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·Original Article·

Involvement of microglia and interleukin-18 in the induction of long-term potentiation of spinal nociceptive responses induced by tetanic sciatic stimulation

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Abstract: Objective The present study aimed to investigate the potential roles of spinal microglia and downstream molecules in the induction of spinal long-term potentiation (LTP) and mechanical allodynia by tetanic stimulation of the sciatic nerve (TSS). **Methods** Spinal LTP was induced in adult male Sprague-Dawley rats by tetanic stimulation of the sciatic nerve (0.5 ms, 100 Hz, 40 V, 10 trains of 2-s duration at 10-s intervals). Mechanical allodynia was determined using von Frey hairs. Immunohistochemical staining and Western blot were used to detect changes in glial expression of interleukin-18 (IL-18) and IL-18 receptor (IL-18R). **Results** TSS induced LTP of C-fiber-evoked field potentials in the spinal cord. Intrathecal administration of the microglial inhibitor minocycline (200 μg/20 μL) 1 h before TSS completely blocked the induction of spinal LTP. Furthermore, after intrathecal injection of minocycline (200 μg/20 μL) by lumbar puncture 1 h before TSS, administration of minocycline for 7 consecutive days (once per day) partly inhibited bilateral allodynia. Immunohistochemistry showed that minocycline inhibited the sequential activation of microglia and astrocytes, and IL-18 was predominantly colocalized with the microglial marker Iba-1 in the spinal superficial dorsal horn. Western blot revealed that repeated intrathecal injection of minocycline significantly inhibited the increased expression of IL-18 and IL-18Rs in microglia induced by TSS. **Conclusion** The IL-18 signaling pathway in microglia is involved in TSS-induced spinal LTP and mechanical allodynia.

Keywords: long-term potentiation; allodynia; glia; interleukin-18

1 Introduction

Tetanic stimulation of the sciatic nerve (TSS) induces long-lasting hyperalgesia and allodynia in the rat[1,2]. In electrophysiological studies, TSS with identical parameters also induces long-term potentiation (LTP) of C-fiber-evoked field potentials in the spinal cord[3]. The induction of spinal LTP is prevented by antagonism of spinal N-methyl-*D*-aspartic acid (NMDA) and neurokinin 1 (NK-1) receptors, both of which play key roles in the sensitization of spinal pain transmission[4-7]. It is, therefore, plausible that spinal LTP may be the substrate of sensitization of the pain pathway in the spinal cord[3,8].

Increasing evidence demonstrates that microglia play an important role in modulating the sensitization of pain transmission in the spinal cord[9-12]. A previous report

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showed that intrathecal administration of minocycline, a microglial inhibitor, dose-dependently reduces the formalin-evoked second phase of flinching responses and the carrageenan-induced thermal hyperalgesia, and completely blocks the hyperalgesia induced by intrathecal delivery of NMDA in rats[13]. Similarly, microglia modulate NMDA-dependent hippocampal LTP[14-16]. Moreover, cytokines released by microglia, including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), modulate the induction and maintenance of hippocampal LTP by increasing the surface expression and phosphorylation of α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid (AMPA) receptors[17,18]. Our studies and others have also revealed that disruption of glial function blocks TSS-induced LTP in the spinal cord[1,19,20]. Taken together, it is likely that spinal microglia contribute to the spinal LTP induced by nociceptive input. The signaling molecules in microglia and the mechanisms mediating this process need to be further explored.

A line of evidence demonstrates that interleukin-18 (IL-18), a proinflammatory mediator predominantly released by microglia[21-23], is likely to be an ideal target for investigating microglial functions. IL-18 is up-regulated in some inflammatory diseases[24], but compared with IL-1β, few studies have focused on the role of the spinal IL-18 signaling pathway in the induction of pathological pain. The present study aimed to explore the potential roles of microglia and IL-18 in the induction of spinal LTP and persistent pain behaviors induced by TSS in rats.

2 Materials and methods

2.1 Animals Adult male Sprague-Dawley rats (180–350 g) (Shanghai Laboratory Animal Center, Chinese Academy of Sciences, Shanghai) were housed separately in plastic cages under a 12:12 light/dark cycle at 23 ± 2°C and fed standard rodent chow and water *ad libitum*. All experiments were performed in accordance with the guidelines of the International Association for the Study of Pain concerning the use of laboratory animals. All procedures were approved by the Institutional Animal Care Committee.

2.2 Electrophysiological recording of spinal LTP The procedures were similar to a previous study[25]. Briefly, rats were initially anesthetized with intraperitoneal (i.p.) urethane (1.5 g/kg). Laminectomy was performed at vertebrae T13–L1 to expose the lumbar enlargement of the spinal cord. An intrathecal catheter (PE-10) filled with ~4 μL sterile saline was inserted into the intervertebral gap between L4 and L5 and extended into the subarachnoid space for drug injection, then the outer end was plugged. During electrophysiological recording, minocycline (Sigma, St. Louis, MO, USA; 200 μg/20 μL, dissolved in saline) or saline alone was injected 1 h before tetanic stimulation. Then the left sciatic nerve was exposed, carefully isolated, and stimulated by bipolar silver electrodes. The spinal column was firmly suspended by vertebral clamps rostral and caudal to the exposed cord. The skin above the sciatic nerve around the incision was raised and fixed to a metal ring to form a skin bath filled with warm paraffin oil. Throughout the experiment, animals were artificially ventilated (Capstar-100, IITC Life Science, USA), and the electrocardiogram, end-tidal CO_2, and rectal temperature (37.5–38°C) were monitored and controlled within the physiological range.

Field potentials were recorded in the ipsilateral dorsal horn at L4–L5, 300–500 μm from the surface of the cord with glass microelectrodes (impedance 3–6 MΩ). The low-pass filter was set at 100 Hz. A single rectangular pulse (0.5 ms, 20–30 V), enough to excite afferent C-fibers, was applied to the sciatic nerve at 1-min intervals as a test stimulus. Stable responses for more than 40 min served as baseline control. A conditioning tetanic stimulation (0.5 ms, 100 Hz, 40 V, 10 trains of 2-s duration at 10-s intervals) was delivered to the sciatic nerve to induce spinal LTP. Four consecutive C-fiber-evoked field potentials were averaged, stored, and analyzed by the SMUP-E data-processing system (Shanghai Medical College, Fudan University, China).

2.3 Lumbar puncture Lumbar puncture was performed 1 h before tetanic stimulation and was repeated daily until day 7 after surgery. Minocycline (200 μg/20 μL) or saline was initially loaded into a 0.25-mL glass syringe with a 27G needle. Under inhalation anesthesia with isoflurane (2% in oxygen), the needle was inserted into the gap be-

tween L4 and L5, and reached the subarachnoid space of the lumbar enlargement. The occurrence of an instantaneous and rapid tail-flick indicated a successful puncture. Drugs were injected over 1 min and then the needle was withdrawn. This acute injection method took 2–3 min to complete, and rats showed full recovery from anesthesia within 10 min. No abnormal motor behavior was observed after any injection.

2.4 Tetanic stimulation of the sciatic nerve Under chloral hydrate anesthesia (0.3 g/kg, i.p.) and aseptic conditions, the left sciatic nerve was carefully exposed at mid-thigh level and separated from neighboring tissues. A pair of silver hooks was placed under the nerve for stimulation. In the sham group, the exposed left sciatic nerve was placed on the hooks without stimulation. The animals were allowed to recover from surgery before behavioral tests.

2.5 von Frey test for mechanical allodynia Allodynia to mechanical stimulation was measured on hind-paws using von Frey hairs (2–26 g bending force) 15 h after minocycline or saline injection on days 3, 5, and 7 after TSS or sham surgery. The baseline response was measured before tetanic stimulation. For the test, each rat was placed in a chamber (20 ×10 × 20 cm^3) with a customized platform made of 3-mm thick Plexiglas containing 1.5-mm diameter holes in a 5-mm grid of perpendicular rows throughout the entire area. Each rat was allowed 30 min for acclimation. After that, a series of von Frey hair stimuli was delivered in ascending order of force to the central region of the plantar surface of the hind-paw. A particular hair was applied until buckling occurred. This was maintained for ~2 s. A withdrawal response was considered valid only if the hind-paw was completely removed from the platform, not just a flinch after a single application. Each trial consisted of 5 tests at 15-s intervals, and only when the hind-paw withdrawal occurred in no less than 3 tests was the value of the particular filament in grams considered to be the 'paw withdrawal threshold' to mechanical stimuli. Otherwise, the next-larger filament was tested until the paw withdrawal threshold was defined.

2.6 Immunofluorescent staining After defined survival times, sham and treated rats were finally anesthetized with urethane (1.5 g/kg, i.p.) and then perfused through the ascending aorta with warm saline, followed by 4% cold paraformaldehyde, pH 7.2–7.4. After perfusion, the L4–L6 segments were removed and postfixed in the same fixative for 2–4 h. After serial immersion in 10%, 20% and 30% sucrose, the lumbar cord was transected into 35-μm thick sections on a cryostat and processed for immunostaining. All the sections were blocked with 10% donkey serum in 0.3% Triton X-100 for 2 h at room temperature (RT) and incubated overnight at 4°C with rabbit anti-ionized Ca^{2+}-binding adaptor protein (Iba-1, a microglial marker, 1:2 000; Wako, Osaka, Japan) or mouse anti-glial fibrillary acidic protein (GFAP, an astrocytic marker, 1:2 000; Sigma). The sections were then incubated for 2 h at 4°C with rhodamine red-X-conjugated donkey anti-rabbit secondary antibody (1:200; Jackson, West Grove, PA, USA) or fluoresceinisothiocyanate (FITC)-conjugated donkey anti-mouse secondary antibody (1:200; Jackson).

All sections were examined with a fluorescence microscope (Leica DMRXA, Germany) and images were captured under 10× magnification, using a computerized image analysis system (Leica Qwin 500, Germany). Fluorescence power and exposure time were fixed for all images. Because the morphology of microglia and astrocytes is complex and immunoreactive staining includes both cell bodies and their processes, cell counts may not sufficiently quantify activation. Therefore, the optical density of immunoreactive staining for Iba-1 and GFAP was measured. The relative density of images was determined by subtracting the background density in each image. For each animal, the corrected density values of six consecutive sections were averaged (n = 4 rats/group). All results are expressed as mean ± SEM. Glial activation was quantified with Image J (version 1.38; NIH, USA). Quantitative analyses were done in a blinded manner.

For double immunofluorescence, spinal sections were firstly immersed in 0.3% H_2O_2 for 30 min and then blocked with TNB buffer (containing 0.1 mol/L Tris-HCl, 0.15 mol/L NaCl and 0.5% blocking reagent) for 2 h at RT. Then the sections were incubated with a mixture of goat anti-IL-18 (1:500; R&D Systems) and rabbit anti-Iba-1 (1:2 000; Wako),

mouse anti-GFAP (1:2 000; Sigma) or monoclonal mouse anti-neuron-specific nuclear protein (NeuN, a neuronal marker, 1:2 000; Chemicon, Temecula, CA, USA) for three days (over three nights) at 4°C, followed by incubation with a mixture of biotinylated secondary antibodies and FITC-conjugated secondary antibodies for 2 h at 4°C. The slices were washed in TNT buffer (containing 0.1 mol/L Tris-HCl, 0.15 mol/L NaCl and 0.05% Tween-20) for 3 × 5 min and then incubated in SA-HRP for 30 min.

After incubation in Fluorophore Tyramide Working Solution for 3–5 min, the slices were washed with TNT buffer for 3 × 5 min, and then examined with a confocal microscopy system (Leica TCS SP2, Germany) with 488 nm (green) and 543 nm (red) laser lines. Overlay of the green and red signals produces a yellow signal. For each experiment, images were processed simultaneously, collected using identical acquisition parameters and analyzed using ImagePro Plus (version 6.0; Media Cybernetics, USA).

2.7 Western blots Under urethane anesthesia (1.5 g/kg, i.p.), the animals were rapidly decapitated at the defined time points. The L4–L6 spinal cord was rapidly removed and homogenized in SDS sample buffer containing a mixture of proteinase and phosphatase inhibitors. Protein concentrations were determined and Western blots were conducted. Samples (8 μL protein and 2 μL 5 × sample buffer) were resolved in 12% SDS-PAGE and electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Blots were blocked with 5% non-fat milk in Tris-buffered saline (pH 7.5) containing 0.1% Tween-20 for 2 h, and then incubated overnight at 4°C with goat anti-IL-18 (1:500; R&D Systems) or goat anti-IL-18 receptor (IL-18R) (1:500; R&D Systems). After washes, the blots were incubated with horseradish peroxidase-conjugated donkey anti-goat IgG (1:1 000; Pierce, Rockford, IL, USA) 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. Proteins were visualized by ECL (Pierce). The densities of specific IL-18 and IL-18R bands were measured with a computer-assisted imaging analysis system (Photoshop).

2.8 Statistical analysis In each experiment, the areas of four consecutive field potentials recorded at 1-min intervals were averaged. The mean area before drug or saline application served as the baseline. The area of C-fiber-evoked field potentials was expressed as percentage of baseline. The summary data from different animals in the same group are expressed as mean ± SEM. Statistical tests were carried out with SPSS (version 13.0, SPSS Inc., USA) or SigmaStat (version 3.5, Systat Software Inc., USA). The values (% of baseline) shown in the results were obtained at 1 h after TSS or immediately before TSS. The effects of electrical stimulation or drugs were analyzed by one-way repeated measures ANOVA followed by Tukey's *post-hoc* test when compared within the group and by two-way repeated measures ANOVA when compared between groups. In immunohistochemistry experiments, the effects of tetanic stimulation with or without pre-injection of minocycline on the expression of the microglial marker Iba-1 and the astrocytic marker GFAP were analyzed by one-way ANOVA followed by the LSD *post-hoc* test. In Western blot experiments, the expression of IL-18 and IL-18R following tetanic stimulation was analyzed by one-way ANOVA followed by the LSD *post-hoc* test. The effects of minocycline on the expression of IL-18 and IL-18R were analyzed using the independent samples *t*-test.

3 Results

3.1 Inhibition of TSS-induced spinal LTP and mechanical allodynia by glia inhibitor minocycline Consistent with our previous studies[1,2,25], C-fiber-evoked field potentials were evoked by testing stimulation of the sciatic nerve (0.5 ms, 20–30 V) at a depth of 300–500 μm from the surface of the cord. The average area of C-fiber-evoked field potentials significantly increased (198.47 ± 19.52% of baseline control, $n = 5$) following TSS (0.5 ms, 100 Hz, 40 V, 10 trains of 2-s duration at 10-s intervals), demonstrating the occurrence of spinal LTP (Fig. 1A).

Intrathecal (i.t.) injection of minocycline (200 μg/20 μL) 1 h before TSS completely blocked the induction of LTP (106.13 ± 11.82% of control, $n = 5$) without affecting the

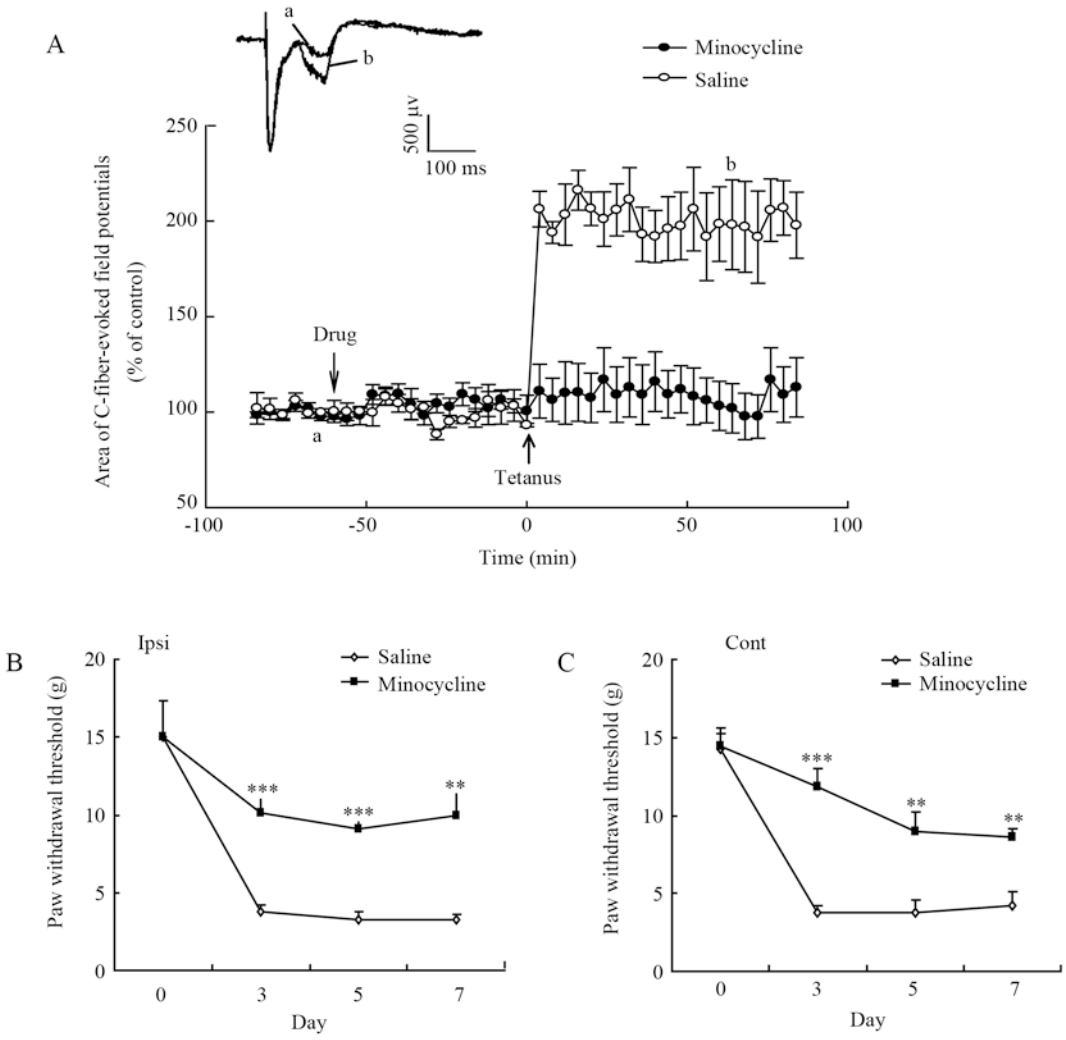

Fig. 1. Minocycline inhibited long-term spinal nociceptive responses induced by tetanic stimulation of the sciatic nerve (TSS). A: Minocycline (200 μg/20 μL, i.t.) completely blocked the induction of spinal LTP of C-fiber-evoked field potentials induced by tetanic stimulation (0.5 ms, 100 Hz, 40 V, 10 trains of 2-s duration at 10-s intervals) (n = 5, P >0.05). Upper insert: The representative C-fiber-evoked field potentials before (a) and after (b) TSS in saline-injection group. B and C are the results of von Frey tests ipsilateral (Ipsi) and contralateral (Cont) to the tetanic stimulation, respectively. $^{**}P$ <0.01, $^{***}P$ <0.001 *vs* saline group (n = 8 in each group).

baseline C-fiber-evoked responses (Fig. 1A), suggesting the involvement of microglial activation in the induction of spinal LTP.

Similar to our previous study[1], bilateral paw withdrawal thresholds (PWTs) to mechanical stimulation significantly decreased following TSS, with identical parameters used in the electrophysiological experiments. To test the effects of the microglial inhibitor minocycline, repeated intrathecal injection of minocycline or saline was made 1 h before and on 7 consecutive days (once per day) following TSS. The PWTs to von Frey stimulation rapidly decreased from 15.00 ± 2.33 to 3.25 ± 0.37 g (P <0.001) ipsilaterally and from 14.25 ± 1.00 to 4.25 ± 0.88 g (P <0.001) contralaterally on day 7 after TSS in the saline group (Fig. 1B, C). However, in the minocycline group, the ipsilateral PWT dropped from 15.00 ± 1.36 to 10.00 ± 1.36 g (P <0.05) on day 7 after TSS. There was a significant difference of PWT compared with the saline group (P <0.01) (Fig. 1B),

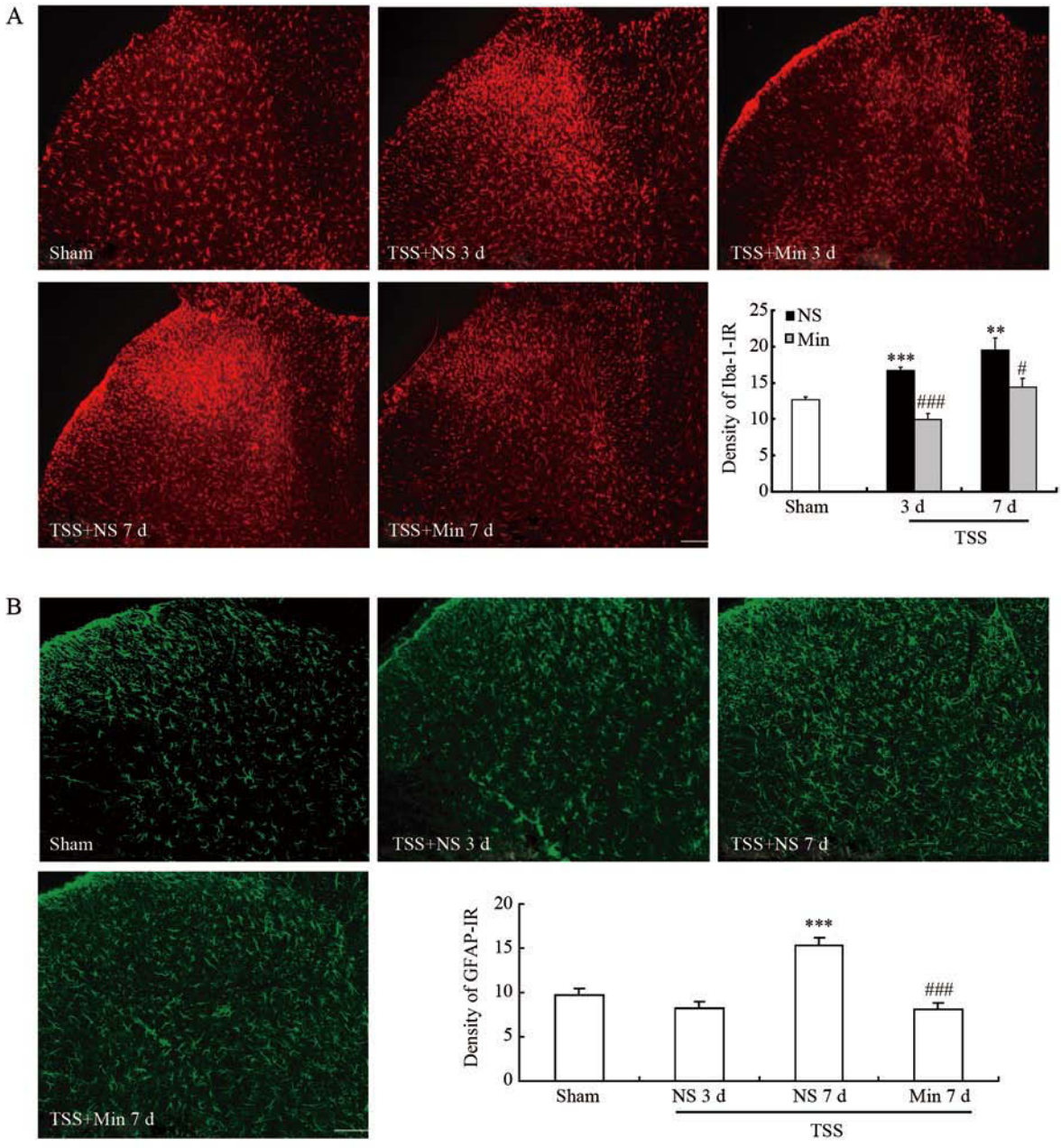

Fig. 2. Inhibition of tetanic stimulation-induced sequential activation of microglia and astrocytes by minocycline. A: Time-course of the microglial marker Iba-1 expression following tetanic stimulation of the sciatic nerve (TSS) in normal saline (NS)-injected group and effects of minocycline (Min) on the ipsilateral side. Bottom-right figure: Inhibition of the increase in Iba-1-immunoreactivity (Iba-1-IR) by minocycline from day 3 to day 7. B: Inhibitory effects of minocycline on the increased expression of the astrocytic marker GFAP-immunoreactivity (GFAP-IR) after tetanus. $^{**}P$ <0.01, $^{***}P$ <0.001 *vs* sham control; $^{\#}P$ <0.05, $^{\#\#\#}P$ <0.001 *vs* NS group on the corresponding day following tetanic stimulation (n = 4/group). Scale bars, 100 μm.

indicating that TSS-induced pain responses were partially alleviated by minocycline. Similar results were obtained for the contralateral PWTs (Fig. 1C).

3.2 Minocycline decreased the TSS-induced activation of microglia and astrocytes Iba-1-positive microglia with a swollen phenotype were markedly increased following

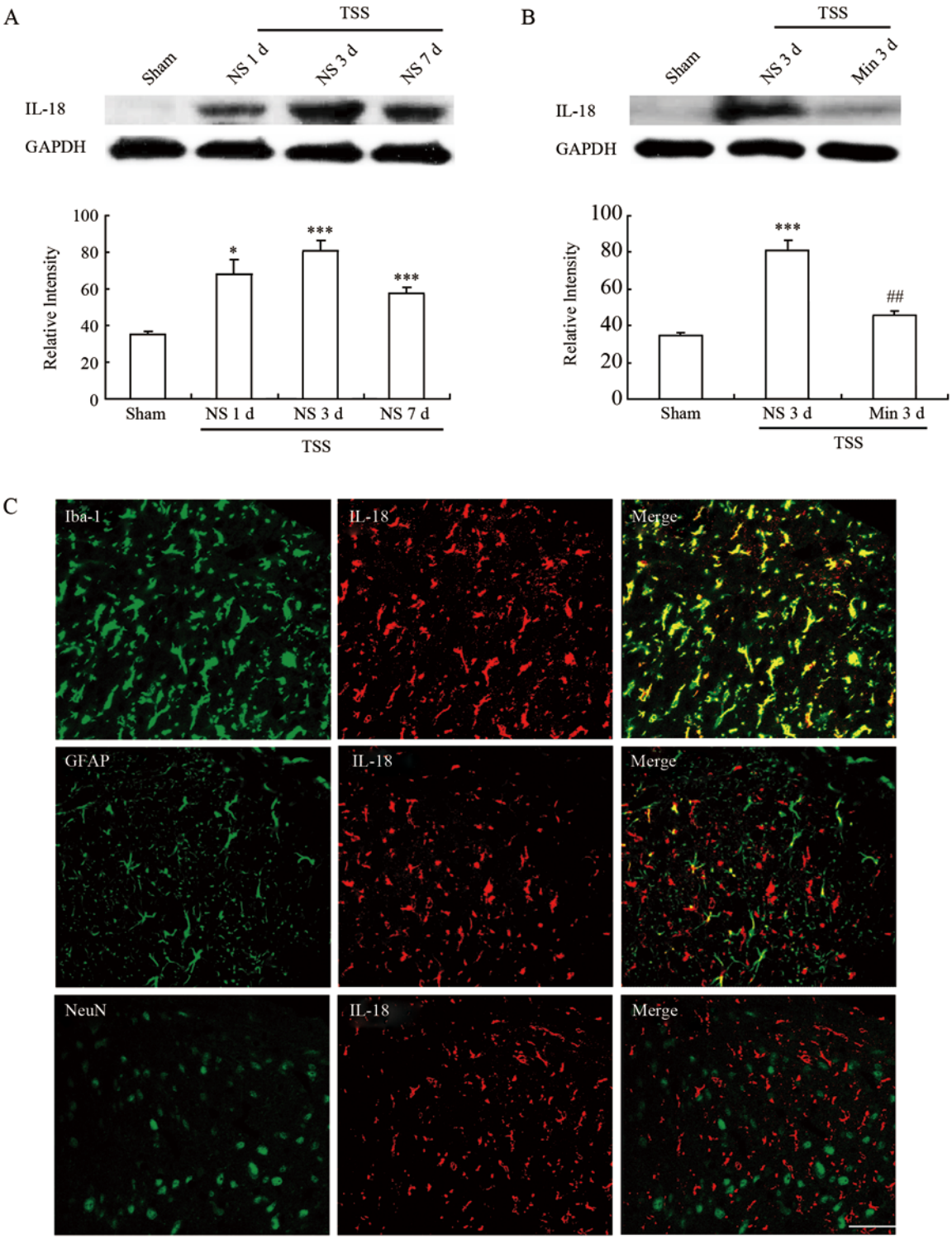

Fig. 3. Inhibition of tetanic stimulation-induced increase in expression of microglial inferleukin-18 (IL-18) by minocycline. A: Time-course of IL-18 expression in the normal saline (NS) group after tetanic stimulation of the sciatic nerve (TSS) using GAPDH as loading control. B: Peak expression of IL-18 on day 3 after TSS was significantly inhibited by repeated administration of minocycline (Min, 200 μg/20 μL). $^{*}P$ <0.05, $^{***}P$ <0.001 *vs* sham (n = 3/group); $^{\#\#}P$ <0.01 *vs* NS (n = 3/group). C: Double immunostaining of IL-18 with Iba-1, GFAP or NeuN on day 3 after TSS. Scale bar, 10 μm.

TSS (Fig. 2A). The microglial activation started on day 3 after TSS, with Iba-1 immunoreactivity density being 16.67 ± 0.52 (P <0.001 *vs* 12.54 ± 0.43 in the sham group) and lasted at least until day 7 (19.50 ± 1.64, P <0.01 *vs* sham). Repeated administration of minocycline reduced the staining intensity of microglia (9.96 ± 0.82 on day 3, P <0.001 *vs* normal saline group; 14.21 ± 1.22 on day 7, P <0.05 *vs* normal saline group). Besides, there was no change in microglial activation on the contralateral side in all groups (data not shown). However, activation of astrocytes occurred on day 7 following TSS (15.33 ± 0.89 *vs* sham 9.74 ± 0.76, P <0.001) and was suppressed by repeated intrathecal injection of minocycline 1 h before and for 7 consecutive days (once per day) following TSS (8.07 ± 0.75, P <0.001 *vs* normal saline group) (Fig. 2B).

3.3 Minocycline attenuated the up-regulation of IL-18 and IL-18R IL-18 has been proposed to be predominantly released from microglia, so the effects of minocycline on the expression of IL-18 were investigated. After TSS, IL-18 expression increased from day 1 (relative intensity, 67.92 ± 7.78, P <0.05 *vs* sham 35.02 ± 1.59), peaked on day 3 (relative intensity, 80.97 ± 5.42, P <0.001 *vs* sham) and was maintained at least to day 7 (relative intensity, 57.23 ± 3.24, P <0.001 *vs* sham) (Fig. 3A). In addition, the

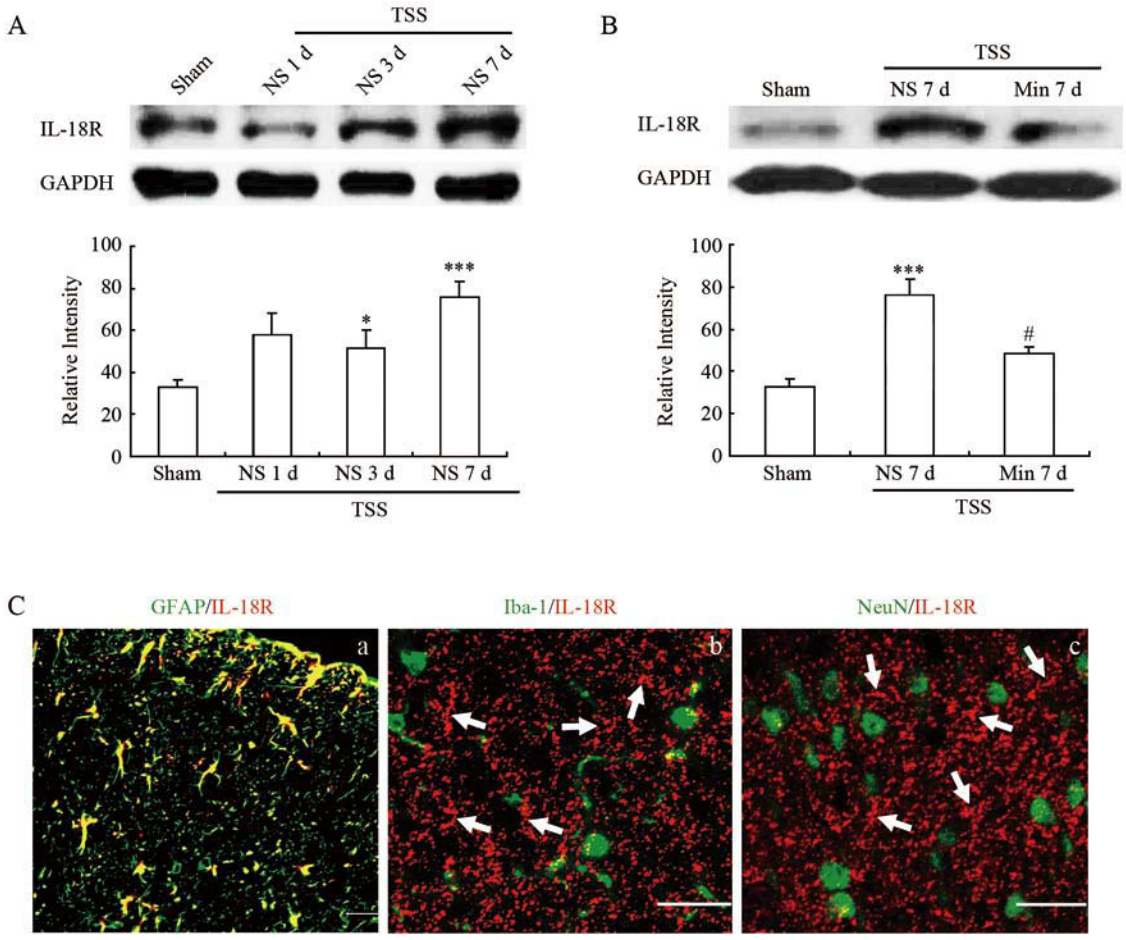

Fig. 4. Inhibition of tetanic stimulation-induced increase in expression of interleukin-18 receptor (IL-18R) by minocycline. A: Western blots show that the expression of IL-18R significantly increased from day 3 to day 7 following tetanic stimulation of the sciatic nerve (TSS) in the normal saline (NS) group. B: The increase in IL-18R expression on day 7 was inhibited by repeated administration of minocycline (Min, 200 μg/20 μL). $^{*}P$ <0.05, $^{***}P$ <0.001 *vs* sham (n = 3/group); $^{\#}P$ <0.05 *vs* NS (n = 3/group). C: Double immunostaining results show that IL-18R colocalized with GFAP but not with Iba-1 or NeuN. Arrows indicate positive staining for IL-18R. Scale bars, 10 μm.

increased IL-18 expression was inhibited by minocycline on day 3 (relative intensity, 45.78 ± 2.27, P <0.01 *vs* saline) (Fig. 3B). To identify the cell type that expressed IL-18, spinal sections were double immunostained with mixed antibodies to IL-18 and Iba-1, GFAP or NeuN on day 3 following TSS in the saline group. The results showed co-localization of IL-18 predominantly with Iba-1 and slightly with GFAP, but not with NeuN (Fig. 3C).

It has been reported that IL-18 mediates the interaction between microglia and astrocytes via binding to IL-18Rs[21,26], so the effects of minocycline on IL-18R expression were investigated. Compared with the sham group, IL-18R expression gradually increased from day 3 (relative intensity, 51.51 ± 8.71, P <0.05 *vs* sham 32.87 ± 3.57) to day 7 (relative intensity, 75.9 ± 7.58, P <0.001 *vs* sham) following TSS (Fig. 4A). However, this increase was inhibited on day 7 by repeated injection of minocycline (relative intensity, 48.44 ± 2.92, P <0.05 *vs* saline) (Fig. 4B).

4 Discussion

Here, we mainly found that the disruption of microglia prevented the induction of spinal LTP and alleviated mechanical allodynia induced by TSS, and that the microglial inhibitor minocycline inhibited the TSS-induced increase in IL-18 expression, which occurred mainly in microglia in the dorsal horn. This study revealed for the first time that IL-18 and its receptor are implicated in TSS-induced spinal LTP and allodynia, supporting the hypothesis that chronic pain is mediated by the sequential activation of microglia first and then astrocytes[27-30].

TSS-induced LTP of C-fiber-evoked field potentials in the spinal cord has been proposed to be the process of central sensitization underlying spinal nociception[25,31,32]. In the present work, we found that disruption of microglial actions blocked spinal LTP and inhibited mechanical allodynia. This is consistent with previous reports that minocycline attenuates the hyperexcitation of dorsal horn neurons following traumatic spinal cord injury and significantly attenuates the allodynia and hyperalgesia following chronic constriction injury in rats[33,34]. Our results provide new evidence for the involvement of microglial activation in the potentiation of synaptic plasticity in the spinal pain pathway. However, neurochemical mechanisms underlying the modulation of pain plasticity by microglia are poorly understood.

Compelling evidence suggests that activation of microglia plays a vital role in the induction of hippocampal LTP by releasing cytokines[35-37]. It has been shown that minocycline reverses β-amyloid-mediated inhibition of NMDA receptor-dependent hippocampal LTP by inhibiting microglial activation[38]. Moreover, proinflammatory cytokines released mainly from microglia, including IL-1β, IL-18 and TNF-α, contribute to the modulation of hippocampal LTP by increasing the translocation and trafficking of AMPA receptors[39,40]. Recent reports suggest that spinal LTP may share a similar mechanism with hippocampal LTP[40,41]. Hippocampal LTP underlies memory and learning. Central sensitization means an increase in synaptic efficacy upon nerve injury or inflammation. These two phenomena have some striking similarities in the expression and functional changes of NMDA and AMPA receptors. However, there are still many differences, such as different roles of neurokinin 1 and cyclooxygenase 2[8]. In addition, under pathological conditions, over-activated glia and up-regulated proinflammatory cytokines cause impairment of LTP in the hippocampus but facilitate LTP in the spinal dorsal horn. Therefore, further investigation is required to provide a reasonable explanation for these differences.

ATP is an activity-dependent signaling molecule in the synaptic transmission of peripheral and spinal nociception and in communication between neurons and glia by activating multiple P2X receptors in glia[42-44]. It has been reported that ATP released from presynaptic nerve terminals contributes to the induction of hippocampal LTP, suggesting a role for ATP in the modulation of synaptic efficiency[45]. Our recent study showed that in the spinal cord, $P2X_7$ receptors ($P2X_7Rs$) are predominantly expressed in microglia, and that $P2X_7R$ antagonists and $P2X_7$-siRNA prevent TSS-induced spinal LTP and reduce mechanical allodynia[46], suggesting that microglial $P2X_7Rs$ are required for plastic changes in pain-sensitive neurons in the dorsal horn.

It has been shown that IL-18, a downstream mol-

ecule of $P2X_7R$, is mainly produced by microglia[47,48]. The present results showed that the microglial inhibitor minocycline not only inhibited TSS-induced spinal LTP but also reduced the increase in microglial IL-18 expression induced by TSS. Notably, we found increases in the expression of both IL-18 in microglia on day 3 and IL-18Rs on day 7 after TSS. This is consistent with previous reports that microglial IL-18 expression significantly increases on day 3 after excitotoxic damage and IL-18-mediated interaction between spinal microglia and astrocytes enhances neuropathic pain[21,26]. Therefore, it is conceivable that the inhibition of TSS-induced spinal LTP and allodynia by minocycline may be attributed to the inhibition of microglial IL-18 release and in turn the inactivation of astrocytes via decreased binding to IL-18Rs. Also, $P2X_4$ receptors ($P2X_4Rs$) are specifically expressed in microglia and involved in the induction of spinal LTP and neuropathic pain[49,50]. Consequently, in addition to $P2X_7Rs$, $P2X_4Rs$ may be at least partly implicated in the minocycline-induced blockade of TSS-induced spinal LTP.

In agreement with our previous study[1], TSS induced bilateral mechanical allodynia, also called mirror-image pain. It has been proposed that glia and proinflammatory cytokines play key roles in the creation of mirror-image pain[51]. We showed that both ipsi- and contralateral mechanical allodynia were alleviated by spinal administration of minocycline, strongly suggesting an important role of microglia in TSS-induced mirror-image pain. In support of this view, a study showed that minocycline relieves the long-lasting contralateral allodynia and the increase in microglial expression induced by carrageenan in mice[52].

Taking these findings together, we speculate that microglia are activated by TSS to release proinflammatory cytokines, including IL-18, which in turn activates astrocytes by binding to IL-18Rs to release more cytokines in the spinal cord. All of these cytokines released from microglia and astrocytes, including IL-1β, TNF-α and IL-18, act on the postsynaptic neuronal membrane to increase the insertion and phosphorylation of AMPA receptors. Thus, the net effects strengthen the synaptic efficacy of nociceptive transmission in the spinal cord.

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