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TRAF6 upregulation in spinal astrocytes maintains neuropathic pain by integrating TNF- α and IL-1 β signaling

Ying Lu^{1,2,&}, Bao-Chun Jiang^{1,&}, De-Li Cao¹, Zhi-Jun Zhang¹, Xin Zhang¹, Ru-Rong Ji³, and Yong-Jing Gao^{1,*}

¹Pain Research Laboratory, Institute of, Nautical Medicine, Jiangsu Key Laboratory of Inflammation and Molecular Drug Target, Nantong University, Nantong, Jiangsu, 226001, China

²Department of Nutrition, School of Public Health, Nantong University, Nantong, Jiangsu 226001, China

³Departments of Anesthesiology and Neurobiology, Duke University Medical Center, Durham, NC 27710, USA

Abstract

The proinflammatory cytokines TNF- α and IL-1 β have been strongly implicated in the pathogenesis of neuropathic pain, but the intracellular signaling of these cytokines in glial cells are not fully understood. Tumor necrosis factor receptor associated factor 6 (TRAF6) plays a key role in signal transduction in the TNF receptor superfamily and the interleukin-1 receptor superfamily. In this study, we investigated the role of TRAF6 in neuropathic pain in mice following spinal nerve ligation (SNL). SNL induced persistent TRAF6 upregulation in the spinal cord. Interestingly, TRAF6 was mainly colocalized with the astrocytic marker GFAP on SNL day 10 and partially expressed in microglia on SNL day 3. In cultured astrocytes, TRAF6 was up-regulated after exposure to TNF- α or IL-1 β . TNF- α or IL-1 β also increased CCL2 expression, which was suppressed by both siRNA and shRNA targeting TRAF6. TRAF6 siRNA treatment also inhibited the phosphorylation of c-Jun N-terminal kinase (JNK) in astrocytes induced by TNF- α or IL-1 β . JNK inhibitor D-NKI-1 dose-dependently decreased IL-1 β -induced CCL2 expression. Moreover, spinal injection of TRAF6 siRNA decreased intrathecal TNF- α -or IL-1 β -induced allodynia and hyperalgesia. Spinal TRAF6 inhibition via TRAF6 siRNA, shRNA lentivirus, or antisense oligodeoxynucleotides partially reversed SNL-induced neuropathic pain and spinal CCL2 expression. Finally, intrathecal injection of TNF- α -activated astrocytes induced mechanical allodynia, which was attenuated by pretreatment of astrocytes with TRAF6 siRNA. Taken together, the results suggest that TRAF6, upregulated in spinal cord astrocytes in the late

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*Correspondence: Yong-Jing Gao, Institute of Nautical Medicine, Nantong University, 19 Qixiu Road, Nantong, Jiangsu 226001, China., Tel: +86-513-85051799; Fax: +86-513-85051796; gaoyongjing@hotmail.com.

&Ying Lu and Bao-Chun Jiang contributed equally to this work.

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Conflict of interest statement

The authors declare no conflict of interest.

phase after nerve injury, maintains neuropathic pain by integrating TNF- α and IL-1 β signaling and activating the JNK/CCL2 pathway in astrocytes.

The tumor necrosis factor (TNF) receptor associated factors (TRAFs) are a family of genetically conserved adapter proteins, which consists of six members (TRAF1-6) in mammals [2]. Among all the family members, TRAF6 is unique in that it not only mediates signaling from the tumor necrosis factor receptor (TNFR) superfamily, but also participates in signal transduction in the interleukin-1 receptor/Toll-like receptor (IL-1R/TLR) superfamily [3,27]. By linking the activation of these receptors to downstream signaling events, TRAF6 exerts critical functions in a wide range of physiological and pathological processes, such as adaptive and innate immunity, inflammation and tissue homeostasis [50].

Neuropathic pain is caused by lesion or dysfunction of the nervous system. It is one of the most intractable human complaints and largely resistant to the current treatments [8]. It has become clear that activation of spinal cord glial cells such as microglia and astrocytes play an important role in the establishment and maintenance of neuropathic pain [19,35,44]. After tissue or nerve damage, the proinflammatory cytokines (e.g., TNF- α and IL-1 β) and chemokines (e.g. CCL2) are upregulated in spinal cord glial cells [19,41]. Targeting glia-expressing signaling molecules such as TNF- α , IL-1 β , and CCL2 has been shown to alleviate neuropathic pain [12,33,48]. Given the fact that TRAF6 is a key signaling integrator of TNF- α and IL-1 β , it is conceivable to postulate that TRAF6 would play a critical role in the pathogenesis of neuropathic pain.

It has been well documented that many of the biological effects of TRAF signaling are mediated by the activation of MAP kinase (MAPK) and NF- κ B pathways [50]. Accumulating evidence has shown that MAPKs (ERK, p38 and JNK) are differentially activated in spinal glial cells after nerve injury and contribute to the generation of neuropathic pain [18,21,56]. Particularly, activation of JNK in spinal cord astrocytes, following TNF- α stimulation and nerve injury, produces CCL2, which is critical for the development and maintenance of neuropathic pain [12,56]. Therefore, we reasoned that TRAF6 in the spinal cord would enhance neuropathic pain through the JNK/CCL2 pathway.

In this study, we investigated the expression of TRAF6 and its role in the spinal cord in spinal nerve ligation (SNL)-induced neuropathic pain in mice. We also examined the upstream and downstream signaling mechanisms of TRAF6 in cultured astrocytes. Our results demonstrated that TRAF6 regulates SNL-induced mechanical allodynia through the JNK/CCL2 pathway in spinal cord astrocytes.

2. Materials and Methods

2.1 Animals and surgery

Adult ICR mice (male, 8 weeks) were purchased from Experimental Animal Center of Nantong University. The animals were maintained on a 12:12 light–dark cycle at a room temperature of $22 \pm 1^\circ\text{C}$ with free access to food and water. The experimental procedures were approved by the Animal Care and Use Committee of Nantong University and

performed in accordance with the guidelines of the International Association for the Study of Pain. To produce a spinal nerve ligation, animals were anesthetized with isoflurane and the L6 transverse process was removed to expose the L4 and L5 spinal nerves. The L5 spinal nerve was then isolated and tightly ligated with 6-0 silk thread [22]. For sham operations, the L5 spinal nerve was exposed but not ligated.

2.2. Drugs and administration

TNF- α and IL-1 β were purchased from R&D systems (Minneapolis, MN). TRAF6 siRNA was purchased from Santa Cruz (Santa Cruz, CA). Before cell transfection or intrathecal injection, siRNA was mixed with X-tremeGENE siRNA transfection reagent (Roche, Carlsbad, CA) for in vitro experiments and polyethyleneimine (PEI, Fermentas Inc) for in vivo experiments, respectively. Non-targeting (NT) siRNA was synthesized by Dharmacon Research Incorporation as a control siRNA. TRAF6 antisense-oligodeoxynucleotide (ODN) (5'-CCA CAG GCC CTT CAA ATG-3') or mismatch ODN (5'-CCA CAT GAA CTT AAA AAG-3') were synthesized by IDT Integrated DNA Technologies (Coralville, IA). Intrathecal injection was made with a 30G needle between the L5 and L6 intervertebral spaces to deliver the reagents (10 μ l) into the cerebral spinal fluid [16]. For sustained spinal drug infusion, osmotic pumps (0.5 μ l/h for 7 days, Alzet, Cupertino, CA) were implanted under the back skin. Each pump was connected to a polyethylene (PE8) catheter that was inserted into subarachnoid space between L5-L6 vertebrate. The drug filled pumps were soaked in saline for 3 h before implantation.

2.3. Lentiviral vectors production and intraspinal injection

Three shRNAs targeting the sequence of murine TRAF6 (Gene Bank Accession: 3 NM_009424.2) were designed (shRNA1: 5'-TAA CAT TAT GAA CAG CCT G-3'; shRNA2: 5'-TAT GGA TTT GAT GAT GCA G -3'; shRNA3: 5'-TAA TGT GTG TAT -3'). An additional scrambled sequence was also designed as a negative control (NC) (5'-TTC TCC GAA CGT GTC ACG T-3'). The recombinant lentivirus containing TRAF6 shRNA (LV-TRAF6 shRNA) or NC shRNA (LV-NC) was packaged using pGCSIL-GFP vector by Shanghai GeneChem (China). The final titers of LV-TRAF6 shRNA1, LV-TRAF6 shRNA2, LV-TRAF6 shRNA3, and LV-NC were 2×10^8 TU/mL, 2×10^8 TU/mL, 6×10^8 TU/mL, and 1.5×10^9 TU/mL, respectively. The knockdown effect of the 3 shRNAs was examined on cultured astrocytes. Because LV-TRAF6 shRNA2 showed the best knockdown effect, we chose it for intraspinal injection and also chose the same sequence of pLenti TRAF6 shRNA for astrocytes treatment.

The intraspinal injection was performed as described previously [55]. In brief, animals were anesthetized with pentobarbital sodium (40–50 mg/kg, i.p.) and underwent hemilaminectomy at the L1-L2 vertebral segments. Intraspinal injection was performed unilaterally on the left side. After exposure of the spinal cord, each animal received 2 injections (8×10^5 TU per injection; 0.8 mm apart and 0.5 mm deep) of LV-TRAF6 shRNA or LV-NC along the L4-L5 dorsal root entry zone using a glass micropipette (diameter 60 μ m). The tip of glass micropipette reached to the depth of lamina II-IV of the spinal cord. The dorsal muscle and skin were then sutured.

2.4. Primary astrocytes cultures

Primary astrocytes cultures were prepared from cerebral cortexes of neonatal mice (P2) [12]. The cerebral hemispheres were isolated and transferred to ice-cold Hank's buffer and the meninges were carefully removed. Tissues were then minced into ~1 mm pieces, triturated, filtered through a 60 µm nylon screen, and collected by centrifugation at ~3000 g for 5 min. The cell pellets were dispersed with a pipette and resuspended in a medium containing 10% FBS (fetal bovine serum) in low glucose DMEM (Dulbecco's Modified Eagle's Medium). After trituration, the cells were filtered through a 10 µm screen and then plated into 6-well plates at a density of 2.5×10^5 cells/cm², and cultured for about 10 days. The medium was replaced twice a week with 10% FBS. Dibutyl cAMP (d-cAMP, 0.15 mM, Sigma, St Louis, MO) was added to induce differentiation when the cells were grown to 95% confluence. Prior to transfection or stimulation, Opti-MEM was replaced. Astrocytes were incubated for different time periods ranging from 1 h to 4 h depending on the experiment. The treatment of the siRNA or pLenti shRNA was given 24 h or 72 h prior to exposure cells to proinflammatory cytokine, respectively. After the treatment, the astrocytes were collected for ELISA or Western blotting.

For astrocytes injection, astrocytes were treated with TRAF6 siRNA or vehicle for 24 h, then stimulated with TNF-α for 1 h [11]. Astrocytes were washed with 0.01 M PBS for three times, centrifuged for 5 min at 3,000 g, and resuspended in PBS. About $2\sim4 \times 10^4$ astrocytes in 10 µl PBS were injected intrathecally.

2.5. ELISA

Mouse CCL2 ELISA kit was purchased from R&D systems. For *in vitro* experiments, cultured cells were collected after treatment. For *in vivo* experiments, animals were transcardially perfused with PBS. The L5 spinal cord was dissected. Astrocytes and spinal cord tissues were homogenized in a lysis buffer containing protease and phosphatase inhibitors (Sigma, St Louis, MO). Protein concentrations were determined by BCA Protein Assay (Pierce, Rockford, IL). For each reaction in a 96-well plate, 100 µg of proteins were used, and ELISA was performed according to manufacturer's protocol. The standard curve was included in each experiment.

2.6. Western blotting

Protein samples were prepared in the same way as for ELISA analysis. Protein samples (30 µg) were separated on SDS-PAGE gel and transferred to nitrocellulose blots. The blots were blocked with 5% milk and incubated overnight at 4°C with antibody against TRAF6 (1:500, anti-rabbit, Santa Cruz) or pJNK (1:500, anti-rabbit, Cell Signaling, Beverly, MA). For loading control, the blots were incubated with GAPDH antibody (1:20000, anti-mouse, Millipore, Billerica, MA). These blots were further incubated with HRP-conjugated secondary antibody, developed in ECL solution, and exposed onto Hyperfilm (Millipore) for 1–5 min. Specific bands were evaluated by apparent molecular size. The intensity of the selected bands was analyzed using Image J software (NIH, Bethesda, MD).

2.7. Immunohistochemistry and immunocytochemistry

Animals were deeply anesthetized with isoflurane and perfused through the ascending aorta with PBS followed by 4% paraformaldehyde with 1.5% picric acid in 0.16 M PB. After the perfusion, the L5 spinal cord segment was removed and postfixed in the same fixative overnight. Spinal cord sections (30 μ m, free-floating) were cut in a cryostat and processed for immunofluorescence as we described previously [12]. The sections were first blocked with 2% goat serum for 1 h at room temperature, then incubated overnight at 4°C with the following primary antibodies: TRAF6 (rabbit, 1:500, Millipore), glial fibrillary acidic protein (GFAP, mouse, 1:5000, Millipore), OX-42 (mouse, 1:100; Serotec, Kidlington, UK), TNF receptor 1 (TNFR1, rabbit, 1:100, Boster, China), and IL-1 receptor (IL-1R, rabbit, 1:100, Boster, China). The sections were then incubated for 1 h at room temperature with Cy3- or FITC-conjugated secondary antibodies (1:1000, Jackson ImmunoResearch, West Grove, PA). For double immunofluorescence, sections were incubated with a mixture of mouse and rabbit primary antibodies followed by a mixture of FITC- and Cy3-conjugated secondary antibodies. The stained sections were examined with a Leica fluorescence microscope, and images were captured with a CCD Spot camera. Some sections with double staining of TRAF6 and GFAP were imaged with Leica SP8 Gated STED confocal microscope.

For immunocytochemistry, cultured astrocytes were fixed with 4% paraformaldehyde for 20 min, and processed for immunofluorescence with TRAF6 (rabbit, 1:500, Millipore) and GFAP (mouse, 1:5000, Millipore) antibody as shown above. After immunostaining, DAPI (0.1 μ g/ml, Sigma) was added for 5 min at room temperature to stain all the nuclei of cells in the cultures.

2.8. Real-time quantitative PCR (qPCR)

For mRNA detection, the total RNA of the spinal cord was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). One microgram of total RNA was reversely transcribed using an oligo (dT) primer according to the manufacturer's protocol (Takara, Shiga, Japan). QPCR analysis was performed in the Real-time Detection System (Rotor-Gene 6000, Hamburg, Germany) by SYBR green I dye detection (Takara). The cDNA was amplified using the following primers: TRAF6-forward (5'-TCA TTA TGA TCT GGA CTG CCC AAC-3') and TRAF6-reverse (5'-TGC AAG TGT CGT GCC AAG TG-3'), GAPDH-forward (5'-TGT TCC TAC CCC CAA TGT G-3') and GAPDH-reverse (5'-GTG TAG CCC AAG ATG CCC T-3'). The PCR amplifications were performed at 95 °C for 30 s, followed by 40 cycles of thermal cycling at 95 °C for 5 s and 60 °C for 45 s. GAPDH was used as endogenous control to normalize differences. Melt curves were performed on completion of the cycles to ensure that nonspecific products were absent. Quantification was performed by normalizing Ct (cycle threshold) values with GAPDH Ct and analyzed with the 2^{-Ct} method.

2.9. Behavioral analysis

Animals were habituated to the testing environment daily for at least 2 days before baseline testing. The behavioral tests were done by the observers blinded to the treatment. For testing mechanical allodynia, animals were put in boxes on an elevated metal mesh floor and allowed 30 min for habituation before examination. The plantar surface of the hindpaw was

stimulated with a series of von Frey hairs with logarithmically incrementing stiffness (0.02–2.56 grams, Stoelting, Wood Dale, IL), presented perpendicular to the plantar surface (2–3 seconds for each hair). The 50% paw withdrawal threshold was determined using Dixon's up-down method [7]. For testing heat sensitivity, animals were put in plastic boxes and allowed 30 min for habituation before examination. Heat sensitivity was tested by radiant heat using Hargreaves apparatus (IITC Life Science Inc., Woodland Hills, CA) and expressed as paw withdrawal latency (PWL). The test was repeated at least three times with a 5-min interval between stimuli. The radiant heat intensity was adjusted so that basal PWL is between 10–14 seconds, with a cut-off of 18 seconds to prevent tissue damage.

2.10. Quantification and statistics

All data were expressed as mean $\pm$ SEM. The behavioral data was analyzed by two-way repeated measures ANOVA followed by Bonferroni test as the multiple comparison analysis. For Western blotting, the density of specific bands was measured with Image J. TRAF6 or pJNK levels were normalized to loading control (GAPDH) [34]. Differences between two groups were compared using Student's *t*-test. The criterion for statistical significance was $P < 0.05$.

3. Results

3.1. Spinal nerve ligation increases TRAF6 expression in spinal astrocytes

SNL produces rapid and persistent neuropathic pain, starting from 1 day and lasting for more than 3 weeks [22]. To examine TRAF6 expression in the spinal cord, we harvested L5 spinal cord from naïve, sham-treated, and SNL-operated mice and did Western blotting. As shown in Fig. 1A, SNL rapidly and persistently increased TRAF6 expression, starting at 1 day ($P < 0.05$, compared to naïve), peaking at 10 days ($P < 0.001$, compared to naïve), and maintaining at 21 days ($P < 0.05$, compared to naïve). Moreover, at 3 days and 10 days after operation, sham surgery had no significant effect on TRAF6 expression in the spinal cord compared to that in naïve animals (Fig. 1B). However, TRAF6 expression in SNL mice was significantly higher than that in sham-operated mice ($P < 0.05$, Fig. 1B).

We then examined TRAF6 expression and distribution in the spinal cord by immunofluorescence staining. TRAF6 had a very low basal expression in naïve animals (Fig. 1C). SNL induced a marked increase of TRAF6-immunoreactivity (IR) in the ipsilateral side of the lumbar spinal cord at 3 days (Fig. 1D) and 10 days (Fig. 1E). The TRAF6-IR cells had small cell bodies and long, thin processes (Fig. 1E). To check if TRAF6 is expressed in glial cells, we did double staining of TRAF6 with the astrocytic marker GFAP or microglial marker OX-42. As shown in Fig. 1F–H, at 3 days after SNL, TRAF6-IR was largely colocalized with GFAP, with some colocalization with OX-42 (Fig. 1I–K). At 10 days after SNL, TRAF6-IR was primarily localized in spinal astrocytes (Fig. 1L–N, scanned under confocal microscope). In addition, GFAP-positive astrocytes appeared to be in reactive state at 10 days after SNL (Fig. 1N).

3.2. TNF- α and IL-1 β increase TRAF6 expression in cultured astrocytes

As TRAF6 was mainly expressed in spinal astrocytes, especially at 10 days after SNL, we prepared primary astrocytes cultures to characterize the upstream and downstream signaling of TRAF6. We induced differentiation of these astrocytes by d-cAMP before the experiment. Immunostaining showed a basal expression of TRAF6 in astrocytes (Fig. 2Aa). Strikingly, a brief exposure to TNF- α (10 ng/ml) for 15 min induced a rapid increase in TRAF6 levels (Fig. 2Ae). Immunostaining also showed > 98% of cells in cultures expressed the astrocyte marker GFAP (Fig. 2Ab–d, f–h). Western blotting further confirmed TRAF6 up-regulation induced by TNF- α (Fig. 2B). The upregulation was evident at 15 min and persisted for more than 4 hours. Similarly, IL-1 β (10 ng/ml, Fig. 2C) also elicited rapid increase in TRAF6 levels, which maintained for more than 4 hours.

3.3. TNF- α and IL-1 β increase CCL2 expression via TRAF6

Our previous studies have shown that TNF- α increases CCL2 expression in cultured astrocytes [12]. Here we show that besides TNF- α (Fig. 3A), IL-1 β also increased CCL2 expression in a time-dependent manner (Fig. 3B). To determine the role of TRAF6 in CCL2 expression, we treated the cultures with a specific siRNA targeting to TRAF6. Pretreatment with TRAF6 siRNA at a low concentration (0.1 μ g/ml) reduced TNF- α -induced CCL2 upregulation by 72% at 1 h after TNF- α treatment (Fig. 3C). At a higher concentration (1 μ g/ml) TRAF6 siRNA decreased TNF- α -induced CCL2 upregulation by 95%. When compared to non-target (NT) siRNA, TRAF6 siRNA also elicited significant reduction of CCL2 levels (Fig. 3C). Western Blotting showed an effective knockdown of TRAF6 expression by TRAF6 siRNA at the concentration of 1 μ g/ml ($44 \pm 5\%$ reduction, compared to NT siRNA, Fig. 3C). We further checked the effects of TRAF6 siRNA on IL-1 β -induced CCL2 upregulation at 1 h after IL-1 β incubation. As shown in Fig. 3D, TRAF6 siRNA at the dose of 0.1 μ g/ml did not change CCL2 expression. But 1 μ g/ml TRAF6 siRNA significantly decreased IL-1 β -induced CCL2 upregulation by 67%. In contrast, 1 μ g/ml NT siRNA had no effect on IL-1 β -induced CCL2 upregulation (Fig. 3D).

To further confirm the role of TRAF6 in CCL2 production after incubation with TNF- α or IL-1 β , we treated the cultured astrocytes with pLenti TRAF6 shRNA or pLenti negative control (NC) (2 μ g) for 72 h, followed by TNF- α or IL-1 β incubation. ELISA analysis showed that transfection with pLenti TRAF6 shRNA reduced CCL2 level by 45% ($P < 0.001$, compared to TNF- α treatment). pLenti NC only caused a mild reduction (8%, $P > 0.05$) of TNF- α -induced CCL2 upregulation (Fig. 3E). IL-1 β also increased CCL2 expression, which was significantly decreased by pretreatment with pLenti TRAF6 shRNA (26%, $P < 0.001$, compared to IL-1 β treatment), while pLenti NC slightly decreased IL-1 β -induced CCL2 upregulation (4%, $P > 0.05$, Fig. 3F).

3.4. TNF- α and IL-1 β activate the JNK-CCL2 pathway via TRAF6

How can TRAF6 induce CCL2 expression? Our previous study has shown (i) TNF- α elicited a rapid and transient activation of JNK in astrocytes, and (ii) TNF- α also induced JNK-dependent CCL2 upregulation in both cultured astrocytes and intact spinal cord [12]. We further checked whether TRAF6 could mediate TNF- α - and IL-1 β -induced JNK activation in astrocytes. TNF- α and IL-1 β induced rapid and transient phosphorylation of

JNK, including pJNK1 (46 kDa) and pJNK2 (52 kDa) (Fig. 4A,B). pJNK increases peaked at 15 min and declined at 30 min after incubation (Fig. 4A,B). Pretreatment of astrocytes with TRAF6 siRNA (1 μ g/ml) inhibited both TNF- α - and IL-1 β -induced JNK activation (Fig. 4C), suggesting that JNK activation is a downstream event of TRAF6 signaling.

To check if JNK pathway is involved in IL-1 β -induced CCL2 upregulation, we examined the effect of a highly selective and potent JNK inhibitor, D-JNKI-1 [10,56] on CCL2 expression using ELISA. Pretreatment with D-JNKI-1 dose-dependently reduced IL-1 β -induced CCL2 expression by 26%, 73%, and 98% at the dose of 10 μ M, 20 μ M, and 50 μ M, respectively. D-JNKI-1 alone at the dose of 50 μ M did not show much change on basal CCL2 expression (Fig. 4D).

3.5. Intrathecal injection of TRAF6 siRNA decreases intrathecal TNF- α - or IL-1 β -induced pain hypersensitivity

Our previous results showed that intrathecal injection of TNF- α (20 ng) induces TNFR1/JNK-dependent pain hypersensitivity via CCL2 secretion [12]. To check if TNF- α -induced pain was also dependent on TRAF6, we first checked the distribution of TNFR1 in the spinal dorsal horn by fluorescence double staining. The results showed that at 10 days after SNL, majority of TNFR1-IR cells express GFAP (Fig. 5A–C). Some TNFR1-IR cells were neurons (Fig. 5A) or microglia (data not shown). Interestingly, in GFAP-positive astrocytes, majority of them ($84.6 \pm 2.5\%$) expressed TNFR1, indicating that TNFR1 is primarily localized in spinal astrocytes. We also checked the IL-1R expression in the spinal cord at 10 days after SNL. As shown in Fig. 5D–F, IL-1R was also mainly expressed in astrocytes, and only some neurons but not microglia expressed IL-1R (data not shown). Statistical data showed that in GFAP-positive astrocytes, $53.9 \pm 3.8\%$ of them express IL-1R in the spinal cord.

To test whether TRAF6 siRNA pre-treatment would reduce TNF- α - and IL-1 β -induced allodynia and hyperalgesia, we intrathecally injected TRAF6 siRNA (3 μ g/10 μ l) or NT siRNA, following by intrathecal injection of TNF- α (20 ng) and IL-1 β (20 ng) 6 h later. TRAF6 siRNA treatment partially prevented intrathecal TNF- α -induced mechanical allodynia (Fig. 5G) and heat hyperalgesia (Fig. 5H) at 1 h and 3 h, as well as intrathecal IL-1 β -induced mechanical allodynia (Fig. 5I) and heat hyperalgesia (Fig. 5J). To examine the knockdown effect of TRAF6 siRNA, we dissected the spinal cord after behavioral testing and checked TRAF6 mRNA expression by qPCR. The results showed that compared to NT siRNA treatment, TRAF6 siRNA significantly decreased TRAF6 mRNA expression in the dorsal horn by $32 \pm 4\%$ ($P < 0.01$). These data suggest that TRAF6 is involved in TNF- α - or IL-1 β -induced pain hypersensitivity.

3.6. Knockdown the expression of TRAF6 by siRNA, shRNA lentivirus or antisense oligodeoxynucleotide (ODN) attenuates SNL-induced mechanical allodynia

To examine whether TRAF6 is involved in the maintenance of neuropathic pain, we checked the effects of TRAF6 knockdown on SNL-induced mechanical allodynia by employing three different approaches. First, we tested TRAF6 siRNA, which effectively inhibited TNF- α - and IL-1 β -induced CCL2 production in vitro (Fig. 3) and TNF- α - and

IL-1 β -induced pain hypersensitivity in vivo (Fig. 5). As shown in Fig. 6A, intrathecal injections of TRAF6 siRNA (5 μ g/10 μ l, 2 injections, 12 h apart) significantly reversed SNL-induced mechanical allodynia at 2 h ($P < 0.05$, compared to NT siRNA) and 4 h ($P < 0.05$, compared to NT siRNA), but not at 24 h. The same dose of NT siRNA did not show any effect (Fig. 6A).

It has been demonstrated that viral vector-mediated RNAi can induce stable and long-term gene silencing and is effective in inhibiting gene expression in a number of diseases, including chronic pain [5,30,55]. To persistently inhibit TRAF6 expression, we made intraspinal injection of TRAF6 shRNA lentivirus (LV-TRAF6 shRNA) and negative control lentivirus (LV-NC) with GFP tag into the L5 spinal cord at 3 days after SNL. Notably, SNL-induced mechanical allodynia was attenuated at 7 d, 10 d, and 14 d in animals treated with TRAF6 shRNA lentivirus (Treatment, $F_{2,65} = 12.03$ and $P < 0.01$; Time, $F_{5,65} = 83.63$ and $P < 0.0001$; Interaction, $F_{10,65} = 2.12$ and $P < 0.05$, Fig. 6B). NC lentivirus had no effect on SNL-induced mechanical allodynia (Fig. 6B). We also checked the effect of LV-TRAF6 shRNA on paw withdrawal threshold in sham-operated animals. The results showed that intraspinal injection of LV-TRAF6 shRNA at 3 days after operation did not change paw withdrawal threshold at 7 days, 10 days, 14 days, and 21 days (data not shown). To examine the knockdown effect of TRAF6 shRNA, we dissected the spinal cord at 10 days after SNL (7 days after lentivirus injection) and examined TRAF6 expression by real-time PCR. The results showed that TRAF6 mRNA was not significantly changed after LV-NC treatment (1.01 ± 0.09 vs. 1.11 ± 0.07 , SNL only group vs. SNL + LV-NC, $P > 0.05$). TRAF6 shRNA lentivirus injection decreased TRAF6 mRNA by 41.4% as compared to LV-NC treatment, confirming the knockdown effect of TRAF6 shRNA.

To further confirm the role of TRAF6 in the maintenance of neuropathic pain, we infused TRAF6 antisense ODN or mismatch ODN intrathecally via an osmotic pump 5 d after establishment of SNL-induced mechanical allodynia (Fig. 6C). Two-way ANOVA showed TRAF6 antisense ODN had significant effect on mechanical allodynia during drug infusion (Treatment, $F_{4,100} = 102.67$ and $P < 0.0001$; Time, $F_{4,100} = 12.81$ and $P < 0.0001$; Interaction, $F_{16,100} = 2.14$ and $P < 0.05$). The Bonferroni posttests showed that 0.4 nmol/ μ l TRAF6 antisense ODN significantly attenuated SNL-induced mechanical allodynia at days 9 and 11, but not at day 7 (Fig. 6C). A higher dose of TRAF6 antisense ODN at 1 nmol/ μ l showed stronger effect than the low dose (0.4 nmol/ μ l) on reversing mechanical allodynia. Especially, 1 nmol/ μ l TRAF6 antisense ODN effectively attenuated mechanical allodynia at day 7 (Fig. 6C). Western blotting showed that TRAF6 antisense ODN treatment decreased TRAF6 expression by 33% in the spinal cord, compared to mismatch ODN treatment ($P < 0.05$, $n = 3$, data not shown). These results suggest TRAF6 is involved in the maintenance of SNL-induced neuropathic pain.

As shown by in vitro data, TRAF6 is an upstream signaling of CCL2 in cultured astrocytes. To ascertain whether it regulates CCL2 expression induced by SNL, we examined CCL2 level in the spinal cord after TRAF6 antisense ODN treatment (1 nmol/ μ l, 11 days after SNL). The ELISA results showed that TRAF6 antisense ODN decreased CCL2 expression from 32.6 ± 1.6 pg/ml in mismatch treatment group to 16.8 ± 2 pg/ml in antisense-treatment

group ($P < 0.05$), suggesting that TRAF6 is required for CCL2 expression under neuropathic pain.

3.7. Knockdown of TRAF6 by siRNA decreases intrathecal TNF- α -activated astrocytes-induced mechanical allodynia

Our previous results showed that intrathecal injection of TNF- α -stimulated astrocytes induced mechanical allodynia via CCL2 secretion [11]. To further check the role of TRAF6/CCL2 signaling in mechanical allodynia, we stimulated astrocytes cultures with TNF- α (10 ng/ml) for 1 h and washed cells with PBS for 3 times, and then collected cells for intrathecal injection in naïve mice. As shown in Fig. 7A, intrathecal injection of TNF- α -stimulated astrocytes caused a dramatic reduction in paw withdrawal threshold, indicating the development of mechanical allodynia. This allodynia developed at 3 h and lasted for more than 24 h after the injection. However, this reduction by TNF- α -stimulated astrocytes was prevented if astrocytes were pre-treated with TRAF6 siRNA (1 μ g/ml) 24 h before TNF- α stimulation (Fig. 7A). These results suggest that TRAF6 is important for intrathecal TNF- α -activated astrocytes-induced mechanical allodynia in naïve mice.

To check if the TRAF6-mediated release of CCL2 is involved in the astrocytes injection-induced mechanical allodynia, we examined CCL2 expression and release in cultures using an experimental protocol to mimic *in vivo* conditions. The astrocytes were incubated with TNF- α for 1 h and then washed with PBS for 3 times followed by incubation with fresh medium for 3 hours. As shown in Fig. 7B, CCL2 expression maintained at high level at 3 h following initial TNF- α stimulation. This increase was prevented when the astrocytes were pretreated with TRAF6 siRNA at the concentration of 1 μ g/ml (Fig. 7B). The CCL2 release in culture medium was also reduced by TRAF6 siRNA pretreatment (Fig. 7B).

4. Discussion

Astrocytes are the most abundant cells in term of their number and volume in the central nervous system (CNS). It has become increasingly apparent that astrocytes, by producing neuromodulators/pain mediators, such as cytokines, chemokines, and growth factors play an important role in the development and maintenance of chronic pain [9,20,32,40,47]. Yet, the intracellular mechanisms mediating the production of these cytokines and chemokines are not fully understood. Here, we demonstrate that a member of the TRAF family, TRAF6, which was expressed in spinal cord astrocytes and modulated by TNF- α and IL-1 β , is essential for JNK activation and subsequent CCL2 expression in astrocytes. Notably, after nerve injury TRAF6 was induced in both microglia and astrocytes at 3 days but predominantly induced in astrocytes in the spinal cord at 10 days. Spinal injection of TRAF6 siRNA partially prevented TNF- α and IL-1 β induced-pain hypersensitivity. Knockdown of the TRAF6 expression by siRNA, shRNA, or antisense ODN attenuated neuropathic pain and CCL2 expression. Finally, pretreatment of TRAF6 siRNA not only reduced TNF- α -induced CCL2 expression and release in astrocytes, but also blocked intrathecal TNF- α -activated astrocytes-induced mechanical allodynia. Taken together, these results suggest that TRAF6, as a convergence point for upstream TNFR and IL-1R and downstream JNK

signaling in astrocytes, is critical for the chemokines' production and the maintenance of neuropathic pain (Fig. 8).

4.1. TRAF6 is a key linker between upstream TNFR and IL-1R activation and downstream JNK/CCL2 signaling in astrocytes

TRAF6 is the only TRAF family member that participates in signal transduction of both the TNFR superfamily and the IL-1R/TLR superfamily [50,54]. Overexpression of a dominant-negative mutant of TRAF6 in human 293 cells was found to inhibit IL-1-induced NF κ B activation [3]. TRAF6 dominant-negative mutant also impairs TLR4-induced NF κ B activity [36]. These studies suggest that TRAF6 is involved in IL-1R/TLR4 signaling. The TNFR superfamily includes CD30, CD40 and receptors for TNF, lymphotoxin- α (LT- α), and LT- β . Studies have shown that the TRAF6-binding region is required for CD40-mediated NF κ B activation [17]. However, it was reported that TNF-induced NF κ B activation is not affected when cells are transfected with TRAF6 mutant vectors. In contrast, TRAF2 dominant-negative mutant completely blocked TNF-mediated NF κ B activation [3]. These results indicate that TRAF6 may not be involved in TNF signaling in human 293 cells. But in the present study, both IL-1 β and TNF- α increased TRAF6 expression in murine astrocytes. Meanwhile, TRAF6 siRNA treatment inhibited TNF- α - or IL-1 β -induced JNK activation and CCL2 production, suggesting that TRAF6 is required for the activation of the JNK/CCL2 pathway initiated by the activation of TNFR and IL-1R in astrocytes. Moreover, the increase of TRAF6 by TNF- α and IL-1 β was very rapid (15 min). In agreement with our results, Wu et al showed that TRAF6 was significantly increased at 15 min after incubation with lipopolysaccharides in macrophages [51]. The rapid induction of TRAF6 may be attributed to translational regulation, which needs to be investigated in the future.

TRAF6 has been shown essential for both NF κ B and JNK activation in several cell types [3,17,27,37]. In TRAF6 knockout embryonic fibroblasts, IL-1 β fails to activate JNK [27]. TRAF6 knockdown significantly blocked TLR4-induced JNK1/2 phosphorylation in macrophages [28] and endothelial cells [15]. In this study, we showed that TNF- α - or IL-1 β -induced JNK was decreased by pretreatment with TRAF6 siRNA in astrocytes. In addition, TRAF6 siRNA or shRNA also dramatically decreased TNF- α - or IL-1 β -induced CCL2 production. Our previous results have shown that JNK inhibitors D-JNKI-1 and SP600125 dose-dependently reduced TNF- α -induced CCL2 upregulation in astrocytes [12]. Consistently, it was reported that CD40 stimulates CCL2 production via TRAF6 recruitment and MAPK activation in proximal tubule cells [24]. Thus, TRAF6 is an important upstream activator of JNK/CCL2 signaling in various cell types, including astrocytes.

4.2. TRAF6 contributes to neuropathic pain via production of CCL2 in spinal astrocytes

In vivo studies have shown that TRAF6 is important for regulating adaptive immunity, innate immunity, and bone metabolism, as TRAF6-deficient mice exhibit deficiency in lymph node organogenesis and severe osteopetrosis [27,37]. Recent studies showed that TRAF6 expression in the brain is regulated in Parkinson's disease [57], Huntington disease [58], Alzheimer's disease [1,13], stroke [26], and trauma [4]. However, the role of spinal TRAF6 in chronic pain is unclear.

In the present study, we for the first time revealed a rapid and persistent increase of TRAF6 expression in the spinal cord in a neuropathic pain model. The upregulation of TRAF6 may be induced by TNF- α and/or IL-1 β released from primary afferents and spinal glial cell, as TNF- α and IL-1 β are upregulated in the damaged tissues, DRG, and spinal cord after nerve damage [6,42,46,49]. Particularly, TNF- α , which is rapidly (1 d) increased in the spinal cord after SNL [55], is thought as an essential trigger for an inflammatory cascade that underlies the development of neuropathic pain after nerve injury [23,31,52]. Our previous studies demonstrated that pretreatment with TNF- α inhibitor partially prevents SNL-induced neuropathic pain [55]. Conversely, intrathecal injection of TNF- α induces marked mechanical allodynia and heat hyperalgesia [12]. In this study we further showed TRAF6 siRNA pretreatment decreased pain hypersensitivity induced by intrathecal TNF- α or IL-1 β . In addition, TNFR1 (not TNFR2) is essential for the TNF- α -induced JNK1 activation in astrocytes, as well as spinal TNF- α -induced heat hyperalgesia and mechanical allodynia [12]. Our immunofluorescence double staining showed about 85% astrocytes express TNFR1 at 10 days after SNL. Similarly, IL-1R was mainly expressed in spinal astrocytes, with a few neurons expressing IL-1R. It was reported that TNFR1 is upregulated in astrocytes and neurons in the spinal cord after sciatic nerve injury [38] or spinal cord injury [53]. TNFR1 is also induced in some microglia after L5 ventral root transection [52]. It was shown that IL-1R is expressed in both glia and neurons in the DRG [25] but only in neurons in the rostral ventromedial medulla [48]. Collectively, our data indicate that TRAF6 is important to mediate TNF- α and IL-1 β signaling in the spinal cord, especially in spinal astrocytes.

Our double staining results showed TRAF6 was expressed in astrocytes and microglia at 3 days after SNL, but mainly expressed in spinal astrocytes at 10 days after SNL. TRAF6 was also found to be expressed in astrocytes and neurons in the brain [4,39]. Consistently, pJNK and CCL2 are also primarily induced in spinal astrocytes after SNL [12]. These data support a critical role of the TRAF6 signaling pathway in spinal astrocytes. As both TRAF6 and TNFR1 were also expressed in some microglia, they may also contribute to microglia-mediated pain hypersensitivity in the spinal cord.

JNK is an important intracellular kinase in astrocytes in chronic pain [10,29,56]. Spinal inhibition of JNK by its inhibitor SP600125 or D-JNKI-1 not only reversed neuropathic pain [56], but also decreased SNL-induced CCL2 increase in the spinal cord [12]. Direct inhibition of CCL2 also attenuated SNL-induced mechanical allodynia and heat hyperalgesia [12]. Our results demonstrated that TRAF6 is an upstream activator of the JNK/CCL2 pathway in spinal astrocytes in neuropathic pain. First, intrathecal injection of TRAF6 siRNA transiently attenuated SNL-induced mechanical allodynia. Second, intraspinal injection of TRAF6 shRNA lentivirus at 3 days after SNL persistently attenuated SNL-induced mechanical allodynia. Third, spinal infusion of TRAF6 antisense ODN also showed long-lasting anti-allodynic effects and reduced CCL2 expression in the spinal cord. Finally, intrathecal injection of activated astrocytes-induced-mechanical allodynia was reduced by pre-treatment of astrocytes with TRAF6 siRNA.

4.3. Activated astrocytes produce mechanical allodynia via TRAF6/CCL2

Accumulating evidence has shown that astrocytes may be sufficient to produce chronic pain symptoms. It was reported that implantation of neural stem cells into the injured spinal cord evoked allodynia-like hypersensitivity in the forepaws by generating astrocytes [14]. In addition, transplantation glial-restricted precursor-derived astrocytes promoted the onset of mechanical allodynia [45]. Our previous [11] and present studies showed that intrathecal injection of TNF- α -activated astrocytes is sufficient to induce persistent mechanical allodynia in naïve animals. Pretreatment with TRAF6 siRNA increased the paw withdrawal threshold for more than 24 h, suggesting TRAF6 contributes to mechanical allodynia induced by TNF- α -activated astrocytes. Our *in vitro* data further showed exposure of astrocytes with TNF- α for 1 h induced a substantial and persistent increase in the expression and release of CCL2 in astrocyte cultures. Our previous data has shown that intrathecal injection of TNF- α -activated astrocytes-induced mechanical allodynia was attenuated by both the CCL2 neutralizing antibody and CCL2 siRNA [11]. *In vivo* study also showed that intrathecal TNF- α markedly increased CCL2 expression in spinal cord astrocytes [11]. Intrathecal injection of CCL2 has been shown to induce chronic pain symptoms such as heat hyperalgesia [12] and mechanical allodynia [43]. These data suggest that TRAF6 may play a role in astrocytes-induced mechanical allodynia via regulating the expression and release of CCL2.

4.4. Conclusions

Our findings demonstrated that TRAF6 is an important signaling protein between upstream TNFR and IL-1R and downstream JNK activation and CCL2 production in astrocytes. Particularly, TRAF6 is mainly upregulated in spinal cord astrocytes in the late phase after nerve injury and plays an important role in the maintenance of neuropathic pain. Therefore, targeting the TRAF6 signaling may provide a novel therapeutic approach for the treatment of neuropathic pain.

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Summary

TRAF6, upregulated in spinal cord astrocytes in the late phase after nerve injury, maintains neuropathic pain by integrating TNF- α and IL-1 β signaling and activating the JNK/CCL2 pathway in astrocytes

Figure 1.

SNL induces TRAF6 upregulation in spinal astrocytes. (A) Western blotting shows that SNL increases TRAF6 expression from 1 day to 21 days. * $P < 0.05$, *** $P < 0.001$, compared to naïve. $n=4/\text{group}$. (B) TRAF6 protein is significant higher in SNL animals than that in sham-operated animals at 3 days and 10 days. * $P < 0.05$, SNL vs. sham. $n=4/\text{group}$. (C–E) Immunohistochemistry shows that TRAF6-IR is very low in naïve animals (C) but increased at 3 days (D) and 10 days (E) after SNL. (F–H) Double staining shows that TRAF6-IR (F) is largely colocalized with astrocytic marker GFAP (G) in the dorsal horn at 3 days after SNL.

(I–K) Double staining shows that TRAF6-IR (I) is partially colocalized with microglial marker OX-42 (J) in the dorsal horn at 3 days after SNL. (L–N) At 10 days after SNL, TRAF6-IR is primarily localized in spinal astrocytes.

Figure 2.

TNF- α and IL-1 β increase TRAF6 expression in primary cultures of astrocytes. (A) TRAF6 is constitutively expressed in control (Aa-d) astrocytes and upregulated by TNF- α (Ae-h). Triple staining of TRAF6 (Aa, Ae), GFAP (Ab, Af) and DAPI (nuclear marker, Ac, Ag) shows that DAPI-positive cells also express GFAP-IR and TRAF6. (B, C) Western blotting shows that TNF- α (10 ng/ml, B) or IL-1 β (10 ng/ml, C) incubation rapidly (15 min) increases TRAF6 expression, which maintains for more than 4 h. ** $P < 0.01$, *** $P < 0.001$, compared to control. $n=4$ /treatment.

Figure 3.

TNF- α and IL-1 β increase CCL2 production via TRAF6 in astrocytes. (A–B) TNF- α (A) or IL-1 β (B) time-dependently increases CCL2 expression in astrocytes. *** $P < 0.001$, compared to control. $n=4$ /treatment. (C–D) TRAF6 siRNA dose-dependently decreased TNF- α -(C) or IL-1 β -induced (D) CCL2 upregulation. TRAF6 siRNA pretreatment decreased TRAF6 expression in astrocytes (Insert, C). T, TRAF6. G, GAPDH. Lane 1, NT siRNA treatment. Lane 2, TRAF6 siRNA treatment. (E–F) pLenti TRAF6 shRNA pretreatment decreased TNF- α - or IL-1 β -induced CCL2 upregulation. pLenti NC had no effect. *** $P < 0.001$. $n=3$ –4/treatment.

Figure 4.

TNF- α and IL-1 β induce TRAF6-dependent JNK/CCL2 signaling activation in astrocytes. (A, B) TNF- α (A) or IL-1 β (B) induces rapid and transient JNK activation in astrocytes (15–30 min). (C) Pretreatment of astrocytes with TRAF6 siRNA (1 μ g/ml) reduced TNF- α - or IL-1 β -induced JNK activation. * P < 0.05, compared to control, # P < 0.05, ## P < 0.01, compared to vehicle (Veh). n =4/treatment. (D) JNK inhibitor D-JNKI-1 dose-dependently inhibited IL-1 β -induced CCL2 upregulation. +++ P < 0.001, compared to control; *** P < 0.001, compared to IL-1 β treatment (positive control). n =3/treatment.

Figure 5.

Intrathecal TRAF6 siRNA pretreatment decreases intrathecal TNF- α - or IL-1 β -induced pain hypersensitivity. (A–C) Double staining shows that TNFR1 (A) is largely colocalized with astrocytic marker GFAP (B) in the dorsal horn at 10 days after SNL. TNFR1 is also expressed in a few neuron-like cells (arrow). (D–F) IL-1R is colocalized with GFAP, with some expression in a few neuron-like cells (arrow). (G–H) Paw withdrawal threshold (G) and paw withdrawal latency (H) after intrathecal injection of TNF- α in animals pretreated with intrathecal TRAF6 siRNA or NT siRNA. * $P < 0.05$, ** $P < 0.01$, compared to NT

siRNA. (I–J) Paw withdrawal threshold (I) and paw withdrawal latency (J) after intrathecal injection of IL-1 β in animals pretreated with intrathecal TRAF6 siRNA or NT siRNA. *P < 0.05, compared to NT siRNA.

Figure 6.

Knockdown of the expression of TRAF6 by siRNA, shRNA or antisense ODN attenuates SNL-induced mechanical allodynia. (A) Intrathecal injection of TRAF6 siRNA at 3 days after SNL attenuated mechanical allodynia at 2 h and 4 h. NT siRNA had no effect. * $P < 0.05$, compared to NT siRNA. (B) Intraspinal infusion of TRAF6 shRNA lentiviral vectors 3 days after SNL alleviated SNL-induced mechanical allodynia at 7 d, 10 d, and 14 d. ** $P < 0.01$, compared to SNL, ## $P < 0.01$, compared to SNL + LV-NC. (C) Intrathecal infusion of

TRAF6 antisense ODN (0.4 nmol/ μ l and 1 nmol/ μ l) attenuated SNL-induced mechanical allodynia from 7 days to 11 days. * $P < 0.05$.

Figure 7.

TRAF6 siRNA treatment attenuated intrathecal TNF- α -activated astrocytes-induced mechanical allodynia. (A) Paw withdrawal thresholds after intrathecal injection of TNF- α -treated astrocytes pretreated with TRAF6 siRNA (1 μ g/ml) or non-target siRNA (1 μ g/ml). * P < 0.05, ** P < 0.01. n =6/group. (B) TRAF6 siRNA pretreatment (1 μ g/ml) reduces TNF- α -triggered CCL2 expression and release. * P < 0.05, ** P < 0.01. n =3/treatment.

Figure 8.

Schematic shows how TRAF6 in spinal astrocytes maintains neuropathic pain. Spinal nerve ligation (SNL) increases the expression of proinflammatory cytokines (e.g. TNF- α and IL-1 β). The activation of TNFR1 and IL-1R receptors by TNF- α and IL-1 β increases TRAF6 expression, leading to increased JNK activation and CCL2 production in astrocytes. Astrocyte-secreted CCL2 activates CCR2 on neurons to increase neuronal excitability and enhance neuropathic pain.