF-α and IL-1β levels in the spinal cord. Based on these findings, it was evident that central pain sensitization in DNP occurs via the microglia P2X4R/p38 signaling pathway, while p-p38 MAPK activates microglia to release BDNF, TNF-α and IL-1β in spinal cords. These results demonstrate that P2X4R/p38 signaling pathway in microglia residing in the spinal cord contribute to the establishment and long term maintenance of DNP and that these represent potential targets for pain therapy.


# Introduction

The number of diabetic patients has been exponentially growing in the past decades globally, is projected to reach about 629 million by 2045 [1]. One-third of all diabetic patients experience painful neuropathic symptomatology, such as paresthesia, hyperalgesia, and allodynia [2], which seriously affect their daily life, sleep, and mental health. Although diabetic neuropathic pain (DNP) is a prevalent clinical symptom of diabetes, there is no fundamental treatment of DNP as its underlying mechanism is not sufficiently clarified.

The spinal cord has a vital role in integrating pain signals as well as central pain sensitization through the reception of pain signal inputs from sensory neurons in the periphery. Previous results have showed that activation of microglia in spinal cords, arising from injuries to the nervous system [3] and a correlation was found between STZ-induced hypersensitivity and microglia activation in rats [4, 5]. P2X4 receptor (P2X4R) is widely distributed on activated microglia in the rat spinal cord, which is essential for the induction of allodynia. However, the signal transduction pathway mediated by activated P2X4R in microglia in DNP needs further study.

p38 mitogen-activated protein kinase (p38 MAPK) is a family of serine/threonine kinases widely expressed [6]. A great deal of evidence indicates that activation of p38 MAPK in spinal microglia mediates pain processing, which is confirmed in different pain models [7–9]. Thus, p-p38 MAPK stands in a central place in microglia signaling. Previous studies have shown that activated microglia P2X4R successfully initiated p38 MAPK phosphorylation, which subsequently promoted release of brain-derived neurotrophic factor (BDNF) [10]. More than this, It seems likely that the activation of pro-inflammatory

factors such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are mediated by p-p38 MAPK [11]. Nevertheless, p-p38 MAPK mediated downstream events that may contribute to pain have yet to be explored in spinal microglia in DNP model.

The aim of the present study is to reveal the mechanism of DNP pathogenesis as well as identify novel strategies for its prevention and treatment. Here, we investigated a rat model of DNP to examine the impact of P2X4R/p38 signaling on the development of DNP. Mean while, DNP-associated behavioural and neurochemical changes were studied in the DNP model. The effect of P2X4R antagonist 5-BDBD and microglia inhibitor minocycline (mino) against DNP was also determined.

# Materials And Methods

### Animals

Male Sprague-Dawley (SPF-grade) rats (160-210 g in weight), were acquired from Shanghai Laboratory Animal Center of Chinese Academy of Sciences (SCXK (hu) 2018-0006). All rats were maintained at a temperature of 25 ± 2 ℃, 55 ± 5% humidity and a 12 h dark/light cycle, with ad libitum supply of water and food. The Animal Welfare Committee of Zhejiang Chinese Medical University permitted this study (Approval number: IACUC-20190805-04).

### Experimental design

We explored the significance of spinal microglia P2X4R/p38 signaling in DNP. The study was conducted in two steps. Step one entailed analysis of the effects of STZ on induction of neuropathic pain. Rats were randomized into two groups: control ($n$ = 10) and model ($n$ = 30). Two rats failed to become DNP model. Body weight (BW), fasting blood glucose (FBG) and paw withdrawl latency (PWL) were tested as described in Figure 1a in control group, all rats were sacrificed for tissue collection at day 21; in model group, 8, 10 and 10 rats were sacrificed for tissue collection at day 7, 14, and 21, respectively. Next, we performed immunofluorescence staining and western blot analysis to analyze P2X4R, microglia, p-p38 MAPK, BDNF, TNF-α and IL-1β levels in the spinal cord.

In step two, we evaluated the role of P2X4R and microglia in DNP by treating rats with a P2X4R-selective antagonist 5-BDBD and the microglia suppressor, mino. Rats in step two were randomized into four groups: control + vehicle ($n$ = 5); model + vehicle ($n$ = 5); model + 5-BDBD ($n$ = 5); and model + mino ($n$ = 5). Next, the rats were administered with 5-BDBD or mino at day 15 post-STZ injection, with treatment repeated every two days until day 21. BW, FBG and PWL were tested as described in **Figure 4a**. After the fourth intrathecal injection, rats were sacrificed and tissues were collected, then analyzed to identify changes in P2X4R, microglia, p-p38 MAPK, BDNF, IL-1β, TNF-α in spinal cords relative to controls.

### Establishment of STZ-induced DNP rat model

Rats were subjected to 16 h of fasting, then intraperitoneally injected with 65 mg/kg streptozotocin (STZ) (S0130, Sigma-Aldrich, USA) in 0.1 M citrate buffer at pH 4.5 for diabetes induction [12, 13] . Control

group rats were administered with a comparable amount of citrate buffer. 7 days after injection, blood was collected from caudal vein and FBG levels were measured. Rats with FBG levels >13.9 mmol/L were considered type 1 diabetic models [14] . Next, we measured the PWL in diabetic rats, and those with a 15% decline in PWL considered as type 1 DNP model. These were selected for further studies.

### Determination of paw withdrawal latency

PWL was assessed via plantar tests (37370, Ugo Basile, Italy). In all steps of the experiment, PWL was measured 1 day prior to STZ injection (base), then at 7, 14, and 21 days after injection. In addition to these 4 time points, PWL was also measured prior to intraperitoneal injection of 5-BDBD and mino, on day 15 as well as 0.5, 1 and 1.5 h post-intraperitoneal injection in step two. Animals were kept in testing chambers, placed on a glass plate at least 30 min for acclimatization. After becoming quiet, an infrared radiant heat source was focused on a plantar surface of each rat's hind paw, and time taken for PWL measured automatically. To avoid possible thermal injury, illumination was performed for only 30 s while exposure to a radiant heat was for 40 s. Three measurements were obtained at 5 min intervals and were averaged for each test. All the behavioral tests were assessed by a investigator that was blinded to both the experimental groups and study hypotheses.

### Drug-based treatment

The P2X4R antagonist 5-BDBD (505736, Sigma-Aldrich, U.S.A.) and the microglia suppressor, minocycline hydrochloride (M9511, Sigma-Aldrich, U.S.A.) were first dissolved in 5% dimethyl sulfoxide (DMSO), to prepare a stock solution after which it was diluted to appropriate concentrations before injection. Specifically, 5-BDBD was diluted to 542 μg, 10 μl, while mino was100 μg, 10μl. Rats in control + vehicle group and model + vehicle group were administered with similar 5% DMSO volumes. The drugs were administered through intrathecal injection, once every other day, for four days.

### Immunofluorescence

Pentobarbital (40 mg/kg) was used to anesthetize rats. Next, their hearts and ascending aorta perfused with 4 ℃ pre-cooled normal saline via the left ventricular apex, until the liver turned white. The animals were subjected to a bolus injection of 4% paraformaldehyde. and the spinal cord was dissected out, then post-fixed in 4% paraformaldehyde for 6 h. Tissues were sequentially hydrated in 15% as well as 30% sucrose solution, before being frozen in liquid nitrogen and kept at -80 °C. Frozen lumbar spinal cord tissues were sliced into 30-μm-thick sections, washed thrice with TBST (10 minutes for each wash), blocked using 10% normal donkey serum in TBST (0.3% triton) at 37 ℃ for 1 h. Next, incubation of sections was done overnight at 4 ℃ with the following primary antibodies: rabbit anti-P2X4R (1:200, APR-002, Alomone, Israel), or rabbit anti-phospho-p38 MAPK (Thr180/Tyr182) (1:400, 4631S, Cell Signaling Technology, USA) or rabbit anti-BDNF (1:400, ANT-010, Alomone, Israel) respectively and mouse anti-CD11b (1:400, MA1-90756, Thermo, USA). Rewarming the section at 37 ℃ for 1 h on the second day, Thereafter, sections were washed six times using TBST (10 min for each wash), and incubated for 1 h at 37 ℃ with the following secondary antibodies: Alexa Fluor 594 donkey anti-Mouse IgG (1:200, A-21203,

Thermo, USA) and Alexa Fluor 488 donkey anti-rabbit IgG (1:400, A-21206, Thermo, USA). Sections were washed 6 times using TBST, and images captured by Imager M2 microscopy (ZEISS, Germany). Mean intensity were calculated in 3 spinal slices from at least three per group using Image J.

### Western blotting

Pentobarbital (40 mg/kg) was used to anesthetize rats after which their lumbar spinal cord isolated and immediately stored at -80 ℃. Proteins were extracted from the samples using the RIPA lysis buffer (P0013B, Beyotime, China), supplemented with 2% protease inhibitors (P1050, Beyotime, China) and 2% of the phosphatase inhibitor (P1050, Beyotime, China). Protein levels were determined using the BCA Protein Assay Kit (23225, Thermo Fisher, USA), then 20 μg of proteins from every sample were separated by SDS-PAGE after which they were transferred into polyvinylidene difluoride membranes, and blocked for 1 h using 5% nonfat milk. Incubation of membranes was done overnight at 4 ℃ with the following primary antibodies: rabbit anti-P2X4R (1:1000, APR-002, Alomone, Israel), rabbit anti-Iba1(1:1000, ab178846, Abcam, England), rabbit anti-phospho-p38 MAPK (Thr180/Tyr182) (1:1000, 4631S, Cell Signaling Technology, USA), rabbit anti-TNF-α (1:1000, ab66579, Abcam, England), rabbit anti-IL-1β (1:1000, ab9787, Abcam, England), rabbit anti-BDNF(1:1000, NB98682, Novus, USA) and *β*-actin (HRP-conjugated) (1:5000, 12262S, Cell Signaling Technology, USA). Next, membranes were washed thrice with TBST (pH 7.5), then incubated with HRP-linked antibody anti-rabbit IgG (1:5000, 7074S, Cell Signaling technology, USA) for 2 h at room temperature. Detection was done by enhanced chemiluminescence (ECL Plus, Beyotime, China) kit, and band intensities measured on the Image Quant LAS 4000 system. Protein expression from target bands was analyzed by Image J, followed normalization to that of *β*-actin.

### Statistical analysis

The SPSS 21.0 was used for data analyses. Data are shown as means ± standard errors of the mean ( ± SEM). Comparisons of means between groups was done by the independent-samples *t*-test, while those across multiple groups was achieved using one-way ANOVA followed by post hoc (LSD or Dunnett's) test. $P < 0.05$ denoted significance.

## Results

### STZ injection modulated upregulation of P2X4R and microglia in spinal cords

A summary of experimental procedures is presented in **Figure 1a**. Firstly, we developed DNP rat models via high-dose intraperitoneal injection of STZ as previously described [12] . Results showed that 65 mg/kg STZ resulted in significantly lower BW and higher FBG at day 7, relative to rats in control group (**Figures 1b,** $P < 0.01$; **Figures 1c**, $P < 0.01$). Moreover, rats in the model group had significantly lower PWL at day 14, relative to rats in control group (**Figure 1d**, $P < 0.01$), and thermal hyperalgesia lasted till the end of observation (day 21). Collectively, these observations indicated that DNP models had been successfully established at day 14. CD11b and Iba1 are microglia activation markers [15] . Results from immunofluorescence staining revealed the effects of STZ injection on P2X4R and CD11b levels in the

spinal cord (**Figure 2a**). Specifically, P2X4R was significantly upregulated from day 7 to 21(**Figure 2b**, $P < 0.05$, $P < 0.01$, $P < 0.01$), while CD11b levels were upregulated from day 14 to 21 after STZ injection, relative to control group (**Figure 2c**, $P < 0.01$, $P < 0.01$). Results from co-localization experiment indicated that P2X4R was co-localized with CD11b in the spinal cord (**Figure 2a**). Similarly, western blots revealed that STZ injection significantly upregulated P2X4R and Iba1 proteins in the spinal cord from day 14 to 21 (**Figure 2d**, $P < 0.01$, $P < 0.01$; **Figure 2e**, $P < 0.05$, $P < 0.01$).

### STZ injection upregulated p-p38 MAPK, TNF-α, BDNF, and IL-1β levels in the spinal cord

Immunofluorescence staining revealed the effect of STZ injection on p-p38 MAPK and BDNF levels in spinal cords (**Figure 3a, d**). Both, p-p38 MAPK and BDNF were markedly upregulated day 7 to 21 (**Figure 3b,** $P < 0.05$, $P < 0.01$, $P < 0.01$; **Figure 3c,** $P < 0.05$, $P < 0.01$, $P < 0.01$) after STZ injection relative to the control group. Results from co-localization experiment indicated that p-p38 MAPK was co-localized with CD11b in the spinal cord.(**Figure 3a**). Western blots revealed significant elevation of spinal TNF-α and IL-1β from day 14 to 21 (**Figure 3e**, $P < 0.01$, $P < 0.05$) and from day 7 to 21 (**Figure 3f**, $P < 0.01$, $P < 0.01$, $P < 0.05$) after STZ administration, relative to the control group.

### The P2X4R antagonist 5-BDBD/the microglia inhibitor mino alleviated Diabetes-induced pain

The procedure for this experiment is summarized in **Figure 4a**. To determine whether changes in the microglia P2X4R was involved in DNP development, we treated rats with P2X4R antagonist 5-BDBD and microglia suppressor mino via intrathecal injection. Relative to control + vehicle group, a high dose of STZ resulted in significantly lower BW (**Figure 4b**, $P < 0.05$, $P < 0.05$, $P < 0.01$), but elevated FBG from day 7 to 21(**Figure 4c**, $P < 0.01$, $P < 0.01$, $P < 0.01$) in model + vehicle group. On the other hand, PWL of model + vehicle group was significantly reduced from day 14 to 21 relative to control + vehicle group (**Figure 4e**, $P < 0.01$, $P < 0.01$). Analysis of PWL, at 0.5, 1, and 1.5 h after either 5-BDBD or mino intrathecal injection, revealed that both antagonists alleviated thermal hyperalgesia relative to model + vehicle group. The effects on PWL lasted 0.5 h in model +5BDBD group after 5-BDBD intrathecal injection (**Figure 4d**. $P < 0.01$), while the effects lasted at least 1.5 h in model + mino group rats after mino intrathecal injection (**Figure 4d**, $P < 0.01$, $P < 0.01$, $P < 0.05$). Four cycles of 5-BDBD or mino treatment improved PWL significantly in model + 5-BDBD and model + mino groups relative to model + vehicle group at 21 days after STZ injection (**Figure 4e**, $P < 0.01$, $P < 0.01$).

### The P2X4R antagonist 5-BDBD or the microglia inhibitor mino downregulated P2X4R, microglia, p-p38 MAPK, BDNF, TNF-α and IL-1β in spinal cords

Western blots revealed that P2X4R and Iba1 were markedly upregulated in the model + vehicle group, relative to control + vehicle group (**Figure 5a,** $P < 0.01$; **Figure 5b,** $P < 0.01$), and we found that p-p38 MAPK, TNF-α, BDNF and IL-1β were elevated in the model + vehicle group, relative to the control + vehicle group (**Figure 5e,** $P < 0.01$**; Figure 5c,** $P < 0.01$; **Figure 5f,** $P < 0.01$; **Figure 5g,** $P < 0.05$). Treatment with 5-BDBD effectively reversed both levels of P2X4R and Iba1 in the spinal cord (**Figure 5a,** $P < 0.01$; **Figure 5b,** $P < 0.05$). We evaluated the effects of 5-BDBD on p-p38 MAPK, BDNF, TNF-α and IL-1β levels (**Figure 5d**).

Levels of p-p38 MAPK, TNF-α, BDNF and IL-1β in model + 5-BDBD group were markedly lower than in model + vehicle group in the spinal cord (**Figure 5e,** $P < 0.05$**; Figure 5c,** $P < 0.05$; **Figure 5f,** $P < 0.01$; **Figure 5g,** $P < 0.05$). As shown in **Figure 5**, after mino treatment, the overexpression of P2X4R and Iba1 were downregulated in the model + vehicle group, relative to control + vehicle group (**Figure 5a,** $P < 0.01$; **Figure 5b,** $P < 0.01$), and p-p38 MAPK, TNF-α, BDNF and IL-1β levels in the spinal cord of DNP rats are also reversed by mino treatment (**Figure 5e,** $P < 0.05$**; Figure 5c,** $P < 0.01$; **Figure 5f,** $P < 0.01$; **Figure 5g,** $P < 0.01$). Collectively, both 5-BDBD and mino suppressed microglia P2X4R/p38 signaling in the spinal cord of DNP rats.

# Discussion

We successfully build DNP rat models using STZ-induced and verified the pain-like behavior by measuring PWL. Next, we explored the role of microglia P2X4R/p38 signaling pathway in DNP. We established that microglia, P2X4R, p-p38 MAPK, its downstream signaling BDNF, as well as IL-1β and TNF-α were all upregulated in spinal cords of DNP rats. Notably, P2X4R antagonist and microglia inhibitor not only significantly attenuated STZ-induced nociceptive thermal hyperalgesia, but also reversed the upregulation of microglia P2X4/p38 signaling in spinal cords post STZ injection. Taken together, these findings indicated that microglia P2X4/p38 signaling plays a vital function in DNP development.

STZ acts as a nitrosourea analogue derivative and shows selective cytotoxicity to pancreatic β cells by damaging its DNA [16]. Because of the convenience of STZ in establishing the diabetes rat model, it has been frequently used to study diabetes and associated complications in experimental animals. The diabetes model can be successfully established with one-time high-dose injection intraperitoneally [17]. Previous studies have clearly shown that hyperglycemia and BW changes occurred early, while diabetic-induced peripheral neuropathy is a later event that occurs after STZ injection [18]. Our results showed that intraperitoneal administration of a high-dose injection of STZ elicited lower BW and higher FBG from day 7. The PWL of the rats was significantly decreased 14 days post STZ injection, indicative of thermal hyperalgesia establishment. These observations showed that DNP rat models were successfully established 14 days post STZ injection.

Previous studies have shown that microglia, the immunocompetent cells in the CNS, playing a crucial function in pain signal transmission at spinal levels [19]. Several studies revealed significant activation of spinal microglia in neuropathic pain models after injuries to peripheral nerves [20–22]. Currently, CD11b and Iba1 are more widely recognized as microglia markers [23, 24]. We established that CD11b and Iba1 were significantly upregulated in spinal cords of STZ-treated rats, a phenomenon consistent with findings from earlier studies [25, 26]. Systemic propentofylline or intrathecal delivery of mino, compounds that inhibit microglia activation, relieves development of induced sensory hypersensitivity in nerve injury models [27, 28]. In this study, intrathecal delivery of 100 μg mino hydrochloride significantly alleviated thermal hyperalgesia in the hind paw after DNP.

Apart from microglia, upregulation of P2X4R is closely associated with occurrence of neuropathic pain [29–31]. Consequently, targeted therapy based on P2X4R have attracted numerous research interest and became the aim of developing effective neuropathic pain treatment approaches [32, 33]. Tsuda found that P2RX4(-/-) mice reduced pain responses both in inflammatory as well as neuropathic pain models, highlighting the potential role of P2X4R in chronic pain [34]. In this study, STZ injection mediated a marked elevation of P2X4R in spinal cords of rats. 5-BDBD, a specific P2X4R antagonist, not only alleviated thermal hyperalgesia, but also down-regulated P2X4R expression in the spinal cord.

P2X4R is expressed on several cell types, including neurons, microglia, astrocytes, oligodendrocytes and macrophages, among others [35]. Tsuda reported that P2X4R was upregulated exclusively in microglia in spinal cords in response to neuronal injury or disease [36]. Immediately posterior, several possible routes underlying the role of P2X4R in microgliosis, have been suggested. For example, lipopolysaccharide or interferon γ was implicated in upregulation of P2X4R in microglia [37, 38]. Although the mode of recruitment of the P2X4R protein on the cell surface of microglia remains uncertain, there is no doubt that P2X4R regulates the fate of activated microglia and its survival. Interestingly, activated microglia were implicated in upregulation of P2X4R [39, 40]. Basis for the hypothesis is that this pathway is regulated by the release of interferon regulatory factor-5 (IRF5) by activated microglia [41]. Upon stimulation of microglia by fibronectin, IRF5 directly binds the promoter region of P2X4R gene, which initiates de novo P2X4R expressions [42]. Our results were consistent with this finding, as evidenced by increased co-expressions of P2X4R as well as microglia in spinal cords of rats post STZ injection. An intrathecal injection of a P2X4R or microglia inhibitor down-regulated both P2X4R and microglia expressions in spinal cords. Overall, our results suggest existence of a bidirectional regulatory relationship between P2X4R and microglia. Nonetheless, the exact cellular interactions between P2X4R and microglia under DNP condition require further research explorations.

The relationship between p-p38 MAPK and microglia under pain conditions, including DNP, has been reported [43, 44]. Notably, p-p38 MAPK is only co-expressed with microglia in spinal cords under pain conditions [45, 46], while recent research evidences have shown that P2X4R can promote significant elevation of p-p38 MAPK levels from the perspective of the central and peripheral nervous system, indicating that P2X4R-mediated p-p38 MAPK activation functions in pain development [47, 48]. Furthermore, Gong demonstrated that pharmacological blocking of P2X4R significantly prevented p-p38 MAPK activation in line with results from the present study [49]. Our results presented here showed that there was coexpression of microglia and p-p38 MAPK proteins in DNP rats. Both the inhibitor of microglia and the antagonist of P2X4R effectively inhibited p-p38 MAPK levels in spinal cords.

With regards to downstream signaling, p-p38 MAPK causes inflammatory cytokine secretion, including TNF-α as well as IL-1β from microglia via transcriptional regulation [50, 51], One article reported p38 inhibitor could attenuated IL-1β-induced sensitization of nociception in rats [52]. And another article reported that activation of p38 MAPK in the spinal cord could downregulate the glucocorticoid receptors expression and thereby promote the release of IL-6 and TNF-α and participating in neuropathic pain [53]. Recent literature reported that BDNF aggravated neuroinflammation and leaded to mechanical allodynia

through BDNF-TrkB-p38 signaling in cystitis model [54]. While p-p38 MAPK have also been identified as an important mediator which leads to the synthesis and exocytotic release of BDNF from microglia [10]. Although the relationship between p-p38 MAPK and BDNF remains controversial, There is undeniably a strong relationship between p-p38 MAPK and BDNF. This study revealed enhanced P2X4R in the spinal cord upregulated expression of BDNF and TNF-α, IL-1β in rat microglia, which are dependent on p-p38 MAPK mediated signaling under DNP condition. Based on these findings, we develop a model of P2X4R/p38 signaling pathway in microglia.

This study had some limitations. Firstly, how P2X4R protein is recruited to the cell surface of microglia remains unknown. Secondly, more proofs are needed to be validate the role of p-p38 MAPK in mediating to promote BDNF,TNF-α and IL-1β in DNP.

# Conclusion

Overall, our study showed that activation of P2X4R induces both BDNF, TNF-α and IL-1β release in the spinal cord, which possibly mediated by p-p38 MAPK In summary, activation of microglia P2X4R/p38 signaling in spinal cords probably contributes to DNP development.

# Abbreviations

BW Body weight

BDNF Brain-derived neurotrophic factor

DNP Diabetic neuropathic pain

DMSO Dimethyl sulfoxide

FBG Fasting blood glucose

IL-1β Interleukin-1β

P2X4R P2X4 receptor

p-p38 MAPK p-p38 mitogen-activated protein kinase

PWL Paw withdrawal latency

STZ Streptozotocin

TNF-α Tumor necrosis factor-α

# Declarations

### Acknowledgments

This research was supported by the National Natural Science Foundation of China (81804181 to X.F.H. and 81774389 to Y.L.J.), the Zhejiang Provincial Natural Science Foundation (LY22H270006 to Y.L.J.), the National Undergraduate Innovation and Entrepreneurship Training Program (202110344013), and the Key Laboratory of Acupuncture and Neurology of Zhejiang Province (2019E10011).

### Authors' contributions

Xiaofen He, Jianqiao Fang and Yongliang Jiang conceived and designed the experiments. Siying Qu, Hanzhi Wang, Qunqi Hu, Yurong Kang and Liqian Ma performed animal experiments. Qunqi Hu, Yiqi Ma and Liqian Ma performed western blot assays. Siying Qu, Xiang Li and Hanzhi Wang performed immunofluorescence labeling experiments. Luhang Chen, Boyu Liu and Hongli Zhao analyzed the data. Xiaofen He, Siying Qu, Hanzhi Wang wrote the manuscript. Xiaomei Shao, Yi Liang, Junying Du and Boyi Liu revised the manuscript. All authors read and approved the final version of the manuscript.

### Funding

The study was supported by grants from the National Natural Science Foundation of China (Grant Nos. 8180418, 81774389), the Zhejiang Provincial Natural Science Foundation (Grant No. LY22H270006), and the Key Laboratory of Acupuncture and Neurology of Zhejiang Province (Grant No. 2019E10011).

### Conflict of Interest

The authors declare that there is no conflict of interest.

### Ethical approval

The study was approved by the ethics committee of Zhejiang Chinese Medical University, Hangzhou, China (Approval No. IACUC-20190805-04).

### Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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# Figures

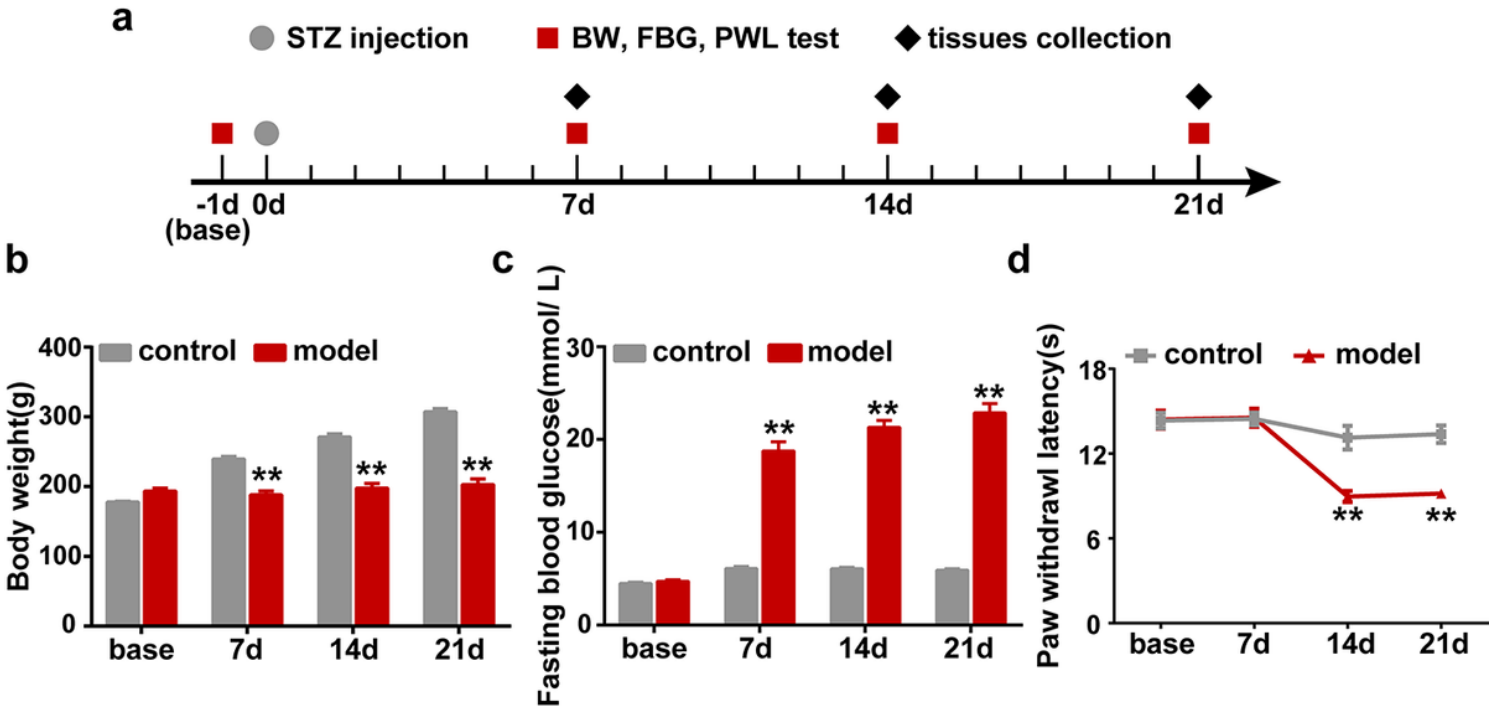
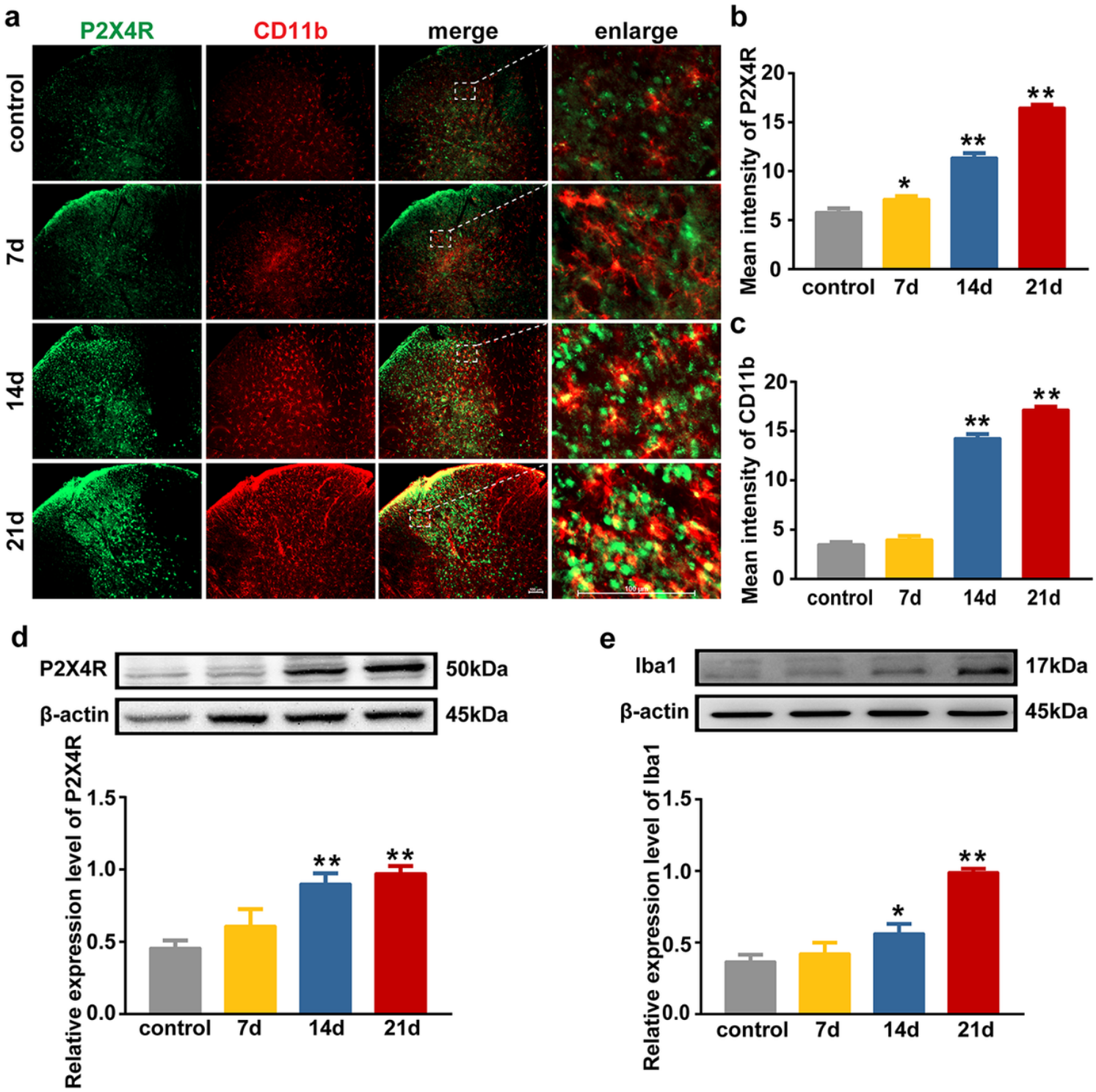


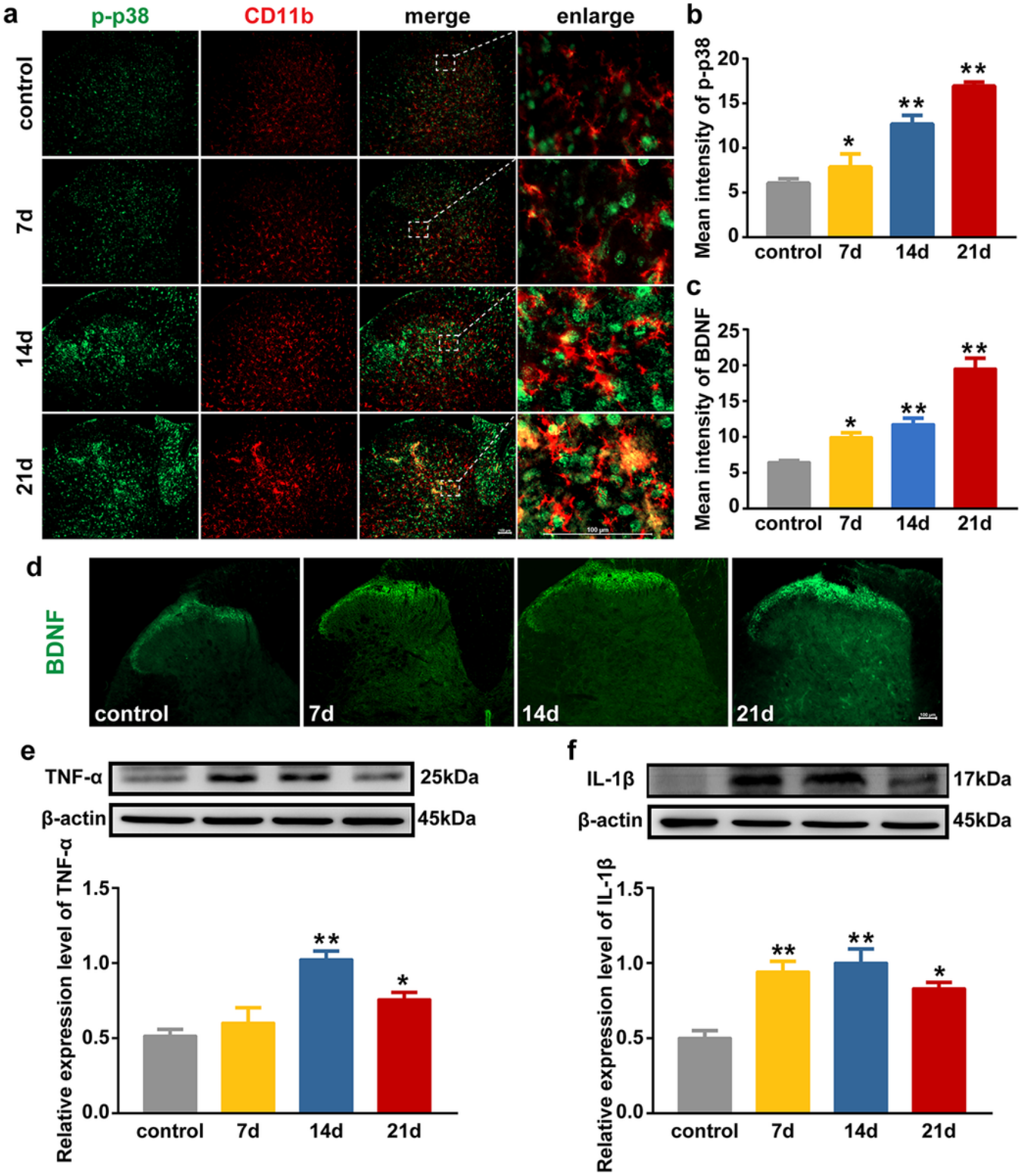

**Figure 1**

The rat model of DNP was successfully established by intraperitoneal injection of STZ. **(a)** The schedule for establishment of DNP rat models. **(b)** Time course effects of STZ injection on BW (*n*=10). **(c)** Time course effects of STZ injection on FBG (*n*=10). **(d)** Time course effects of STZ injection on PWL (*n*=10). **P* < 0.05, ***P* < 0.01 vs. control group.




## Figure 2

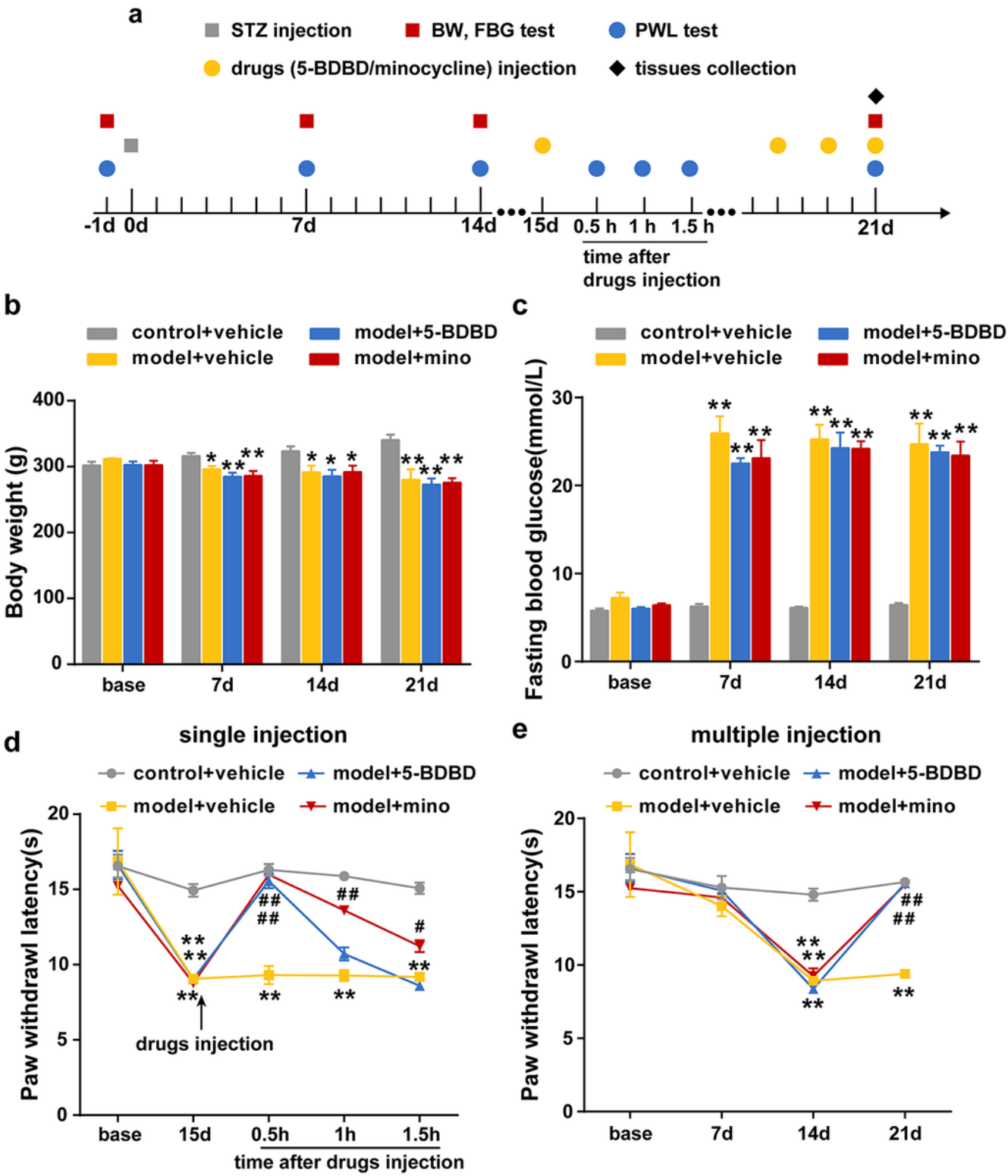
STZ injection upregulated P2X4R and microglia expression in spinal cords of rats. **(a)** Representative immunofluorescence-stained images and their enlarged images of P2X4R (green) and CD11b (red) in control, 7, 14, and 21 d groups. **(b)** Summary of mean intensity of P2X4R immunostaining ($n$=3). **(c)** Summary of mean intensity of CD11b immunostaining ($n$=3). **(d)** Profiles of P2X4R protein level in the four groups ($n$=5). **(e)** Expression levels of Iba1 protein in the four groups. $\beta$-actin was the loading control ($n$=5). *$P$ < 0.05, **$P$ < 0.01 vs. control group.




## Figure 3

STZ injection upregulated p-p38 MAPK, BDNF, IL-1β and TNF-α expression in spinal cords. **(a)** Representative immunofluorescence-stained images and their enlarged images of p-p38 MAPK (green) and CD11b (red) in control, 7, 14, and 21 d groups. **(b)** Summary of mean intensity of p-p38 MAPK immunostaining (*n*=3). **(c)** Summary of mean intensity of BDNF immunostaining (*n*=3). **(d)** Representative immunofluorescence-stained images of BDNF (green) in control, 7, 14, and 21 d groups.

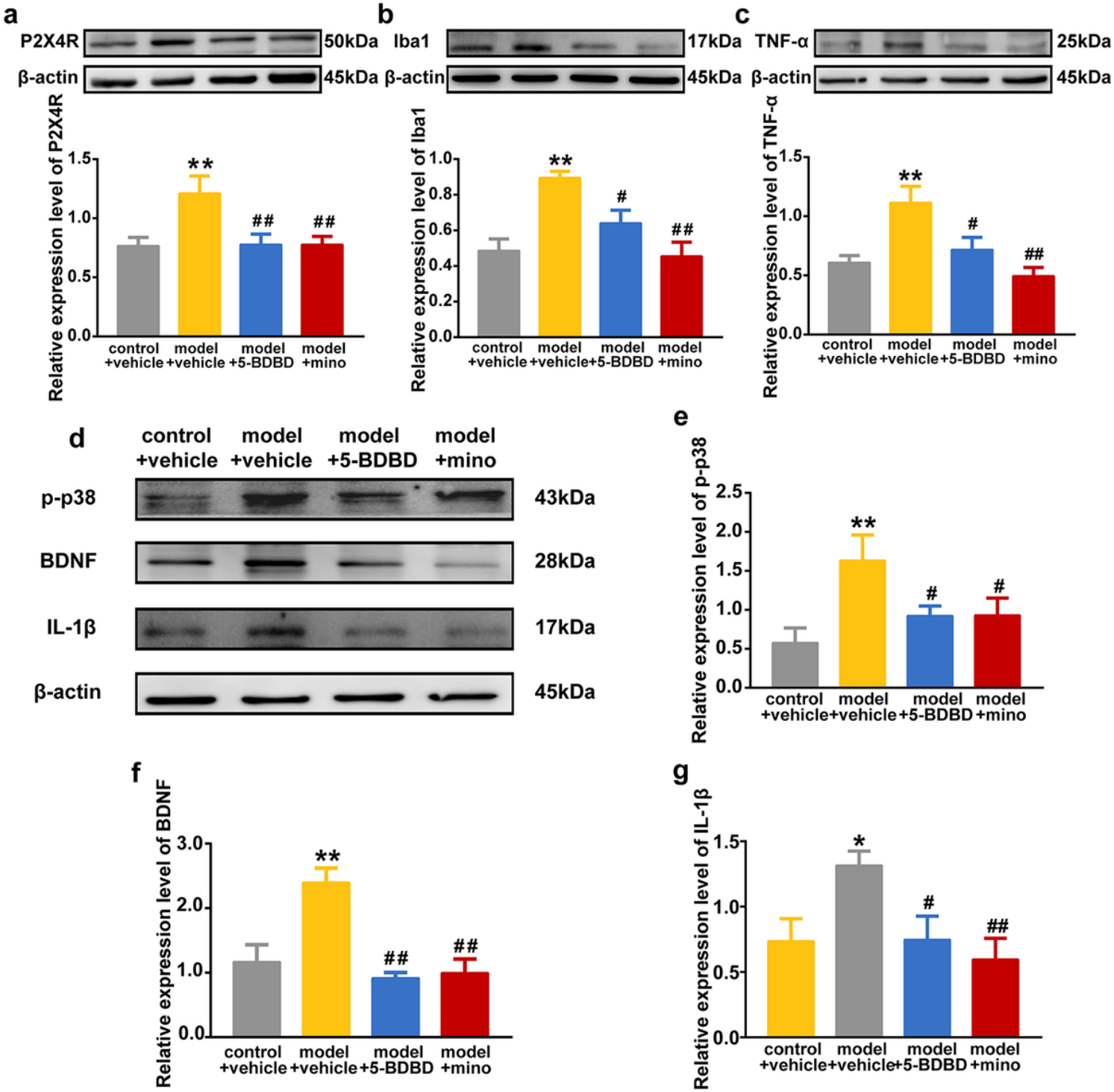
**(e)** Levels of TNF-α protein across the four groups (*n*=5). (f) IL-1β protein levels in the four groups (*n*=5). β-actin was the loading control. *$P < 0.05$, **$P < 0.01$ vs. control group.




## Figure 4

Single and multiple 5-BDBD/mino intrathecal injection relieved DNP. **(a)** Schematic presentation of generation of DNP rat models and 5-BDBD/mino injection. **(b)** Time-course effects of 5-BDBD/mino on

BW ($n$=5). **(c)** Time-course effects of 5-BDBD/mino on FBG ($n$=5). **(d)** Effect of single 5-BDBD/mino intrathecal injection on PWL ($n$=5). **(e)** Effect of multiple 5-BDBD/mino intrathecal injection on PWL($n$=5). *$P$ < 0.05, **$P$ < 0.01 vs. Control + vehicle group. #$P$ < 0.05, ##$P$ < 0.01 vs. model + vehicle group.




## Figure 5

Multiple 5-BDBD/mino intrathecal injection supressed spinal cords microglia P2X4R/p38 signaling. **(a)** Levels of P2X4R protein across the four groups ($n$=5). **(b)** Levels of Iba1 protein across the four groups ($n$=5). **(c)** TNF-α protein levels in the four groups (n=5). **(d)** Western blotting of p-p38 MAPK, BDNF, IL-1β.

**(e)** Levels of p-p38 MAPK protein across the four groups ($n$=5). **(f)** BDNF protein levels in the four groups ($n$=5). **(g)** Levels of IL-1β protein across the four groups ($n$=5). $\beta$-actin was the loading control. *$P$ < 0.05, **$P$ < 0.01 vs. Control + vehicle group. $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01 vs. model + vehicle group.