DNA Hydroxymethylation by Ten-eleven Translocation Methylcytosine Dioxygenase 1 and 3 Regulates Nociceptive Sensitization in a Chronic Inflammatory Pain Model

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ABSTRACT

Background: Ten-eleven translocation methylcytosine dioxygenase converts 5-methylcytosine to 5-hydroxymethylcytosine, which plays an important role in gene transcription. Although 5-hydroxymethylcytosine is enriched in mammalian neurons, its regulatory function in nociceptive information processing is unknown.

Methods: The global levels of 5-hydroxymethylcytosine and ten-eleven translocation methylcytosine dioxygenase were measured in spinal cords in mice treated with complete Freund’s adjuvant. Immunoblotting, immunohistochemistry, and behavioral tests were used to explore the downstream ten-eleven translocation methylcytosine dioxygenase-dependent signaling pathway.

Results: Complete Freund’s adjuvant-induced nociception increased the mean levels (± SD) of spinal 5-hydroxymethylcytosine (178 ± 34 vs. 100 ± 21; \( P = 0.0019 \)), ten-eleven translocation methylcytosine dioxygenase-1 (0.52 ± 0.11 vs. 0.36 ± 0.064; \( P = 0.0088 \)), and ten-eleven translocation methylcytosine dioxygenase-3 (0.61 ± 0.13 vs. 0.39 ± 0.08; \( P = 0.0083 \)) compared with levels in control mice (\( n = 6 \)/group). The knockdown of ten-eleven translocation methylcytosine dioxygenase-1 or ten-eleven translocation methylcytosine dioxygenase-3 alleviated thermal hyperalgesia and mechanical allodynia, whereas overexpression cytosinethem in naïve mice (\( n = 6 \)/group). Down-regulation of spinal ten-eleven translocation methylcytosine dioxygenase-1 and ten-eleven translocation methylcytosine dioxygenase-3 also reversed the increases in Fos expression (123 ± 26 vs. 294 ± 64; \( P = 0.0031 \); and 140 ± 21 vs. 294 ± 60; \( P = 0.0043 \), respectively; \( n = 6 \)/group), 5-hydroxymethylcytosine levels in the Stat3 promoter (75 ± 16.1 vs. 156 ± 28.9; \( P = 0.0043 \); and 91 ± 19.1 vs. 156 ± 28.9; \( P = 0.0066 \), respectively; \( n = 5 \)/group), and consequent Stat3 expression (93 ± 19.6 vs. 137 ± 27.5; \( P = 0.035 \); and 72 ± 15.2 vs. 137 ± 27.5; \( P = 0.028 \), respectively; \( n = 5 \)/group) in complete Freund’s adjuvant-treated mice.

Conclusions: This study reveals a novel epigenetic mechanism for ten-eleven translocation methylcytosine dioxygenase-1 and ten-eleven translocation methylcytosine dioxygenase-3 in the modulation of spinal nociceptive information via targeting of Stat3. (Anesthesiology 2017; 127:147-63)

What We Already Know about This Topic

• Epigenetic changes serve to control gene expression and may contribute to the persistence of pain
• The methylation of DNA is one such epigenetic mechanism

What This Article Tells Us That Is New

• The knockdown of key DNA demethylating enzyme ten-eleven translocation methylcytosine dioxygenases (TET1, TET3) reduces nociceptive sensitization induced by inflammation
• The effects of TET1/TET3 knockdown may result from alterations in spinal signal transducer and activator of transcription 3 expression
two major enzymes for de novo DNA methylation, suggesting an underlying active mechanism in the demethylation of aberrantly expressed genes in response to chronic pain. The functional role of DNA demethylation in nociceptive information processing, however, remains poorly understood.

Recent reports have verified that all three of the known ten-eleven translocation methylcytosine dioxygenases (TET1, TET2, and TET3) act as intermediaries between 5mC and 5-hydroxymethylcytosine (5hmC) in different mammalian cells. Increasing numbers of studies reveal that TET and 5hmC are proficiently enriched at transcription start sites and CpG sites in gene bodies. Furthermore, the distribution of 5hmC affects transcription efficiency by altering chromatin structure or recruiting/excluding DNA-binding proteins. Several studies have identified an enrichment of 5hmC within mammalian neurons. For example, the 5hmC content in brain cells is approximately 10-fold that in embryonic stem cells, suggesting 5hmC may be a stable epigenetic modification.

TET-mediated hydroxymethylation in the aberrant expression of genes has not been explored in chronic pain. The functional role of DNA demethylation in nociceptive information processing, however, remains poorly understood. Thus, we hypothesized that the TET-mediated increase of 5hmC affects transcription efficiency by altering chromatin structure or recruiting/excluding DNA-binding proteins. Several studies have identified an enrichment of 5hmC within mammalian neurons. For example, the 5hmC content in brain cells is approximately 10-fold that in embryonic stem cells, suggesting 5hmC may be a stable epigenetic modification.

In this study, we found significant increases in the levels of 5hmC, TET1, and TET3 in mice spinal cords in a CFA-induced inflammation pain model that mimics nociceptive sensitization in patients with inflammatory pain. Moreover, the level of 5hmC in the Stat3 promoter was up-regulated. Thus, we hypothesized that the TET-mediated increase of Stat3 5hmC in spinal cord contributed to nociceptive information processing in the development of chronic inflammatory pain. Here, we show that TET1 and TET3 regulate Stat3 expression as a novel epigenetic mechanism in nociceptive information processing in a CFA-induced inflammation pain model.

Materials and Methods

Animals, Pain Model, and Behavior Testing

Adult male Shanghai populations of Kunming mice (20 to 25 g) were used in this study. For each experiment, the animals were randomized to either a control or an experimental group. Nociceptive sensitization was induced by subcutaneous administration of CFA (40 μl; Sigma-Aldrich, USA) into the plantar surface of the hind paw. A 0.9% saline solution was used as a control for CFA. Paw withdrawal latency to a thermal stimulus and paw withdrawal thresholds to a mechanical stimulus were used to measure hyperalgesia and allodynia, respectively. Before nociceptive behavior testing, mice were acclimatized to the environment for 1 h. Thermal hyperalgesia was measured by focusing a beam of light on the plantar surface of the hind paw to generate heat, and the time required for the stimulus to elicit a withdrawal of the hind paw was recorded. The radiant heat intensity was adjusted to obtain basal paw withdrawal latency of 11 to 14 s. An automatic 20-s cutoff was used to prevent tissue damage. Thermal stimuli were delivered three times to each hind paw at 5-min intervals. Mechanical allodynia was assessed with the use of von Frey filaments, starting with a 0.31-g and ending with a 5.0-g filament. The filaments were presented, in ascending order of strength, perpendicular to the plantar surface with sufficient force to cause slight bending against the paw. A brisk withdrawal or flinching of the paw was considered a positive response. In climbing tests, a 0.5-mm-diameter metal wire mesh with a 5-mm-wide grid was placed vertically 30 cm above the table. Each mouse started at the bottom of the mesh with its head facing downward. After the mouse was released, the time to climb all the way to the top was recorded. Behavioral testing was performed in a double-blind trial fashion. All animal procedures were approved by the animal care committee of Xuzhou Medical University (Xuzhou, China).

Sample Collection

Blood samples were collected from the facial veins of the mice. To summarize in brief, mice were picked up firmly by the scruff via the thumb and first finger, and the hairless freckle on the side of the jaw was pricked with a needle. A 200-μl sample of blood was collected from each mouse and placed in tubes containing EDTA anticoagulant for storage at −80°C. For harvesting tissue, mice were anesthetized with 10% chloral hydrate, and the spinal cord within the lumbar segments (L3–L5) was removed rapidly. The dorsal spinal cord ipsilateral to CFA injections was separated for subsequent analyses. Peripheral blood was obtained after the eyeballs were removed and snap-frozen in liquid nitrogen before they were stored at −80°C.

DNA Dot-Blot

Genomic DNA was extracted from tissue and blood samples with a QIAamp DNA mini kit (51306; QIAGEN, Xuzhou, China).
Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed by the use of a ChIP assay kit (17 to 295; Millipore) according to the manufacturer’s instructions (345818; Millipore, USA). Equal DNA loading amounts were verified by staining the membranes with 0.2% methylene blue. The reference DNA fragments containing 5hmC were used as the positive standard. The dot-blot densities were analyzed with Image J software (USA).

Sequencing of 5hmC- and 5mC-enriched Genomic DNA

The capture, sequencing, and analyses for the regions of DNA enriched in 5hmC and 5mC were carried out in accordance with the Illumina sequencing kit. Genomic DNA was sonicated to achieve a 200- to 900-base pair (bp) size with a Covaris instrument, and 800 ng of the fragmented sample was end-repaired, A-tailed, and ligated to single-end genomic adapters with a Genomic DNA sample kit (FC-102-1002; Illumina, USA) according to the manufacturer’s instructions. The ligated DNA fragments of 300 to 1,000 bp in size were separated by agarose gel electrophoresis. The DNA was heat-denatured at 94°C for 10 min, rapidly cooled on ice, and immunoprecipitated with 1 μl monoclonal anti-5mC (C15200081; Diagenode) or anti-5hmC (C15200200; Diagenode) antibodies in 400-μl immunoprecipitation buffer (0.5% bovine serum albumin in PBS) overnight at 4°C with rocking agitation. To recover the immunoprecipitated DNA fragments, 20 μl magnetic beads (Life Technologies, Inc., USA) were added and incubated for an additional 2 h at 4°C with agitation.

Five washes were performed with ice-cold immunoprecipitation buffer. A nontarget mouse IgG immunoprecipitation was performed in parallel with methylated or hydroxymethylated DNA immunoprecipitation as a negative control. Washed beads were resuspended in Tris-EDTA buffer with 0.25% SDS and 0.25 mg/ml proteinase K for 2 h at 65°C and were then allowed to cool to room temperature. Methylated DNA immunoprecipitates or hydroxymethylated DNA immunoprecipitates (hMeDIP) and supernatant DNA were purified with the use of QIAGEN MinElute columns and eluted in 16 μl elution buffer. The pull-down DNA or input DNA was used for preparing sequencing libraries. The libraries were generated in accordance with the Illumina protocol for Preparing Samples for ChIP Sequencing of DNA (111257047; Rev. A) with 25 ng 5mC- or 5hmC-captured DNA. Fourteen cycles of PCR were performed on 5 μl immunoprecipitated DNA with the single-end Illumina PCR primers. The resulting reactions were purified with QIAGEN MinElute columns, after which a final size selection (300 to 1,000 bp) was performed by electrophoresis in 2% agarose. PCR-amplified DNA libraries were quality controlled with an Agilent 2100 Bioanalyzer (Agilent, USA) and finally diluted in elution buffer to 5 ng/μl. Then, 1 μl was used in real-time PCRs to confirm enrichment for the hydroxymethylated region. The library was denatured with 0.1 M NaOH to generate single-stranded DNA molecules, was loaded onto channels of the flow cell at an 8-pM concentration, and amplified in situ with the TrueSeq rapid PE cluster kit (PE-402-4001; Illumina) to generate the sequencing polymerase chain reaction (qPCR) analyses. We used Scf and Scr primers to amplify ChIP DNA and input DNA (see table S1, Supplemental Digital Content 1, http://links.lww.com/ALN/B419). ChIP PCR products were normalized to input products amplified using genomic DNA.

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Quantification of 5mC and 5hmC

The bisulfite conversion method for 5mC quantification was performed as described previously.2 To summarize, genomic DNA was subjected to bisulfite conversion with an EZ DNA methylation-gold kit (D5005; Zymo, USA). Bisulfite-modified DNAs were amplified using BS primers listed in table S1. PCR products were ligated to a vector with a TA cloning kit (SK2214; Shanghai Sangon Biotech), and we quantitatively analyzed the 5mC levels after Sanger sequencing. 5hmC quantification was determined with an EpiMark 5hmC assay kit (E3317S; NEB, USA). An outline of the assay and sample calculations can be seen in the manufacturer’s instructions. In brief, DNA is treated with T4 β-glucosyltransferase, enabling an estimation of the 5hmC level at the T4 cleavage site. The 5hmC level before and after treatment with T4 β-glucosyltransferase, enabling an estimation of the 5hmC level as those of the first round. A negative control was obtained from pipettes that were submerged in the bath solution only. Gapdh was used as the reference gene.

Plasmid Construction

All constructs were produced by the use of standard molecular methods and confirmed by DNA sequencing. To construct TET1 and TET3 knockdown vectors, pLVTTHM was digested with MluI (R0198S; NEB) and CiaI (R0197S; NEB) and then ligated with the double-strand sif1/sir1 (Lenti-T1-siRNA) and sif3/sir3 (Lenti-T3-siRNA) oligos, respectively (see table S1). To construct TET1 and TET3 overexpression vectors, Gibson DNA Assembly reactions (E5510S; NEB) were used to generate Lenti-T1 and Lenti-T3 constructs according to the manufacturer’s instructions. In brief, three Tet1 or four Tet3 overlapping inserts were prepared by PCR with the PCR primers listed in table S1. Gibson DNA Assembly reactions containing 100 ng of each insert and 50 ng of the pWPXL vector digested with BamHI (R0136L; NEB) were carried out at 50°C for 45 min.

To construct the promoter reporter vectors, a defined region of the Stat3 promoter was amplified from mouse genomic DNA using S3-P6F1/S3-P6R1 (products with transcription start site [TSS]) and S3-P6F2/S3-P6R2 (without TSS) primer pairs. The products were then cloned into a pGL6 plasmid via XhoI (R0146S; NEB) and HindIII (R3104S; NEB) digestion to produce pGL6-Stat3-TSS and pGL6-Stat3.25

Real-time qPCR

Total RNA was isolated with a Trizol reagent (15596-026; Invitrogen, USA) to generate cDNA templates by reverse transcription reactions with oligo (dT)18 and reverse transcriptase M-MLV (2641A; Takara Bio, Japan) at 42°C for 60 min. cDNA products were used as templates to detect Tet1, Tet2, Tet3, and Stat3 expression via real-time qPCR (RT-qPCR) with SYBR Premix Ex TaqII (RR820A; Takara Bio) according to the manufacturer’s instructions. RT-qPCR primers are listed in table S1. Reactions were performed in triplicate. The expression levels of the target genes were quantified relative to glyceraldehyde-3-phosphate dehydrogenase (Gapdh) expression (cycle threshold, C_t) using the 2-ΔΔCt method, as described previously,26 where ΔC_t was the difference in C_t values derived from the detected sample and Gapdh control, and ΔΔC_t represented the difference between paired samples calculated by subtracting the sample ΔC_t from the control ΔC_t. Any value among triplicates that had a marked difference (21.00) compared with the average of the other two was omitted.

Single-cell RT-PCR

Single-cell RT-PCR for spinal neurons was performed as described previously.27 In brief, the contents of dissociated spinal neurons from CFA mice were harvested into patch pipettes with tip diameters of ~35 μm, placed gently into reaction tubes with Dnase I at 37°C for 30 min, and heated to 80°C for 5 min to remove genomic DNA. Reverse transcriptase (SuperScript III Platinum; Invitrogen) was added, the sample was incubated at 50°C for 50 min, and the reaction was terminated at 70°C for 15 min. The cDNA products were used in gene-specific nested PCR. The primers are shown in table S1. The first-round PCR was performed with the outer primer pair in the FastStart universal SYBR green master kit (Roche, Switzerland). PCR conditions were as follows: 1 cycle of 3 min at 94°C; 35 cycles of 15 s at 95°C and 15 s and 60°C, and 1 cycle of 10 min at 72°C. The second round of PCR was carried out using 0.5 μl of the first PCR product as the template and with inner PCR primers. The amplification reagents and procedure were the same as those of the first round. A negative control was obtained from pipettes that were submerged in the bath solution only. Gapdh was used as the reference gene.

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**Lentivirus Production and Infection**

HEK 293T cells were cultured in Dulbecco modified Eagle medium with 10% fetal bovine serum (FBS). The cells were cotransfected with plasmid constructs and 16 μg of the core plasmid, 12 μg PSPAX2, and 4.8 μg PMD2G envelope plasmid in 6-well plates with Lipofectamine® 2000 (ThermoFisher Scientific, USA) according to the manufacturer’s instructions (11668-027; Invitrogen). The viral supernatant was collected 48 h after transfection and concentrated with a Centricon Plus-70 filter unit (UFC910096; Millipore). Lentivirus titers were measured at >10^5 transduction units/ml. The assays for *in vitro* and *in vivo* lentivirus infection were performed according to the method described previously. To summarize, 20 and lentivirus infection in vivo units/ml. The assays for from samples of the ipsilateral dorsal spinal cord taken 72 h siRNA, and -siRNA was confirmed with RT-qPCR -siRNA, Lenti-T1-siRNA, -siRNA, Lenti-T3- via T et1

**Reporter Methylation and Hydroxymethylation of the Promoter Construction Sequences**

Report Plasmid constructs and 16 μg PMD2G envelope plasmid in 6-well plates with Lipofectamine® 2000 (Ther- moFisher Scientific, USA) according to the manufactur- er’s instructions (11668-027; Invitrogen). The viral superna- tant was collected 48 h after transfection and concentrated with a Centricon Plus-70 filter unit (UFC910096; Milli- pore). Lentivirus titers were measured at >10^5 transduction units/ml. The assays for *in vitro* and *in vivo* lentivirus infection were performed according to the method described previously. To summarize, 20 μl lentivirus and 1.5 μl polybrene (1.4 μg/μl; H9268; Sigma-Aldrich) were added in a 24-well plate containing 1 × 10^5 HEK 293T cells and 400 μl Dulbecco modified Eagle medium without FBS. After 24 h, the transfection medium was replaced with 500 μl fresh complete medium containing 10% FBS. Cells were collected after 48 h in culture.

**Short-interfering RNA and Lenti–short-interfering RNA Delivery**

Injections were performed by holding the mouse firmly by the pelvic girdle and inserting a 30-gauge needle attached to a 25-μl microsyringe between L5 and L6 vertebral. Proper insertion of the needle into the subarachnoid space was verified by a slight flick of the tail after a sudden advancement of the needle. Injections of 5 μl of 20 μM short-interfering (si)RNAs for Tet1, Tet3, and Stat3 or 1 μl Lenti-T1-siRNA or Lenti-T3-siRNA were performed daily for 3 days in a double-blind trial fashion. Knockdown via Tet1-siRNA, Lenti-T1-siRNA, Tet3-siRNA, Lenti-T3- siRNA, and Stat3-siRNA was confirmed with RT-qPCR from samples of the ipsilateral dorsal spinal cord taken 72 h after the last injection. Animals receiving intrathecal injections of scrambled siRNA or an empty vector were used as control groups. The siRNA sequences and siRNA-vector construction sequences are listed in table S1.

**Methylation and Hydroxymethylation of the Promoter Reporter**

CpGs (5C group) in pGL6-Stat3-TSS or pGL6-Stat3 were methylated with the use of CpG methyltransferase (M0226S; NEB) at 37°C for 1 h and then purified with a Wizard Plus SV miniprep DNA purification system (A1330; Promega, USA) according to the manufacturer’s instructions. The methylated CpGs (5mC group) in reporter plasmids were hydroxymethylated (5hmC group) with recombinant TET1 protein (31363; Active Motif) in 50 mM HEPES (pH 8) with 50 μM Fe(NH4)2(SO4)2, 2 mM ascorbate, and 1 mM α-ketoglutarate for 3 h at 37°C. An empty pGL6 vector was used as the negative control, and the pRL-TK plasmid was used as an internal control (Promega).

**Luciferase Reporter Assay**

HEK 293T cells were seeded at 1 × 10^5 cells per well of a 24-well plate. Cells were transfected with 200 ng of the methylated and the hydroxymethylated pGL6-Stat3-TSS, pGL6-Stat3, empty pGL6, or control pRL-TK vectors with Lipofectamine® 2000 (11668-027; Invitrogen). Cell lysates were prepared and subjected to luciferase assays by the use of the Dual-Luciferase® reporter kit (Promega) 48 h after transfection.

**Immunohistochemistry**

Spinal cords were dissected rapidly from mice perfused with 4% formaldehyde and postfixed in the same solution, cryo- protected in 30% sucrose, and then sectioned into 35-μm slices. For 5hmC immunofluorescence staining, an additional process was performed: the sections were treated with 1 M HCl at 37°C for 3 min followed by blocking with 5% FBS in PBS as described previously. The treated slices were incubated with anti-5hmC (1:2,000; 39999; Active Motif), anti-TET1 (1:200; 61741; Active Motif), anti-TET3 (1:200; 61743; Active Motif), anti-IBA1 (1:500; 19-19741; Wako, USA), anti-GFAP (1:300; 3670; Cell Signaling, USA), or anti-NeuN (MAB377; Millipore) antibo-odies at 4°C overnight. Sections were then washed twice in 0.4% Triton X-100 in PBS at room temperature for 10 min, incubated with fluorescent-conjugated secondary antibo-odies (AB10113, AB10081, or AB10053; Shanghai Sangon Biotech) at room temperature for 60 min, washed twice, and then sealed after drying. Fos immunohistochemistry was performed as described previously. In brief, a series of 30-μm transverse sections were cut on a cryostat and stored in PBS. After they were washed in PBS, the tissue sections were incubated in PBS containing 5% normal goat serum and 0.3% Triton X-100 at room temperature for 30 min. The sections were then incubated in primary rabbit anti-Fos antibody (1:1,000; Santa Cruz Biotechnology) at 4°C for 48 h. The sections were then incubated in biotinylated goat anti-rabbit antibody (1:200) at 37°C for 1 h and with an avi- din–biotin–peroxidase complex (1:100; Vector Labs, USA) at 37°C for 2 h. Finally, sections were treated with 0.05% diaminobenzidine for 5 to 10 min. Sections were rinsed in PBS to stop the reaction, mounted on gelatin-coated slides, air dried, dehydrated with 70 to 100% alcohol, cleared with xylene, and coverslipped for microscopic examination. For analyzing changes in Fos expression, we examined five L3–L5 spinal cord sections per animal, selecting the sec- tions with the greatest number of positive neurons. For each animal, we recorded the total number of positive neurons in bilateral I–V and X laminae of the spinal cord. All positive neurons were counted without considering the intensity of the staining. Immunostaining images were acquired with a confocal microscope (FluoView FV1000; Olympus, Japan). Immunohistochemistry slides were analyzed via bright field on a Nikon Eclipse E600 microscope, and images were
obtained with a Nikon Digital Sight camera (DS-Fi1) and NIS Elements (Nikon Instruments, Japan).

**Western Blot Analysis**
Proteins (20 to 50 μg/sample) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Due to the large difference in molecular weights between the target protein and internal reference protein, they were separated from blot membranes and incubated simultaneously at 4°C overnight in the corresponding antibodies against: TET1 (1:100; 61,443; Active Motif), TET2 (1:100; sc-136926; Santa Cruz Biotechnology), TET3 (1:100; sc-139186; Santa Cruz Biotechnology), and STAT3 (1:1,000; Santa Cruz Biotechnology) or control β-actin (1:1,000; TA-09; ZSBG-Bio, China). The membranes were then washed twice in tris-buffered saline with Tween-20 at room temperature for 10 min, incubated with anti-rabbit IgG secondary antibodies (1:1000; A0208; Beyotime, China) at room temperature for 1 h, and washed twice again in tris-buffered saline with Tween-20 at room temperature for 10 min. The immune complexes were detected with an NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate) assay kit (72091; Sigma-Aldrich). Band analyses were performed in ImageJ software, with the intensities of the target signals normalized to those of β-actin for statistical analyses.

**Statistical Analysis**
On the basis of previous experience,29 we used five to six mice per group for each experiment. Data are presented as mean values ± SD. The results from behavioral testing, luciferase reporter assay, 5hmC levels after TET knockdown or overexpression, 5hmC, protein, DNA dot-blot, Fos immunohistochemistry staining, qRT-PCR, and climbing test were analyzed statistically with a one-way or two-way ANOVA or paired or unpaired Student’s t test. When ANOVA showed a significant difference, pairwise comparisons between means were tested by the post hoc Tukey method. Statistical analyses were performed with Prism (GraphPad 5.00, USA). P < 0.05 was considered statistically significant.

**Results**

**CFA-induced Nociception Increases Spinal 5hmC Levels**
5hmC has been identified as a novel epigenetic modification in mammals, and its change becomes an epigenetic feature in CNS diseases.28 As the spinal cord plays critical roles in transducing and transmitting pain-related gene signaling, we explored the potential modulatory role of spinal 5hmC in nociceptive sensitization. We first examined the changes in total levels of spinal 5hmC in CFA-induced nociceptive sensitization. Dot-blot results revealed that the total 5hmC content increased to the highest level at 3 days (178 ± 34 μg/ml, 100 ± 21; P = 0.0019) during the observed 14 days after CFA injection (fig. 1A), suggesting CFA-induced nociception increased the total level of spinal 5hmC, which is dynamically regulated in a time-dependent manner. The strong correlation of epigenetic marks between spinal cord and blood may be useful for diagnostic and therapeutic applications in chronic pain. Therefore, we asked whether changes in 5hmC in whole blood are similar to those in the spinal cord underlying CFA-induced nociceptive sensitization. We found that blood 5hmC was markedly increased from 1 to 5 days (all P < 0.02), reached a peak value at day 5 (615 ± 106 μg/ml).

[Fig. 1. Complete Freund’s adjuvant (CFA)-induced nociceptive sensitization increases global 5-hydroxymethylcytosine (5hmC) levels in spinal cords. (A) Time course of dot-blot assay for spinal 5hmC from 1, 3, 7, and 14 days after CFA injection. Spinal DNA samples from 1, 3, 7, and 14 days after CFA injection were dot-blotted with a 5hmC-specific antibody; n = 6 mice at each time; one-way ANOVA (expression vs. time point) followed by post hoc Tukey test, F time (4, 25) = 41.48, *P < 0.05, **P < 0.01. (B) Time course of dot-blot assay from mouse peripheral blood for 5hmC from 1, 3, 5, and 7 days after CFA injection; n = 6 mice at each time; one-way ANOVA (expression vs. time point) followed by post hoc Tukey test, F time (4, 25) = 123.3, *P < 0.05, **P < 0.01, ***P < 0.001. (C) Combined 5hmC (red) and NeuN, GFAP, or IBA1 (green) immunofluorescence staining in ipsilateral spinal cord 3 days after CFA or saline injection. Scale bar, 25 μm. Arrows indicate the positive 5hmC signal. Acquisition parameters of images: laser intensity, 45% for 488 (green) and 50% for 543 (red); detector voltage (high voltage), 550 for green and 600 for red; gain, 0 for green and 0 for red; offset, 53% for green and 72% for red; scanning speed, 20.0 μm/pixel. Ctrl = control; NS = not significant.]
100 ± 20; \( P = 0.00035 \), and then returned almost to the basal level at 7 days after CFA injection (127 ± 24 vs. 100 ± 20; \( P = 0.096; \) fig. 1B), indicating the gain of 5hmC in mouse spinal cord and blood is a novel epigenetic feature in the nociceptive information processing.

To further explore the mechanism underlying the regulation of spinal 5hmC during nociceptive information processing, we investigated the genomic characteristics of changes in spinal 5hmC at 3 days after CFA injection. First, we used immunofluorescence staining to verify the increase in global spinal 5hmC in mice treated with CFA (fig. 1C) compared with that of the control group. Immunofluorescence staining showed an ~85.4% overlap between the 5hmC signal and NeuN (a neuron marker) in the control group and 90% overlap in the CFA group, whereas 5hmC signals rarely overlapped with GFAP (an astrocyte marker) and IBA1 (a microglial marker) in both groups (fig. 1C), suggesting that 5hmC may occur in the mouse spinal neurons. Then, we carried out a genome-scale evaluation of spinal 5hmC distribution at 3 days after CFA injection using a genome DNA immunoprecipitation sequencing method with an anti-5hmC antibody (hMeDIP-Seq). Before sequencing, the specificities of anti-5hmC and anti-5mC antibodies were evaluated against a negative control IgG antibody. We determined that DNAs were pulled down by anti-5hmC and anti-5mC antibodies but not by IgG via gel staining and NanoDrop2000 spectrophotometry, indicating that anti-5hmC and anti-5mC antibodies are specific for hMeDIP. In the subsequent sequencing, the means of 40.1 million and 35.5 million reads were obtained from the spinal cords of control and CFA groups, respectively. When the reads from the two groups were mapped to a mouse reference genome and filtered out, 20.9 million and 18.6 million uniquely mapped reads, respectively, remained that were used for subsequent analysis (data not shown). Genome-scale densities of 5hmC reads from each sample were determined in an Integrated Genomics Viewer browser (Broad Institute, USA). Global 5hmC density in CFA mice differed from that of control animals due to obvious alterations of 5hmC at numerous gene loci (fig. 2A). The normalized spinal 5hmC read densities across the transcript units of all the reference genes in the control and CFA groups showed different features and genome-wide coverages (see fig. S1A, Supplemental Digital Content 2, http://links.lww.com/ALN/B420). In addition, the clustering analysis clearly showed distinctive patterns of specific 5hmC enrichment between control and CFA-treated mice (see fig. S1B, Supplemental Digital Content 2, http://links.lww.com/ALN/B420).

Because cytosine methylation of CpG dinucleotides frequently leads to transcriptional silencing, CpG islands have been recognized as a crucial regulation region in DNA methylation. To systematically identify the downstream genes...
regulated by TET1 and TET3, we performed a genome-wide analysis for spinal 5hmC density of CpG island (CGI) in individual genes. In total, we identified 11,990 5hmC peak regions from spinal cords of mice in the control group and 11,844 peaks from the CFA group. Although the gross chromosomal distributions of 5hmC peak regions were equivalent between control and CFA groups, we found that 12 chromosomes had relatively low distributions, but the Y sex chromosome had extremely high 5hmC enrichment (fig. 2B). To achieve 5hmC peak regions with relatively high differentiation under saline and CFA-treated conditions, we also identified ~9,810 CGI regions with 2-fold differential spinal 5hmC densities after CFA treatment. These 5hmC differential regions were enriched heavily in promoters (~62.64%) and gene bodies (~26.94%), as determined by calculating the percentages of differential region counts across all genomic features (fig. 2C). In addition, the densities of 5hmC changes across various genomic regions were measured, and we found that the highest occurred in CGI in promoters (fig. 2D). As 5hmC in promoter CGI is associated with high levels of transcription, we speculated that 5hmC might function in promoter regions during CFA-induced nociceptive sensitization. Taken together, these results suggest that the global 5hmC contents were increased in the spinal cords during nociceptive sensitization and that 5hmC modification may be involved in the process of chronic pain.

**Spinal TET1 and TET3 Contribute to CFA-induced Nociception and Spinal Neuron Sensitization**

TET proteins catalyze 5mC toward 5hmC; therefore, we next investigated Tet expression and its roles in regulating nociceptive responses using a CFA model. Tet expression was quantified at the messenger (m)RNA and protein levels by RT-qPCR and Western blotting, respectively. We found that protein and mRNA levels of TET1 and TET3, but not TET2, were significantly increased at 3 days after CFA injection compared with those in the control group; Tet1 and Tet3 mRNAs were increased by 180% (280 ± 35 vs. 100 ± 18; P = 0.0026) and 121% (219 ± 35 vs. 98 ± 14; P = 0.017), respectively (fig. 3A). Correspondingly, TET1 and TET3 proteins were increased by 47% (145 ± 28 vs. 98 ± 19; P = 0.0088) and 55% (157 ± 34 vs. 102 ± 22; P = 0.0083), respectively (fig. 3B); TET2 proteins were almost not detected (data not shown). These results suggest that increases in TET1 and TET3, but not TET2, may be involved in the nociceptive responses induced by CFA. The distribution of 5hmC in spinal neurons prompted us to determine whether TET1 and/or TET3 also are expressed in spinal neurons. Immunofluorescence double staining showed that TET1 and TET3 were highly expressed in the spinal cords of CFA-treated mice. There was 85.5% overlap between NeuN and TET1 staining and 64.3% overlap between NeuN and TET3 staining in the CFA-treated mice (fig. 3C); however, TET1 was rarely expressed in spinal glial cells, whereas TET3 was expressed in some astrocytes, but not in microglial cells (data not shown). In addition, single-cell PCR showed that five of six spinal neurons expressed TET1 and four of six neurons expressed TET3 in CFA mice (fig. 3D), further evidence that most spinal neurons express TET1 or TET3.

We explored the potential effects of manipulating spinal TET1 or TET3 expression on nociceptive responses. For this purpose, we used siRNAs (T1-siRNA or T3-siRNA for exogenous down-regulation) and lentiviruses (Lenti-T1-siRNA or Lenti-T3-siRNA for endogenous down-regulation, and Lenti-T1 or Lenti-T3 for endogenous up-regulation). The lentivirus constructs were expressed mainly in spinal neurons but also were expressed in some spinal glial cells (data not shown). The transfection efficiencies of siRNAs and lentiviruses were validated in the spinal cords of naïve and CFA mice by RT-qPCR (all P < 0.031; see fig. S2A-D, Supplemental Digital Content 2, http://links.lww.com/ALN/B420). Before nociceptive behaviors were tested, mice performed vertical climbing tests to verify that TET1 and TET3 manipulation did not affect motor behaviors (data not shown); however, knockdown of TET1 and TET3 via

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**Fig. 3.** Complete Freund's adjuvant (CFA)-induced noiceptive sensitization increases the expression of spinal ten-eleven translocation methylcytosine dioxygenase (TET1) and TET3. (A) Messenger (m)RNA expression measured by real-time quantitative polymerase chain reaction at 3 days after CFA injection. All samples were normalized to Gapdh. n = 6/group; **P < 0.01 versus the corresponding control group (Ctrl) by two-tailed unpaired Student's t test. (B) Protein expression by Western-blotting after normalizing to β-actin. n = 6/group; **P < 0.01 versus the corresponding control group by two-tailed unpaired Student's t test. (C) Double staining of TET1 or TET3 with NeuN. Scale bar, 25 μm. Arrows indicate the positive overlayed signal. (D) Single-cell real-time polymerase chain reaction showing colocalization of TET1 or TET3 with NeuN. Nos. 1–6 represent six different neurons; No. 7 (N) is a negative control.

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intradiscal injections for 3 consecutive days of T1-siRNA or T3-siRNA and Lenti-T1-siRNA or Lenti-T3-siRNA reversed CFA-induced thermal hyperalgesia and mechanical allodynia (all \( P < 0.039 \)), but no effect was observed from injections of scrambled siRNA or Lenti-vector (fig. 4, A and B). To further determine the contribution of TET1 and TET3 in initiating nociceptive sensitization, we pretreated animals with Lenti-T1-siRNA or Lenti-T3-siRNA for 3 days before CFA injection and then assessed the preventive effect on nociceptive responses. We found that this pretreatment inhibited CFA-induced thermal hyperalgesia and mechanical allodynia (all \( P < 0.037 \); fig. 4C). Finally, we found that overexpressing TET1 and TET3 via intradiscal injections of Lenti-T1 and Lenti-T3, respectively, promoted thermal hyperalgesia and mechanical allodynia (all \( P < 0.047 \); fig. 4D). These findings suggest that spinal TET1 and TET3 contribute to the modulation of nociceptive sensitization.

Fos, the protein encoded by the protooncogene *c-fos*, has been used extensively as a marker for activity in activated nociceptive neurons in the spinal cord.\(^{31}\) In our genomic 5hmC profiling, we found no change in spinal 5hmC in the promoter of *c-fos* after CFA-induced nociceptive sensitization. Moreover, the knockdown of TET1 or TET3 with siRNA did not alter 5hmC content in the promoter of *c-fos* (data not shown), suggesting that Fos expression is not regulated by hydroxymethylation. Therefore, we further investigated the effect of TET1 and TET3 on CFA-induced spinal neuron activation by detecting spinal Fos expression. The results showed Lenti-T1-siRNA and Lenti-T3-siRNA, but not Lenti-vector, inhibited CFA-induced increases of Fos expression in the superficial and deep dorsal horn at 7 days after CFA injection (2 days after 3 continuous days of lentivirus injection; 123 ± 26 vs. 294 ± 6; \( P = 0.0031 \); and 140 ± 21 vs. 294 ± 60; \( P = 0.0043 \), respectively; fig. 5A). Pretreating animals with Lenti-T1-siRNA or Lenti-T3-siRNA for 3 days before the CFA injection reversed the increase in spinal Fos expression (220 ± 44 vs. 464 ± 98; \( P = 0.0041 \); and 264 ± 54 vs. 464 ± 98; \( P = 0.0063 \), respectively; fig. 5B), suggesting that knockdown of TET1 and TET3 reversed nociceptive sensitization by inhibiting spinal neuron activation. Conversely, overexpression of TET1 and TET3 with Lenti-T1 and Lenti-T3, respectively, increased spinal Fos expression in naïve mice (189 ± 37.6 vs. 100 ± 21.5; \( P = 0.0045 \); and 186 ± 38.7 vs. 100 ± 21.5; \( P = 0.0048 \), respectively; fig. 5C). Collectively, these findings suggest that spinal TET1 and TET3 contribute to nociceptive sensitization via regulating neuronal activation.

**TET1 and TET3 Regulate Nociception by Targeting Stat3**

TET proteins convert 5mC to 5hmC; therefore, we further investigated the role of TET1 and TET3 in producing spinal

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**Fig. 4.** The regulation of ten-eleven translocation methylcytosine dioxygenase (TET)1 and TET3 on nociceptive responses. (A, B) Intradiscal injection of Tet1-siRNA and Tet3-siRNA (A) or Lenti-T1-siRNA and Lenti-T3-siRNA (B) for 3 consecutive days reversed complete Freund’s adjuvant (CFA)-induced thermal hyperalgesia (paw withdrawal latency, PWL) and mechanical allodynia (paw withdrawal threshold, PWT). n = 6/group; two-way ANOVA (effect vs. group × time interaction) followed by post hoc Tukey test, PWL: \( F_{\text{group}} (40, 270) = 44.61 \), PWT: \( F_{\text{group}} (40, 216) = 6.91 \) (A); PWL: \( F_{\text{group}} (40, 270) = 44.78 \), PWT: \( F_{\text{group}} (40, 270) = 7.55 \) (B), \( * P < 0.05 \), ** \( P < 0.01 \). (C) Pretreatment with Lenti-T1-siRNA and Lenti-T3-siRNA for 3 consecutive days prevented CFA-induced thermal hyperalgesia and mechanical allodynia. Blue arrow indicates CFA or saline injection; black arrow indicates Tet1- and Tet3-siRNA/Scr or Lenti-T1-siRNA and Lenti-T3-siRNA/Lenti-vector injection. n = 6/group; two-way ANOVA (effect vs. group × time interaction) followed by post hoc Tukey test, PWL: \( F_{\text{group}} (45, 300) = 54.87 \), PWT: \( F_{\text{group}} (45, 300) = 24.82 \), \( * P < 0.05 \), ** \( P < 0.01 \). (D) Intradiscal injection of Lenti-T1 and Lenti-T3 for 3 consecutive days produced thermal hyperalgesia and mechanical allodynia in naïve mice. Black arrow indicates Lenti-T1 and Lenti-T3 or vector injection. n = 6/group; two-way ANOVA (effect vs. group × time interaction) followed by post hoc Tukey test, PWL: \( F_{\text{group}} (14, 120) = 18.4 \), PWT: \( F_{\text{group}} (14, 120) = 9.18 \), \( * P < 0.05 \), ** \( P < 0.01 \). Scr = scrambled; T1 = TET1; T3 = TET3.
5hmC in specific gene loci. First, we carried out a cross-analysis for 5hmC and 5mC densities in promoter CGI with 2-fold changes in CFA and control groups. We identified 337 genes with a greater than 2-fold 5hmC gain (hMeDIP-Seq score > 30) and 3,295 genes with a greater than 2-fold 5mC loss after crossover analysis and obtained 103 genes with 5hmC gain and 5mC loss (fig. 6A). Among these 103 genes (data not shown), there were only two transcript factors, Gata2 and Stat3, that play important roles in turning gene transcription on and off by binding to gene promoters. RT-qPCR analysis revealed that mRNA levels of Gata2 and Stat3 were up-regulated in spinal cords of CFA-treated mice (247 ± 52.1 vs. 100 ± 21.2; P = 0.00057; fig. 6B). Stat3, however, showed greater differences than Gata2 in 5hmC and 5mC levels (data not shown) and mRNA expression (fig. 6B) between CFA-treated and control groups. Furthermore, we
found that 5hmC was increased but 5mC decreased in a 238-bp CGI in the Stat3 promoter in CFA versus control groups via hMeDIP-Seq profiling (fig. 6C). These results were verified by ChIP-qPCR (data not shown). A previous finding indicated that STAT3 may be involved in the development of chronic pain.20 How the expression of Stat3 is regulated during nociceptive sensitization, however, remains unclear. Therefore, we chose to further investigate the role of Stat3 in regulating nociceptive sensitization mediated by TET1 and TET3.

We examined the colocalizations of STAT3 with TET1 and TET3 and the binding capacities of TET1 and TET3 to the Stat3 promoter in spinal neurons. We first examined the expression of Stat3 in spinal neurons via immunofluorescence double staining and single-cell PCR. Double-staining results showed that there was ~28.0% overlap between NeuN and STAT3 staining in the control group and 64.7% overlap between NeuN and STAT3 in the CFA group (fig. 6D). These findings were supported by the results of single-cell PCR showing that three of six spinal neurons expressed Stat3, that these three cells coexpressed TET1, and that two of them coexpressed TET3 (fig. 6E). ChIP-PCR using TET1 and TET3 antibodies showed a 2-fold increase in the amounts of Stat3 that were pulled down in CFA versus control groups; however, no differences were observed in the input or negative control sample (see fig. S3A, Supplemental Digital Content 2, http://links.lww.com/ALN/B420). Together, these results suggest Stat3 is coexpressed with TET1 or TET3 in spinal neurons and the promoter of Stat3 is bound by TET1 and/or TET3 in spinal cells.

To determine whether 5hmC in the Stat3 promoter is catalyzed by TET, we used in vitro and in vivo strategies (fig. 7, A and G). First, we evaluated whether hydroxymethylation of the Stat3 promoter increases gene transcription in vitro. We cloned two segments of the Stat3 promoter, including a 1,511-bp segment containing the TSS (~1365 to
DNA Hydroxymethylation Regulates Nociception

Fig. 7. Ten-eleven translocation methylcytosine dioxygenase (TET)1- and TET3-mediated DNA hydroxymethylation of Stat3 promoter regulates spinal signal transducer and activator of transcription (STAT3) expression and nociceptive behavior. (A) The activities of methylated or demethylated promoter of the cloned Stat3 promoter encompassing transcription start site (TSS) or not were detected by firefly luciferase (LuC) reporter assays in HEK 293T cells. Empty vector (pGL6) plasmid was used as the negative control. pGL6-Stat3-TSS and pGL6-Stat3 are plasmids with a Stat3 promoter containing the ATG transcript site or not, respectively. Values of luciferase activities for each plasmid were normalized for transfection efficiency by cotransfecting with pRL-TK plasmid. n = 3 per group; two-way ANOVA (effect vs. plasmid × treated interaction) followed by post hoc Tukey test, Fgroup (4, 18) = 13.63, *P < 0.05, **P < 0.01, ***P < 0.001. (B, C) CFA increased spinal 5-hydroxymethylcytosine (5hmC) levels and decreased spinal 5-methylcytosine (5mC) level of Stat3 promoter, which was reversed by Tet1-siRNA and Tet3-siRNA delivered by lentivirus. White and black dots represent demethylated and methylated CpG dinucleotides, respectively. Each line indicates an individual sequence. 5hmC represents the ratio of (CC hmGG) to (CC hmGG, CC mGG, and CCGG) in the detection fragment; n = 5/group; one-way ANOVA (expression vs. the treated groups) followed by post hoc Tukey test, Ftime (5, 24) = 28.06, *P < 0.05, **P < 0.01. (D, E) Lentivirus-mediated overexpression of TET1 and TET3 increased the 5hmC level and decreased the 5mC level in naïve mice. n = 5/group; one-way ANOVA (expression vs. the treated groups) followed by post hoc Tukey test, Fgroup (2, 12) = 9.1, *P < 0.05, **P < 0.01. (F) The down-regulation of TET1 and TET3 mediated by lentivirus reversed the increased STAT3 expression in CFA-treated mice. n = 5/group; one-way ANOVA (expression vs. the treated groups) followed by post hoc Tukey test, Ftime (4, 20) = 8.03, *P < 0.05, **P < 0.01. (G) TET1 and TET3 overexpression via lentivirus infection increased Stat3 expression in naïve mice. n = 5/group; one-way ANOVA (expression vs. the treated groups) followed by post hoc Tukey test, Ftime (3, 16) = 7.52, *P < 0.05. (H) Intrathecal injection of Stat3-siRNA for 2 consecutive days reversed CFA-induced thermal hyperalgesia and mechanical allodynia. Blue arrow indicates CFA or saline injection; black arrow indicates Stat3-siRNA or scrambled (Scr) injection. n = 6/group; two-way ANOVA (effect vs. group × time interaction) followed by post hoc Tukey test, paw withdrawal latency (PWL): Fgroup (18, 140) = 56.56, paw withdrawal threshold (PWT): Fgroup (18, 140) = 11.29, **P < 0.01. (I) Stat3-siRNA markedly inhibited thermal hyperalgesia and mechanical allodynia induced by Lenti-T1 and Lenti-T3 injection in naïve mice. Stat3-siRNA was injected intrathecally 2 days after pain behavior testing. Blue arrow indicates Stat3-siRNA or Scr injection; black arrow indicates Lenti-T1 and Lenti-T3 injection. n = 6/group; two-way ANOVA (effect vs. group × time interaction) followed by post hoc Tukey test, PWL: Fgroup (10, 90) = 19.72, PWT: Fgroup (10, 90) = 9.84, *P < 0.05, **P < 0.01. 5C = unmethylated promoter; SV40 = SV40 promoter.

+146bp) and a 1,361-bp segment without the TSS (−1365 to −5bp), into a pGL6 luciferase reporter (pGL6-S3-TSS and pGL6-S3, respectively) and examined their abilities to drive luciferase expression in HEK 293T cells. We found that both segments produced greater luciferase activities than the empty vector; however, pGL6-S3-TSS generated stronger activity than pGL6-S3 (pGL6-S3-TSS: 248.1 ± 47.7 vs. 100 ± 19.5; P = 0.00068; fig. 7A), indicating the cloned
pGL6-S3-TSS region contains the efficient regulatory element and controls Stat3 expression. Moreover, the methylation of pGL6-S3-TSS in vitro by the CpG methyltransferase before introducing it into cells abolished the enhancement in luciferase activity (105 ± 20.5 vs. 248.1 ± 47.7; P = 0.0032; fig. 7A). This effect of methylation was reversed by hydroxymethylation, which was generated in vitro from the methylated pGL6-S3-TSS via TET protein catalysis (186.2 ± 36.6 vs. 105 ± 20.5; P = 0.006; fig. 7A). These results suggest a direct positive role of hydroxymethylation of the pGL6-S3-TSS promoter in transcriptional efficiency. Second, we quantitatively analyzed in vivo the changes of 5hmC and 5mC in the Stat3 promoter after Tet1 and Tet3 knockdown in CFA-treated mice and after overexpression in naïve mice. We found that lentivirus-mediated knockdown of TET1 and TET3 reversed the increase of 5hmC (75 ± 16.1 vs. 156 ± 28.9; P = 0.0043; and 91 ± 19.1 vs. 156 ± 28.9; P = 0.0066, respectively; fig. 7B) and the decrease of 5mC in spinal cords of CFA-treated mice (fig. 7C), which was accompanied by an inhibition of STAT3 expression at the mRNA (data not shown) and protein (93 ± 19.6 vs. 137 ± 27.5; P = 0.035; and 72 ± 15.2 vs. 137 ± 27.5; P = 0.0028, respectively; fig. 7F) levels. Lentivirus-mediated overexpression of spinal TET1 and TET3 significantly increased 5hmC levels (148 ± 28.7 vs. 100 ± 18.8; P = 0.0085; and 132 ± 25.5 vs. 100 ± 18.8; P = 0.046, respectively; fig. 7D) but reduced the 5mC content (fig. 7E) and increased the expression of STAT3 at the mRNA (data not shown) and protein (93 ± 19.6 vs. 137 ± 27.5; P = 0.040; and 72 ± 15.2 vs. 137 ± 27.5; P = 0.038, respectively; fig. 7G) levels in naïve mice. Collectively, these results suggest that TET1 and TET3 regulate spinal Stat3 expression by converting 5mC to 5hmC in its promoter during nociceptive sensitization. To further determine whether the increased expression of STAT3 contributed to the regulation of nociceptive sensitization, we tested pain behaviors after knockdown of STAT3 via intrathecal injections of siRNA in CFA-treated mice. We found that Stat3-siRNA not only knocked down the mRNA and protein expressions of STAT3 in naïve mice but also reversed the increase in STAT3 protein in CFA-treated mice, demonstrating the efficiency of Stat3-siRNA knockdown (see fig. S3B, Supplemental Digital Content 2, http://links.lww.com/ALN/B420). Climbing tests showed that the knockdown of Stat3 with siRNA did not affect motor function (data not shown). Compared with in the scramble group, however, Stat3-siRNA significantly alleviated pain induced by CFA (all P < 0.042; fig. 7H), suggesting that spinal STAT3 plays an important role in nociceptive sensitization.

Finally, to explore the role of STAT3 in mediating pain via TET1 and TET3, we pretreated or posttreated animals with siRNA to knockdown STAT3 before or after, respectively, intrathecal injections of Lenti-T1 and/or Lenti-T3, and then measured the behavioral responses. We found that knockdown of STAT3 significantly inhibited or reversed nociceptive responses induced by TET1 and/or TET3 overexpression in naïve mice (all P < 0.046; fig. 7I), suggesting that STAT3 mediates the regulation of pain by TET. Taken together, these results indicate that hydroxymethylation of the Stat3 promoter mediated by TET1 and TET3 regulates nociceptive hypersensitivity via spinal neuron sensitization (fig. 8). In this study, 16 mice had to be excluded from the statistical analysis because of poor health or accidental death caused by anesthesia.

Discussion

Central sensitization describes a state in which central synapses become hyperresponsive to extracellular nociceptive and/or nonnociceptive stimuli, and it is thought to play a critical role in the pathogenesis of chronic pain. The induction and maintenance of central sensitization is associated closely with the release of neurotransmitters and the activation of spinal ion channels, receptors, and intracellular signal

Fig. 8. Schematic of regulation of nociceptive sensitization by ten-eleven translocation methlysytosine dioxygenase (TET)1 and TET3-mediated hydroxymethylation of the Stat3 promoter. 5mC = 5-methylcytosine; RNAPII = RNA polymerase II; STAT3 = signal transducer and activator of transcription 3.
transduction pathways. Aberrant pain-related gene expression is the molecular basis for central sensitization of spinal networks. Therefore, uncovering the mechanisms of gene regulation that underlie central sensitization will improve our understanding of chronic pain and may provide potential targets for developing new therapeutic strategies. DNA demethylation mediated by pharmacologic inhibitors, as a disruptive active mechanism of DNA methylation, has attracted a great deal of attention for its regulatory function in not only diverse neurologic diseases but also neuro-inflammation processes. 7,34 For example, treatment with the demethylating agent 5′-aza-2′-deoxycytidine, as a passive demethylation strategy, markedly attenuates nociceptive behaviors and spinal neuronal sensitization by reducing the methylation and subsequently increasing the expression of spinal miRNA-219. 2 Interestingly, in this study, we demonstrated that knockdown of TET, an active demethylating enzyme, significantly reversed nociceptive behavior by decreasing the sensitivity of spinal neurons mediated by an increase in Stat3 methylation. In addition, to our knowledge, this is the first evidence for an active demethylating mechanism in a chronic inflammatory pain model.

Indeed, recent growing evidence has confirmed a dependent linkage between global 5hmC alteration and neurologic processes and psychiatric diseases. Global 5hmC is reduced markedly both in terminally differentiated mouse Purkinje neurons and in mouse brain tissues of the YAC128 model of Huntington disease. The loss of 5hmC impairs neurogenesis in Huntington disease brain. 28 In contrast, gains in global 5hmC have been found in different CNS areas, including in embryonic mouse brains, aging mouse hippocampi, and spinal cords from amyotrophic lateral sclerosis disease. 3 By demonstrating a determining function of genomic 5hmC in the development of CNS disease, these studies provide the rationale to further study the role of 5hmC in neuronal dysfunction in a variety of CNS diseases. Interestingly, the loss or gain of 5hmC has been confirmed as a novel epigenetic feature in CNS diseases.

For example, mutant huntingtin is associated with a marked reduction in the 5hmC landscape, indicating that the loss of the 5hmC marker is a new epigenetic feature of Huntington disease. Therefore, reestablishing 5hmC levels and landscape may slow/halt the progression of Huntington disease. 28 In this study, we observed a change in spinal 5hmC that was similar to the increase seen in peripheral blood during nociceptive sensitization. Interestingly, 5hmC immunostaining was distributed among the neurons of the superficial and deep dorsal horn and the ventral horn in spinal slices, suggesting 5hmC in dorsal and ventral horn spinal neurons may be involved in nociceptive information processing. Whether the alteration of 5hmC in deep dorsal or ventral horn neurons contributes to nociceptive sensitization deserves further study in the future. Interestingly, there was extremely high 5hmC in the Y sex chromosome after CFA injection. As all of the experiments were performed in male mice, future study is needed to determine if there are sex differences in 5hmC in response to nociception.

TET family proteins have been shown to specifically catalyze the demethylation of 5mC to 5hmC, as well as its further oxidation into 5-formylcytosine and 5-carboxycytosine. 38,39 TET1 and TET3 are abundantly expressed in mouse embryonic stem cells and CNS neurons, including in the inferior parietal lobule (BA39-40) in psychotic patients. 40 TET1 knockout in mice reduced the 5hmC content in hippocampal neurons, resulting in abnormal hippocampus long-term depression and impaired memory extinction or spatial memory deficits. 34 Although TET2 is enriched in several CNS regions, the association between TET2 and CNS-related physiologic and pathologic processes remains unclear. TET2 is a tumor suppressor and is implicated specifically in the production and development of hematopoietic stem cells from animals and humans. 41-43 It is possible that TET2 protein was expressed in low levels in mouse spinal cords in this study. In addition, it is demonstrated that TET3 plays a critical role in neural progenitor cell maintenance, terminal differentiation, and neuronal development. Embryonic stem cells lacking Tet3 have normal self-renewal and maintenance but impaired neuronal differentiation. 44,45 In contrast, TET3 overexpression rescues miRNA-15b-induced impairment of cortical neural progenitor cell proliferation, which is responsible for neuronal differentiation. 46 Moreover, a behavioral study reveals that neocortical TET3-mediated accumulation of 5hmC contributes to a rapid behavioral adaptation in extinction learning. 47 In addition, TET1 and TET3 are increased significantly in the cerebella of autistics and are accompanied by increases in global 5hmC, suggesting a cooperative role of TET1 and TET3 in autism disease. 48 In our previous study, we found that nociceptive behavior induced by formalin increased the expression of spinal TET1 and TET3, and manipulating TET1 and TET3 alleviated nociceptive injury via miRNA-365-3p hydroxymethylation. 19 It was not known, however, whether TET proteins regulate chronic inflammatory pain. The results of this study show that CFA-induced nociceptive sensitization increased the expression of TET1 and TET3 in the spinal cords of mice. Conversely, knockdown of TET1 and TET3 via endogenous or exogenous siRNA alleviated nociceptive responses. These results are consistent with those from a recently published report in which spinal nerve ligation up-regulated TET1 expression in rat spinal neurons and knockdown alleviated nociceptive responses induced by nerve injury. 49 These data reveal a close correlation between TET1 or TET3 and nociceptive processes; consequently, our data expand the functional role of TET in the modulation of CNS diseases. It is worth noting, however, that the recent accumulating data have shown that intrathecal injections of the DNA methyltransferase inhibitor 5′-aza-2′-deoxycytidine significantly attenuated hyperalgesia behaviors induced by CFA and chronic constrictive injury. 2,3,50 Thus, compared with inhibitors of the demethylating enzyme TET in analgesic function, two proteins with converse functions in DNA methylation have the same effect of inducing hyperalgesia behaviors.
Therefore, we speculated that different methylation enzymes may be epigenetically responsible for differential gene regulation in nociceptive processes and analgesic responses, suggesting that the epigenetic mechanism underlying the pain process comprises a complicated integrated network.

In addition, in this study, our genomic Integrated Genomics Viewer results indicated that 5hmC-enriched loci occurred mainly in the promoters and bodies of different genes, suggesting that intragenic 5hmC enrichment is likely a positive epigenetic regulator for gene expression. These findings are in agreement with those from a recent report showing that 5hmC is associated with activated genes and actively transcribed genes in CNS tissues, such as the cerebellum and hippocampus of mouse brains. As transcription factors regulate the rate that genetic information is transcribed from DNA to mRNA via binding to specific DNA sequences, they are key players in gene expression. We analyzed the 5hmC level of transcription factors among 103 screened genes and found that Stat3 was actively transcribed by the enriched 5hmC in its promoter during nociceptive sensitization. STAT3, as a primary signal transducer and activator of transcription, plays an important role in a variety of neurophysiologic and neuropathologic processes, such as neuronal development, plasticity, and CNS diseases. Emerging lines of evidence suggest a potential role for STAT3 in developing and maintaining nociceptive sensitization in various types of chronic pain models, including neuropathic pain, inflammatory pain, and cancer pain. Rat spinal nerve ligation or bilateral chronic constriction injury increases the mRNA and protein levels of spinal Stat3 and Janus kinase, an upstream key active enzyme of STAT3; the increased Janus kinase further amplifies the level of active phosphorylated STAT3. Intrathecal administration of STAT3 inhibitors (AG490 or WP1066) or Janus kinase inhibitor I significantly reduced established thermal hyperalgesia and mechanical allodynia. Although a large body of molecular and functional evidence links STAT3 with nociceptive information processing, little is known about how Stat3 expression is regulated in these processes. In this study, our data indicated that knockdown of TET1 and TET3 markedly reversed the enhanced 5hmC level, which was accompanied by a decrease in STAT3 expression and an alleviation of nociceptive behavior in CFA-treated mice. TET-mediated demethylation of Stat3 and its functional significance in nociceptive sensitization provide novel potential pharmacotherapeutic target. Interestingly, STAT3 is found to be frequently activated in microglial or astrocyte cells of the spinal cord in a neuropathic pain model induced by spared nerve injury, spinal nerve ligation, or inflammatory pain by lipopolysaccharide. In this study, Stat3 was found via single-cell PCR to be coexpressed with TET1 and TET3 in spinal neurons of mice. Furthermore, manipulating TET1 and TET3 expression significantly changed the 5hmC level of the Stat3 promoter and its subsequent expression in the spinal cord; however, we found that TET3 also was expressed in some astrocytes. Whether TET3 functionally regulates 5hmC production in astrocytes in nociceptive processes needs to be investigated in the future.

In conclusion, our findings not only reveal an unknown epigenetic mechanism involved in a chronic inflammatory pain model but also provide a new insight into how TET1 and TET3 epigenetically regulate Stat3 expression in the spinal cord. It may significantly contribute to potential treatment strategies for chronic pain.

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Competing Interests

The authors declare no competing interests.

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