Research Article

MOLECULAR PAIN

Molecular Pain
Volume 16: 1–9

DOI: 10.1177/1744806920918059
journals.sagepub.com/home/mpx
SAGE

Targeting spinal TRAF6 expression attenuates chronic visceral pain in adult rats with neonatal colonic inflammation

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Abstract

Background: Irritable bowel syndrome is one of the most common gastrointestinal disorders. It is featured by abdominal pain in conjunction with altered bowel habits. However, the pathophysiology of the syndrome remains largely unknown. Tumor necrosis factor receptor-associated factor 6 (TRAF6) has been reported to be involved in neuropathic pain. The aim of this study was to investigate roles and mechanisms of TRAF6 in the chronic visceral hypersensitivity.

Methods: Visceral hypersensitivity was induced by neonatal colonic inflammation and was identified by colorectal distention. The protein level, RNA level, and cellular distribution of TRAF6 and its related molecules were detected with Western blot, quantitative polymerase chain reaction, and immunofluorescence. In vitro spinal cord slice recording technique was performed to determine the synaptic transmission activities.

Results: Neonatal colonic inflammation rats displayed visceral hypersensitivity at the age of six weeks. The expression of TRAF6 was obviously upregulated in spinal cord dorsal horn of neonatal colonic inflammation rats at the age of six weeks. Immunofluorescence study showed that TRAF6 was dominantly expressed in spinal astrocytes. Intrathecal injection of TRAF6 small interfering RNA (siRNA) significantly reduced the amplitude of spontaneous excitatory postsynaptic currents at the spinal dorsal horn level. Furthermore, knockdown of TRAF6 led to a significant downregulation of cystathionine β synthetase expression in the spinal dorsal horn of neonatal colonic inflammation rats. Importantly, intrathecal injection of TRAF6 siRNA remarkably alleviated visceral hypersensitivity of neonatal colonic inflammation rats.

Conclusions: Our results suggested that the upregulation of TRAF6 contributed to visceral pain hypersensitivity, which is likely mediated by regulating cystathionine β synthetase expression in the spinal dorsal horn. Our findings suggest that TRAF6 might act as a potential target for the treatment of chronic visceral pain in irritable bowel syndrome patients.

Keywords

Irritable bowel syndrome, tumor necrosis factor receptor-associated factor 6, cystathionine β synthetase, spinal cord, visceral pain

Date Received: 21 January 2020; Revised 19 February 2020; accepted: 6 March 2020

Introduction

Irritable bowel syndrome (IBS) is one of the most common functional gastrointestinal disorders, which affects approximately 20% of population worldwide.[1,2] Chronic abdominal pain is one of its features. The exact causes and pathogenesis of abdominal pain are not clear. Recent studies have shown that early-life trauma or infection such as acute bacterial gastroenteritis plays a critical role in the development of visceral sensory,[3–5]

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which has been reported to generate visceral hyperalgesia in adult rats.[6,7] Therefore, neonatal colonic inflammation (NCI) was used in this study as an adverse early-life stimulation to induce visceral hypersensitivity.

Mechanisms underlying the chronic visceral hypersensitivity include peripheral sensitization and central sensitization. Peripheral sensitization was characterized by enhanced excitability of primary sensory neurons innervating the colon[3] and increased release of neurotransmitters at the spinal cord levels.[8–11] As to central sensitization, many studies have been focused on the characterization of neurons in specific brain areas such as amygdala,[12] the anterior cingulate cortex,[13] and insular cortex.[14] However, relatively few studies are focusing on the spinal dorsal horn. The spinal dorsal horn is an important center and bridge for processing and relaying the nociceptive signalings from the periphery to the center.[15,16] It is, therefore, important to investigate the roles and mechanisms of spinal cord in the development and maintenance of chronic visceral pain. Our previous study has shown that NCI enhanced spinal synaptic transmission, which is modified by hydrogen sulfide.[17] It is proved that the NCI-induced visceral hyperalgesia is associated with the upregulated expression of cystathionine β synthase (CBS) both at peripheral sensory neurons[7] and spinal cord levels.[17] However, it is not clear how the CBS expression is upregulated in the spinal cord of rats with visceral pain.

Tumor necrosis factor (TNF) receptor-associated factor (TRAF) is an important binding protein of TNF and the toll/IL-1 receptor (TIR) superfamily, which plays a crucial role in innate and acquired immunity. TRAF family has seven members, of which TRAF6 has special facture and biological function. TRAF6 has been reported to be able to integrate with multiple kinases and to regulate signaling pathway function by acting NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways.[18–20] Recent studies have increasingly demonstrated that TRAF6 is closely related to central nervous system diseases, such as stroke, traumatic brain injury, neurodegenerative diseases, and neuropathic pain.[21] However, whether TRAF6 is participated in visceral pain remains unknown.

In this study, we aimed to investigate the role of TRAF6 and its downstream pathways in the spinal cord in a rat model of NCI-induced visceral hypersensitivity. The expressions of TRAF6 and CBS in the spinal cord were examined by Western blot techniques from control and NCI rats. The effects of TRAF6-siRNA on molecular expression, synaptic transmission, and visceral hypersensitivity were also explored. Our data suggest that TRAF6 might act as a potential target for the treatment of visceral pain in IBS patients.

Materials and methods

Induction of chronic visceral pain

A total of 52 male Sprague-Dawley rats (150 ± 20 g) were used in this study. Care and handling of these rats were approved by the Institutional Animal Care and Use Committee of the Soochow University and also were in accordance with the guidelines of the International Association for the Study of Pain. Chronic visceral pain was established in rats by NCI, as described previously.[3] In brief, 10-day-old pups received an injection of 0.2 ml of 0.5% acetic acid solution into the colon 2 cm from the anus. The age-matched control rats received an equal volume of normal saline (NS). Behavior testing, molecular detection, and patch clamp recordings were performed in these rats at the age of six weeks.

Measurement of visceral hypersensitivity

Visceral sensitivity was determined by colorectal distention (CRD) threshold as described previously.[3,22]

Western blotting

The expression of TRAF6 in spinal dorsal horn corresponding to afferent nerve fibers from dorsal root ganglions (DRGs, T_{13}-L_2) for adult NCI and control rats (six- to eight-week-old) were determined using Western blot analyses as described previously.[17] In brief, the tissue of spinal dorsal horn was lysed by ultrasonic cracker in the ice bath. The lysates were then microfuged for 30 min at 4°C. After fractionating of spinal cord protein extract on 4% and 10% polyacrylamide gels, proteins were transferred to polyvinylidene difluoride membranes (Millipore). Membranes were then blocked in Tris-buffered saline (TBS) containing 5% dilution of non-fat milk powder under room temperature (RT). Membrane was incubated with anti-TRAF6 antibody (1:500; Biotechnology, INC), anti-CBS (1:1000; Abnova, Taiwan, China), anti-CSE (1:500; Abnova, Taiwan, China), or anti-GAPDH (1:2000; Goodhere Biotechnology Co., Ltd, Hangzhou, China). After washed in TBS containing 0.5% Tween-20 (TBST), membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:4000; Jackson) in TBS containing 1% milk for 2 h at RT. The membranes were then washed with TBST and the immunoreactive proteins were detected by enhanced chemiluminescence (ECL kit; Amersham Biosciences, Arlington Heights, IL) and appropriate exposure to chemiluminescent imaging system (ChemiDoc XRS, Biorad). Band intensities were calculated using Image J software. All samples were normalized to GAPDH as loading control.

Real-time quantitative polymerase chain reaction

Total RNA was exacted from the dorsal horn of spinal cord in control and NCI rats as followed by Trizol method. cDNA was synthesized from total RNA using EasyScript First-Strand cDNA Synthesis SuperMix kit (Transgen Biotech, Beijing, P.R. China) following the instructions. The primer sequences using in quantitative polymerase chain reaction (qPCR) are shown in Table 1. Negative control (NC) reactions were performed by omitting cDNA temple. The relative expression level for each target gene was normalized via $2^{-\Delta\Delta Ct}$ methods.

Table 1. The primer sequences used in qPCR.

Primers	Sequences (5′ to 3′)
TRAF6-F	CCATAAGGGATGCAGGTCACA
TRAF6-R	TGGGCACAGCACAGTTTACA
GAPDH-F	TGGAGTCTACTGGCGTCTT
GAPDH-R	TGTCATATTTCTCGTGGTTCA

Immunofluorescence study

After deeply anesthetized, rat was perfused transcardially with 300 ml 0.9% NS followed by pre-cooling 4% paraformaldehyde (PFA). T_{13}-L_2 spinal cord segments were rapidly removed and post-fixed in PFA for 3 h and then immersed in the increasing phosphate-buffered sucrose to gradient dehydrate. For double labeling, 14 μm sections of spinal cord were simultaneously incubated with TRAF6 (1:100, Abcam, Cambridge, MA) and NeuN (1:50, Merk Millpore, MAB377) or glial fibrillary acidic protein (GFAP; 1:300, Cell Signaling Technology, 3670S) or CD11b (1:100, Bio-Rad, MCA275R) antibodies at 4°C overnight and then incubated with Alexa Fluor 488 (1:500, Molecular Probes, A21206) and 555 (1:100, Molecular Probes, A31570) for 2 h at RT. NCs were performed by omitting primary antibodies.

Preparation of colon-related spinal cord slice

Spinal cord slices were prepared from adult rats as described previously.[17,23] In brief, after anesthesia, the rat spinal cord was exposed. The colon-related spinal cord segment was indicated by the afferent nerve fibers of T_{13}-L_2 DRGs. The isolated colon spinal cord segment was immediately placed in the ice-cold pre-oxygenated Krebs solution with 95% O_2 and 5% CO_2 in a glass dish. The composition of Krebs solution contains (in mM): 95 NaCl, 1.8 KCl, 1.2 KH_2PO_4, 0.5 $CaCl_2$, 7 $MgSO_4$, 26 $NaHCO_3$, 15 glucose, and 50 sucrose, at pH of 7.3–7.4 and an osmolarity of 310–320 mOsm. Several transverse slices (450 μm thickness) were cut with a Vibratome (Leica, VT1200S, Germany). The slices were transferred to oxygenated Krebs solution at 31°C until use.

Patch-clamp recordings from spinal substantia gelatinosa neurons

Whole-cell voltage-clamp recordings were made from substantia gelatinosa (SG) neurons as previously described.[17,24] Briefly, the slice was continuously perfused with oxygenated recording solution at the speed of 10–15 ml/min at RT. The composition of recording solution contains (in mM): 127 NaCl, 1.8 KCl, 1.2 KH2PO4, 2.4 CaCl2, 1.3 MgSO4, 26 NaHCO3, and 15 glucose, at pH of 7.3–7.4 and an osmolarity of 300–310 mOsm. The SG (lamina II) and its neurons were indentified under the 5× objective (NA 0.10) or a 40× magnification water-immersion objective (NA 0.80) of an upright microscope (BX51WI, Olympus, Japan) with the help of infrared differential interference contrast optics. The image of the slice was enhanced with a CCD camera (IR1000E, DAGE MTI) and was displayed on a computer monitor. The recording patch pipette, made by a puller (Sutter-P97, USA), was filled with internal solution (in mM): 140 K-Gluconate, 3 KCl, 10 HEPES, 0.2 EGTA, 4 NaCl, and 2 Ma-ATP. The tip with resistance of 3–8 MΩ was driven down to the slice by a micromanipulator (MP-225, Sutter-P97, USA). The neurons were holding at −70 mV for recording spontaneous excitatory postsynaptic currents (sEPSCs). Signals were acquired using a Multiclamp 700B amplifier filtered at 5 kHz with Bessel filter, Digidata 1440A interface and ClampEx10.3 software (Molecular Devices, Axon, USA). Data were stored on a computer for offline analyses. The amplitude and frequency of sEPSC were calculated as described previously.[12] In brief, the raw sEPSCs recorded in the duration of 4 min were selected and imported into the analysis software (Clampex 10.3, Axon) according to the manufactory instructions. The software automatically calculates the average amplitude and frequency of sEPSCs.

Drug application

To verify the roles of TRAF6, TRAF6 siRNA was used in this study. TRAF6 siRNA was purchased from RIBOBIO (Guangzhou, P.R. China). Before intrathecal (i.t.) injection, 5 nmol TRAF6 and NC siRNA were respectively dissolved in 250 μL DEPC water. Reagents (10 μL) were administered by one injection. To further examine the long-time effects, TRAF6 siRNA (0.5 nmol/10 μL) was injected once every two days for seven consecutive days. CRD thresholds were recorded 1, 2, 4, 8, 12, 24, and 48 h after siRNA treatment.

Statistical analysis

All values are showed as mean ± standard error of the mean. Statistical analyses were done using OriginPro 8 (OriginLab, Northampton, MA) and Prism 6 (Graph Pad, San Diego, California) software. Before analysis, Gaussian distribution test was first carried out. Two sample *t*-test or Mann–Whitney test was conducted to determine significance of changes between two groups. Two-way analysis of variance followed by Tukey's post hoc test were performed when appropriate. $P < 0.05$ was considered statistically significant.

Results

TRAF6 expression was enhanced in spinal dorsal horn of NCI rats

In consistent with our previous report,[3,17] we demonstrated that NCI rats displayed a significant reduction in CRD threshold (Figure 1(a), $^{*}P < 0.05$, $n = 6$ rats for each group). We next examined TRAF6 expression at protein level in colon-related spinal dorsal horn (T_{13}-L_2) of control and NCI rats at the age of six weeks. As shown in Figure 1(b), the protein expression of TRAF6 was significantly increased in NCI rats ($n = 4$ for each group, $^{*}P < 0.05$). In addition, TRAF6 mRNA expression was also significantly upregulated in T13-L2 spinal dorsal horn of NCI rats (Figure 1(c), $n = 4$ for each group, $^{*}P < 0.05$). However, the expression of TRAF6 in L4-L6 spinal cord of NCI rats did not change compared with control rats (Figure 1(d), $n = 4$ for each group, $P > 0.05$), nor in L4-L6 DRGs (Figure 1(e), $n = 4$ for each group, $P > 0.05$). Importantly, TRAF6 expression in T13-L2 DRGs was obviously increased in NCI rats compared with CON ones (Figure 1(f), $n = 4$ for each group, $^{*}P < 0.05$).

TRAF6 was mainly expressed in spinal astrocytes

The distribution of TRAF6 expression in the spinal dorsal horn was then determined by immunofluorescence staining. As shown in Figure 2, double staining showed that TRAF6-Iike immunoreactivities were largely co-localized with astrocyte marker GFAP (Figure 2 (b)). However, TRAF6-Iike immunoreactivities were not co-localized in neurons labeled with NeuN (Figure 2(a)) and not in microglial cells labeled with CD11b (Figure 2 (c)). The higher magnification pictures in the bottom left of each panel to show clearly whether TRAF6 is co-localized with different markers. The yellow staining indicates co-localization.

Knockdown of TRAF6 reduced spinal CBS expression

We have previously reported that the CBS expression was significantly enhanced in the spinal cord of NCI rats.[17] In this study, we investigated the possible cross-talk between TRAF6 and CBS. CBS expression in spinal dorsal horn was examined after TRAF6 siRNA treatment. We first confirmed that spinal TRAF6 expression was indeed reduced after TRAF6 siRNA treatment (Figure 3(a), $^{**}P < 0.01$). The relative densitometry of TRAF6 was 0.98 ± 0.06 ($n = 6$) in NCI rats treated with NC and was 0.66 ± 0.04 ($n = 6$) in NCI rats treated with TRAF6 siRNA. Next, we determined the expression of CBS in the spinal dorsal horn of NCI rats treated with siRNA TRAF6. The relative densitometry of CBS was 0.80 ± 0.16 ($n = 6$) in NCI rats treated with NC and 0.22 ± 0.04 ($n = 6$) in NCI rats treated TRAF6 siRNA. Statistic analysis showed that knockdown of TRAF6 by siRNA significantly reduced the spinal expression of CBS of NCI rats (Figure 3(b), $^{**}P < 0.01$). However, CSE expression was not markedly altered after TRAF6 siRNA injections (Figure 3(c), $P > 0.05$). These data suggest that CBS upregulation is likely a downstream event of TRAF6 signaling.

Knockdown of TRAF6 suppressed spinal synaptic transmission

To determine the effect of inhibiting TRAF6 on sEPSCs of spinal SG neurons, TRAF6 siRNA was intrathecally injected in NCI rats. Data were compared with the sEPSCs of SG neurons in slices from NS-treated NCI rats. The representative traces from two typical neurons of NC- or siTRAF6-treated NCI rats illustrated a significant reduction in amplitude but not frequency of sEPSCs of SG neuron (Figure 4(a)). The average results were also shown in Figure 4(b). The mean peak amplitude of sEPSCs was 20.6 ± 1.0 pA ($n = 8$ cells from three rats) in NC group and 14.7 ± 0.9 pA ($n = 8$ cells from three rats) in TRAF6 siRNA group, respectively (Figure 4(b), $^{**}P < 0.01$, two sample *t*-test). The mean frequency of sEPSCs was 10.2 ± 0.4 Hz ($n = 8$ cells) in NC group and 9.7 ± 0.6 Hz ($n = 8$ cells) in TRAF6 siRNA group, respectively (Figure 4(c), $P > 0.05$, two sample *t*-test). These data suggest that the glutamatergic synaptic activity was significantly decreased in SG neurons after TRAF6 siRNA treatment.

Knockdown of TRAF6 attenuated visceral pain of NCI rats

We next investigated whether intrathecal injection of siRNA TRAF6 increased the distension threshold. As shown in Figure 5(a), one time intrathecal injection of siRNA TRAF6 at the dose of 10 μg/μL obviously enhanced the CRD threshold ($n = 6$ rats for each

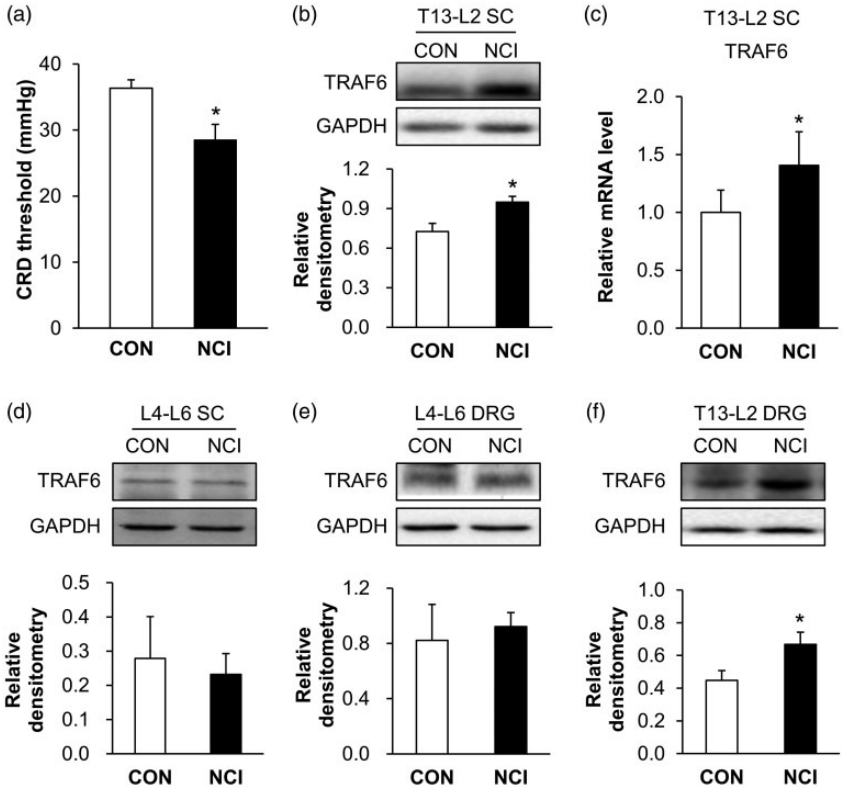

Figure 1. Upregulated expression of TRAF6 in spinal cord of NCI rats. (a) NCI treatment significantly reduced CRD threshold at the age of six weeks compared with the age-matched CON rats (*$P < 0.05$, $n = 6$ rats for each group). (b) Increase in expression of TRAF6 in spinal cord at T13-L2 levels from NCI rats at the age of six weeks compared with control rats (*$P < 0.05$, $n = 4$ for rats each group). (c) Increase in TRAF6 mRNA expression in spinal cord between CON and NCI rats at the age of six weeks (*$P < 0.05$, $n = 4$ for rats each group). (d) No difference of TRAF6 expression in spinal cord at L4-L6 levels between CON and NCI rats at the age of six weeks ($P > 0.05$, $n = 4$ for rats each group). (e) No difference of TRAF6 expression in L4-L6 DRGs between CON and NCI rats at age of six weeks ($P > 0.05$, $n = 4$ for rats each group). (f) Increase in TRAF6 expression in T13-L2 DRGs between CON and NCI rats at age of six weeks (*$P < 0.05$, $n = 4$ for rats each group). CON: control; NCI: neonatal colonic inflammation; CRD: colorectal distention; TRAF6: tumor necrosis factor receptor-associated factor 6.

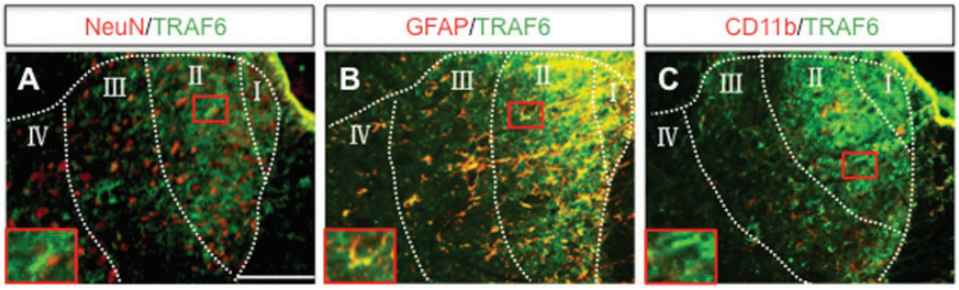

Figure 2. TRAF6 was predominantly expression in spinal astrocytes. TRAF6 was co-localized with GFAP (B) but not CD11b (C) or NeuN (A) by immunofluorescence. Bar = 100 μm for all photos. The higher magnification pictures in the bottom left of each picture to show the extent of co-localization of TRAF6 with different markers. The yellow staining indicates co-localization.

group, **$P < 0.01$). The reverse effect started at 2 h and lasted for 4 h. One time injection of siRNA NC did not produce any effect on CRD thresholds. We then determined the time course of the effects after consecutive administration of siRNA TRAF6 every two days for one week. As shown in Figure 5(b), the analgesia effect was evident at 1 h to 24 h and returned to normal level at 48 h ($n = 6$ rats, **$P < 0.01$, ***$P < 0.001$). These data indicate that TRAF6 in the spinal dorsal horn is obligatory for the development of NCI-induced visceral hypersensitivity. To exclude the possible side effect, the Rota-rod test was performed. There was no significant

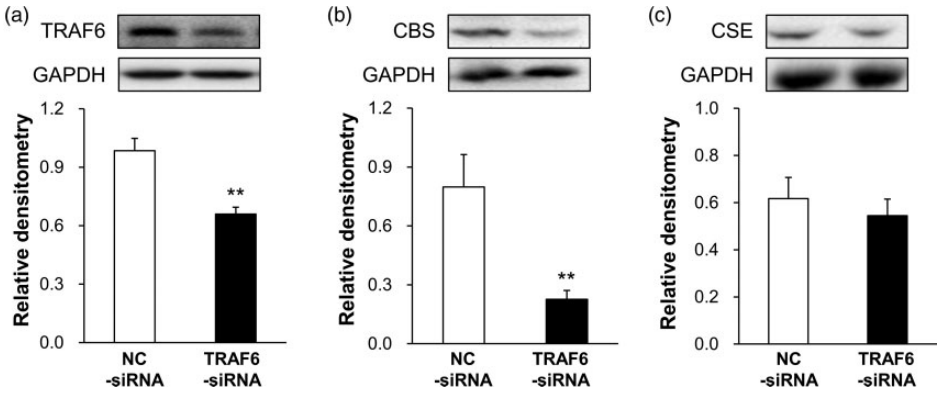

Figure 3. Knockdown of TRAF6 by intrathecal injection of TRAF6 siRNA reduced spinal CBS expression of NCI rats. (a) TRAF6 expression was significantly reduced after intrathecal injection of TRAF6 siRNA ($n = 6$ for each group, $^{**}P < 0.01$ vs. NC). (b) The protein expression of CBS was dramatically reduced after siTRAF6 injection (i.t.) once every two days for consecutive seven days ($n = 6$ for each group, $^{**}P < 0.01$ vs. NC). (c) CSE expression did not change after TRAF6 siRNA treatment ($n = 6$ for each group). CBS: cystathionine β synthase; CSE: cystathionine γ lyase; NC: negative control; siRNA: small interfering RNA; TRAF6: tumor necrosis factor receptor-associated factor 6.

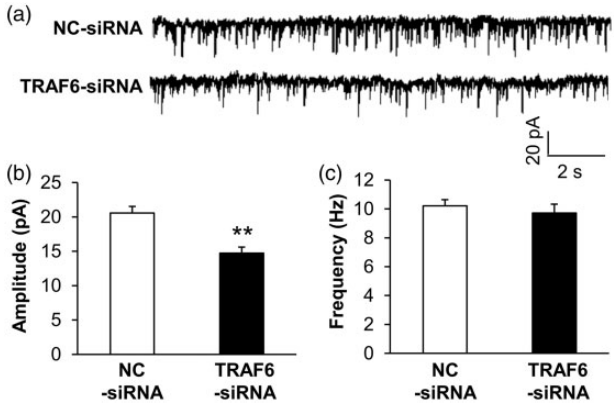

Figure 4. Knockdown of TRAF6 suppressed spinal synaptic transmission. (a) Representative traces of sEPSCs recorded from SG neurons holding at -70 mV in voltage clamp from NCI rats treated NC- and TRAF6-siRNA. (b) Bar plot showing that TRAF6-siRNA treatment significantly reduced the amplitude of sEPSC compared with NC-treated NCI rats. $^{**}P<0.01$, $n=8$ cells for each group of three rats. (c) Bar plot showing that intrathecal injection of TRAF6-siRNA did not alter the frequency of sEPSCs when compared with NC-treated group. $P>0.05$, $n=8$ cells for each group of three rats. NC: negative control; siRNA: small interfering RNA; TRAF6: tumor necrosis factor receptor-associated factor 6.

difference in the time for rats to stay on the rod after siRNA TRAF6 injection (Figure 5(c), $n = 6$ rats, $P > 0.05$). In addition, intrathecal injection of siRNA TRAF6 did not change the paw withdrawal latency (PWL) to the heat stimulation (Figure 5(d), $n = 6$ rats, $P > 0.05$).

Discussion

The pathogenesis of chronic visceral pain in patients with IBS remains largely unknown. This study demonstrates that TRAF6 signaling at spinal cord level produces visceral hypersensitivity in adult rats following the NCI. The conclusion is based on the following observations. Firstly, NCI led to a significant increase in TRAF6 expression specifically in the colon-related spinal dorsal horn where receives colonic sensory inputs from T_{13}-L_2 DRG neurons (Figure 1(b)). Importantly, knockdown TRAF6 expression by intrathecal injection of TRAF6 siRNA greatly attenuated the visceral hypersensitivity of rats with NCI. These findings reveal a central mechanism involved in chronic visceral pain. In addition to neuropathic pain,[21] TRAF6 might be also involved in chronic visceral pain. It is therefore suggested that TRAF6 possibly represents a potential strategy for therapy of chronic visceral pain in patients with IBS. Since TRAF6 siRNA was injected intrathecally, the inhibitors may act on spinal cord and/or DRGs. It is therefore difficult to rule out the contribution of DRGs. However, by considering the data obtained from the electrophysiological recordings from the SG neurons of spinal dorsal horn, it is reasonable to hypothesize that spinal cord might be one of the important action sites for TRAF6 signaling. Since we only observed the sEPSC, it is difficult to determine whether a decline in sEPSC after siRNA treatment is due to decreased input or to decreased sensitivity to the input. Further experiments to record evoked EPSC will be much helpful. Since TRAF6 siRNA did not affect the Rota-rod performance and PWL of NCI rats, these data suggest that TRAF6 siRNA produced effect was not a non-specific analgesic effect. These also suggest that the role of the TRAF6 pathway may not be as important in signaling somatic sensory as in signaling the visceral pain.

The mechanisms by which TRAF6 produced visceral pain remain unknown. Under neuropathic pain

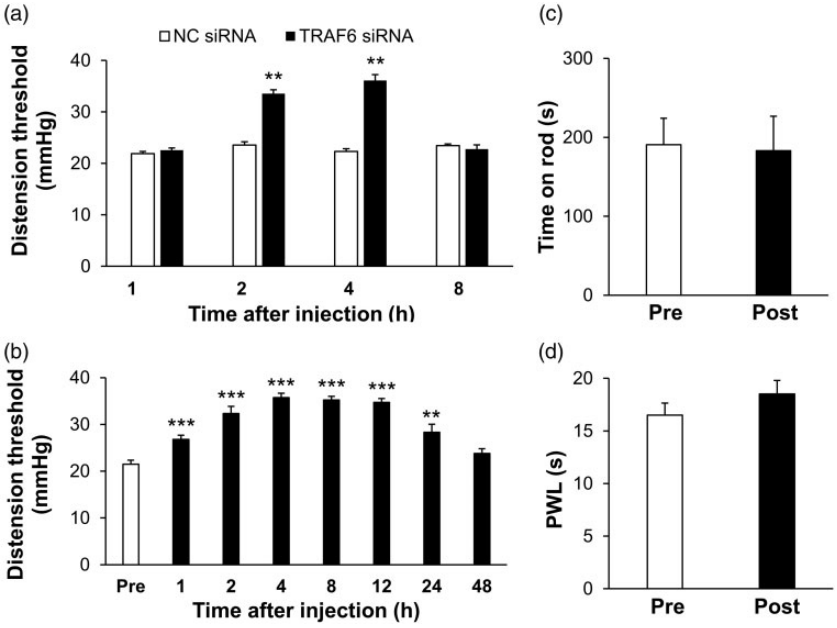

Figure 5. Knockdown of TRAF6 attenuated visceral pain of NCI rats. (a) One time injection of TRAF6 siRNA injection (i.t.) significantly enhanced the CRD threshold of NCI rats from 2 h to 4 h ($n = 6$, $^{**}P < 0.01$ vs. NC siRNA). (b) TRAF6 siRNA injection (i.t.) once every two days for consecutive seven days significantly reversed the CRD threshold of NCI rats from 1 h to 24 h ($n = 6$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Pre). (c) TRAF6 siRNA injection did not alter the time for rats to stay on the Rotarod test ($n = 6$, $P > 0.05$ vs. Pre). (d) TRAF6 siRNA injection did not alter the paw withdrawal latency to heat stimulation of the hindpaw ($n = 6$, $P > 0.05$ vs. Pre). NC: negative control; siRNA: small interfering RNA; TRAF6: tumor necrosis factor receptor-associated factor 6.

conditions, TRAF6 maintains neuropathic pain by integrating TNF-alpha and IL-1beta signaling in spinal astrocytes.[21] However, there is no study to explore the downstream molecules targeted by TRAF6 under chronic visceral pain conditions. As reported previously, CBS-H_2S signaling pathway exerts a critical role in visceral pain.[17,25,26] Therefore, we aimed to investigate whether CBS is a downstream molecule targeted by TRAF6 under chronic visceral pain conditions. We provided new evidence to show that TRAF6 regulates visceral pain by integrating CBS signaling at spinal cord level. As demonstrated previously, CBS expression was significantly upregulated in chronic visceral pain.[25] In this study, knocking down TRAF6 expression by siTRAF6 treatment in adulthood was sufficient to reverse all the changes both at molecular level and at the behavioral phenotypes. Furthermore, we have provided evidence to confirm that the increase of excitability of SG neurons in NCI rats is associated with the upregulation of CBS. Therefore, we hypothesized that the increase of excitability of SG neurons in NCI rats might be associated with the upregulation of TRAF6 and CBS. Indeed, we show here that inhibition of TRAF6 in NCI rats not only reduced the expression of CBS but also suppressed excitability of SG neurons as well. This is confirmed by electrophysiological data recorded from the spinal cord slices of NCI rats treated with TRAF6 siRNA. The two enzymes CBS and CSE are important for endogenous H_2S production. It is reported that both CBS and CSE are expressed in the spinal cord.[27] CBS is reported to be localized to astrocytes-enriched tissues such as hippocampus, temporal lobe, and cortex.[28,29] In the spinal cord, CBS might also be localized to astrocytes, although it needs to be confirmed in the future. Nevertheless, we showed here that TRAF6 siRNA injection dramatically reduced CBS expression but did not significantly alter CSE expression, indicating that CBS might be a major factor contributing to visceral hyperalgesia of NCI rats. TRAF6 exerts critical roles in a wide range of physiological and pathological processes, such as adaptive and innate immunity, inflammation, and tissue homeostasis.[18–20] It is a key intermediary signaling pathway protein that induces the activation of MAPKs and NF-κB to regulate the expression of downstream genes. It is reported that TRAF6 maintains neuropathic pain by integrating TNF-α and IL-1β signaling and activating the JNK/CCL2 pathway in astrocytes.[21,30] Therefore, we speculate that TRAF6 might participate in many types of chronic pain. However, the different downstream signaling pathway might mediate different types of chronic pain, which needs to be further clarified. This study adds additional roles and signaling pathway of TRAF6 in the process of chronic visceral pain conditions.

However, mechanisms by which TRAF6 was upregulated remain to be further investigated. Previous study demonstrated that microRNA-146a-5p attenuates neuropathic pain via suppressing TRAF6 signaling in the spinal cord,[30] indicating microRNAs might be a upstream regulation of TRAF6 expression. In NCI-induced visceral pain, we have previously showed that miR-325-5p expression was significantly decreased.[22] These data indicate a possible upstream mechanism of TRAF6 expression in the spinal dorsal horn. Although the spinal dorsal horn is a key region in the processing of visceral pain, it is also important to note that supraspinal areas such as the brain pain matrix (prefrontal cortex, amygdala, insular, anterior cingulate cortex, etc.) may also underlie the effects of early-life stress and the therapeutic benefit of siTRAF6 seen here. Very interestingly, TRAF6 was predominantly expressed in spinal astrocytes. This is consistent with previous report.[21] Since glial activation is a common mechanism underlying spinal synaptic plasticity,[31–33] future investigation into the roles of TRAF6 on glial-neuronal interaction is very important.

Taken together, this study shows that the upregulation of TRAF6 in spinal cord takes part in visceral hypersensitivity of adult rats with NCI through an enhanced synaptic mechanism by sensitizing the SG neurons. Inhibiting TRAF6 signaling in spinal cord suppresses synaptic transmission and alleviates the visceral pain, which is likely mediated by CBS activation. These results shed light on the mechanisms of TRAF6 signaling in spinal cord in development of visceral hypersensitivity and provide new therapeutic avenue into the treatment of chronic visceral pain in patients with IBS.

Author Contributions

R-XW, WC, and J-NT performed experiments, analyzed data, and prepared figures and the manuscript. QS, ML, XX, and P-AZ performed experiments and analyzed data. YZ and C-YH analyzed data and revised the manuscript. G-YX designed experiments, supervised the experiments, and finalized the manuscript. All the authors have read and approved the paper.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by grants from National Natural Science Foundation of China (81920108016, 31730040, and 81801115), China Postdoctoral Science Foundation Grant (2018M642304), and the Priority Academic Program Development of Jiangsu Higher Education Institutions of China. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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