Original Article
Inhibition of GAP-43 by propentofylline in a rat model of neuropathic pain

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Abstract: Neural plasticity within the spinal nociceptive network may be fundamental to the chronic nature of neuropathic pain. The relation of growth-associated protein-43 (GAP-43), a protein involved in the nerve fiber growth and sprouting, to pain hypersensitivity has been investigated. Glial activation and inflammatory cytokines released by microglia and astrocytes are considered to be involved in the neural sprouting and plasticity. In the present study, the anti-nociception effect of propentofylline, a glial modulating agent, was investigated in a rat chronic constriction injury (CCI) model aiming to explore the role of GAP-43 expression. Our results demonstrated that propentofylline could attenuate the CCI-induced mechanical allodynia and thermal hyperalgesia and inhibit the astrocyte activation and production of IL-1β. GAP-43 expression was also down-regulated by intrathecal propentofylline. These findings suggest that astrocyte activation is involved in the regulation of GAP-43 expression and propentofylline might be used in the treatment of neuropathic pain.

Keywords: Astrocytes, growth-associated protein-43, interleukin 1β, neuropathic pain

Introduction

Neuropathic pain (NP) is a debilitating condition affecting millions of people worldwide. NP remains a prevalent and persistent clinical problem due to incomplete understanding of its pathogenesis [1]. A variety of mechanisms have been proposed for the induction and/or maintenance of NP. In recent years, more attention has been paid to the role of glia activation in NP [2, 3].

It has been demonstrated that glial cells in the central nervous system (CNS) play roles in many aspects of neuronal functions including pain processing [4]. Previous studies have shown that glial modulating agents, such as propentofylline, can exhibit anti-allodynic effect in NP models [5, 6]. Peripheral tissue damage or inflammation initiates signals to alter the functions of microglia and astrocytes, causing the release of factors that regulate the nociceptive neuronal excitability [7]. The inflammatory cytokines, such as interleukin 1β (IL-1β), IL-6 and tumor necrosis factor α (TNF-α), are capa-

ble of activating and sensitizing peripheral nociceptive neurons and thereby contributing to the ongoing pain and hyperalgesia. In addition, Parish et al [8] also showed the roles of IL-1 and IL-6 in the neuronal plasticity and regeneration.

Pathological pain is an expression of neuronal plasticity. In the nervous system, growth associated protein 43 (GAP-43) is considered as an internal decision factor in the nerve regeneration and serves as a marker of neural plasticity [6]. GAP-43 has been regarded as an important factor related to the NP. In the L5 spinal nerve transection model of mechanical pain hypersensitivity, GAP-43 was found to be up-regulated in the spinal dorsal horn from 5 up to 10 days after injury [9]. During the nerve injury, astrocytes were activated and probably contributed to the nerve regeneration [4]. However, whether astrocyte activation involves in the up-regulation of GAP-43 needs to be further elucidated.

In present study, the glial cell activation was inhibited by intrathecal administration of pro-

pentofylline in a rat chronic constriction injury (CCI) model to explore the role of astrocytes and GAP-43 expression in the NP.

Materials and methods

Animals

Male Sprague-Dawley (SD) rats weighing 200-250 g (Shanghai Experimental Animal Center of Chinese Academy of Sciences) were housed in a specific pathogen free (SPF) environment with a 12/12 h light /dark cycle. All experiments were approved by the Administrative Committee of Experimental Animal Care and Use of Shanghai, and in accordance to the Guidelines of National Institute of Health on the ethical use of animals. Animals were randomly assigned into three groups (n=48 per group): sham group, normal saline group (NS group), and propentofylline group (PPF group). Propentofylline (40 μg/40 μl) and NS of equal volume were administered intrathecally once daily starting at 1 day after surgery in the PPF and NS groups, respectively. Propentofylline is an atypical methylxanthine previously shown to attenuate the astrocyte activation. Propentofylline was purchased from the Sigma-Aldrich Shanghai Trading Corporation (Shanghai, China) and was given as described [10].

Chronic constriction injury

The CCI model was established as previously described [11]. Briefly, rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). The common sciatic nerve was exposed at the mid-thigh level. The nerve was ligated loosely with a 4-0 chromic gut suture at 4 sites with an interval of 1 mm, so that the nerve diameter was only slightly reduced. Meanwhile, a sham surgery was performed on the sciatic nerve but ligation was absent in the sham group. Upon recovery from anesthesia, animals were housed individually in clear plastic cages.

Lumbar subarachnoid catheterization

One week prior to CCI, a chronic indwelling catheter was implanted into the subarachnoid space of each rat. Briefly, rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). A PE-10 catheter (Becton Dickinson, Sparks, MD, USA) was inserted into the lumbar

subarachnoid space between 5th and 6th lumbar vertebrae (L5 and L6) [12]. The catheter was slowly implanted and the external portion was protected according to Milligan's method [13].

*Evaluation of thermal hyperalgesia and mechanical allodynia*

Thermal hyperalgesia was assessed by the paw withdrawal latency (PWL) to radiant heat according to the described by Hargreaves et al [14]. Rats were placed on a piece of 3-mm-thick glass plate in an inverted clear plexiglas cage (23×18×13 cm) and allowed to acclimate to the environment for 30 min before testing. Then, the radiant heat source was positioned under the glass floor directly beneath the hind paw. The radiant heat source was a high-intensity projection lamp bulb (8 V, 50 W) which located at 40 mm below the glass floor and projected through a 5×10-mm aperture on the top of a movable case. A digital timer automatically recorded the duration between the stimuli and the paw withdrawal, which was defined as PWL. Experiment was carried out thrice in each rat with a 5-min interval. The heat exposure was confined to 20 sec to avoid tissue damage.

Mechanical allodynia was assessed with the von Frey filaments. Rats were placed on a wire mesh platform, covered with a transparent plastic dome, and allowed to acclimate for 30 min before testing. The filament was applied perpendicularly to the plantar surface of the hindpaw (ipsilateral to the side of CCI). The paw withdrawal threshold (PWT) was determined by sequentially increasing and decreasing the stimulus strength ('up-and-down' method) (in gram, g), and data were analyzed using the nonparametric method of Dixon [15].

Western blot assay

On the 3rd, 5th and 7th day, rats were sacrificed and the lumbar spinal cords (L4-L5) were quickly removed and prepared as previously described [16]. Proteins were separated by 8% SDS-PAGE and then transferred onto a nitrocellulose membrane which was blotted with primary antibody against GAP-43 (1:100, Santa Cruz, CA, USA) and then with horseradish peroxidase conjugated secondary antibody. Protein signals were detected with an ECL system (Amersham Pharmacia Biotech, Uppsala,
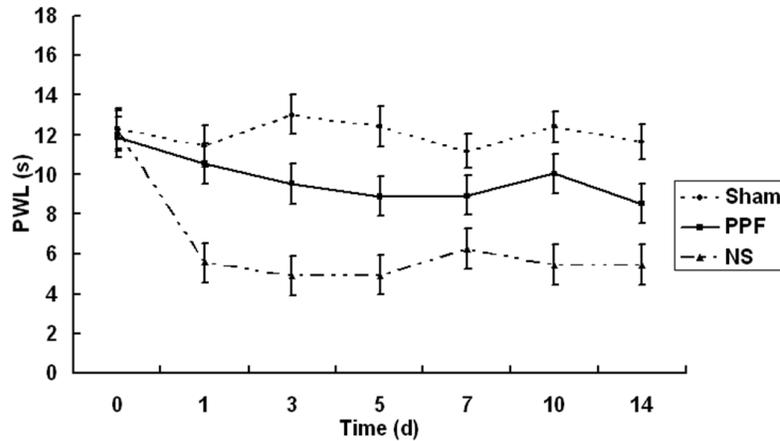
Figure 1. PPF increases PWL. After the sciatic nerve was ligated, the PWL decreased significantly in NS group compared to Sham group (p<0.05). The injection of PPF increased the PWL significantly in PPF group compared to NS group (p<0.05).
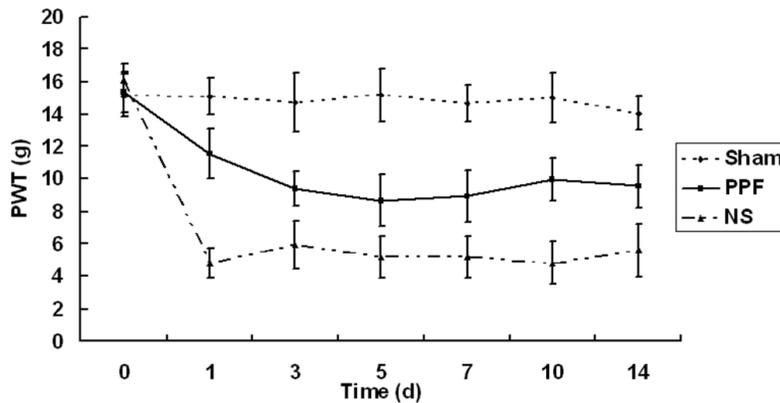
Figure 2. PPF increases PWT. PWT was used to measure mechanical allodynia. Upon NS, but not sham surgery, pain response such as mechanical allodynia was induced, as evidenced by reduced PWT. In PPF group, however, CCI-induced mechanical allodynia was attenuated comparing to NS group (P<0.05).

buffer (pH 7.2-7.4) containing 1.5% picric acid. After perfusion, the L5 spinal cord was collected and fixed in the same fixation solution for 3 h and then in 15% sucrose overnight. Transverse spinal sections (30 μm) were obtained on a cryostat and processed for immunofluorescence assay [16]. All the sections were blocked in 0.3% Triton X-100 containing 2% goat serum for 1 h at room temperature and incubated over two nights at 4°C with anti-GFAP antibody (1:400; Santa Cruz, USA). The sections were incubated for 1 h at room temperature with Cy3-conjugated secondary antibody (1:300; Santa Cruz, USA), and then with a mixture of Alexa Fluor 555-conjugated secondary antibodies for 1 h at room temperature. These sections were examined under an Olympus (Olympus, Japan) fluorescence microscope, and representative images were captured.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using Student's t-test or multiple-factor ANOVA followed by least-significance difference post-hoc comparison. A value of P<0.05 was considered statistically significant.

Sweden). GAPDH (Sigma, St. Louis, MO, USA, 1:500) served as an internal control.

Enzyme linked immunosorbent assay (ELISA)

Dorsal spinal cord tissue samples were prepared as previously described [17]. IL-1β in spinal cords was detected by ELISA according to the manufacturer's instructions (Peprotech, UK).

Immunofluorescence assay

Rats were anesthetized and perfused through the ascending aorta with saline, and then with 4% paraformaldehyde in 0.16 M phosphate

Results

PPF attenuates nociception in CCI rats

After the sciatic nerve was ligated, the PWL, representing the threshold of thermal pain, decreased significantly (P<0.05). This indicated that thermal hyperalgesia was induced by the ligation. PPF gradually and significantly increase the PWL (P<0.05), but the PWL remained unchanged in the NS group (P>0.05) (**Figure 1**).
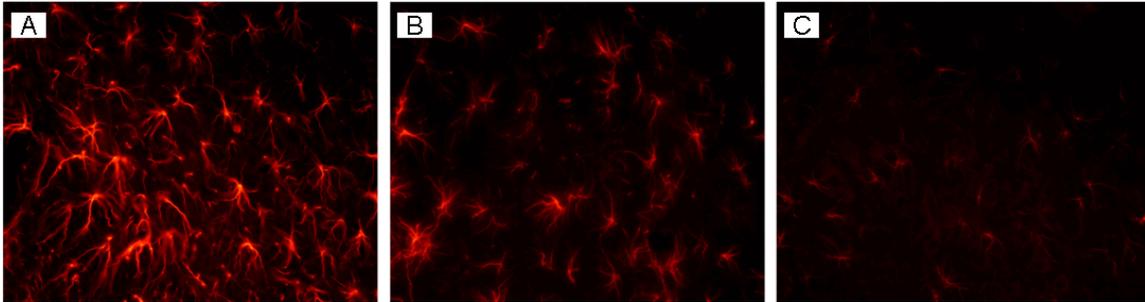
Figure 3. Inhibition of astrocytes by PPF. Immunohistochemistry shows GFAP expression in spinal cord. In the sham group, the astrocytes were in a quiescent state. After sciatic nerve ligation, the astrocytes in the NS were activated and characterized by enlargement of GFAP positive cells, increase and enlargement of in processes and strong positive for GFAP. After PPF treatment, the number of activated astrocytes reduced and the fluorescence intensity decreased accompanied by reduction in processes. A: NS; B: PPF; C: Sham.
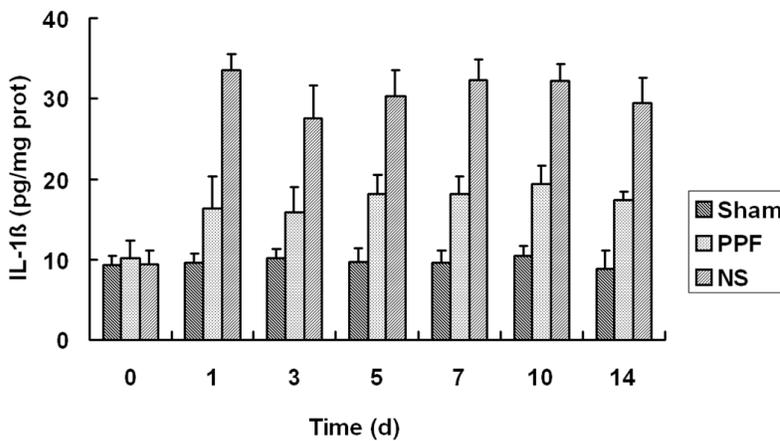
Figure 4. Down-regulation of IL-1β in spinal cord. Tissues from dorsal spinal cord were prepared and ELISA was performed as described. IL-1β was up-regulated in the dorsal spinal cord tissues of CCI rats, and compared with the NS group (P<0.05), the production of IL-1β in spinal cord was significantly decreased during the course of PPF treatment (P<0.05). ELASA showed the IL-1β expression was at a low level in the Sham group. The IL-1β expression began to increased at 1 day after sciatic nerve ligation and remained at a high level during the experiment, which was significantly higher than that in the sham group (P<0.05). After PPF treatment, the IL-1β expression reduced significantly (P<0.05 vs NS group). Thus, we speculated that PPF could inhibit the astrocyte activation to reduce the IL-1β expression.

tribute to the development of sprouting. In the Sham group, the astrocytes in the spinal cord were in a quiescent state. After sciatic nerve ligation, the astrocytes were activated in the NS group, demonstrated by enlargement of GFAP positive cells, increase and enlargement of processes and increase in fluorescence intensity (**Figure 3**). After PPF treatment, the activated astrocytes reduced, fluorescence intensity decreased and processes reduced. These findings suggest that sciatic nerve ligation induced NP is related to the activation of astrocytes, which may be inhibited by PPF.

Down-regulation of IL-1β in spinal cord

PWT was used to measure the mechanical allodynia. Upon NS, but not sham surgery, pain response such as mechanical allodynia was induced, as evidenced by reduced PWT. CCI rats receiving intrathecal PPF, however, presented with attenuated CCI-induced mechanical allodynia (P<0.05), as compared to the CCI rats treated with NS (**Figure 2**).

Inhibition of astrocytes by PPF

GFAP expression decreased significantly after PPF treatment. Astrocyte activation may con-

IL-1β was up-regulated in the dorsal spinal cord of CCI rats. When compared with the NS group, the IL-1β in the spinal cord was significantly lowered after PPF treatment (P<0.05), indicating that intrathecal PPF significantly attenuates the IL-1β expression in the spinal cord of CCI rats (**Figure 4**).

Detection of GAP-43 expression by immuno-fluorescence and western blot assays

As shown in **Figure 5**, the GAP-43 was massively expressed in the ipsilateral dorsal horn
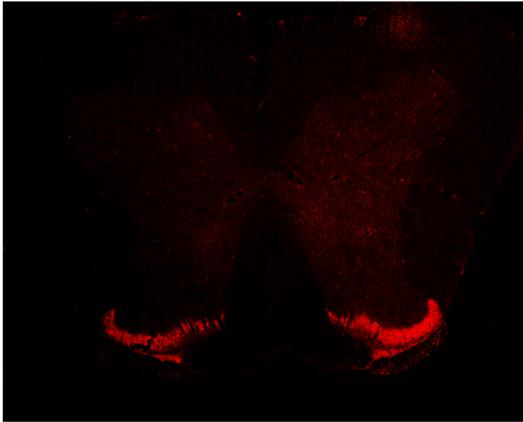
Figure 5. Detection of GAP-43 expression by immunofluorescence. Immunofluorescence revealed the GAP-43 expression in the spinal cord of CCI rats. The GAP-43 was massively expressed in the ipsilateral dorsal horn of spinal cord (red) following ligation, but less found in the contralateral side without ligation (×40).

(red) following sciatic nerve ligation, but less found in the contralateral spinal cord with intact sciatic nerve. Western blot assay revealed the GAP-43 expression in the PPF group was significantly lower than that in the NS group at 3, 5 and 7 days after surgery (**Figure 6**). These results indicate that inhibition of astrocyte activation may reduce the GAP-43 expression.

Discussion

In this study, we investigated the spatiotemporal expression of GAP-43 in the dorsal horn of CCI rats and the therapeutic effect of PPF was also investigated. Our results showed the GAP-43 expression was significantly up-regulated in the L4-5 dorsal horn after sciatic nerve ligation. Treatment with PPF, a glial modulator, effectively attenuated the CCI-induced thermal and mechanical pain hypersensitivity. In addition, the GFAP expression, IL-1β up-regulation and spinal GAP-43 expression were also inhibited by PPF.

Glia, such as microglia and astrocyte, plays key roles in the development, inflammation and repair of CNS by secreting a variety of cytokines, chemokines, and growth factors [18-20]. Many studies have shown that the glia mediated central sensitization in the spinal dorsal horn is a mechanism for the pain hypersensitivity [18, 21]. Anti-nocicepion effects have been found in the pharmaceutical reagents such as CNI-1493 and PPF which may inhibit the astrocyte activation [22-24]. Meanwhile, many studies also reveal that astrocytes play an important role in the nerve fiber growth and sprouting. During the sprouting, astrocytes are activated as shown by the up-regulation of GFAP, a marker of astrocyte activation [25]. Furthermore, the pro-inflammatory cytokines, such as IL-1 and IL-6, which are released after astrocyte activation, also promote the sprouting in an inflammation-independent manner [8]. In the present study, the astrocytes were observed in the spinal cord of CCI rats. Our results showed a large amount of astrocytes were activated in the spinal cord of CCI rats, and these cells were enlarged and had increased and enlarged processes. In addition, the GFAP expression increased significantly, and the IL-1β secreted by these cells remained at a continuous high level. The thermal hyperalgesia and mechanical allodynia also markedly increased in CCI rats but significantly decreased by PPF, which indicate that the astrocyte activation and the subsequent release of IL-1β may involve in the nerve ligation induced NP.

Increase in the GAP-43 expression is most likely an early indicator of structural changes in the nociceptive network, and a return of GAP-43 expression to the normal level may point to the establishment of synaptic contacts in the dorsal horn, as developmental studies have shown a down-regulation of GAP-43 expression after fibers reaching their targets [26]. Such structural changes may consequently form the basis for a persistent sensitization [9]. In our previous work, results showed that GAP-43 expression in the ipsilateral dorsal horn of spinal cord with ligation was significantly increased when compared with the contralateral spinal cord [27], which is consistent with findings in the present study. This increase was also observed in the dorsal horn of lumbar spinal cord in the present study. Our results showed that the glial inhibition decreased the GAP-43 expression in the dorsal horn after CCI. After inhibition of astrocyte activation by PPF, the activated astrocytes reduced accompanied by reduction in processes and expressions of IL-1β and GAP-43. In addition, hyperalgesia was also attenuated. We speculate that sciatic nerve ligation induced activation of astrocytes and subsequent IL-1β up-regulation may increase the GAP-43 expression. The neuronal plasticity
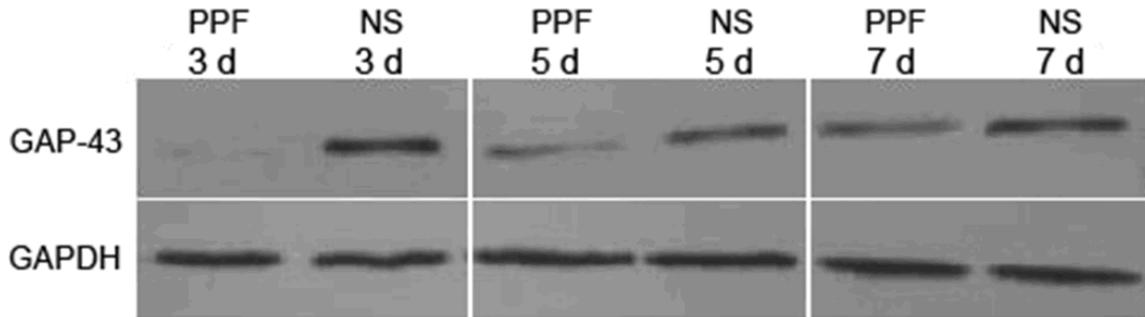
Figure 6. Detection of GAP-43 expression by western blot assay. Western blot assay showed the GAP-43 expression in the PPF group was significantly decreased compared to that in the NS group on the 3rd, 5th and 7th day after surgery, which indicated the down-regulation of GAP-43 by inhibition of astrocytes.

changes and the sprouting may result in persistent sensitization in CCI rats. However, it is necessary to study the direct relationship between neuronal sprouting and pain hypersensitivity aiming to increase the understanding of cellular and/or molecular mechanisms underlying the neuronal sprouting.

Conclusion

In summary, our results confirm the up-regulation of GAP-43 in the spinal cord following sciatic nerve ligation, and also demonstrate that inhibition of glia with intrathecal PPF can alleviate the NP in CCI rats. In addition, GAP-43 expression decreased after the inhibition of astrocyte activation. These suggest that glial activation is related to the increase in GAP-43 expression, which leads to a consistent hypersensitivity status in NP.

Acknowledgements

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