Daphnetin Ameliorates Neuropathic Pain in CCI Rats by Interfering With Microglia P2X₄ - P38 MAPK (Daphnetin Improves Neuropathic Pain)

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Abstract

Background: Neuropathic pain is a common chronic pain caused by a lesion or diseases of somatosensory nervous system, which has a significant impact on the quality of life. It has been found that its underlying pathological mechanism is related to inflammation. Daphnetin is a natural product with a variety of biological activities such as anti-inflammatory and analgesic. It has been shown to be effective in the treatment of chronic inflammatory pain, but its underlying mechanisms are not completely understood. We aimed to evaluate the effect and regulatory mechanism of daphnetin on neuropathic pain.

Methods: The experimental animals were divided into 8 groups: control group, model group, sham group, positive group (0.0375mg/kg), DAPH group (0.0625mg/kg), DAPL group (0.025mg/kg), P2X₄ blocker group (1mg/kg) and P38 blocker group (5mg/kg). After constructing the subdural drug delivery system in rats, the neuropathic pain model was established by chronic constrictive injury (CCI), and the pain sensitivity was evaluated by measuring mechanical withdrawal threshold (MWT) and thermal withdrawal threshold (TWT). The protein expression of inflammatory factors and related pathways was measured by Western Blot. The activation of microglia and neurons and the expression of target protein in spinal dorsal horn were measured by immunofluorescence staining.

Results: Compared with the sham group, MWT and TWT decreased significantly in model group, and the expression of IL-1 β , IL-6 and TNF- α increased. After intrathecal injection of daphnetin, the MWT and TWT of rats were significantly increased, and the protein expression of IL-1 β , IL-6 and TNF- α was significantly decreased compared with the model group. Compared with sham group, the expression of P2X₄, IRF8, IRF5, BDNF, p-P38 / P38, p-JNK / JNK and p-ERK1/2 / ERK1/2 in model group increased significantly. After intrathecal injection of daphnetin, the expression of the above proteins was significantly down-regulated compared with model group. Compared with sham group, microglia and neurons in the spinal dorsal horn of model group were activated, and the expression of P2X₄ and P38 in microglia increased. After intrathecal injection of daphnetin, compared with the model group, the activation of microglia and neurons in the spinal dorsal horn decreased significantly, and the expression of P2X₄ and P38 in microglia decreased. Importantly, daphnetin could not improve neuropathic pain in CCI rats under the action of P2X₄ and P38 blocker.

Conclusion: daphnetin can alleviate neuropathic pain in CCI model rats by interfering with P2X₄-P38 MAPK pathway of microglia, suggesting that daphnetin is a potential drug for the treatment of neuropathic pain.

Introduction

Neuropathic pain is defined as "pain caused by primary lesions, dysfunction or transient disturbance of the peripheral or central nervous system"[1]. In most cases, neuropathic pain is caused by peripheral or central nerve lesions. Epidemiological study have shown that the prevalence of neuropathic pain in the

general population may be as high as 7–8%, accounting for 20–25% of patients with chronic pain[2]. Continuous stimulation such as tissue or nerve injury will reshape nociceptors and make the body engender pain sensitization, including hyperalgesia and allodynia[3]. Peripheral sensitization of dorsal root ganglion (DRG) and central sensitization of spinal cord and supraspinal cord are still the main mechanisms and research hotspots of neuropathic pain[4].

In recent years, with the in-depth study of neuropathic pain, in addition to the abnormal excitation of neurons, non-neural cells of the nervous system, including monocytes, macrophages, T cells and glial cells, play an important role in the study of neuropathic pain[5]. In 2003, when P2X purine receptor 4 (P2X₄) and P38 mitogen activated protein kinase (P38 MAPK) were identified as microglia specific molecules up-regulated and activated after neuropathic pain, the causal role of spinal microglia in neuropathic pain was determined for the first time[6]. It has been reported that the deletion of P2X₄ receptor and targeted intervention can reduce hyperalgesia in animal models of pain [7]. Therefore, P2X₄-P38 MAPK pathway in microglia can be used as a potential therapeutic target for neuropathic pain.

Daphnetin (7,8-dihydroxycoumarin, structural formula is shown in Figure 1A), a natural coumarin compound isolated from the stem and root bark of *Daphne giraldii* Nitsche, a plant of Daphne family, has a wide range of biological activities, including antioxidant, analgesic and anti-inflammatory effects[8]. It was found that daphnetin inhibited the production of cytokines IL-1 β , TNF- α and macrophage migration inhibitory factor (MIF) when exposed to pro-inflammatory stimuli; In addition, daphnetin inhibited the infiltration of inflammatory cells and alleviated the inflammatory injury in mice[9]. In animal studies, daphnetin has been widely used in the treatment of clinical autoimmune diseases, including experimental autoimmune encephalomyelitis (EAE) and rheumatoid arthritis (RA), which are related to its analgesic function[10]. Therefore, in this study, we aimed to judge whether daphnetin can treat neuropathic pain through spinal cord administration, explore the effects of daphnetin on P2X₄-P38 pathway of microglia and activated neurons under central sensitization, and find its potential analgesic mechanism.

Materials And Methods

Establishment of neuropathic pain model

Healthy Sprague Dawley (SD) rats aged 6-8 weeks ($n= 60$), Male, weighing 180-200g, provided by Beijing SBF Experimental Animal Technology Co., Ltd. Animal license No.: SCXK2020-0033. All rats were maintained under 12 hours of light/12 hours of dark cycle, constant temperature ($23\pm1^{\circ}\text{C}$) and relative humidity ($60\%\pm5\%$) under standardized conditions, and food and water were freely available. All rats were allowed to adapt to the environment for a week before the experiment. The animal experimental protocol of this study was reviewed and approved by the medical and experimental animal ethics committee of Beijing University of Chinese Medicine (No: BUCM4-202103201-1078).

After one week of adaptive feeding, the rats were anesthetized with pentobarbital sodium (Beijing Chemical Reagent Company, 020402) (1.5%, i.p.) and then underwent spinal cord subdural intubation. A

longitudinal 1cm incision was made at the intersection of iliac bone and spine of the rat to separate fascia and muscle, and fully expose caudal vertebra, iliac bone and sacrum. Intervertebral foramen can be seen on the side of the first caudal vertebra, and clear cerebrospinal fluid can be seen after puncture. Mark 2cm of EP-10 catheter (Smiths medical, UK, 302021) filled with normal saline and insert it forward from the level of intervertebral foramen. Suture the muscle and skin, disinfect, close the catheter, and feed in a single cage. Lidocaine hydrochloride (Shandong Hualu Pharmaceutical Co., Ltd., XB19H26) was injected intrathecally on the fifth day after operation, and the lower limbs of rats were paralyzed immediately after injection, which proved that the catheter was located at L4-L5 of the subdural cavity [11]. The rats with successful catheterization were randomly divided into seven groups: model group, sham group, positive group, DapH group and DapL group (9 rats in each group), P2X₄ blocker group and P38 MAPK blocker group (3 rats in each group), and 9 blank rats were selected as the control group.

On the seventh day after the operation, except for the control group and the sham group, the other groups underwent the production of chronic constriction injury (CCI) model[12]. The rats were anesthetized after catheterization for seven days. The rats were prone on the operating table, and the left hind limb was depilated and disinfected. The biceps femoris was separated and the sciatic nerve trunk was exposed and ligated for four time, with the space of each ligation about 1mm. After ligation, the muscles and skin were sutured and disinfected. The criteria for successful modeling were claudication, foot lifting and licking, reduction of heat threshold and pain threshold, and pain sensitization in the affected limb.

Group and administration

DAPH group was intrathecal injected with 0.0625mg/kg daphnetin (Shanghai Yuanye Biotechnology Co., Ltd., B21084) (0.5mg/mL), twice a day for 3 days; DAPL group was intrathecal injected with 0.025mg/kg daphnetin (0.2mg/mL), twice a day for 3 days; Positive group was intrathecal injected with 0.0375mg/kg morphine (Qinghai pharmaceutical factory, 850807) (0.3mg/mL), twice a day for 3 days; P2X₄ blocker group was given intragastrically with P2X₄ blocker (1mg/kg/day, administration for 3 days, MCE, HY-130605) and intrathecal injected 0.0625mg/kg daphnetin (0.5mg/mL), twice a day for 3 days; P38 MAPK blocker group was given intragastrically with P38 MAPK blocker (5mg/kg/day, administration for 3 days, MCE, HY-10256) and injected 0.0625mg/kg daphnetin (0.5mg/mL), twice a day for 3 days. Control group, model group and sham group were injected with the same dose of normal saline twice a day for 3 days.

Measurement of Mechanical Withdrawal Threshold (MWT)

The rats were placed in a 20*20*20 cm metal grid cage, adapted for 5min before measurement, and began to measure after the search behavior stopped. The rats were stimulated with Von Frey fiber hair, and the changes of plantar response to Von Frey fiber hair stimulation were observed. The Up and Down method[13] was used for determination. 4, 6, 8, 10, 15, 26, 60, 100, 180 and 300g fibers were selected for Von Frey fiber hair. The positive reaction was recorded as O and the negative reaction was recorded as X. In case of positive reaction, the low-grade fiber hair shall be used for stimulation, and in case of negative reaction, the high-grade fiber hair shall be used for stimulation. When there is a reflective change, it is the

first formal data. After that, measure 5 data as the original data, check the data table and calculate the 50% positive reaction amount (ED_{50}).

$$ED_{50} = 10^{\log a + k \times b}$$

a) The corresponding value of hair used in the final official data
b) The mean value of the difference between the logarithm of the corresponding values of adjacent hair
k) Corresponding parameters in the table (see supplementary materials)

Measurement of Thermal Withdrawal Threshold (TWT)

The rats were placed in a transparent glass cover for 5 min. The reference temperature of the cold/hot disk pain meter (Cat.No: PE34, IITC, USA) was adjusted to 26°C, the target temperature was adjusted to 51°C, and the temperature rise rate was set to 5°C/min. After the animal adapts to the environment, start the instrument and start heating up. When the affected limb of the rat shrinks or licks due to burning, immediately press the pause button to record the instantaneous temperature displayed by the instrument. Each rat is measured three times with an interval of 10min. The average value is the TWT value[14].

Immunofluorescence staining

Rat spinal cord tissue was fixed in 10% neutral formalin, dehydrated with ethanol and embedded in wax. The slice thickness was 4µm. Spinal cord sections were incubated with rabbit/mouse antibodies Iba-1 (1:200, ab178846/ab283319, Abcam), P2X₄ (1:200, bs-7690R, BIOSs), c-fos (1:1000, ab208942, Abcam), P38 (1:50, 14064-1-AP, Proteintech) at 4°C overnight. Goat anti rabbit IgG H&L (Alexa Flour 488, 1:200, ab150077, Abcam) and goat anti mouse IgG H&L (Alexa Flour 647, 1:200, ab150115, Abcam) were selected as secondary antibodies. After immunofluorescence staining, the tissue sections were observed and photographed under laser confocal microscope (Sp8, Leica, Germany). Fluorescence quantitative analysis was performed by image J.

Western Blot analysis

Rat spinal cord was homogenized in RIPA lysate buffer (Z2104A, I TEST) supplemented with protease and phosphatase inhibitor (20210401, NCM). The protein concentration was determined by BCA protein detection kit (KGP902, keygen Biotech). Total protein 10µg was separated by SDS-PAGE and electrically transferred to PVDF membrane (R1DB92455, Millipore). After blocking the membrane for 1.5h, incubate with the primary antibody overnight at 4°C. The sources of primary antibodies are as follows: IL-1β (1:1000, ab254360, Abcam), IL-6 (1:1000, ab259431, Abcam), TNF-α (1:1000, ab183218, Abcam), α-Tubulin (1:5000, ab7291, Abcam), P2X₄ (1:1000, bs-7690R, Bioss), BDNF (1:1000, 66292-1-Ig, Proteintech), IRF8 (1:1000, 18977-1-AP, Proteintech), IRF5 (1:1000, ab181553, Abcam), β-actin (1:5000, ab8226, Abcam), P38 (1:1000, 14064-1-AP, Proteintech), p-P38 (1:1000, 4511S, CST), JNK (1:5000, 66210-1-Ig, Proteintech), p-JNK (1:2000, 9255S, CST), ERK1/2 (1:5000, 67170-1-Ig, Proteintech), p-ERK1/2 (1:2000, 4370S, CST). Then the membrane was washed three times and incubated with Goat anti rabbit

IgG HRP (1:5000, S0101, LABLEAD) or Goat anti mouse IgG HRP (1:5000, S0100, LABLEAD) for 1 h at room temperature, the protein band signal was detected by Amersham ECL system (Amersham imager 680). The protein bands were analyzed quantitatively by Image J software.

Statistical method

SAS 8.2 software was used for statistical analysis. All data are expressed as mean $\pm$ standard deviation (SD). One way analysis of variance (ANOVA) and T-test were used to test the differences between sham operation group, CCI group and control group, treatment group and CCI group, and the * symbol was used to indicate $P < 0.05$ or ** to indicate $P < 0.01$, which was considered to be statistically significant.

Result

Daphnetin improves neuropathic pain in CCI rats

In this study, we constructed an intrathecal injection mode before making CCI pain model, as shown in Figure 1C. This method of administration can realize multiple local administration in L4-L5 without damaging the spinal cord through EP-10 catheter. Seven days after subdural intubation, we established a neuropathic pain model CCI rats, as shown in Figure 1B. The model has the characteristics of pain sensitization, including thermal hyperalgesia and mechanical hyperalgesia[15]. We measured TWT and MWT of CCI rats before modeling, 1 hour after administration and 3 days after administration. Before modeling, there was no significant difference between TWT and MWT in each group ($P > 0.05$), which proved that subdural catheterization did not cause pain sensitization in rats. 1 hour after the administration, compared with the sham group, the TWT and MWT of the model group were significantly reduced ($P < 0.05$), which proved that the CCI model was successfully prepared. At this time, the rats have shown thermal hyperalgesia and mechanical hyperalgesia. Similarly, there was no significant difference in TWT and MWT between sham group and control group, which further confirmed that subdural catheterization would not induce pain sensitization in rats; Compared with the model group, the positive group, DAPH group, and DAPL group had a significant increase in TWT and MWT ($P < 0.05$), indicating that daphnetin has the same analgesic effect as morphine. After 3 days of continuous administration, there was still significant difference in TWT and MWT between model group and sham group ($P < 0.05$), which proved that pain sensitization persisted; Compared with the model group, the positive group, DAPH group, and DAPL group showed a significant increase in TWT and MWT ($P < 0.05$) (Figure 1D and E).

Daphnetin inhibits the activation of microglia in spinal dorsal horn of CCI rats

Studies have shown that neuroinflammation caused by microglia activation plays a key role in the pathogenesis and maintenance of neuropathic pain[16]. Microglia are involved in the initial signal regulation of neuronal activation, which is often accompanied by high expression of microglia in the spinal dorsal horn of neuropathic pain model[17]. After perfusion fixation, the spinal cord was taken out and the microglia marker Iba-1 was stained by immunofluorescence. It was found that Iba-1 was highly expressed in the spinal dorsal horn of model group, which was significantly different from that of sham

group ($P<0.01$). Compared with the control group, the expression of Iba-1 in the sham group did not increase significantly ($P>0.05$), suggesting that subdural catheterization did not cause spinal cord injury or activation of microglia. The expression of Iba-1 in DAPH group was significantly lower than that in model group ($P<0.01$) (Figure 2 and Supplementary Figure1A).

Daphnetin inhibits the expression of inflammatory factors in spinal cord of CCI rats

More and more scholars believe that the activation of central nervous immune cells and pain sensitization mediated by inflammatory factors play an important role in neuropathic pain[1]. CCI model can induce microglia to release TNF- α , IL-1 β and IL-6[18]. Therefore, we used Western Blot to analyze the expression of inflammatory factors in the spinal cord of CCI rats in order to explore whether daphnetin can regulate related inflammatory factors. The results showed that, compared with the control group, the expression of IL-1 β , IL-6 and TNF- α in the sham group did not increase significantly, indicating that subdural catheterization did not affect the expression of inflammatory factors in the spinal cord. Compared with sham group, the expression of IL-1 β , IL-6 and TNF- α in the model group was significantly increased ($P<0.01$). Compared with the model group, the expressions of IL-1 β , IL-6 and TNF- α in the DAPH group were significantly decreased ($P<0.05$), suggesting that intrathecal injection of daphnetin can inhibit the expression of related inflammatory factors in the spinal cord of CCI rats (Figure 3A-D).

Daphnetin regulates P2X₄ receptor in spinal microglia of CCI rats by interfering with IRF8 protein

The P2 purinergic receptor family on the surface of microglia is the key to microglia activation[19]. The P2 purinergic receptor family includes ion channel receptor P2X and metabolic G protein coupled receptor P2Y. The subtypes are P2X1-7 and P2Y1, 2, 4, 6, 11, 12, 13 and 14, respectively[20]. The key mechanism of intercellular signal transduction is to change the transmembrane potential and intracellular ion concentration through the ion current of P2XR[21]. P2X₄ and P2X₇ in P2X family allow organic macromolecules to transfer between cells, which can lead to pain sensitization[22]. In this experiment, we used Western Blot to analyze the protein expression of P2X₄ in the L4-L5 spinal cord of rats and found that compared with the sham group, the expression of P2X₄ protein in the model group was significantly increased ($P<0.01$). Compared with the control group, the expression of P2X₄ protein in the sham group did not change significantly, which proved that subdural catheterization failed to affect the expression of P2X₄ protein in the spinal cord of rats. Compared with the model group, the expression of P2X₄ protein in the DAPH group was significantly reduced ($P<0.01$), indicating that intrathecal injection of daphnetin can effectively inhibit the up-regulation of P2X₄ protein in the spinal cord of CCI rats (Figure 4A and 4C).

By immunofluorescence double staining technique, we stained the L4-L5 spinal cord of CCI rats with Iba-1 and P2X₄. Through fluorescence intensity analysis, it was found that the fluorescence intensities of Iba-1 and P2X₄ in the model group were enhanced in the spinal dorsal horn area ($P<0.01$ compared with the control group), and the fluorescence of Iba-1 and P2X₄ overlapped more. The fluorescence intensity of P2X₄ in DAPH group was significantly lower than that in model group ($P<0.01$) and the fluorescence of Iba-1 and P2X₄ did not overlap. This result is consistent with the trend of Western Blot protein results. In

the same sham group, the fluorescence intensity of Iba-1 and P2X₄ did not increase significantly (compared with the control group, $P<0.01$) (Figures 4E, 4H and 4I).

In order to further explore the mechanism of daphnetin intervention on P2X₄ protein, we explored its upstream and downstream target proteins. Masuda[23] recently found that the expression of interferon regulatory factor (IRF) 8 was selectively up-regulated in microglia after nerve injury. It was also found that IRF5 was the target of IRF8. After nerve injury, microglia in the ipsilateral spinal dorsal horn also selectively expressed IRF5[24]. Fibronectin in activated microglia regulates the transfer of IRF5 from cytoplasm to nucleus, and IRF5 induces the expression of P2X₄ receptor by directly binding to the promoter region of P2X₄ gene[25]. Accordingly, we determined the proteins of IRF8 and IRF5 by Western Blot. The results are shown in Figure 4B, 4F and 4G. Compared with the sham group, IRF8 and IRF5 in the model group were significantly increased ($P<0.05$, $P<0.01$). After intrathecal injection of daphnetin, the protein of IRF8 and IRF5 decreased significantly compared with model group ($P<0.01$).

Peripheral nerve injury stimulates microglia activation through P2X₄ receptors, which in turn induces microglia to release brain-derived neurotrophic factor (BDNF)[26]. BDNF can act on nociceptive projection neurons in layer I of spinal cord, which can lead to abnormal neuronal excitation and hypersensitivity to mechanical pain[27]. Therefore, we determined the protein expression of BDNF in the spinal cord, and the results are shown in Figure 4A and D. Compared with the sham group, the protein expression of BDNF in the model group increased significantly ($P<0.01$). After intrathecal injection of daphnetin, the protein of BDNF decreased significantly compared with model group ($P<0.01$). The above experimental results prove that daphnetin regulates the P2X₄ receptor in the spinal cord of CCI rats by interfering with the IRF8-IRF5-P2X₄ pathway, inhibits the activation of microglia, and then interferes with neuropathic pain.

Daphnetin inhibits the activation of MAPK pathway in spinal microglia of CCI rats by interfering with P2X₄ receptors

Mitogen-activated protein kinases (MAPKs) are evolutionarily conserved intracellular signaling molecules serine/threonine protein kinases, which play a key role in regulating gene expression, promoting disease development, and maintaining pain[28]. The MAPK family is divided into three main members: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and P38 MAPK[29]. Microglia phosphorylated by P38 is a common pathway after activation of cell surface receptors on microglia[29]. A variety of purinergic receptors can be used as initiators and regulators of the MAPK pathway to participate in the induction of pain sensitization[30]. After activation of the MAPK pathway, P38 MAPK increases the synthesis and release of a variety of microglia activation mediators, such as TNF- α , IL-1 β , IL-6, and prostaglandin E2 (PGE2), and promotes the development of neuropathic pain[31]. In the early stage of the experiment, it was found that daphnetin inhibits the activation of microglia in the spinal dorsal horn of CCI rats and the up-regulation of inflammatory factors by interfering with P2X₄ receptors. Therefore, we speculate that daphnetin may participate in regulating the related target proteins of MAPKs pathway in microglia to intervene in the synthesis and release of inflammatory factors.

We measured P38, JNK, ERK1/2 and their phosphorylated proteins by Western Blot, and calculated the ratio of phosphorylated protein to total protein to measure the changes of MAPKs pathway related proteins. The results showed that compared with Sham group, the protein expression ratios of p-P38/P38, p-JNK/JNK and p-ERK1/2 / ERK1/2 in Model group were significantly higher ($P<0.05$), indicating the activation of MAPKs pathway in spinal microglia, which is related to the overexpression of inflammatory factors. After intrathecal injection of daphnetin, it was found that the protein expression ratio of p-P38/P38 decreased significantly (compared with model group, $P<0.01$), while the protein expression of p-JNK/JNK and p-ERK1/2/ERK1/2 did not change significantly (Figures 5A, 5B, 5D and 5E).

According to the above experimental results, we stained the L4-L5 spinal cord of CCI rats with Iba-1 and P38 MAPK by immunofluorescence double staining technique. Through fluorescence intensity analysis, it was found that the fluorescence intensity of Iba-1 and P38 in the spinal dorsal horn area of the model group increased (compared with the sham group, $P<0.01$), and the fluorescence of Iba-1 and P38 overlapped more, which further verified that P38 protein was expressed in microglia after MAPK pathway activation. The fluorescence intensity of P38 in the DAPH group was significantly lower than that in the model group ($P<0.01$) and the fluorescence of Iba-1 and P38 did not overlap. The fluorescence intensity was consistent with the protein expression results of Western Blot. The fluorescence intensity of Iba-1 and P38 in the sham group did not increase significantly (compared with the control group, $P>0.05$) (Figure 5B, 5F and 5G).

Daphnetin inhibits the activation of neurons in spinal dorsal horn of CCI rats

As an important signal molecule of neurons, BDNF can combine with tyrosine kinase receptor B (TrkB) on neurons to reduce the expression of K^+-Cl^- cotransporter 2 (KCC2) on neurons, and then regulate the concentration difference of Cl^- inside and outside neurons, trigger the down regulation of GABA, produce depolarization reaction, make neurons continuously activated and induce neuropathic pain[32].

Therefore, we stained the L4-L5 spinal cord of CCI rats with c-fos by immunofluorescence technique. We found that neurons in the spinal dorsal horn of the model group were activated, and the c-fos fluorescence intensity was significantly increased compared with that of the sham group ($P<0.01$). Compared with the model group, the fluorescence intensity of c-fos in the DAPH group was significantly reduced ($P<0.01$), which proved that intrathecal injection of daphnetin can inhibit the activation of spinal dorsal horn neurons, thereby inhibiting the hyperalgesia caused by neuropathic pain (Figure 6 and supplementary Figure 1B).

Daphnetin interferes with P2X₄ receptor and P38 protein to inhibit neuropathic pain in CCI rats

In the previous experiment, we found that daphnetin inhibits microglia activation by interfering with P2X₄ receptors and inhibits the expression of P38 MAPK in microglia, thereby reducing the production of inflammatory factors and inhibiting the activation of neurons. However, it is unknown whether daphnetin can inhibit neuropathic pain by interfering with P2X₄ receptor and P38 protein. For this reason, we introduced P2X₄ and P38 MAPK blockers to verify the effect of daphnetin on pain. After the successful

modeling of CCI rats, the two groups of animals were given P2X₄ and P38 MAPK blockers by intragastric administration, once a day for 3 consecutive days. The TWT and MWT of rats were measured before intrathecal injection of daphnetin, 1 hour after injection and three days after injection. The results can be seen: 1 hour after the injection of daphnetin, there was no significant difference in TWT and MWT between the P2X₄ blocker group and the P38 MAPK blocker group compared with the model group ($P>0.05$), but there was significant difference in TWT compared with DAPH group ($P<0.01$). There was no significant difference between the TWT and MWT of the P2X₄ blocker group and the P38 MAPK blocker group compared with the model group ($P>0.05$) after 3 days of continuous injection of daphnetin, but there was significant difference in TWT and MWT compared with DAPH group ($P<0.05$). The above data showed that daphnetin could not inhibit neuropathic pain in CCI rats under the action of blocker P2X₄ or blocker P38 MAPK.

Discussion

In this experiment, we selected CCI model to simulate neuropathic pain. The selection of model has been confirmed in relevant reference[33]. CCI model is relatively stable and easy to replicate, and involves neuropathy and inflammatory response[34]. Therefore, we used the CCI model to explore the analgesic mechanism of daphnetin. In this experiment, we measured the MWT and TWT of CCI rats to evaluate the hyperalgesia in neuropathic pain. CCI rats showed obvious mechanical hyperalgesia and thermal hyperalgesia, which could be observed on the second day after surgery and continued until the end of the experiment. Our data showed that intrathecal injection of daphnetin could significantly reduce neuropathic pain caused by peripheral nerve injury. Specifically, it effectively reduced TWT and MWT in Daph group. Daphnetin can quickly inhibit mechanical pain sensitization and thermal pain sensitization after nerve injury in rats.

Hiroshi et al. proved that the activation of spinal nerve microglia and the increase of IL-1 β are involved in the process of neuropathic pain through the lumbar nerve root pain model[35]. This research has changed people's perception of neuropathic pain from the activation of a single neuron to the role of glial cells and inflammatory factors. Peripheral nerve injury induces macrophage aggregation and cytokine production, and leads to neuronal sensitization and inflammation in the spinal dorsal horn[36]. Under the action of inflammatory factors, it can also increase the discharge of spinal cord neurons, further aggravating the production of pain[37]. IL-1 β can directly act on sensitizing receptors through transient receptor potential vanilloid type-1 (TRPV1) and IL-1 receptors to induce pain, and it can also indirectly sensitize nociceptors to induce pain by increasing the synthesis of PGE₂[38]. TNF- α induced neuronal sensitization and hyperalgesia are dependent on TRPV1 and TRPA1[39]. TNF- α also changes neuronal excitability through P38 MAPK mediated phosphorylation of Nav1.8 and Nav1.9 sodium channels, thereby inducing rapid regulation of nociceptor sensitivity[40]. After peripheral nerve injury, the mRNA and protein expression levels of spinal cord IL-6 and IL-6 receptors are up-regulated[41]. The function of IL-6 is similar to TNF- α , in addition to enhancing excitatory currents, it can also increase the phosphorylation of cAMP-response element binding protein (CREB) to produce thermal hyperalgesia[42]. In this experiment,

we observed that the spinal cord of model group produced a large amount of IL-1 β , IL-6 and TNF- α inflammatory factors. After 3 consecutive days of intrathecal injection of daphnetin, it was found that the protein expression of IL-1 β , IL-6 and TNF- α in the spinal cord of model group was significantly down-regulated, which proved that daphnetin could effectively inhibit the expression of inflammatory factors in the spinal cord. This may be due to daphnetin is the key to the analgesic effect.

Microglia are immunologically active cells in the central nervous system, which are responsible for regulating the transmission of neuronal excitement[5]. Tsuda's report revealed for the first time the role of microglia in neuropathic pain[6]. Purinergic P2 receptors, especially ionotropic P2 subtype P2X₄ receptors, were found to be key receptors in spinal cord microglia for the development of mechanical hyperalgesia induced by peripheral nerve injury. In the immunofluorescence section of spinal cord, we found that the number of activated microglia in the spinal dorsal horn of rats in model group increased significantly compared with the control group. After intrathecal injection of daphnetin, the activation of microglia in rat of DapH group spinal dorsal horn was effectively inhibited.

Using Western Blot technology, we found that the P2X₄ protein of the L4-L5 spinal cord of rats in model group was highly expressed. At the same time, it was found that intrathecal injection of daphnetin could effectively inhibit the expression of P2X₄ protein of the spinal cord of DAPH group. Immunofluorescence double staining can also prove the above results. Based on this, we speculate that daphnetin inhibits microglia activation by intervening in P2X₄ receptors.

IRF8 is a key regulator that selectively up-regulates the expression of P2X₄ receptor in microglia[43]. Masuda[23] and others recently discovered that in the spinal cord, the expression of IRF8 is selectively upregulated in microglia after nerve injury. They also found that IRF8-deficient mice showed a reduction in tactile allodynia induced by nerve injury. Further analysis showed that IRF5 is a direct transcription regulator of P2X₄ receptor under IRF8 transcription regulation[24]. Therefore, the IRF8-IRF5 transcription axis helps to regulate the P2X₄ receptor activation of the ipsilateral spinal dorsal horn microglia after nerve injury. According to the results of Western blot, the protein expression of IRF8 and IRF5 in spinal cord of rats in model group was significantly up-regulated. Intrathecal injection of daphnetin could significantly inhibit the expression of IRF8 and IRF5 proteins. Therefore, we speculate that daphnetin regulates P2X₄ receptor through IRF8-IRF5 axis.

P2X₄ purinergic receptors can be used as the initiating factor and regulator of the MAPK pathway to participate in the induction of pain sensitization[30]. MAPK is activated by phosphorylation and converts a wide range of extracellular stimuli into a variety of intracellular responses through transcriptional and non-transcriptional regulation. The activation of the MAPK pathway induces the expression of a variety of inflammatory factors[44], which is consistent with our previous experimental data. After intrathecal injection of daphnetin, we found that the expression of inflammatory factors in the spinal cord of rats in DapH group was significantly reduced, which may be related to daphnetin's intervention in the MAPK pathway of microglia in the spinal cord of CCI rats. P38 MAPK is activated by the upstream kinase

MKK3/MMK6 and is considered to be a stress-induced kinase, which plays a key role in the inflammatory response [45]. Asiaticoside reduces neuropathic pain induced by spinal cord injury (SCI) through anti-inflammatory effects and inhibiting the mechanism of P38 MAPK[46]. Intrathecal injection of anti-inflammatory cytokines can reduce the inflammation induced by spinal cord injury and inhibit the activation of P38 MAPK induced by spinal cord injury[47]. ERK activation was observed in spinal microglia 1-2 days after nerve injury[48]. Activated ERK also plays a key role in neuronal plasticity, contributing to the nociceptive response of the spinal dorsal horn after inflammation and/or nerve injury[49]. After peripheral nerve injury, the activation of JNK mainly occurs in C-fiber neurons with smaller diameters, which is very important for the early development of neuropathic pain[50]. To explore how daphnetin interferes with the MAPK pathway, we detected P38, JNK and ERK1/2 and their phosphorylated proteins. In the experimental results, we saw that p-P38/P38 was significantly decreased in DapH group after intrathecal injection of daphnetin. Daphnetin does not have an effect on the three MAPK pathways as envisaged, but can only have a significant inhibitory effect on p-P38/P38. Combined with the results of immunofluorescence double staining, it can also confirm the inhibitory effect of daphnetin on P38 MAPK in microglia. It can be seen that daphnetin reduces the expression of inflammatory factors in the spinal cord by inhibiting the P38 MAPK pathway in microglia.

BDNF is a neurotrophic factor that plays an important role in neuron survival and differentiation. However, in addition to the classic neurotrophic effects, BDNF as a neuromodulator directly participates in the control of neuronal activity and synaptic plasticity[51]. Stimulated by harmful signaling factors, microglia are activated rapidly. According to the involved signaling pathways, this process may lead to the secretion of specific messengers, including BDNF[52]. Once released, BDNF will reshape the excitability of neuronal electrical conduction through the above-mentioned signal cascade. In this experiment, we can observe a large number of activation of neurons in the spinal dorsal horn of rats in model group by immunofluorescence staining, which is related to the large secretion of inflammatory factors and BDNF in the spinal cord of rats. In the presence of BDNF, the plastic rearrangement of spinal dorsal horn neurons can promote central sensitization and help maintain hyperalgesia and hyperalgesia[53]. After intrathecal injection of daphnetin, we found that the expression of BDNF in spinal cord of CCI rats decreased significantly, which may be related to inhibiting the activation of microglia and intervening in p38 MAPK pathway. According to our data, daphnetin can effectively reduce the expression of inflammatory factors and BDNF and inhibit the over activation of neurons, so as to intervene in pain sensitization and finally achieve the analgesic effect.

Conclusion

Daphnetin inhibits the expression of IRF8 and IRF5 proteins, interferes with the P2X₄ receptor, effectively inhibits the activation of microglia and P38 MAPK pathway, reduces the overexpression of inflammatory factors and BDNF in the spinal cord, and greatly reduces the sensitization of neurons, so as to achieve the effect of improving neuropathic pain.

Abbreviations

CCI: chronic constrictive injury; MWT: mechanical withdrawal threshold; TWT: thermal withdrawal threshold; DRG: dorsal root ganglion; P2X4: P2X purine receptor 4; P38 MAPK: P38 mitogen activated protein kinase; MIF: migration inhibitory factor; EAE: experimental autoimmune encephalomyelitis; RA: rheumatoid arthritis; SD rat: Sprague Dawley rat; SD: standard deviation; IRF: interferon regulatory factor; BDNF: brain-derived neurotrophic factor; PGE2: prostaglandin E2; TrkB: tyrosine kinase receptor B; KCC2: K⁺-Cl⁻ cotransporter 2; GABA: Gamma-Amino Butyric Acid; TRPV1: transient receptor potential vanilloid type-1; CREB: cAMP-response element binding protein;

Declarations

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Not applicable.

Authors' contributions

Tianrui Zhang, Mingqian Zhang, Wenjing Ou, Shuang Cui, Wulin Liang, Zhanhong Jia, Xue Fan, Yonghong Wu and Shuofeng Zhang participated in drafting, editing, and writing the manuscript. Dongyu Ge and Ruijuan Dong provided pathological technical support. Tianrui Zhang and Shuofeng Zhang approved the final version of the manuscript. Tianrui Zhang and Shuofeng Zhang designed the table and figures.

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Availability of data and materials

The data used to support the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

All authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interests. The authors declare that they have no competing interest.

References

1. Finnerup NB, Kuner R, Jensen TS. Neuropathic Pain: From Mechanisms to Treatment. *Physiol Rev*. 2021;101(1):259-301.
2. Bouhassira D, Lanteri-Minet M, Attal N, Laurent B, Touboul C. Prevalence of chronic pain with neuropathic characteristics in the general population. *Pain*. 2008;136(3):380-7.
3. Bouhassira D. Neuropathic pain: Definition, assessment and epidemiology. *Rev Neurol (Paris)*. 2019;175(1-2):16-25.
4. Ji RR, Xu ZZ, Gao YJ. Emerging targets in neuroinflammation-driven chronic pain. *Nat Rev Drug Discov*. 2014;13(7):533-48.
5. Inoue K, Tsuda M. Microglia in neuropathic pain: cellular and molecular mechanisms and therapeutic potential. *Nat Rev Neurosci*. 2018;19(3):138-52.
6. Tsuda M, Shigemoto-Mogami Y, Koizumi S, Mizokoshi A, Kohsaka S, Salter MW, et al. P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature*. 2003;424(6950):778-83.
7. Masoodifar M, Hajhashemi S, Pazhoohan S, Nazemi S, Mojadadi MS. Effect of the conditioned medium of mesenchymal stem cells on the expression levels of P2X4 and P2X7 purinergic receptors in the spinal cord of rats with neuropathic pain. *Purinergic Signal*. 2021;17(1):143-50.
8. Yu WW, Lu Z, Zhang H, Kang YH, Mao Y, Wang HH, et al. Anti-inflammatory and protective properties of daphnetin in endotoxin-induced lung injury. *J Agric Food Chem*. 2014;62(51):12315-25.
9. Yu W, Wang H, Ying H, Yu Y, Chen D, Ge W, et al. Daphnetin attenuates microglial activation and proinflammatory factor production via multiple signaling pathways. *Int Immunopharmacol*. 2014;21(1):1-9.
10. Yang Y, Sheng Q, Nie Z, Liu L, Zhang W, Chen G, et al. Daphnetin inhibits spinal glial activation via Nrf2/HO-1/NF-kappaB signaling pathway and attenuates CFA-induced inflammatory pain. *Int Immunopharmacol*. 2021;98:107882.
11. Storkson RV, Kjorsvik A, Tjolsen A, Hole K. Lumbar catheterization of the spinal subarachnoid space in the rat. *J Neurosci Methods*. 1996;65(2):167-72.
12. Bennett GJ, Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain*. 1988;33(1):87-107.
13. Dixon WJ. Efficient analysis of experimental observations. *Annu Rev Pharmacol Toxicol*. 1980;20:441-62.
14. Wang L, Yin C, Liu T, Abdul M, Zhou Y, Cao JL, et al. Pellino1 regulates neuropathic pain as well as microglial activation through the regulation of MAPK/NF-kappaB signaling in the spinal cord. *J Neuroinflammation*. 2020;17(1):83.
15. Li J, Zhu Y, Ma Z, Liu Y, Sun Z, Wu Y. miR-140 ameliorates neuropathic pain in CCI rats by targeting S1PR1. *J Recept Signal Transduct Res*. 2021;41(4):401-7.
16. Zhao H, Alam A, Chen Q, M AE, Pal A, Eguchi S, et al. The role of microglia in the pathobiology of neuropathic pain development: what do we know? *Br J Anaesth*. 2017;118(4):504-16.

17. Chen G, Zhang YQ, Qadri YJ, Serhan CN, Ji RR. Microglia in Pain: Detrimental and Protective Roles in Pathogenesis and Resolution of Pain. *Neuron*. 2018;100(6):1292-311.
18. Chu LW, Cheng KI, Chen JY, Cheng YC, Chang YC, Yeh JL, et al. Loganin prevents chronic constriction injury-provoked neuropathic pain by reducing TNF-alpha/IL-1beta-mediated NF-kappaB activation and Schwann cell demyelination. *Phytomedicine*. 2020;67:153166.
19. Agostinho P, Madeira D, Dias L, Simoes AP, Cunha RA, Canas PM. Purinergic signaling orchestrating neuron-glia communication. *Pharmacol Res*. 2020;162:105253.
20. Tsuda M, Beggs S, Salter MW, Inoue K. Microglia and intractable chronic pain. *Glia*. 2013;61(1):55-61.
21. Khakh BS, North RA. Neuromodulation by extracellular ATP and P2X receptors in the CNS. *Neuron*. 2012;76(1):51-69.
22. Bernier LP, Ase AR, Boue-Grabot E, Seguela P. P2X4 receptor channels form large noncytolytic pores in resting and activated microglia. *Glia*. 2012;60(5):728-37.
23. Masuda T, Tsuda M, Yoshinaga R, Tozaki-Saitoh H, Ozato K, Tamura T, et al. IRF8 is a critical transcription factor for transforming microglia into a reactive phenotype. *Cell Rep*. 2012;1(4):334-40.
24. Masuda T, Iwamoto S, Yoshinaga R, Tozaki-Saitoh H, Nishiyama A, Mak TW, et al. Transcription factor IRF5 drives P2X4R+reactive microglia gating neuropathic pain. *Nat Commun*. 2014;5:3771.
25. Matsumura Y, Yamashita T, Sasaki A, Nakata E, Kohno K, Masuda T, et al. A novel P2X4 receptor-selective antagonist produces anti-allodynic effect in a mouse model of herpetic pain. *Sci Rep*. 2016;6:32461.
26. Trang T, Beggs S, Wan X, Salter MW. P2X4-receptor-mediated synthesis and release of brain-derived neurotrophic factor in microglia is dependent on calcium and p38-mitogen-activated protein kinase activation. *J Neurosci*. 2009;29(11):3518-28.
27. Kohno K, Tsuda M. Role of microglia and P2X4 receptors in chronic pain. *Pain Rep*. 2021;6(1):e864.
28. Mai L, Zhu X, Huang F, He H, Fan W. p38 mitogen-activated protein kinase and pain. *Life Sci*. 2020;256:117885.
29. Kumar S, Boehm J, Lee JC. p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. *Nat Rev Drug Discov*. 2003;2(9):717-26.
30. Burnstock G. Purinergic receptors and pain. *Curr Pharm Des*. 2009;15(15):1717-35.
31. Ji RR, Berta T, Nedergaard M. Glia and pain: is chronic pain a gliopathy? *Pain*. 2013;154 Suppl 1:S10-S28.
32. Hildebrand ME, Xu J, Dedek A, Li Y, Sengar AS, Beggs S, et al. Potentiation of Synaptic GluN2B NMDAR Currents by Fyn Kinase Is Gated through BDNF-Mediated Disinhibition in Spinal Pain Processing. *Cell Rep*. 2016;17(10):2753-65.
33. Wilkerson JL, Jiang J, Felix JS, Bray JK, da Silva L, Gharaibeh RZ, et al. Alterations in mouse spinal cord and sciatic nerve microRNAs after the chronic constriction injury (CCI) model of neuropathic pain. *Neurosci Lett*. 2020;731:135029.

34. Challa SR. Surgical animal models of neuropathic pain: Pros and Cons. *Int J Neurosci*. 2015;125(3):170-4.
35. Hiroshi Hashizume JAD, Raymond W. Colburn, and James N. Weinstein, . Spinal glial activation and cytokine expression after lumbar root injury in the rat. *Spine*. 2000;25:1206-27.
36. Malcangio M. Role of the immune system in neuropathic pain. *Scandinavian Journal of Pain*. 2019;20(1):33-7.
37. Xanthos DN, Sandkühler J. Neurogenic neuroinflammation: inflammatory CNS reactions in response to neuronal activity. *Nature Reviews Neuroscience*. 2013;15(1):43-53.
38. Binshtok AM, Wang H, Zimmermann K, Amaya F, Vardeh D, Shi L, et al. Nociceptors are interleukin-1beta sensors. *J Neurosci*. 2008;28(52):14062-73.
39. Fernandes ES, Russell FA, Spina D, McDougall JJ, Graepel R, Gentry C, et al. A distinct role for transient receptor potential ankyrin 1, in addition to transient receptor potential vanilloid 1, in tumor necrosis factor alpha-induced inflammatory hyperalgesia and Freund's complete adjuvant-induced monarthritis. *Arthritis Rheum*. 2011;63(3):819-29.
40. Gudes S, Barkai O, Caspi Y, Katz B, Lev S, Binshtok AM. The role of slow and persistent TTX-resistant sodium currents in acute tumor necrosis factor-alpha-mediated increase in nociceptors excitability. *J Neurophysiol*. 2015;113(2):601-19.
41. Hung AL, Lim M, Doshi TL. Targeting cytokines for treatment of neuropathic pain. *Scand J Pain*. 2017;17:287-93.
42. Li QY, Xu HY, Yang HJ. [Effect of proinflammatory factors TNF-alpha,IL-1beta, IL-6 on neuropathic pain]. *Zhongguo Zhong Yao Za Zhi*. 2017;42(19):3709-12.
43. Tozaki-Saitoh H, Tsuda M. Microglia-neuron interactions in the models of neuropathic pain. *Biochem Pharmacol*. 2019;169:113614.
44. Arthur JS, Ley SC. Mitogen-activated protein kinases in innate immunity. *Nat Rev Immunol*. 2013;13(9):679-92.
45. Ji RR, Suter MR. p38 MAPK, microglial signaling, and neuropathic pain. *Mol Pain*. 2007;3:33.
46. Luo Y, Fu C, Wang Z, Zhang Z, Wang H, Liu Y. Asiaticoside attenuates the effects of spinal cord injury through antioxidant and antiinflammatory effects, and inhibition of the p38MAPK mechanism. *Mol Med Rep*. 2015;12(6):8294-300.
47. Sun S, Chen D, Lin F, Chen M, Yu H, Hou L, et al. Role of interleukin-4, the chemokine CCL3 and its receptor CCR5 in neuropathic pain. *Mol Immunol*. 2016;77:184-92.
48. Kondo M, Shibuta I. Extracellular signal-regulated kinases (ERK) 1 and 2 as a key molecule in pain research. *J Oral Sci*. 2020;62(2):147-9.
49. Ma W, Quirion R. The ERK/MAPK pathway, as a target for the treatment of neuropathic pain. *Expert Opin Ther Targets*. 2005;9(4):699-713.
50. Gao YJ, Ji RR. Activation of JNK pathway in persistent pain. *Neurosci Lett*. 2008;437(3):180-3.

51. Ferrini F, De Koninck Y. Microglia control neuronal network excitability via BDNF signalling. *Neural Plast.* 2013;2013:429815.
52. Ulmann L, Hatcher JP, Hughes JP, Chaumont S, Green PJ, Conquet F, et al. Up-regulation of P2X4 receptors in spinal microglia after peripheral nerve injury mediates BDNF release and neuropathic pain. *J Neurosci.* 2008;28(44):11263-8.
53. Cappoli N, Tabolacci E, Aceto P, Dello Russo C. The emerging role of the BDNF-TrkB signaling pathway in the modulation of pain perception. *J Neuroimmunol.* 2020;349:577406.

Figures

Figure 1

The effect of daphnetin on the pain sensation of CCI rats. (A) The structural formula of daphnetin. (B) Experimental flowchart. (C) Spinal cord subdural intubation in rats. In the figure, the rats were intubated at L4-L5 spinal cord in the subdural cavity. After injection of lidocaine hydrochloride, they immediately became paralyzed. (D) The change of TWT in CCI rats within different administration time ($n=6$). (E) The

change of MWT in CCI rats within different administration time ($n=6$). $*P<0.05$, $**P<0.01$ vs. model group.

Figure 2

Effect of daphnetin on microglia activation in spinal dorsal horn of CCI rats. The rat spinal cord microglia marker Iba-1 was stained by immunofluorescence ($n=3$).

Figure 3

Effect of daphnetin on inflammatory factors in spinal cord of CCI rats.

(A-D) Protein expression of IL-1 β , IL-6, and TNF- α in the spinal cord ($n=3$). * $P<0.05$, ** $P<0.01$ vs. model group.

Figure 4

Effect of daphnetin on P2X₄ receptor in spinal cord of CCI rats. (A-D, F, G) Protein expression of IRF8, IRF5, P2X₄ and BDNF in spinal cord ($n=3$). (E, H, I) Expression of Iba-1 and P2X₄ receptors in spinal dorsal horn by immunofluorescence double staining ($n=3$), * $P<0.05$, ** $P<0.01$ vs. model group.

Figure 5

The effect of daphnetin on the MAPK pathway of spinal cord microglia in CCI rats. (A, B, D, E) The protein expression of P38/p-P38, JNK/p-JNK, ERK1/2/p-ERK1/2 in the spinal cord ($n=3$). (C, F, G) The expression of Iba-1 and P38 receptors in the spinal dorsal horn in immunofluorescence double staining ($n=3$).

* $P<0.05$, ** $P<0.01$ vs. model group.

Figure 6

Effect of daphnetin on activation of spinal dorsal horn neurons in CCI rats. Immunofluorescence staining was performed on c-fos, a marker of rat spinal cord neurons ($n=3$).

Figure 7

Daphnetin interferes with P2X₄ receptor and P38 protein to improve neuropathic pain in CCI rats. (A) The changes of TWT in different groups of CCI rats in different administration time ($n=3$). (B) The change of MWT in different groups of CCI rats in different administration time ($n=3$). * $P < 0.05$, ** $P < 0.01$ vs. model group; # $P < 0.05$, ## $P < 0.01$ vs. DAPH group.

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