Antiallodynic Effects of Microglial Interleukin-1β Inhibition in the Spinal Cord

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Abstract—**Microglia play a pivotal role in synaptic plasticity of chronic pain. In this study, the potential role of interleukin 1beta (IL-1β), mainly released by microglia in early stage of nerve injury, in mechanical allodynia induced by tetanic stimulation of the sciatic nerve (TSS) was examined. Mechanical allodynia was observed on both ipsilateral and contralateral sides of TSS. Moreover, the expression of the microglial marker Iba-1 and the proinflammatory cytokine IL-1β were significantly increased. Intrathecal injection of the IL-1 receptor antagonist (IL-1ra, 3.5 μg/ml, 20 μl/rat) 30 min before TSS significantly inhibited bilateral mechanical allodynia on day 3, 5 and 7 after TSS. Immunohistochemistry showed that IL-1β was colocalized with the microglial marker OX-42 in the spinal superficial dorsal horn, but not with the astrocytic marker GFAP and the neuronal marker NeuN on day 4 following TSS. The results demonstrate that microglial IL-1β participates in the hypersensitivity of pain behaviors induced by TSS.**

Keywords-allodynia; microglia; interleukin 1beta; spinal cord

I. INTRODUCTION

It has been proposed that microglia play an important role in the induction of synaptic plasticity of pain pathway [1-3]. The microglial inhibitor minocycline dose-dependently reduces formalin-evoked the second phase of flinching responses [4]. Previous study in our lab showed that tetanic stimulation of the sciatic nerve (TSS) produces bilateral mechanical allodynia and these hypersensitive pain behaviors are alleviated by inhibition of glial function [5]. However, the mechanisms employed by microglia for modulating the synaptic plasticity needs to be further explored.

Glial cells in the spinal cord are activated in multiple pathological pain models and the glia-derived proinflammatory cytokines may contribute greatly to pain hypersensitivity [6,7]. TSS can produce long-lasting hyperalgesia and allodynia in rats [5,8]. This study was to further investigate the potential role of microglial interleukin 1beta (IL-1β) in persistent pain using a rat model of neuropathic pain induced by TSS.

II. MATERIALS AND METHODS

A. Animals

Adult male Sprague-Dawley rats (180–220 g) (Animal Center, Chinese Academy of Sciences , Shanghai) were raised in a light (12:12 light/ dark cycle) and temperature (23 ± 2 °C) controlled room. Standard rodent chow and water were available ad libitum. The treatment of the rats conformed to the guidelines of the International Association for the Study of Pain concerning the use of laboratory animals.

B. Tetanic stimulation of the sciatic nerve

Under Chloral Hydrate (0.3 g/kg, i.p.) anesthesia, the left sciatic nerve was exposed. A pair of silver hooks was placed under the sciatic nerve to export electrical stimulation in treated groups but no real electrical current in the sham group.

C. Lumbar puncture（LP）

The LP was performed 30 min before TSS. The IL-1 receptor antagonist (IL-1ra, 3.5 μg/ml, 20 μl/rat) or saline was loaded into a 0.25 ml vitric syringe. Then the pinhead penetrated into intervertebral gap between the L4 and L5, and reached subarachnoid space under inhalation anesthesia with isoflurane (2% in oxygen). An instaneous and fast tail-flick represents a successful puncture. Then the drugs were injected slowly.

D. von Frey test for mechanical allodynia

The baseline and allodynia to mechanical stimulation were measured on hind paws by von Frey filaments before tetanic stimulation and on day 3, 5 and 7 following TSS, respectively. Each rat was placed in a single chamber and acclimated for about 30 min. Then a series of von Frey filament stimuli was applied to the hind paws in an ascending order. A particular hair was applied until the filament buckled and maintained for 2 s. The value of the filament was the 'paw withdrawal threshold (PWT)' if the hind paw withdrew at least three times out of five stimulations. The interval of two stimulations was 15s.

E. Immunofluorescence staining

On day 4, sham and treated rats were finally anesthetized with urethane (1.5 g/kg, i.p.). The rats were perfused with warm saline through the ascending aorta and then 4% cold paraformaldehyde, pH 7.2–7.4. The L4–L6 spinal cord segments was removed quickly and postfixed in the same fixative for 2–4 h. After gradient dehydration in 10%, 20% and 30% sucrose solution, the lumber spinal cord was transectted into pieces of 35 μm thick in a cryostat for immunostaining.

In the double staining experiments, spinal sections were

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firstly immersed in 0.3% H_2O_2 for half an hour and then blocked with TNB buffer (including 0.1 M TRIS-HCL, 0.15 M NaCl and 0.5% Blocking Reagent) for 2 h at RT. The sections were then incubated with a mixture of anti-IL-1β (rabbit, 1:500; R & D Systems) and OX-42 (a microglial marker, mouse; 1:500; Serotec), GFAP (an astrocytic marker, mouse; 1:2000; Sigma) or monoclonal neuronal specific nuclear protein (NeuN, a neuronal marker, mouse; 1:2000; Chemicon) over three nights at 4 °C. The slices were immersed in a solution consisted of biotinylated secondary antibodies and FITC-conjugated secondary antibodies for 2 h at 4 °C. After washing with TNT buffer (including 0.1 M TRIS-HCL, 0.15 M NaCl and 0.05% Tween 20) for 3×5 min, the slices were incubated in SA-HRP for 30 min. Finally, the slices were incubated in Fluorophore Tyramide Working Solution for 3–5 min and then washed with TNT buffer. A confocal microscopy system (Leica TCS SP2, Germany) with 488 nm (green) and 543 nm (red) laser lines was used to examine the staining. For each experiment, images were processed simultaneously and using identical acquisition parameters. The analysis was carried out with the aid of ImagePro Plus (version 6.0; Media Cybernetics, USA).

F. Western blots

Under urethane (1.5 g/kg, i.p.) anesthesia, the animals were killed by decapitation on day 4 following TSS. The L4-L6 lumbar spinal cord was removed and homogenized in an SDS sample. After detecting the protein concentrations, the Western blot was conducted. 10 μl sample consisted by 8 μl protein and 2 μl 5× sample buffer was added to 12% SDS-PAGE and electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore Immobilon-p Transfer Membrane). The membranes were immersed in 5% non-fat milk in Tris-buffered saline (pH 7.5) containing 0.1% Tween 20 for 2 h. Then the membranes were incubated overnight at 4 °C with anti-ionized calcium binding adaptor protein (Iba-1, a microglial marker, rabbit, 1:2000; Wako) or anti-IL-1β (rabbit, 1:1000; R & D Systems). After washing, the blots were immersed in the solution including horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:1000; Pierce) for 2 h at 4 °C. Meanwhile, the membranes with 37 KD protein were incubated with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:10, 000; Cell Signaling) for 1 h at RT. After washing, all the membranes were developed in ECL solution (Pierce) and exposed onto films. The densities of specific Iba-1 or IL-1β bands were measured with the aid of the soft Photoshop.

G. Statistical analysis

All the data were expressed as mean ± SEM. Statistical tests were carried out with SPSS (version 13.0, SPSS Inc., USA). The effects of electrical stimulation or drugs on PWTs were analyzed by one way repeated measures ANOVA followed by LSD post hoc test when compared within the group and by two way repeated measures ANOVA when compared between groups. The expression changes of the microglial marker Iba-1 and IL-1β in western blot experiments were analyzed by independent-sample t test.

III. RESULTS

A. Activation of microglia in the spinal cord following TSS

The expression of Iba-1, a microglial marker, was detected by Western blot. As shown in Fig. 1, the expression of Iba-1 was significantly increased on day 4 following TSS (1.07 ± 0.07 vs. sham 0.50 ± 0.01, $p < 0.05$).

B. Increased expression of IL-1β following TSS

The expression of IL-1β following TSS was investigated. Compared with the sham group, the expression of IL-1β on day 4 following TSS was significantly increased (1.03 ± 0.08 vs. sham 0.77 ± 0.03, $p < 0.05$) (Fig. 2).

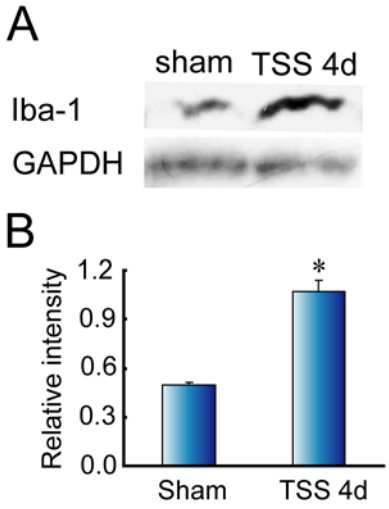

Figure 1. The activation of microglia following tetanic stimulation of the sciatic nerve (TSS). A, Representative bands of Iba-1. B, Histogram indicates the statistical results of the Iba-1 expression using GAPDH as loading control. *$p < 0.05$ vs. sham group ($n = 3$/group).

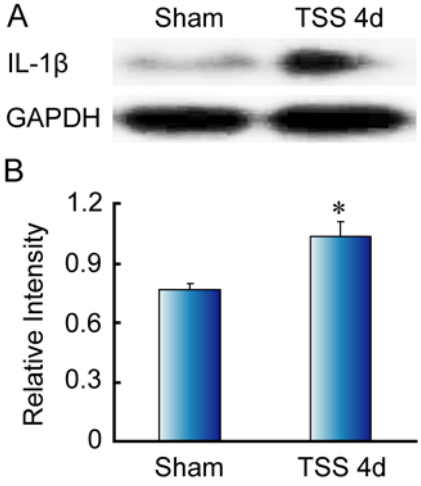

Figure 2. The increased expression of IL-1β in the spinal cord following tetanic stimulation of the sciatic nerve (TSS). A, Representative Western blot bands of IL-1β and GAPDH. B, The histogram shows the statistical results of IL-1β expression in sham and TSS groups. *$p < 0.05$ vs. sham group (n = 3/group).

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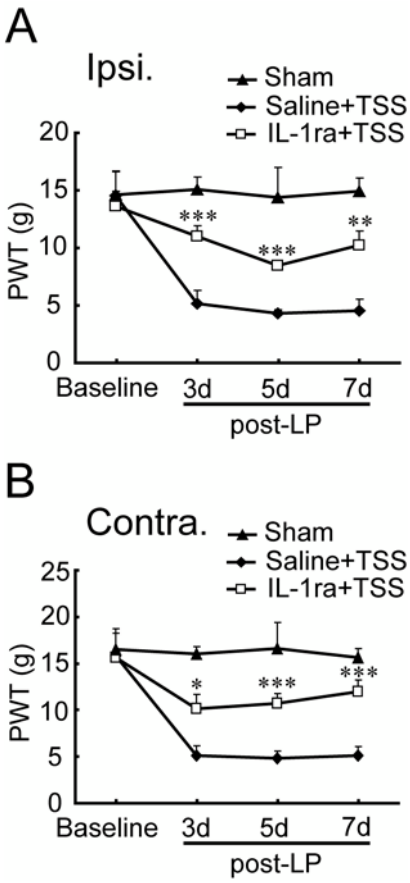

Figure 3. IL-1ra inhibited bilateral mechanical allodynia induced by tetanic stimulation of the sciatic nerve (TSS). Compared with saline-injection group, IL-1ra (3.5 μg/ml, 20 μl/rat) significantly increased the values of paw withdrawal threshold (PWT) on day 3, 5 and 7 following TSS on both the ipsilateral (Ipsi., A) and the contralateral (Contra., B) side of TSS. *p < 0.05, **p < 0.01, ***p < 0.001 vs. saline-injection group (n = 8/group).

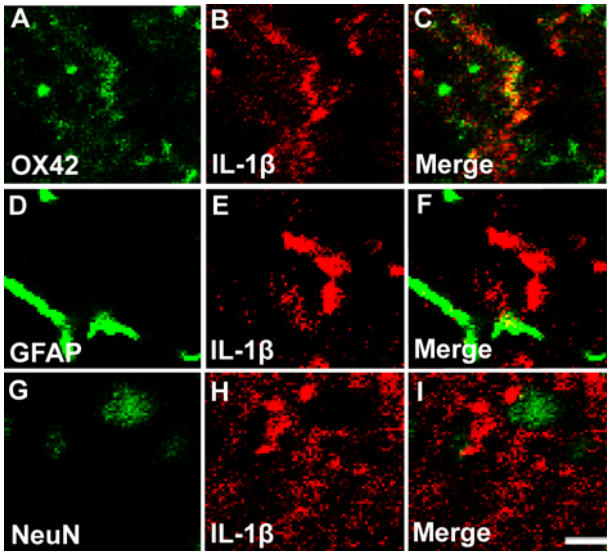

Figure 4. Double immunostaining of IL-1β with OX-42 (A-C), GFAP (D-F) and NeuN (G-I) on day 4 following TSS. Scale bar, 5 μm.

C. *Inhibition of mechanical allodynia following TSS by IL-1 receptor antagonist IL-1ra*

Bilateral PWTs to mechanical stimulation significantly decreased following TSS. As shown in Fig. 3, the ipsilateral PWTs rapidly decreased from 14.89 ± 1.16 g to 4.57 ± 0.95 g (p < 0.001) on day 7 following TSS. Compared with the saline- injection group, PWTs in IL-1ra-injection group on day 3, 5 and 7 following TSS were significantly higher (11.00 ± 0.91 vs. 5.14 ± 1.14, p < 0.001; 8.50 ± 0.33 vs. 4.29 ± 0.29, p < 0.001; 10.25 ± 1.21 vs. 4.57 ± 0.95, p < 0.01) on the ipsilateral side of TSS, indicating that TSS-induced pain hypersensitivity was significantly inhibited by IL-1ra (Fig. 3). A similar phenomenon was observed on the contralateral side of TSS.

D. *Cell location of IL-1β*

To identify the cell type that expresses IL-1β, double immunostaining was carried out on day 4 following TSS. The results showed that IL-1β is almost completely colocalized with OX-42, but not with GFAP and NeuN (Fig. 4).

IV. DISCUSSION

The main findings of this study are: 1) Spinal microglia were activated following TSS and the expression of IL-1β was increased; 2) Inhibition of IL-1β signaling pathway alleviated TSS-induced mechanical allodynia; 3) IL-1β is expressed by microglia in the spinal dorsal horn.

The release of IL-1β in the spinal cord is increased in multiple pathological pain models and plays an important role in the induction of chronic pain [9,10]. In addition, intrathecal administration of IL-1β enhances pain responses [11,12]. This study found that the expression of IL-1β is significantly increased following TSS, a result consistent with previous reports.

As previously reported [5,13], TSS induced the activation of spinal microglia and bilateral mechanical allodynia. Glia and proinflammatory cytokines including IL-1, tumor necrosis factor alpha (TNF-α) and IL-6 have been proposed to contribute greatly to the induction of bilateral mechanical allodynia, also called mirror-image pain [14]. This study showed that intrathecal injection of IL-1ra alleviates bilateral mechanical allodynia, supporting the important role of microglia and IL-1β in mirror-image pain.

Therefore, microglia are activated by TSS to release proinflammatory cytokines, including IL-1β. All of these cytokines released from glial cells in the spinal cord contribute greatly to the modulation of synaptic plasticity of pain pathway.

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