Increased ten-eleven translocation methylcytosine dioxygenase one in dorsal root ganglion contributes to inflammatory pain in CFA rats

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Abstract

DNA hydroxylation catalyzed by Tet dioxygenases occurs abundantly in neurons in mammals. However, effects of ten-eleven translocation methylcytosine dioxygenase 1 (TET1) expression and hydroxymethylation status on neuron injury remain unclear. This study was designed to explore the effects of TET1 and TET2 expression in the inflammatory pain of rats induced by complete Freund's adjuvant (CFA). Mechanical paw withdrawal threshold (PWT) and thermal withdrawal latency (TWL) were detected to assess pain behavior. The expression of TET1 and TET2 were measured in the dorsal root ganglion (DRG) with western blotting analysis. Immunofluorescence staining is employed to detect the expression and co-location of TRPV1 with TET1. Intrathecal administration of Bobcat339 was used to inhibit TET1 function in dorsal root ganglion. The paw withdrawal threshold and thermal withdrawal latency of rats were significantly reduced after CFA Injection. Western blot results showed that the expression of TET1 was significantly increased at 3 days after CFA injection, but TET2 had no statistical difference. Immunofluorescence results showed that TET1 was co-localized with TRPV1. Intrathecal administration of Bobcat339 improved mechanical and thermal pain threshold in CFA rats. Our findings highlight the role of TET1 in chronic inflammatory pain model. The expression of TET1 was increased in CFA rats, and suppression of TET1 will ameliorate inflammatory pain.

Keywords

Ten-eleven translocation methylcytosine dioxygenase 1, inflammatory pain, dorsal root ganglion, hyperalgesia

Introduction

More than 20% of adults worldwide suffer from different kinds of chronic pain, which increases a burden physically and mentally and makes a great deal of adverse impact on quality of life. Chronic inflammation can cause persistent tissue damage and unwarranted pain due to the release of inflammatory mediators, including bradykinin, prostaglandins, and various cytokines, released locally during tissue damage or by inflammatory cells.[1–8] The dorsal root ganglion (DRG) are highly complex structures located on either side of the spinal cord and span the length of the spinal column.[9–11] The neurons located within the DRG are responsible for sensory transduction and modulation from the periphery, including pain perception.[12–15]

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Epigenetic mechanisms, strongly affecting the regulation of gene expression, have been associated with pathophysiological pain.[16–21] Inhibiting DNA methylation prevents and reverses pain behaviors, suggesting a potential role of DNA demethylation in pain.[20] DNA methylation can be actively demethylated by DNA methylcytosine dioxygenases, the 10-11 translocation (TET) proteins.[22–25] The three TET family members (TET1, TET2 and TET3) have distinct expression patterns among different cell types and are tightly regulated during development.[26] TET1 protein is highly expressed in both human and mouse embryonic stem cells (ESCs), whereas TET2 is expressed at extremely low levels in human ESCs similar to TET3 in mouse ESCs.[27,28] Recent studies demonstrated that TET1 overexpression in DRG alleviated pain hypersensitivity during the development and maintenance periods of neuropathic pain.[20] However, the regulation of TET1 and TET2 in inflammatory pain remains unclear.

In this study, we showed a significant increase in the expression of TET1 in DRG of CFA induced inflammatory pain model rats. Persistent inflammation was induced by subcutaneously injection of CFA into the plantar surface of the left hind paw. Our findings suggest that inhibition of TET1 might be a novel epigenetic mechanism in nociceptive information processing in a CFA-induced inflammatory pain model.

Materials and methods

Animals

A total of 108 adult male Sprague–Dawley (SD) rats (180–200 g) were purchased from the Animal Center of Chinese Academy of Sciences, Shanghai, China. For each experiment, the animals were randomly divided into control or experimental group. These rats were individually housed in a temperature-controlled room ($22 \pm 2°C$) on a 12:12 h light-dark cycle (lights on from 8:00 a.m. to 8:00 p.m.) and allowed free access to food and water. The experimental procedures were approved by the Institutional Animal Care and Use Committee of Soochow University. All experimental procedures were strictly conducted under the requirements in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Inflammatory pain model

Intraplantar (i.pl.) injection of CFA is a commonly used method to induce long-lasting inflammatory nociception in animal studies of pain to induce inflammatory pain.[29] The rats were injected in the left paw with CFA (i.pl., 100 μL), while the rats were briefly anesthetized with isoflurane. A 0.9% saline solution (NS) was used as a control for CFA. Rats were randomly divided into two groups with various duration times (8 h, 1 day, 3 days, 7 days and 14 days). Except for measuring the time course of CFA-induced inflammatory pain, all other

experiments were performed at day 3 after NS or CFA injection. All experiments were performed by experimenters who were blind to the treatment condition of the animals. The animals were sacrificed and the DRG (L4-L6) were collected for the subsequent experiments.

Western blot

The animals were euthanized with isoflurane and then ipsilateral L4-L6 DRG tissues was immediately isolated and frozen in liquid nitrogen. The tissue specimens were collected and homogenized in the RIPA lysis buffer containing protease inhibitor. The protein was extracted and the BCA protein assay kit was used to measure the concentration. Proteins were separated by 8% SDS polyacrylamide gel electrophoresis and transferred onto PVDF membranes. Then the membrane was blocked in 5% defatted milk for 2 h at room temperature and subsequently incubated overnight at 4°C with the following primary antibodies: TET1 (abcam, ab191698, 1:1000), TET2 (Millipore, ABE364, 1:1000), GAPDH (Simaikang, SMK-q, 1:10000), Tubulin (Proteintech, 66240-1-Ig, 1:10000). The next day, after the membrane was washed, appropriate secondary antibodies (Invitrogen) were added and incubated at room temperature for 2 h. The target band signals were detected using an enhanced chemiluminescence (ECL) detection system. The signals were quantified using Image J software. GAPDH or Tubulin was used for normalization.

Intrathecal injection of drugs

Bobcat339 hydrochloride, a potent and selective cytosine-based inhibitor of TET enzyme, was delivered intrathecally into rats. The rats were lightly anesthetized by isoflurane. Hairs were shaven in the midline of the back. All rats were placed in the prone position, a Hamilton syringe with a 30 gauge needle was inserted into the subarachnoid space of the cauda equina. Tail flick was a sign to identify successful intrathecal injection. 10 μL of drugs or vehicle was delivered into the intrathecal space over 1 min. Bobcat339 hydrochloride (MCE) was dissolved in normal saline (NS) and NS was delivered as a negative control. For intrathecal injections, 0.1 pM., 1 pM and 10 pM of Bobcat339 were used for each group of rats. The animals were placed in a recovery cage to wake up and monitored until they resumed normal activity.

Pain threshold assessment

Mechanical paw withdrawal threshold (PWT) to mechanical stimuli was determined by delivering von Frey filaments (VFF) to the plantar surface. A series of calibrated filament (0.6, 1, 1.4, 2, 4, 6, 8, 10, 15 and 26 g) was perpendicularly applied to the plantar surface of the hind paw. A series of gradually increasing force was applied. To avoid the potential injury, 26 g of the VFF force was set as a cutoff. The pressure was applied for 10 times each filament. The minimal force

that initiates paw withdrawal of rats was defined as threshold. A light beam (Plantar Test 37370, Ugo Basile srl, ITALY) was focused onto the plantar surface of one hind paw through the glass plate from below, and the latency from onset of the light to brisk withdrawal of the stimulated paw was measured as thermal withdrawal latency (TWL). The radiant heat intensity was adjusted to obtain basal paw withdrawal latency of 11–14 s. An automatic 20 s cutoff was used to prevent tissue damage. Thermal stimuli was delivered three to five times at 10-min intervals. Before nociceptive behavior testing, rats were acclimatized to the environment for 1 h. Two-blinding procedure was followed during the experiments. The investigator was blinded as to the chemical injected. The third person carried on the statistics.

Immunofluorescence assay

The rats were sacrificed for immunostaining 3 days after CFA injection. All rats were anesthetized with isoflurane and then perfused with 0.9% saline and 4% paraformaldehyde (PFA) systemically. The L4-L6 segments of the DRG were carefully separated, fixed in the 4% PFA at 4°C for 2 h and then transferred into 15% and 30% sucrose solution for gradient dehydration. The DRG tissues were then cut into 15 μm thick sections. The sections were rinsed in phosphate-buffered saline (PBS) with 0.3% Triton X-100 for 10 min, repeat three times. The sections were blocked in 10% normal horse serum for 1 h at room temperature and incubated with primary antibody overnight at 4°C and then incubated with secondary antibody for 2 h at room temperature in the dark. Negative controls were performed without the primary antibody. The primary antibody used in our experiment is TET1 (abcam, ab191698, 1:200) and TRPV1 (Proteintech, 66983-1-Ig, 1:200). Then after washing for three times, the sections were covered with DAPI Fluoromount-G. Fluorescent images were obtained using a laser confocal microscope (Leica, TCS SP8).

Statistical analysis

All values are expressed as MEAN ± SEM. The normality test was conducted in IBM SPSS Statistic 21 and all the data were normal. The data were analysed using IBM SPSS Statistic 21 and Graphpad Prism 5. Student's t-test was used to analyze the difference between two groups. Differences among 3 or more groups were compared by analysis of variance (ANOVA), followed by the Bonferroni post-hoc test for multiple comparisons. $p < 0.05$ was considered statistically significant.

Results

Establishment of the chronic inflammatory pain rat model and behavioral assessment

Inflammatory pain was induced in model group rats by intra-plantar injection of CFA. PWT and TWL were performed to verify whether CFA injection caused mechanical and thermal allodynia in the ipsilateral hindpaw of rats (Figure 1(a)). We assessed baseline values for the PWT and TWL of rats before CFA injection. The values of PWT were 21.60 ± 0.17 ($n = 6$ rats) and 20.5 ± 0.23 ($n = 6$ rats) for NS-treated (CON) rats and CFA-treated (CFA) rats, while the values of TWL were 12.8 ± 0.08 ($n = 6$ rats) and 14.3 ± 0.14 ($n = 6$ rats) for CON rats and CFA rats. Statistical analysis showed that they did not significantly differ. The PWT of rats treated with CFA were 3.80 ± 0.15 g, 4.90 ± 0.21 g, 5.25 ± 0.06 g, 8.50 ± 0.23 g, 18.90 ± 0.18 g at 8 h and 1, 3, 7, 14 days, respectively. The PWT of rats treated with NS was 15.00 ± 0.08 g, 20.60 ± 0.14 g, 22.80 ± 0.33 g, 19.20 ± 0.21 g, 19.48 ± 0.24 g at 8 h and 1, 3, 7, 14 days, respectively. The PWT of CFA rats was reduced significantly from 8 h, and maintained at 1, 3 and 7 days compared with CON rats (Figure 1(b), $p < 0.05$ vs CON, two-way ANOVA). Meanwhile, the TWLs of CFA rats were 4.1 ± 0.14 s, 7.7 ± 0.09 s, 9.5 ± 0.15 s, 12.9 ± 0.21 s, 14.3 ± 0.07 s at 8 h and 1, 3, 7, 14 days, respectively. The TWLs of CON rats was 11.2 ± 0.26 s, 14.9 ± 0.15 s, 15.1 ± 0.16 s, 16.5 ± 0.06 s, 13.3 ± 0.21 s at 8 h and 1, 3, 7, 14 days, respectively. The TWL of CFA rats decreased significantly from 8 h, and maintained at 1 and 3 days compared with CON rats (Figure 1(c), $p < 0.05$ vs CON, two-way ANOVA). The results revealed a statistically significant difference between the CON and CFA groups ($p < 0.05$) at 8 h, 1 day, 3 days and 7 days, which indicated that the chronic inflammatory pain model was successfully established.

TET1 expression was increased in L4-L6 DRGs of rats with chronic inflammatory pain while TET2 expression did not show statistically difference

To investigate the potential relationship between TET1, TET2 and inflammatory pain, samples of L4-L6 DRG were harvested at different time point after CFA/NS injection. The relative values of TET1 in CFA rats were 0.55 ± 0.24, 1.20 ± 0.57, 1.85 ± 0.10, 0.89 ± 0.64 and 1.51 ± 0.17 at 8 h and 1, 3, 7, 14 days, respectively (Figure 2(a)–(e)). The relative values of TET2 in CFA rats were 1.08 ± 0.25, 1.55 ± 0.63, 0.69 ± 0.08, 1.43 ± 0.04 and 1.21 ± 0.14 at 8 h and 1, 3, 7, 14 days, respectively (Figure 3(a)–(e)). The relative expression level of TET1 was significantly increased at 3 days after CFA injection (Figure 2(c), $*p < 0.05$ vs CON, two sample t-test). TET2 expression did not show statistically difference (Figure 3(a)–(e)). To confirm TET1 expression in L4-L6 DRG, immunofluorescence assay was conducted to detect their location in L4-L6 DRG. As shown in Figure 4(a) and (b), the expression of TET1 (green) was increased in CFA rats, and it was mostly co-localized with TRPV1 (red). These results indicate that TET1 may be involved in CFA-induced inflammatory pain by acting on TRPV1.
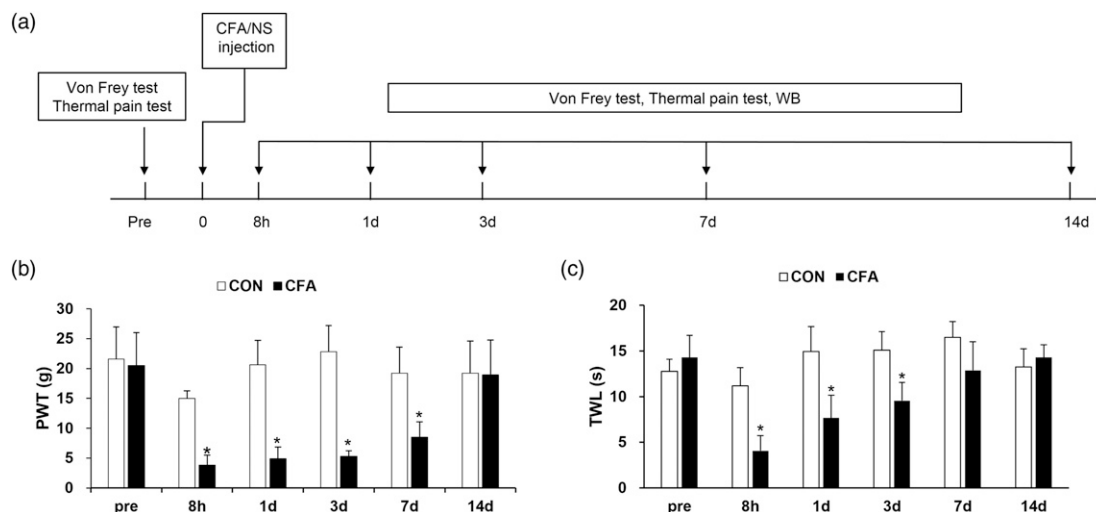
Figure 1. The chronic inflammatory pain model was successfully established. (a) PWT and TWL baseline values were assessed before CFA/NS injection. At 8 h, 1, 3, 7 and 14 days after CFA or NS injection, PWT and TWL values were assessed, TET1 and TET2 expression was detected with WB. (b) The PWT of CFA rats was reduced significantly from 8 h, and maintained at 1, 3 and 7 days compared with CON rats ($n = 6$ rats for each group, $*p < 0.05$ vs CON, two-way ANOVA). (c) The TWL of CFA rats decreased significantly from 8 h, and maintained at 1 and 3 days compared with CON rats ($n = 6$ rats for each group, $*p < 0.05$ vs CON, two-way ANOVA).
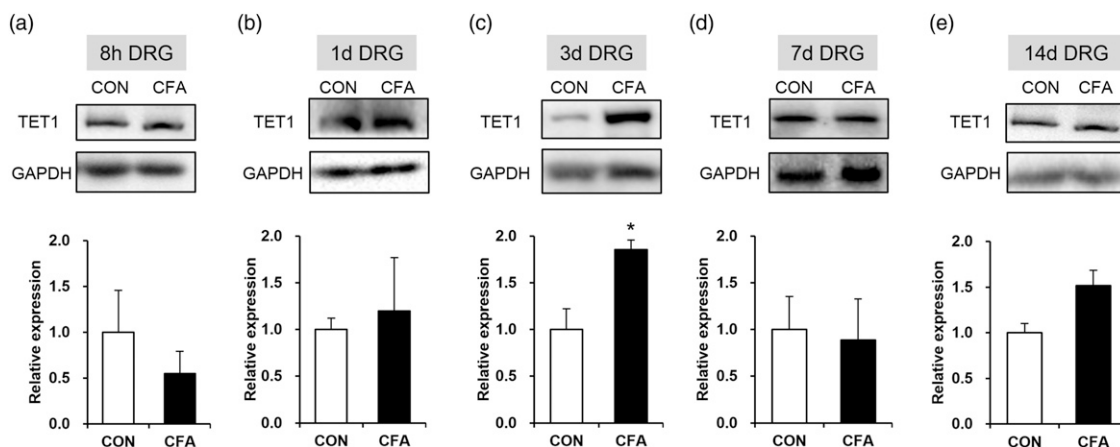

Figure 2. TET1 expression was increased in L4-L6 DRGs of rats with chronic inflammatory pain. (a–e) The relative expression level of TET1 significantly increased at 3 days after CFA injection ($n = 3$ rats for each group, $*p < 0.05$ vs CON, two sample t-test).

Single injection of TET enzyme inhibitor increased mechanical pain threshold of CFA rats at 4 h after intrathecal injection

Bobcat339 hydrochloride is a potent and selective cytosine-based inhibitor of TET enzyme. To find out the effect of Bobcat339 by intrathecal injection on CFA-induced inflammatory pain, we divided CFA rats into four groups. At the third day after CFA injection, the four groups of rats received

intrathecal injection with different concentration of Bobcat339 as CFA + NS, CFA + Bobcat339 0.1 pM, CFA + Bobcat339 1 pM and CFA + Bobcat339 10 pM (Figure 5(a)). In the CFA + NS group, PWT values were 1.10 ± 0.10 g, 1.30 ± 0.10 g, 1.20 ± 0.12 g, 1.45 ± 0.41 g, 1.45 ± 0.21 g and 2.85 ± 1.72 g at pre, 0.5 h, 1 h, 2 h, 4 h and 8 h after intrathecal injection, respectively. While the TWL values were 7.19 ± 0.34 s, 8.63 ± 1.26 s, 6.71 ± 0.71 s, 8.40 ± 0.58 s, 8.91 ± 1.77 s and 8.52 ± 1.05 s. In the CFA + Bobcat339 0.1 p.m. group,
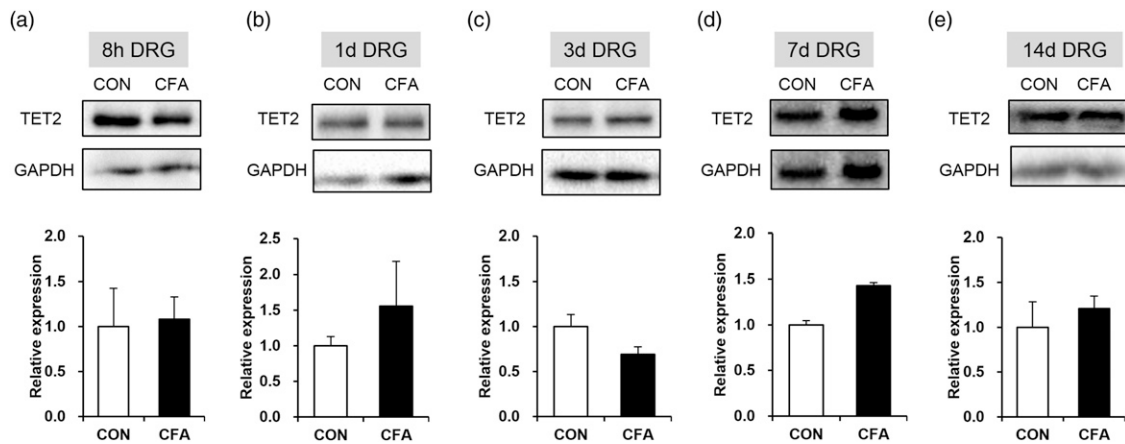
Figure 3. (a–e) TET2 expression did not show statistically difference in L4-L6 DRGs of rats with chronic inflammatory pain (*n* = 3 rats for each group, *p* < 0.05 vs CON, two sample *t*-test).
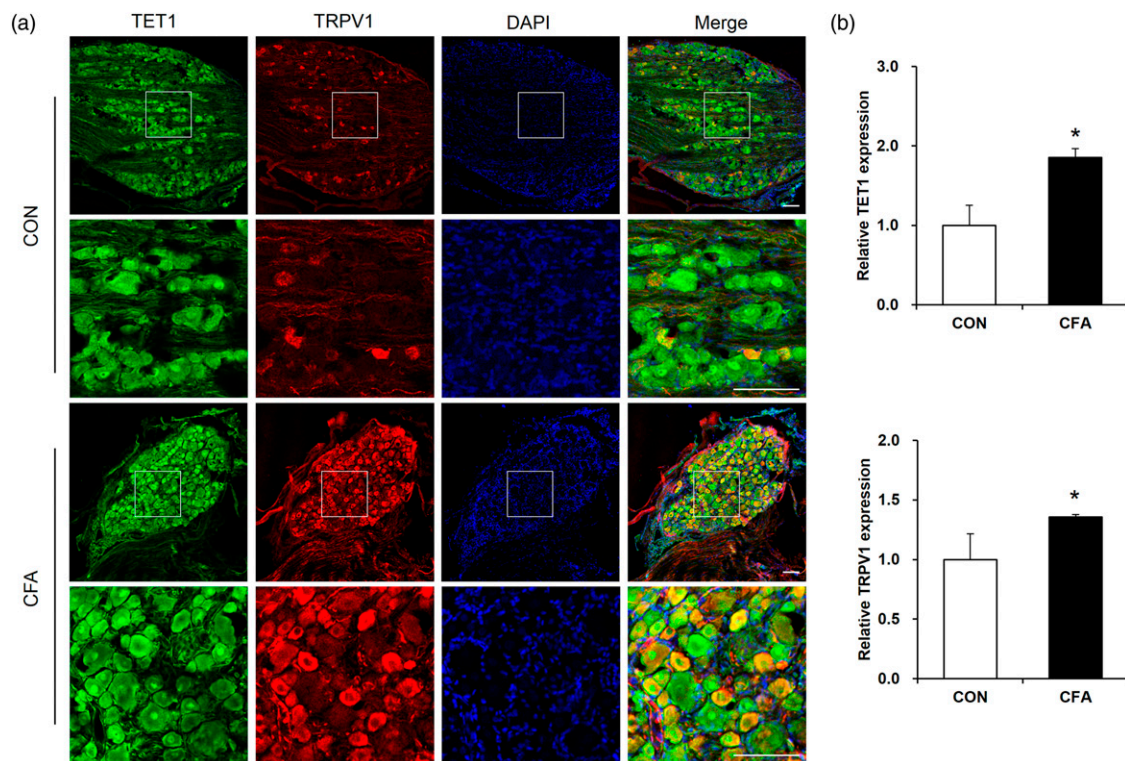

Figure 4. The expression of TET1 was increased in CFA rats, and it was mostly co-localized with TRPV1. (a) The representative pictures of TET1 (green), TRPV1 (red) and DAPI (blue) staining in DRG of CON and CFA rats. (b) The statistical analysis of TET1 and TRPV1 expression (*n* = 3 for each group, *p* < 0.05 vs. CON, two sample *t*-test, scale bar = 100 μm).

PWT values were 1.20 ± 0.12 g, 1.30 ± 0.10 g, 1.45 ± 0.21 g, 1.10 ± 0.10 g, 1.30 ± 0.10 g and 4.0 ± 0.96 g at pre, 0.5 h, 1 h, 2 h, 4 h and 8 h after intrathecal injection, respectively. While the TWL values were 7.74 ± 0.64 s, 9.33 ± 1.37 s, 8.82 ± 0.81 s, 9.49 ± 1.11 s, 7.84 ± 0.30 s and 7.46 ± 0.89 s. In the CFA + Bobcat339 1 p.m. group, PWT values were 1.60 ± 0.24 g, 1.27 ± 0.12 g, 1.33 ± 0.29 g, 1.47 ± 0.25 g, 5.58 ± 2.93 g and 2.5 ± 1.49 g at pre, 0.5 h, 1 h, 2 h, 4 h and 8 h after intrathecal injection,

respectively. While the TWL values were 6.20 ± 0.83 s, 8.48 ± 0.62 s, 8.53 ± 1.99 s, 8.06 ± 1.01 s, 5.96 ± 0.79 s and 8.75 ± 0.93 s. In the CFA + Bobcat339 10 p.m. group, PWT values were 1.10 ± 0.10 g, 3.00 ± 1.22 g, 1.45 ± 1.22 g, 3.60 ± 1.39 g, 6.85 ± 1.88 g and 5.00 ± 0.82 g at pre, 0.5 h, 1 h, 2 h, 4 h and 8 h after intrathecal injection, respectively. While the TWL values were 7.44 ± 0.35 s, 11.23 ± 2.63 s, 10.93 ± 1.64 s, 10.53 ± 1.59 s, 8.07 ± 0.96 and 8.26 ± 1.12 s, respectively. These results showed
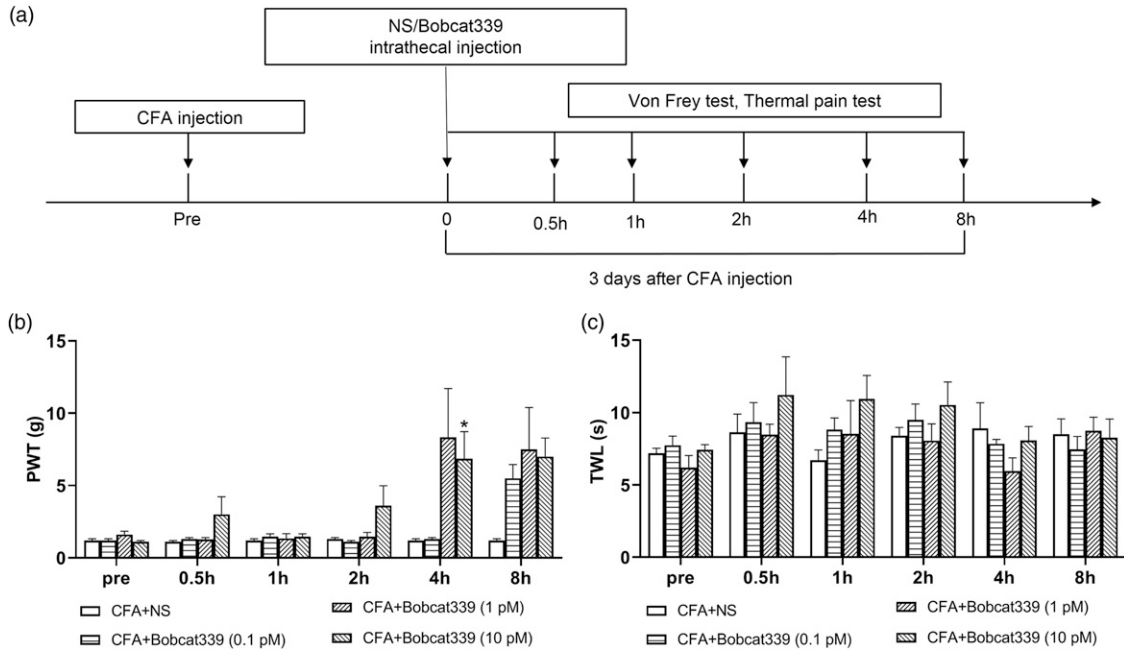
Figure 5. Single injection of TET enzyme inhibitor Bobcat339 increased PWT value at 4 h after intrathecal injection. (a) Schematic flow diagram of the intrathecal injection of NS and Bobcat339. (b) PWT value increased in the CFA + Bobcat339 10 p.m. group at 4 h after intrathecal injection. (c) TWL values showed no statistically difference ($n = 3$ for each group, $*p < 0.05$ vs CFA + NS group, two sample t-test).
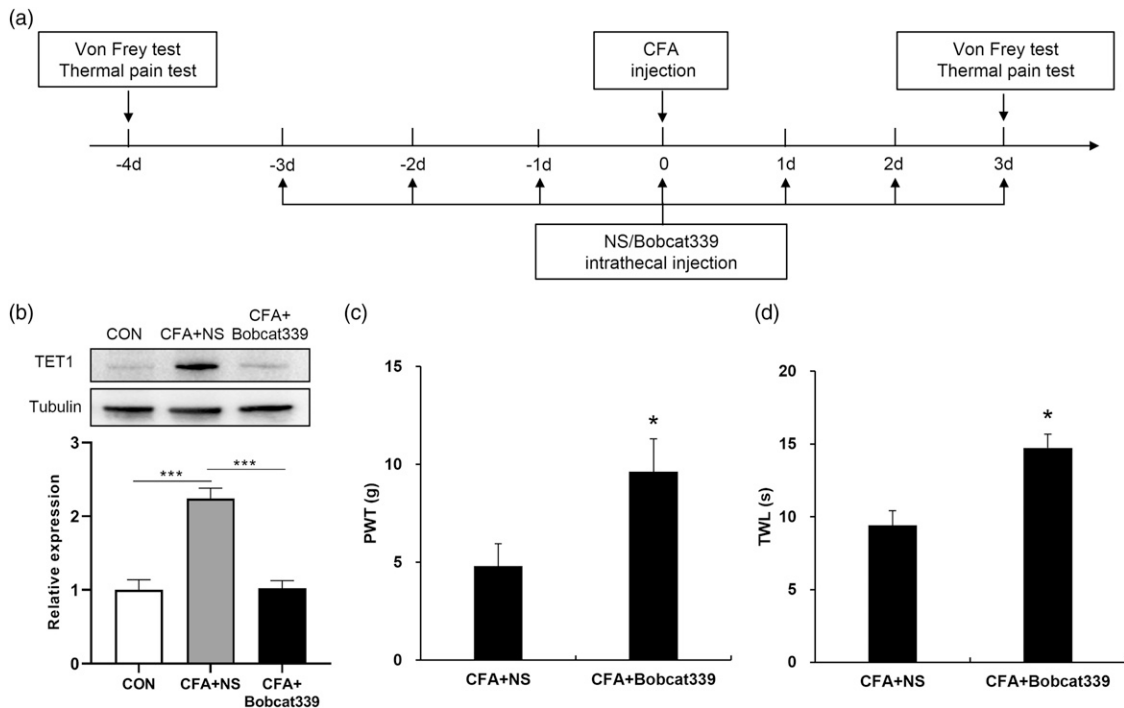

Figure 6. Continuously intrathecal injection of TET enzyme inhibitor Bobcat339 for 7 days alleviated inflammatory pain in CFA rats. (a) Schematic flow diagram of the intrathecal injection of NS and Bobcat339. (b) Increased TET1 expression was inhibited by Bobcat339 injection in CFA rats ($n = 4$ for each group, $***p < 0.001$, two sample t-test). (c and d) PWT and TWL values of CFA + Bobcat339 group were significantly increased at 3 days after CFA injection ($n = 4$ for each group, $*p < 0.05$ vs CFA + NS group, two sample t-test).

that intrathecal injection of Bobcat339 10 pM significantly increased mechanical pain threshold of CFA rats at 4 h after single injection (Figure 5(b)). But there is no significant difference in thermal pain threshold (Figure 5(c)).

Intrathecal injection of TET enzyme inhibitor for 7 consecutive days increased mechanical and thermal pain threshold of CFA rats

To explore the long-term effect of Bobcat339, we administrated it daily with a concentration of 10 pM for 3 days in advance before CFA injection and 4 days following CFA injection. The control group was injected with normal saline intrathecally. At 3 days after CFA or NS injection, the PWT and TWL were assessed (Figure 6(a)). Western blot results demonstrated that Bobcat339 injection significantly attenuated the expression of TET1 (Figure 6(b), ***$p < 0.001$, two sample t-test). Consistently, behavioral tests showed that the PWTs of CFA + Bobcat339 and CFA + NS group were 9.63 ± 1.68 g and 4.80 ± 1.15 g, respectively, and the TWLs of the two groups were 14.73 ± 0.95 s and 9.41 ± 1.01 s, respectively (Figure 6(c) and (d), *$p < 0.05$, two sample t-test). These results showed that intrathecal injection of Bobcat339 for seven consecutive days significantly increased the PWT and TWL at 3 days after CFA injection compared to the control group, suggesting that decrease of TET1 alleviated CFA-induced inflammatory pain.

Discussion

TET1 is expressed in the neurons of the DRG and spinal cord dorsal horn, two major pain related regions in the nervous system.[30,31] Some studies found peripheral SNL and paw injection of formalin or CFA increased TET1 expression in ipsilateral spinal cord dorsal horn, but not in the DRG.[20] The present study demonstrated that TET1 overexpression in DRG aggravated pain hypersensitivity during the development and maintenance periods of inflammatory pain. We demonstrated that TET1 was involved in inflammatory pain in the present study based on the following observed results. Firstly, we showed that the mechanical allodynia of CFA rats occurred at 8 h after injection and lasted for at least 7 days. Western blot results showed that TET1 was upregulated in L4-L6 DRGs at the time of 3 days after CFA injection, which suggesting a potential role of TET1 in inflammatory pain. Additionally, immunofluorescence assay was conducted to detect the location and expression of TET1 in L4-L6 DRG. The results showed that TET1 was co-localized with TRPV1 in L4-L6 DRG, which was increased at 3 days after CFA injection, indicating a possibility of TRPV1 involved in regulation of TET1 expression.

This result was then further confirmed by application of TET1 inhibitor which significantly reversed paw withdrawal response and thermal withdrawal latency threshold of CFA rats. Firstly, we administrated a single injection of TET

enzyme inhibitor, leading to an increase of PWT value 4 h after intrathecal injection while no change of TWL value at all time points. Then to explore the long-term effect of Bobcat339, we administrated it for seven continuous days. We found both of the PWT and TWL values were increased 3 days after CFA injection.

The mechanism by which the TET1 contributes to inflammatory pain remains largely unknown. In the present study, we showed that TRPV1 is co-expressed in TET1 positive DRG neurons, suggesting a possible role of TET1-TRPV1 signal transduction pathway in inflammatory pain. TRPV1 is an integrator for diverse painful stimuli.[32–34] The contribution of presynaptic TRPV1 at the spinal cord level to regulating nociceptive drive in chronic inflammatory pain induced by CFA injection in rats has been determined.[35] In DRGs, TRPV1 was involved in developing and maintaining inflammatory heat hypersensitivity.[36] However, whether TET1 regulates TRPV1 expression and function in DRG neurons needs to be investigated in the future studies. In addition to TRPV1, other channels such as Na(v)1.8 sodium channels and voltage-gated potassium channels may be also involved in the TET1-mediated inflammatory pain.[37,38] A recent study showed that overexpressing methyltransferase-like 3 (Mettl3) reversed a loss of m6A in TET1 mRNA and blocked the CFA-induced increase of TET1 in the spinal cord, resulting in the attenuation of pain behaviors.[39] This study suggests that Mettl3 regulates the expression of TET1 in the spinal cord neurons.

In summary, the present study demonstrated that TET1 overexpression in DRG significantly enhanced pain behaviors under inflammatory pain conditions. The suppression of TET1 expression may be an attractive strategy for inflammatory pain therapy.

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