Brain, Behavior, and Immunity 24 (2010) 874–880

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Named Series: Biology of Microglia

The direction of synaptic plasticity mediated by C-fibers in spinal dorsal horn is decided by Src-family kinases in microglia: The role of tumor necrosis factor-α

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ARTICLE INFO

Article history:
Received 6 October 2009
Received in revised form 16 January 2010
Accepted 19 January 2010
Available online 29 January 2010

Keywords:
Long-term potentiation
Long-term depression
Spinal dorsal horn
High frequency stimulation
Microglia
Pro-inflammatory cytokines
TNF-α
Src-family kinases
Minocycline
Neuropathic pain

ABSTRACT

Previous studies have shown that Src-family kinases (SFKs) are selectively activated in spinal microglia following peripheral nerve injury and the activated SFKs play a key role for the development of neuropathic pain. To investigate the underlying mechanism, in the present study the effect of SFKs on long-term potentiation (LTP) at C-fiber synapses in spinal dorsal horn, which is believed as central mechanism of neuropathic pain, was investigated in adult rats. Electrophysiological data revealed that pretreatment with either microglia inhibitor (minocycline, 200 μM) or SFKs inhibitors (PP2, 100 μM and SU6656, 200 μM) reversed the effect of high frequency stimulation (HFS), that is, HFS, which induces long-term potentiation (LTP) normally, induced long-term depression (LTD) after inhibition of either microglia or SFKs. Western blotting analysis showed that the level of phosphorylated SFKs (p-SFKs) in ipsilateral spinal dorsal horn was transiently increased after LTP induced by HFS, starting at 15 min and returning to control level at 60 min after HFS. Double-labeled immunofluorescence staining demonstrated that p-SFKs were highly restricted to microglia. Furthermore, we found that the inhibitory effects of minocycline or SU6656 on spinal LTP were reversed by spinal application of rat recombinant tumor necrosis factor-α (TNF-α 0.5 ng/ml, 200 μl). HFS failed to induce LTP of C-fiber evoked field potentials in TNF receptor-1 knockout mice and in rats pretreated with TNF-α neutralization antibody (0.6 μg/ml, 200 μl). The results suggested that in spinal dorsal horn activation of SFKs in microglia might control the direction of plastic changes at C-fiber synapses and TNF-α might be involved in the process.

1. Introduction

Long-term potentiation (LTP), which refers to a long-lasting enhancement in efficacy of synaptic transmission, is first observed in hippocampus and is intensively studied as a synaptic model of learning and memory (Bliss and Lomo, 1973; Bliss and Collingridge, 1993). Previously, we have demonstrated that LTP of C-fiber evoked field potentials in spinal dorsal horn can be induced by electrical stimulation of afferent C-fibers, as well as by peripheral nerve injury (Liu and Sandkühler, 1995; Zhang et al., 2004). The spinal LTP is considered as a form of pain memory (Ji et al., 2003; Willis, 2002), as afferent C-fibers, which transfer nociceptive signals, form synapses with neurons in superficial lamina of spinal dorsal horn and LTP-inducing stimuli produces a long-lasting hyperalgesia and allodynia in human (Klein et al., 2004).

Several lines of evidence have demonstrated that the activation of spinal microglia after nerve injury is crucial for the pathogenesis of neuropathic pain (Marchand et al., 2005; Tsuda et al., 2003, 2005) The activated microglia may contribute to pain hypersensitivity by releasing pro-inflammatory cytokines, such as TNF-α (DeLeo and Yezierski, 2001; Watkins et al., 2001, 2003; Watkins and Maier, 2003). Previously, we have shown that TNF-α is essential for initiation of neuropathic pain following nerve injury (Xu et al., 2006). However, whether and how activated microglia affects the synaptic plasticity mediated by afferent C-fibers is largely unknown.

Src-family kinases (SFKs) are expressed widely throughout the mammalian central nervous system (CNS) and have been implicated in proliferation and differentiation in the developing CNS (Kuo et al., 1997; Hoffman-Kim et al., 2002). A recent study has shown that peripheral nerve injury activates SFKs only in microglia but not in neurons and astrocytes in spinal dorsal horn and that intrathecal administration of the SFKs inhibitor PP2 suppresses nerve injury-induced mechanical hypersensitivity (Katsura et al., 2006), suggesting SFKs in spinal microglia is critical for neuropathic pain.

In the present work the role of SFKs in microglia for plastic changes at C-fiber synapses in spinal dorsal horn was evaluated in adult rats. We found that the kinases controlled the direction of synaptic plasticity and TNF-α was involved in the process.

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doi:10.1016/j.bbi.2010.01.007

2. Materials and methods

2.1. Animal preparation

Experiments were carried out on male Sprague–Dawley rats (250–280 g) and the TNFR1 knockout mice (C57BL/6-TNFR1$^{-/-}$) that were purchased from The Jackson Laboratory. All mice were maintained in a specific pathogen-free environment. Urethane (1.5 g/kg, i.p.) was used to induce and maintain anesthesia. Additional doses of the drug (0.5 g/kg) were given if needed. Surgical level of anesthesia was verified by the stable mean arterial blood pressure and constant heart rate during noxious stimulation. The trachea was cannulated, and the animal breathed spontaneously. One carotid artery was cannulated to continuously monitor the mean arterial blood pressure, which was maintained from 80 to 120 mm Hg. A laminectomy was carried out to expose the lumbar enlargement of spinal cord, and the dura mater was incised longitudinally. The left sciatic nerve was dissected free for bipolar electrical stimulation with platinum hook electrodes. The rats were placed on a stereotaxic apparatus. All exposed nervous tissues were covered with warm paraffin oil, except for the spinal lumbar enlargement, onto which the drugs will be applied. Colorectal temperature was kept constant (37–38 °C) by means of a feedback-controlled heating blanket. At the end of the experiments, animals were killed with an overdose of urethane. The local animal care committee has approved all experiments.

2.2. Electrophysiologic recording

Recording of C-fiber evoked field potentials in spinal dorsal horn has been described previously (Liu and Sandkühler, 1997). Briefly, following electrical stimulation of the left sciatic nerve with a bipolar silver chloride hook-electrode, field potentials were recorded at a depth of 100–500 μm from the surface of the spinal cord in ipsilateral lumbar enlargement (L4 and L5 segments) with a glass microelectrode (filled with 0.5 M sodium acetate, impedance 0.5–1 MΩ), which was driven by an electronically controlled microstepping motor (Narishige Scientific Instrument Laboratory). An A/D converter card (ADC-42. PICO) was used to digitize and store data at a sampling rate of 10 kHz. The test stimuli (0.5 ms duration, every 1 min) delivered to the sciatic nerve was used to evoke field potentials in spinal dorsal horn. The strength of the test stimulation was adjusted to 1.5–2 times of threshold for C-fiber response. High frequency stimulation (HFS, 40 V, 0.5 ms, 100 Hz, given in four trains of 1 s duration at 10 s intervals) was used to induce LTP of C-fiber evoked field potentials. Only one experiment was conducted in each animal.

2.3. Drug preparation

Minocycline hydrochloride (Sigma, St. Louis, MO) was dissolved in DMSO and diluted with double-distilled water to the working concentration (50, 100, 200 μM) just prior to use. PP2 (4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3,4-d] pyrimidine, Calbiochem), SU6656 (2-oxo-3-(4,5,6,7-tetrahydro-1*H*-indol-2-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonic acid dimenthylamide, Calbiochem) and PP3 (4-Amino-7-phenylpyrazolo [3,4-d] pyrimidine, Calbiochem) were first dissolved in DMSO to make a stock concentration of 50 mM, aliquoted in small volumes and stored at −80 °C. The stock solution was subsequently diluted with 0.9% saline to make final concentrations immediately before administration. Recombinant rat TNF-α (rr-TNF, R&D Systems, Minneapolis, MN) and TNF-α neutralization antibody (R&D Systems, Minneapolis, MN) were first dissolved as a concentrated stock solution (10 mg/ml) in 0.1% BSA/PBS (bovine serum albumin/phosphate buffer saline), the stock solution was diluted with 0.9% saline to make final concentrations immediately before administration. The final concentration of DMSO (dimethyl sulphoxide) was <0.5%.

2.4. Westerning blotting

The dorsal quadrants of L4-L5 spinal cord on the stimulated side were separated and were put into liquid nitrogen immediately, followed by homogenization in 15 mmol/l Tris buffer, pH 7.6 [250 mmol/l sucrose, 1 mM MgCl, 1 mM DTT, 2.5 mM EDTA, 1 mM EGTA, 50 mM NaF, 10 μg/ml leupeptin, 1.25 μg/ml pepstatin, 2.5 μg/ml Aprotin, 2 mM sodium pyrophosphate, 0.1 mM $NaVO_4$, 0.5 mM PMSF, and protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN)]. The tissues were sonicated on ice, and then centrifuged at 13,000*g* for 15 min at 4 °C to isolate the supernatant containing protein samples. The proteins samples were stored at −80 °C until assayed.

Proteins were separated by gel electrophoresis (SDS–PAGE) and transferred onto a PVDF membrane (Bio-Rad, Hercules, CA). The blots were blocked with 5% w/v non-fat dry milk in TBST (20 mM Tris-base, pH 7.6, 137 mM NaCl and 0.1% Tween 20) for 1 h at room temperature and then incubated with primary antibodies, including rabbit polyclonal anti-rat p-SFKs (Tyr416) antibody (1:1000, Cell Signaling Technology, Beverly, MA) and rabbit polyclonal anti-rat β-actin antibody (1:1000, Cell Signaling Technology), overnight at 4 °C with gentle shaking. The blots were washed three times for 15 min each with washing buffer (TBST) and then incubated with secondary antibody horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:1000, Santa cruz) for 2 h at room temperature. After incubation with the secondary antibody, the membrane was washed, again, as above. The immune complex was detected by ECL (Amersham, USA) and exposed to X-ray film (Kodak, Rochester, NY). The band intensities on the film were analyzed by densitometry with a computer-assisted imaging analysis system (Germany, KONTRON IBAS 2.0). To calculate the phosphorylated versus non-phosphorylated form of protein, the same membrane was stripped with stripping buffer (67.5 mM Tris, pH 6.8, 2% SDS, and 0.7% β-mercaptoethanol) for 30 min at 50 °C and reprobed with rabbit polyclonal anti-rat SFKs antibody (1:1000, Cell Signaling Technology), and detected as above.

2.5. Immunohistochemistry

After electrophysiological recordings, the rats were perfused with 300 ml saline followed by 350 ml cold 4% paraformaldehyde in phosphate buffer (PB, 0.1 M, pH 7.4). The L4–L5 spinal cord were removed, post-fixed with 4% paraformaldehyde in PB for 4 h, and then replaced with 30% sucrose in PBS for 2 days at 4 °C. Transverse spinal sections (25 μm) were cut in a cryostat (Leica CM3035 S; −20 to −22 °C) and processed for immunofluorescence according to the methods as described previously (Katsura et al., 2006) Briefly, all sections were blocked with 3% donkey serum in 0.3% Triton X-100 for 1 h at room temperature and incubated with rabbit anti-rat polyclonal p-SFKs antibody (1:200; Cell Signaling) overnight at 4 °C, followed by incubation with CY3-conjugated secondary antibody (1:200; Jackson Immunolab) for 1 h at room temperature. For double-labeled immunofluorence, spinal sections were incubated with a mixture of the p-SFKs antibody and monoclonal NeuN (neuronal marker, 1:500; Chemicon), GFAP (astrocyte marker, 1:500, Chemicon) or OX-42 (microglia marker, 1:500; Serotec), followed by a mixture of FITC- and CY3-conjugated secondary antibodies (1:200; Jackson Immunolab) for 1 h at room temperature. The stained sections were transferred to glass microscope slides, stretched and arranged using a small paintbrush, then covered with coverslips and sealed by painting around edges of coverslips with nail polish. Sections were immediately examined

with an Olympus IX71 fluorescence microscope (Olympus Optical, Tokyo, Japan) and images were captured with a CCD spot camera.

For double staining, with use of software IM50 the images of red signal (E_{max} = 570 nm, A_{max} = 550 nm) and green signal (E_{max} = 520 nm, A_{max} = 492 nm) were overlayed, this resulted in yellow signal, if both red and green signals expressed in the same place. Magnification of all the images is 200 times.

2.6. Experimental designs

2.6.1. Experiment I

To evaluate the role of microglia in synaptic plasticity in spinal dorsal horn mediated by afferent C-fibers, minocycline, an inhibitor of microglia, at different concentrations (50, 100, 200 μM, 200 μl in volume, n = 5–6 in each group) were applied onto the spinal dorsal horn 60 min prior to LTP induction by HFS of the sciatic nerve. In control experiments, either minocycline (200 μM, 200 μl) or DMSO (0.4%), which was used to resolve minocycline, PP2, PP3 and SU6656 was added to the spinal dorsal horn to test whether the drugs affect the baseline of C-fiber response (n = 5 for each drug). We also tested whether DMSO (0.4%) affect LTP induction (n = 5).

2.6.2. Experiment II

To investigate the role of SFKs on spinal synaptic plasticity mediated by afferent C-fibers, a selective Src inhibitor PP2 (100 μM, 200 μl in volume,), a non selective SFKs inhibitor SU6656 (200 μM, 200 μl in volume,) or PP3 (an inactive form of PP2; 100 μM, 200 μl in volume,) was applied to spinal dorsal surface 60 min before HFS (n = 5–6 in each group). We also tested if the drugs affect the baseline of C-fiber evoked responses (n = 5 in each group).

2.6.3. Experiment III

The activation of SFKs in spinal dorsal horn following LTP induction was accessed by evaluation of p-SFKs levels at different time points (15, 30, 60, 180 min, n = 3 in each time points) after HFS with use of Western blotting. The activation of SFKs was also evaluated in control animals (n = 3), in which C-fiber evoked field potentials were recorded for 180 min but no HFS was delivered and in naïve animals (without operation and recordings, n = 3). The expression of p-SFKs in control animals (n = 2) and animals undergoing HFS (n = 2) were also detected by immunohistochemistry. To verify the specificity of p-SFKs antibody used in the present study (Western blotting and immunohistochemistry), negative controls were performed in which only secondary antibody (CY3-conjugated donkey anti-rabbit antibody) but no primary antibody was added (n = 2). To determine the cell types that express p-SFKs following HFS, double-labeled immunofluorescence staining was carried out at 15 min after LTP induction in six rats.

2.6.4. Experiment IV

To test the possibility that activated microglia may facilitate LTP induction in spinal dorsal horn by release of TNF-α, rr-TNF (0.5 ng/ml, 200 μl) was applied to spinal dorsal surface 30 min after spinal application of either minocycline or SU6656, and 30 min later HFS was delivered to the sciatic nerve.

2.7. Statistical analysis

The amplitudes of C-fiber evoked field potentials, measured as the maximal distance from the baseline (Fig. 1A, top), were determined on-line by LTP program (www.ltp-program.com). In each experiment, amplitudes of five consecutive field potentials recorded at 1 min intervals were averaged. The mean amplitudes of the averaged responses before drug application or HFS served as baseline. The amplitude of C-fiber evoked field potential was expressed as percentage of baseline. The summary data from different animals in the same group were expressed as mean ± SE. Statistical tests were carried out with SPSS 10.0 (SPSS Inc.). The effects of electrical stimulation or drug were analyzed by repeated measures ANOVA followed by Tukey post hoc test. The mean amplitude of C-fiber evoked field potentials (% of baseline) immediately before HFS or drug application was compared with that recorded in 30 min, 1 h and 3 h after HFS or drug application. $p < 0.05$ was considered significant. The relative densities of Western blots at different time points after LTP induction were compared to those in control animals, using ANOVA with the least significant difference test (LSD-t). The data were expressed as mean ± SE.

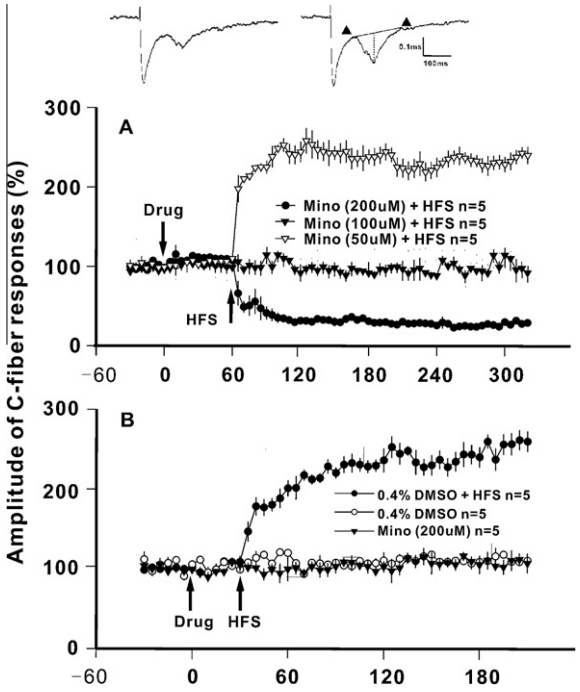

Fig. 1. Spinal application of minocycline affects synaptic plasticity in a dose-dependent manner. (A) The effect of minocycline at different concentrations (50, 100, 200 μM) on the synaptic plasticity mediated by afferent C-fibers are shown. Traces at top were recorded before (left) and after (right) HFS (100 Hz, 40 V, 0.5 ms, given in four trains of 1 s duration at 10 s interval) in rats treated with 50 μM minocycline. In the trace on the right the horizontal dotted line indicates the baseline and the vertical one the maximal amplitude of C-fiber evoked response, as determined by parameter extraction software. (B) DMSO (0.4%), which was used to dissolve minocycline, affected neither the baseline of C-fiber response (open circle) nor LTP (closed circle) in spinal dorsal horn. Minocycline at 200 μM did not affect the baseline of C-fiber responses (closed triangle). Each data point represents the mean amplitude of five consecutive field potentials recorded at 1 min intervals. The mean amplitudes of the averaged responses before drug application served as baseline.

3. Result

3.1. The effects of minocycline on the synaptic plasticity in spinal dorsal horn

As shown in Fig. 1A, spinal application of minocycline 60 min before HFS affected synaptic plasticity in a dose-dependent manner. In the presence of 50 μM minocycline HFS led to a significant increase in the amplitude of C-fiber responses (open triangle, $F(3, 16) = 653.77$, $p < 0.05$ vs. baseline, n = 5, ANOVA), persisting for at least 4 h, indicating LTP was induced, which is comparable to that induced in presence of saline (Yang et al., 2005). When the concentration of the drug increased to 100 μM, HFS did not af-

fect the amplitude of C-fiber responses (closed triangle, $F(3, 16) = 2.41$, $p > 0.05$ vs. baseline; $n = 5$, ANOVA), indicating that the LTP was completely blocked. Furthermore, in the presence of 200 μM minocycline, HFS induced a significant and long-lasting decrease in the amplitude of C-fiber responses (closed circle, $F(3, 16) = 321.58$, $p < 0.05$ vs. baseline; $n = 5$, ANOVA), indicating that long-term depression (LTD) was induced. In control experiments, minocycline at 200 μM did not affect the amplitude of C-fiber responses (Fig. 1B, closed triangle; $F(3, 16) = 1.34$, $p > 0.05$ vs. baseline; $n = 5$, ANOVA). DMSO (0.4%), which was used to dissolve minocycline affected neither the baseline of C-fiber responses (Fig. 1B, open circle; $F(3, 16) = 1.85$, $p > 0.05$ vs. baseline; $n = 5$, ANOVA) nor LTP induction (Fig. 1B, closed circle; $F(3, 16) = 701.85$, $p < 0.05$ vs. baseline; $n = 5$, ANOVA). These results indicate that activated microglia may control the direction of synaptic plasticity at C-fiber synapses in spinal dorsal horn, but not affect basal synaptic transmission.

3.2. The effect of SFKs inhibitors on the synaptic plasticity in spinal dorsal horn

As SFKs were activated only in spinal microglia following peripheral nerve injury (Katsura et al., 2006), we next tested whether the kinases play a role in synaptic plasticity by spinal application of SFKs inhibitors 60 min before HFS. We found that similar to minocycline, PP2 (100 μM) did not affect C-fiber responses (Fig. 2A, open circle; $F(3, 16) = 1.48$, $p > 0.05$ vs. baseline; $n = 5$, ANOVA), but enabled HFS to induce LTD (Fig. 2A, closed circle; $F(3, 20) = 914.68$, $p < 0.05$ vs. baseline; $n = 6$, ANOVA). Whereas PP3 (100 μM), an inactive form of PP2, had no effect on LTP induction (Fig. 2A, closed triangle; $F(3, 16) = 671.73$, $p < 0.05$ vs. baseline; $n = 5$, ANOVA). To confirm the role of SFKs in spinal synaptic plasticity, another SFKs inhibitor SU6656 was tested with the same protocol. We found that in the rats pretreated with SU6656 (200 μM), HFS induced LTD of C-fiber evoked field potentials too (Fig. 2B, closed circle; $F(3, 16) = 475.93$, $p < 0.05$ vs. baseline; $n = 5$). The results indicate that the effect of activated microglia on spinal synaptic plasticity may be mediated by activation of SFKs.

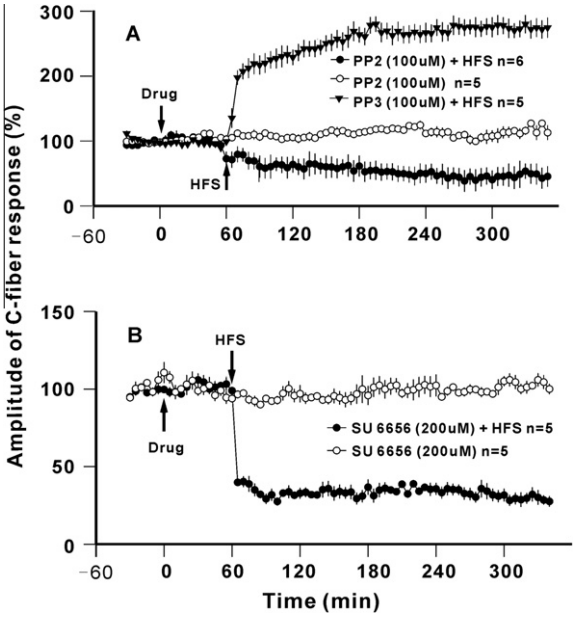

Fig. 2. High frequency stimulation induces LTD in animals pretreated with SFKs inhibitors (PP2 or SU6656). A: Spinal application of PP2 (100 μM, filled circle) but not its inactive form PP3 (100 μM, filled triangle) enabled HFS to induce LTD. The same dose of PP2 did not affect baseline of C-fiber responses (open circle). (B) Pretreatment with SU6656 (200 μM), which did not affect basal synaptic transmission (open circle), also reversed the effect of HFS (closed circle).

3.3. SFKs are activated in spinal microglia after LTP induction

To further evaluate the role of SFKs in microglia in spinal synaptic plasticity, the levels of p-SFKs were evaluated using Western blotting at different time points after LTP induction by HFS. As shown in Fig. 3A and B, the p-SFKs levels after HFS were different from that in naïve and control animals ($F(5, 12) = 788.73$, $p < 0.05$, ANOVA). The following LST test showed the p-SFKs increased significantly at 15 min ($p < 0.001$; $n = 3$) and 30 min ($p < 0.01$; $n = 3$) but not at 60 min ($p > 0.05$; $n = 3$) and 180 min ($p > 0.05$; $n = 3$) after LTP induction, compared with that in naïve and control animals.

Immunofluorescence staining revealed that the expression of p-SFKs increased in spinal dorsal horn of animals undergoing HFS, compared to that in control animals (Fig. 3D and E). No signals were detected in the negative control groups, in which only secondary antibody but no primary antibody was added to tissues (Fig. 3C). To determine the cell types that express p-SFKs following HFS, immunofluorescence staining was carried out at 15 min after LTP induction in 6 rats. We found that p-SFKs were co-localized only with OX-42 (Fig. 3G), a microglia marker, but not with NeuN (Fig. 3F), a neuron marker, or with GFAP (Fig. 3H), an astrocyte marker, which is in line with a previous work showing that p-SFKs is exclusively expressed in microglia following nerve injury (Katsura et al., 2006). Together, results indicated that SFKs in microglia of spinal dorsal horn were activated transiently after induction of LTP of C-fiber evoked field potentials.

3.4. TNF-α is critically involved in the induction of spinal LTP

As previous works have shown that activated microglia may contribute to neuropathic pain by release of TNF-α (Xu et al., 2006; Watkins et al., 2003), we tested the possibility that activated microglia may facilitate LTP induction in spinal dorsal horn by release of TNF-α. As shown in Fig. 4A, spinal application of TNF-α at a dose as low as 0.5 ng/ml (200 μl) enable HFS to induce LTP in rats pretreated with either minocycline (closed circle; $F(3, 20) = 2185.59$, $p < 0.05$ vs. baseline; $n = 6$, ANOVA) or SU6656 (open circle; $F(3, 16) = 1944.01$, $p < 0.05$ vs. baseline; $n = 5$, ANOVA). To confirm the role of TNF-α in spinal LTP, we perform experiments in wild type and TNFR1 knock out mice and found that HFS induced spinal LTP of C-fiber evoked field potentials in wild type mice (Fig. 4B, closed circle; $F(3, 16) = 914.53$, $p < 0.05$ vs. baseline; $n = 5$, ANOVA) but not in the TNFR1 knock out mice (Fig. 4B, open circle; $F(3, 20) = 1.88$, $p > 0.05$ vs. baseline; $n = 6$, ANOVA). Furthermore, we found that spinal application of anti-rat TNF-α neutralization antibody (0.6 μg/ml, 200 μl) 30 min before HFS inhibited spinal LTP in rats (Fig. 4C; closed circle; $F(3, 20) = 1.35$, $p > 0.05$ vs. baseline; $n = 6$, one-way ANOVA), while PBS (0.01 M, 200 μl), which was used to dissolve TNF-α and TNF-α neutralization antibody, affected neither amplitude of C-fiber response (Fig. 4C; open triangle; $F(3, 16) = 2.95$, $p > 0.05$ vs. baseline; $n = 5$, ANOVA nor LTP by HFS (Fig. 4C; closed triangle; $F(3, 16) = 1345.23$, $p < 0.05$ vs. baseline; $n = 5$, ANOVA). The data suggested that TNF-α released from activated microglia might be critical for spinal LTP induction.

4. Discussion

In hippocampus, the activation of microglia results in impairment of LTP (Maher et al., 2006). It has been shown that the age-related or amyloidβ-mediated attenuation in LTP and memory

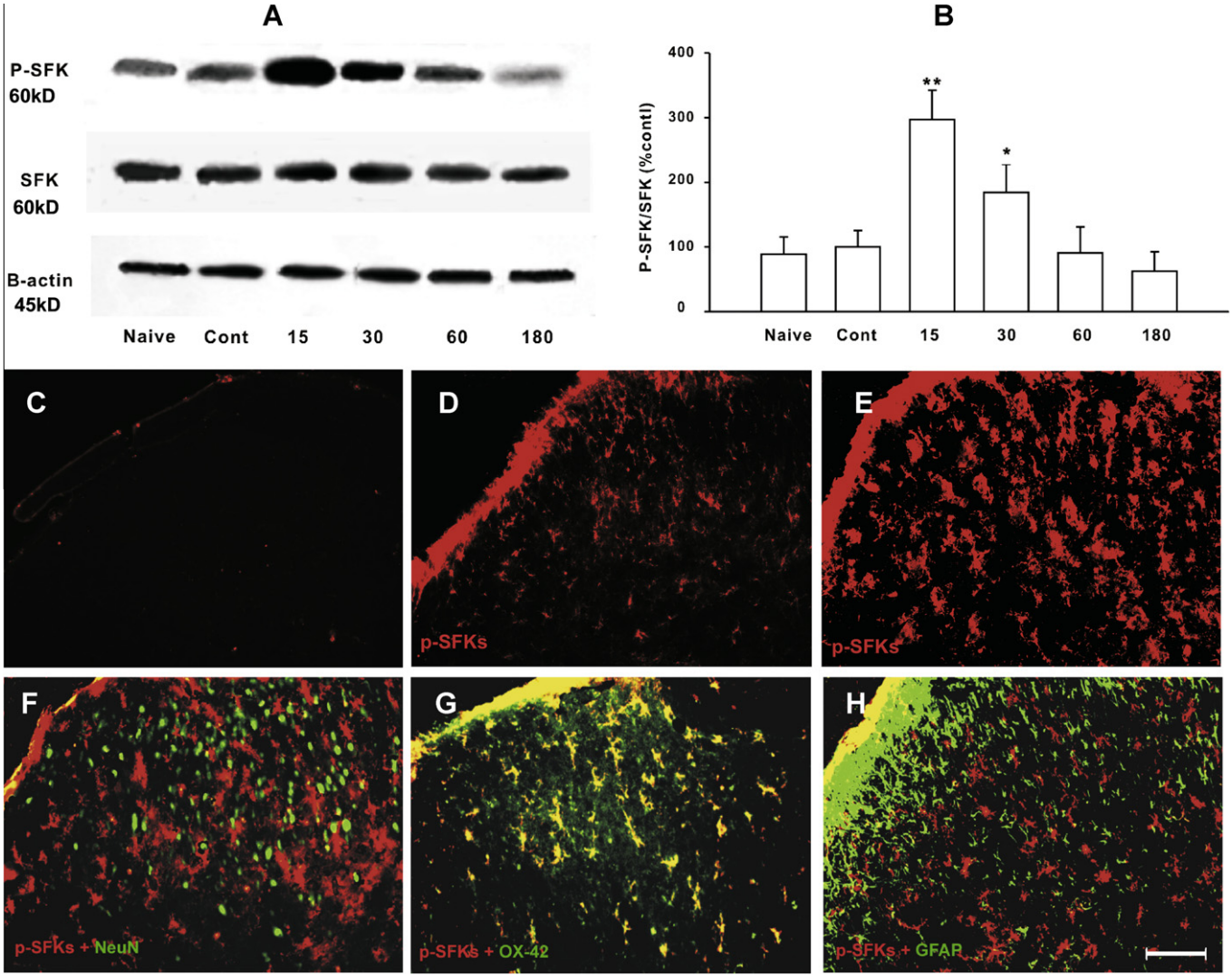

Fig. 3. The Src-family kinases in spinal microglia are activated after LTP induction in spinal dorsal horn. (A) The bands showed the phosphorylated and total SFKs and β-actin from protein samples of ipsilateral spinal dorsal horn (L4-L5) at 15, 30, 60 and 180 min after LTP induction by HFS. In control animals C-fiber responses were recorded but no HFS was delivered. (B) The histogram showed the p-SFKs levels that were normalized by total SFKs levels and expressed as percentages of their controls.*$P < .01$, **$P < 0.001$, $n = 3$ in each group. (C) The photograph showed no signal was detected in negative control experiments, in which only secondary antibody but no primary antibody was added ($n = 2$). (D, E) The photographs showed the expression of p-SFKs in spinal dorsal horn 15 min after HFS and that in control animals, in which only test stimuli but no HFS were delivered. (F–H) The photographs showed that p-SFKs (G, red) were co-localized with OX-42 (microglia marker, G, green), but not with NeuN (neuron marker, F, green,) or GFAP (astrocytes marker, H, green,). The yellow cells indicated double-labeled cells. Scale bar = 100 μm (C–H). (For interpretation of color mentioned in this figure legend the reader is referred to the web version of the article.)

deficit are associated with microglia activation (Griffin et al., 2006; Wang et al., 2004). In the present study, however, we reported for the first time that in spinal dorsal horn inhibition of microglia by spinal application of minocycline depressed LTP induction at C-fiber synapses in a dose-dependent manner. Minocycline at 50 μM did not affect LTP of C-fiber evoked field potentials induced by HFS of the sciatic nerve, at 100 μM blocked the spinal LTP and at 200 μM reversed LTP to LTD. Clearly, microglia plays a totally different role for synaptic plasticity in hippocampus and in spinal dorsal horn. Therefore, inhibition of activated microglia may not only treat neuropathic pain but also improve memory in hippocampus.

In hippocampus SFKs are expressed at the postsynaptic density (PSD) and are activated 1–5 min after LTP induction (see Soderling and Derkach, 2000 for a review). Inhibition of SFKs blocks LTP but not LTD induction, while infusion of Src results in an enhancement of AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid)-receptor mediated current that is dependent on the influx of Ca^{2+} through the NMDA (*N*-methyl-D-aspartate)-receptor channel (Lu et al., 1998). In the present work, we found that in spinal dorsal horn SFKs were activated only in microglia but not in neurons after LTP induction. In the presence of SFKs inhibitors, HFS induced LTD rather than LTP. Taken together, SFKs may affect synaptic plasticity in different ways.

Following nerve injury, spinal microglia is rapidly activated and the activated microglia may contribute to chronic pain by releasing a variety of pro-inflammatory cytokines, such as TNF-α (Watkins and Maier, 2003; Watkins et al., 2003; Sommer and Kress, 2004; Wieseler-Frank et al., 2005), interleukin-1β (IL-1β) (DeLeo and Yezierski, 2001) and interleukin- 6 (IL-6) (Watkins et al., 2001). TNF-α and TNFR1 are upregulated in spinal dorsal horn and DRG in several models of neuropathic pain (Ohtori et al., 2004; Xu et al., 2006; Wei et al., 2007). Our previous work has shown that spinal application of TNF-α (4.5–100 ng/ml) affects neither basal synaptic transmission mediated by C-fibers nor spinal LTP of C-fiber evoked field potentials induced by HFS in naïve rats. How-

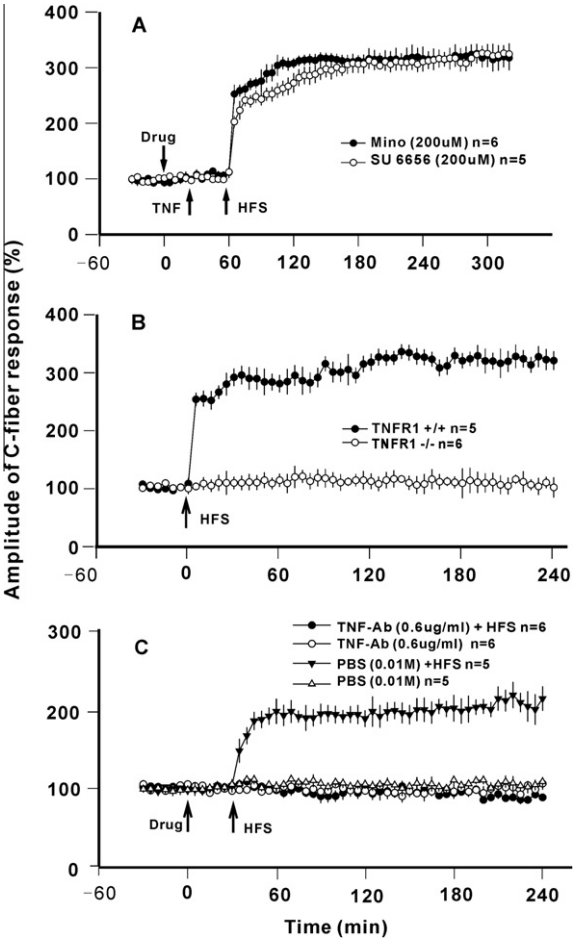

Fig. 4. The effect of TNF-α on spinal LTP induction induced by HFS. (A) In the rats pretreated with microglia inhibitor (minocycline, filled circle) or SFKs inhibitor (SU6656, open circle), spinal application of TNF-α (0.5 ng/ml, 200 μl) at recording segments 30 min before HFS rescued spinal LTP. (B) HFS induced spinal LTP of C-fiber evoked field potentials in wild type mice (filled circle) but not in TNFR1 knock out mice (open circle). (C) Spinal application of anti-rat TNF-α neutrolization antibody (0.6 μg/ml, 200 μl) had no effect on baseline of C-fiber responses (opened circle) but inhibited spinal LTP by HFS (filled circle). PBS (0.01 M, 200 μl), which was used to dissolve TNF-α and TNF-α neutralization antibody, affected neither baseline of C-fiber response (open triangle) nor LTP (closed triangle) by HFS.

ever, in rats with nerve injury, TNF-α at a dose as low as 10 pg/ml is able to induce spinal LTP (Liu et al., 2007). It seemed that TNF-α might be involved in synaptic plasticity in spinal dorsal horn only in pathological conditions. However, in the present work we showed that inhibitory effect of either microglia inhibitor or SFKs inhibitors on spinal LTP was completely reversed by spinal application of TNF-α (0.5 ng/ml, 200 μl). Furthermore, we found that in the animals pretreated with TNF-α neutralization antibody or genetic deletion of TNFR1, HFS failed to induce spinal LTP. Accordingly, TNF-α may be involved in the spinal LTP induction in both physiological and pathological conditions. Our recent work has demonstrated that IL-1βaffected spinal LTP of C-fiber evoked field potentials in the same manner as TNF-α , i.e., the cytokine does not affect spinal LTP in naïve animals but induces LTP in rats with nerve injury (Zhong et al., 2009). It is likely that IL-1βmay be also involved in the LTP induction in normal conditions. In the present work we showed that in the case of genetic deletion of TNFR1 or neutralization of TNF-α, HFS failed to induce LTP but was unable to induce LTD. The results indicated that TNF-α may be an important, but not a unique molecule for controlling the direction of the synaptic plasticity in spinal dorsal horn and other cytokines, such as IL-1β, IL-6 etc. might be involved in the process, as well.

How the pro-inflammatory cytokines facilitate spinal LTP induction is largely unknown. According to the previous studies and the present results, we speculated that inducible nitric oxide synthase (iNOS) might play a role in the pathological process, as spinal LTP at C-fiber synapses is dependent on nitric oxide production (Ruscheweyh et al., 2006) and is associated with the increase in the expression of iNOS (Pedersen et al., 2009). Further studies are needed to elucidate how activation of SFKs in spinal microglia regulates plastic change in the synapses between afferent C-fibers and spinal dorsal horn neurons.

Conflict of interest statement

All authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported by grants from the National Nature Science Foundation of China (Nos. 30770705, 30970957) and from the Ministry of Education of China (20060558001).

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