Methyltransferase-like 3 contributes to inflammatory pain by targeting TET1 in YTHDF2-dependent manner

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Abstract
The methyltransferase-like 3 (Mettl3) is a key component of the large N6-adenosine-methyltransferase complex in mammalian responsible for RNA N6-methyladenosine (m6A) modification, which plays an important role in gene post-transcription modulation. Although RNA m6A is enriched in mammalian neurons, its regulatory function in nociceptive information processing remains elusive. Here, we reported that Complete Freund’s Adjuvant (CFA)-induced inflammatory pain significantly decreased global m6A level and m6A writer Mettl3 in the spinal cord. Mimicking this decrease by knocking down or conditionally deleting spinal Mettl3 elevated the levels of m6A in ten-eleven translocation methylcytosine dioxygenases 1 (Tet1) mRNA and TET1 protein in the spinal cord, leading to production of pain hypersensitivity. By contrast, overexpressing 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 behavior. Furthermore, the decreased level of spinal YTS21-B homology domain family protein 2 (YTHDF2), an RNA m6A reader, stabilized upregulation of spinal TET1 because of the reduction of Tet1 mRNA decay by the binding to m6A in Tet1 mRNA in the spinal cord after CFA. This study reveals a novel mechanism for downregulated spinal cord METTL3 coordinating with YTHDF2 contributes to the modulation of inflammatory pain through stabilizing upregulation of TET1 in spinal neurons.

Keywords: Inflammatory pain, Mettl3, Tet1, Ythdf2, Spinal dorsal horn

1. Introduction
N6-methyladenosine (m6A) is one of the most abundant RNA modification1,2,5,6,5 found in over 25% of mammalian mRNA.3,4,24,35 Growing bodies of studies confirm the linkage between m6A and mRNA splicing in nucleus57 or mRNA degradation12,55,65 and translation efficiency.47,53 m6A has been proven to be associated with adult neurogenesis,22 neuronal synapses formation,1 brain development, memory formation and consolidation,49,59 and peripheral nerve injury process.55 However, whether and how m6A modification regulates nociception remains unknown.

RNA m6A is wrote by the large RNA methyltransferase complex containing methyltransferase-like 3 (Mettl3), METTL14,32 WTAP,42 and erased by demethylases including fat-mass and obesity-associated proteins (FTO)22 and ALKBH5.66 Peripheral nerve injury increases the expression of FTO and reduces the level of FTO-controlled m6A in Ehm2 mRNA in the injured dorsal root ganglia (DRG).29 Blocking this increase rescues the nerve injury-induced loss of m6A and alleviates nerve injury-associated pain hypersensitivities, suggesting that FTO contributes to neuropathic pain in RNA m6A-dependent manner.39 Methyltransferase-like 3 is identified as a key catalytic component of m6A “writer” complex. Knockdown (KD) of Mettl3 recapitulates hippocampal deficiency, resulting in dysfunction of memory formation and consolidation.45 Mice lacking Mettl3 display the loss of m6A in cortical and cerebellar regions, resulting in the half-lives extension of development-associated and apoptosis-associated genes mRNAs. Consequently, METTL3-mediated m6A serves as an important regulator in the mammal central nervous system.50

RNA m6A is read by YTS21-B homology domain family (YTHDF) proteins, which can exert different functions in m6A modifications.34,53,65 YTS21-B homology domain family contains 3 members: YTHDF1, 2, and 3, with an evolutionarily conserved YTH domain selectively recognizing and binding m6A.59 YTS21-B homology domain family or 3 is believed to enhance translation efficiency,5,26,55 YTHDF2 to destabilize or degrade RNA.28,63 Increasing studies implicate YTHDF proteins in regulating central nervous system (CNS) function. Ythdf1-deficiency attenuates injury-induced global protein translation and reduces functional axon...
Ten-eleven translocation methylcytosine dioxygenases 1 (Tet1) is an endogenous nociception initiator by converting 5-methylcytosine in DNA to 5-hydroxymethylcytosine. Increased levels of m6A-modified mRNAs in the neural stem/progenitor cell, displaying the delayed cortical neurogenesis during embryonic mouse development. However, it remains unknown whether Ythdf2 can participate in nociceptive processing in the spinal cord.

In the current work, we found significant decreases of RNA m6A, Mettl3, and Ythdf2 in mice spinal cords in a Complete Freund’s Adjuvant (CFA)-induced inflammatory pain model that mimics clinical symptoms in arthritic patients. Moreover, the m6A level of Tet1 mRNA was decreased. Therefore, we hypothesized that downregulated spinal Mettl3 contributes to inflammatory pain by increasing m6A level of Tet1 mRNA in a Ythdf2-dependent manner. Here, we show that Mettl3 regulates Tet1 as a novel epigenetic mechanism under chronic inflammatory pain conditions.

2. Material and methods

2.1. Conditional knockout mice

Three strains’ mice were used: Shanghai populations of Kunming mice, Tet1fl/fl C57BL/6 mice, and Mettl3fl/fl C57BL/6 mice (Cyagen Biosciences, Guangzhou, China). Pups were kept with their dams after birth and weaned at postnatal day 21, then group housed by sex with 4 to 5 mice per cage. The genotype of each mouse was determined by the genomic DNA extracted from tail tip tissue. All mice used in the experiment were 6–8-week-old male mice, maintained at 23 ±3°C with 35% w10% relative humidity, on a 12 hours light/dark cycle (lights on 6:00, lights off 18:00), and libitum accessed to food and water.

2.2. Pain models

Complete Freund’s Adjuvant-induced chronic inflammatory pain and chronic constriction injury (CCI) of sciatic nerve were performed as described previously.36

2.3. Behavioral tests

Hyperalgesia and alldynia were measured using paw withdrawal latency to a thermal stimulus and paw withdrawal thresholds to a mechanical stimulus as described previously.37 Cold response to hypersensitivity was performed as described.19 The conditional place preference (CPP) test was performed as described.30 All behavioral tests were performed in a double-blind trial fashion.

2.4. Locomotor function

Three reflex tests, including grasping, placing, and righting reflex, and their scores analysis were performed as described before.36,49,63

2.5. Spinal tissue collection

Mice were anesthetized with 10% chloral hydrate, and the spinal cord within the lumbar segments (L3-L5) was removed rapidly. The ipsilateral or contralateral dorsal spinal cord was separated and snap frozen in liquid nitrogen and stored at −80°C.

2.6. RNA extraction and quantitative real-time polymerase chain reaction

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) were performed according to described previously.37 In brief, total RNA was extracted with Trizol (9190, Takara) and reversely transcribed into cDNA with oligo (dT) or random oligo for Mettl3, Mettl14, Alkbh5, Fto, Wtap, Tet1, Dnmt1, Dnmt3a, and Dnmt3b and reverse transcription-M-MLV (2641A, Takara). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene. All PCR primers were seen in Table S1, http://links.lww.com/PAIN/B283. The expression levels of the target genes were quantified using the 2−ΔΔCt analysis.

2.7. RNA m6A dot-blot assay

RNA m6A dot-blot assay was performed as described before55,61 with minor modification. mRNA was harvested from homogenized dorsal spinal cord tissue of mice by the use of Dynabeads mRNA Direct Purification Kit (61011, Ambion, Austin, TX). The primary anti-m6A antibody (ABE572, Millipore, Billerica, MA) was used to pull down the m6A-modified RNA fragments. Quantified m6A levels were normalized to the amount of mRNA loaded.

2.8. Plasmid construction

Plasmid constructions were prepared according to previously described methods.36,37 To construct Mettl3 and Ythdf2 overexpression (OE) vectors, one insert prepared by PCR using primer pairs (Table S1, http://links.lww.com/PAIN/B283) and PWPXL vector were digested by corresponding double restriction endonucleases (NEB), and then ligated with T4 ligase. To construct Mettl3 KD vector, the synthesized LV-M3F and LV-M3R oligos (Table S1, http://links.lww.com/PAIN/B283) were annealed and ligated to the digested PLVTHM vector. Our Tet1 KD vector37 was used in this study. All constructs were confirmed by Sanger sequencing.

2.9. Lentivirus or recombinant adeno-associated virus

Lentivirus production and verification were performed as described before.46 Lentivirus with titers 1 Le106 TU/mL was used in the experiment. The assays of lentivirus in vitro and in vivo infection were performed according to a previous study.39 The recombinant adeno-associated virus rAAV-hSyn-Cre and rAAV-hSyn-Gfp with 1015/g/mL litter were bought from Brainvta Brain Science and Technology Co, Ltd, Wuhan, China.

2.10. Small interfering RNA and virus delivery

Intrathecal injections of Small interfering RNA (siRNA) or virus were performed according to previously described methods.37 Injections of 5 μL of 20 μM siRNAs for Mettl3, Tet1, Ythdf2, or Scramble were used here. All siRNA sequences were seen in Table S1, http://links.lww.com/PAIN/B283. One microtitre Lentivirus or rAAV were performed daily. Knockdown by Mettl3-siRNA, Tet1-siRNA, Ythdf1-siRNA, and LV-Mettl3 was confirmed.
with qRT-PCR from samples of the ipsilateral dorsal spinal cord. Mice receiving intrathecal injection of scrambled siRNA or empty vector were used as control groups.

2.11. RNA immunoprecipitation
RNA immunoprecipitation was performed according to the previously described with a few modifications. In brief, equal weight mice spinal cord tissues from 3 different individuals were washed in precooled PBS buffer and then digested in nuclear isolation buffer. Tissues were mechanically sheared using a homogenizer. Nuclei were precipitated and resuspended in RIP buffer. After centrifugation, the supernatant was collected and split into 3 fractions for input, IP, and negative control. Antibody to Tet1 was added into a 12 mL tube (Beckman) gently. Each weight mice spinal cord tissues from 3 different individuals were washed in freshly prepared lysis buffer. Tissues were mechanically sheared using a precooled PBS buffer for 3 times and then digested in freshly prepared lysis buffer. Each group was added to the supernatant and incubated overnight. Protein G Magnetic Beads (S1430S, NEB, Beijing, China) were added and incubated. Beads were collected and resuspended in RIP buffer for 3 times followed by one wash in PBS. Coprecipitated RNAs were isolated by RNA Isolator extraction reagent (R401-01, Vazyme, Nanjing, China). qRT-PCR was used to detect the m6A level in different regions of the Tet1 mRNA. 8 pairs of PCR primers for the Tet1 CDS region and 6 pairs of PCR primers for the Tet1 3' UTR region were seen in Table S1, http://links.lww.com/PAIN/B283. The fifth region primer pairs in 3'UTR was used to evaluate the binding level of YTHDF2 to Tet1 mRNA.

2.12. Measurement of newly synthesized protein
Measurement of global protein synthesis was described previously. To summarize, puromycin (10 mg/kg) were intraperitoneally injected 1 hour before spinal tissues collection for Western blot. Extracted protein samples (40 μg/sample) were separated by 10% SDS polyacrylamide gel electrophoresis and transferred onto PVDF membranes. Membranes were blocked in 5% no-fat dry milk for 1.5 hours and incubated simultaneously in mouse antipuromycin antibody (1: 1000; MABE343, Millipore) at 4°C overnight. HRP-labeled Goat Antimouse IgG (1:2000; A0216, Beyotime) was then applied at room temperature for 1.5 hours. Membranes were washed by stripping buffer and reblotted with rabbit anti-GAPDH antibodies (1:2000; 10494-1-AP, Proteintech) followed by HRP-labeled Goat AntiRabbit IgG (1:2000; A0208, Beyotime) as the loading control. Newly synthesized protein was quantified by detecting immune complexes signal intensities approximately ranged from 15 to 220KD. Band intensities of the target signals normalized to those of GAPDH for statistical analyses.

2.13 Polyrribosome fractionation assay
According to the previously described with few modifications, 5 different sucrose gradient solutions (47, 37, 27, 17, and 7% in Tris-HCl) were added into a 12 mL tube (Beckman) gently. Each gradient was 2.2 mL with a progressively decreasing sucrose concentration from the bottom of the tube. Equal weight mice spinal cord tissues from 3 different individuals were washed in precooled PBS buffer for 3 times and then digested in freshly prepared lysis buffer. Tissues were mechanically sheared using a homogenizer and centrifuged, and 500 μL supernatant were added on the top of the tube. SW41Ti rotor was installed for 36,000 rpm centrifugation which lasted for 2hours at 4°C. After centrifugation, solutions in the tube were split into 15 equal fractions of 760 μL each. Components were fully mixed, and 400 μL of which was subjected to 1.2 mL RNA Isolator total RNA extraction reagent (R401-01, Vazyme) for RNA extraction. The above fifth region primer pairs in 3'UTR was used to measure the polyrribosome fractionation level.

2.14. Spinal neuron culture
The primary culture of spinal neurons was performed as described previously. In brief, after decapitation of 3-d-old to 4-d-old mice under deep anesthesia, a laminectomy was performed, and the lumbar L3 to L5 segments of the dorsal spinal cord was cut with a razor blade. After enzymatic digestion with papan and mechanical dissociation, the homogenate was centrifugated; the supernatant was removed and replaced with culture medium. After trituration with a fire-polished Pasteur pipette, the cells were plated on plastic culture dishes. Cultures were maintained in a water-saturated atmosphere (95% air, 5% CO2) at 37°C until used (10-15 d). Two days after the cells were seeded, cytosine arabinoside was added to the culture medium for 24 hours to reduce glial proliferation.

2.15. Single-cell reverse transcription polymerase chain reaction
Single-cell RT-PCR for spinal neurons was performed according to previously described method with minimal modifications. In brief, the contents of dissociated spinal neurons from mice were harvested into patch pipettes with the tip, placed gently into reaction tubes with DNase I, and heated to remove genomic DNA. Reverse transcriptase (SuperScript III Platinum, Invitrogen) and specific reverse outer primer was added to reversely transcribe. The cDNA products were used in gene-specific nested PCR. The first-round PCR was performed with the outer primer pair (outF and outR). The second round of PCR was performed using the first PCR product as the template and with inner PCR primers (inF and inR). A negative control was obtained from pipettes that were submerged in the bath solution alone. Gapdh was used as the reference gene. All single-cell PCR primers are shown in Table S1, http://links.lww.com/PAIN/B283.

2.16. Western blot analysis
According to our described method, proteins (20–50 μg/sample) were separated, transferred onto nitrocellulose membranes, and incubated in the corresponding antibodies against the following: METTL3 antibody (1:1000; ab195352; Abcam, Cambridge, MA), TET1 antibody (1:1000; GTX627420; Genetex, San Antonio, MA), TET3 antibody (1:1000; 61744; Active motif, Carlsbad, CA), DNMT1 antibody (1:1000; A16729; AbClonla, DNMT3a antibody (1:1000; A16834; AbClonla, Wuhan, China), DNMT3b antibody (1:1000; A11079; AbClonla), YTHDF2 (1:2000; 24744-1-AP; Proteintech), STAT3 antibody (1:1000; 9139; Cell Signal Technology, Danvers, MA), C-FOS (1:1000; 2250; Cell Signal Technology, Danvers, MA), C-FOS antibody (1:2000; 2250; Cell Signal Technology), or control tubulin antibody (1:2000; 10094-1-AP; Proteintech) or GAPDH antibody (1:2000; 10494-1-AP; Proteintech). The membranes were then washed and incubated with HRP-labeled Goat Antimouse IgG (1:2000; A0216; Beyotime, Haimen, China) or HRP-labeled Goat AntiRabbit IgG (1:1000; A0208, Beyotime). The immune complexes were detected with a nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phospho-phosphate assay kit (72091, Sigma-Aldrich, St. Louis, MO).
2.17. Double-labeling immunofluorescence
L3-L5 spinal cord were rapidly dissected from mice perfused with 4% paraformaldehyde, and postfixed with 4% paraformaldehyde for overnight, followed by dehydration in 30% sucrose overnight, and were cut transversely and consecutively sectioned into 15-μm slices. After blocking at room temperature for 1 hour and PBS washing, the slices were incubated with mouse METTL3 antibody (1:1000; ab195352; Abcam) mixed with NeuN antibody (1:2000; MAB377; Millipore), or GFAP antibody (1:2000; 3670S; Cell Signal Technology) or IBA1 antibody (1:1000; GTX632426; Genetex), or TET1 antibody (1:1000; GTX627420, Genetex), respectively, at 4°C overnight or 48 hours. After PBS washing, slices were finally incubated with fluorescent-conjugated secondary antibodies Alexa Fluor 488 donkey antirabbit IgG (A21206; Molecular Probes, Eugene, OR) and Alexa Fluor 546 donkey antimouse IgG (A10036; Molecular Probes). The slides were mounted on a mounting medium with DAPI and scanned by Zeiss LSM 880 confocal microscope (Zeiss, Jena, Germany).

2.18. Statistical analysis
Statistical analysis was performed as described. All data were presented as mean ± SEM. The statistical analysis was performed with a 1-way or 2-way analysis of variance or paired or unpaired Student’s t test. P < 0.05 was considered statistically significance in all analyses.

3. Results
3.1. Complete Freund’s Adjuvant-induced inflammatory pain decreases methyltransferase like 3 level in the spinal cord
To detect the role of spinal RNA m^6^A modification in inflammatory pain, we first analyzed the change of m^6^A abundance, methyltransferases—Mettl3, Mettl14, and Wtap, and demethylases—Fto and Alkbh5 in the spinal cord after subcutaneous injection of CFA into the plantar surface of the left hind paw as described previously. This model can mimic some key features of human arthritic pain. Dot-blot revealed m^6^A was changed in a time-dependent manner after CFA injection: not altered in 2 hours and day 1 and decreased by 36% on day 3, and 29% on day 7 in the ipsilateral L3-L5 (L3/5) spinal cord (Fig. 1A). The level of methyltransferase Mettl3, but not of Mettl14 and Wtap mRNA, was decreased in a time-dependent manner, reaching the valley in the ipsilateral L3/5 spinal cord on day 3 post-CFA (Fig. 1B and Figs. 1A and B, available at http://links.lww.com/PAIN/B283). The amount of demethylase Alkbh5 was slightly elevated only on day 1 post-CFA, but the level of Fto was not changed in the ipsilateral L3/5 spinal cord after CFA (Fig. S1C and D, available at http://links.lww.com/PAIN/B283). None of these mRNA displayed the changes in the contralateral spinal cord (Fig. S1E-I, available at http://links.lww.com/PAIN/B283). However, the levels of Mettl3, Wtap, Alkbh5, and Fto were unchanged, but the amount of Mettl4 was slightly reduced in ipsilateral L3/5 DRG day 3 after CFA injection (Fig. S2A-E, available at http://links.lww.com/PAIN/B283). These mRNAs were not changed in the contralateral L3/5 DRG day 3 after CFA injection (Fig. S2F-J, available at http://links.lww.com/PAIN/B283). Considering that Mettl3 functions as a predominant catalytic subunit in RNA methyltransferase complex (Mettl3, Mettl14, and Wtap), and that its change has a consistent trend with spinal m^6^A, Mettl3 was chose to be further examined. The level of METTL3 protein was decreased by 19% day 1, reached the lowest (25%) day 3, and slightly back (18%) day 7 in the ipsilateral L3/5 spinal cord (Fig. 1C). Interestingly, the amount of Mettl3 mRNA and protein were decreased only on day 7 after CCI in the ipsilateral L3/5 spinal cord (Figs. 1D and E), a preclinical model that mimics nerve trauma–induced neuropathic pain in the clinic, suggesting Mettl3 may be a temporary response to peripheral nerve injury.

The distribution pattern of METTL3 in the spinal cord was identified using double labeling assays. METTL3 immunoreactivity was decreased in ipsilateral than that in contralateral L3/5 dorsal horn 3 days after CFA injection (Fig. 1F). This decrease also was observed when comparing METTL3 immunoreactivity between CFA-injection and its control saline-injection mice (Fig. 1G). METTL3 mainly coexisted with NeuN (a specific neuronal marker): approximately 85.5% of METTL3-positive cells were labeled in saline mice and 74% in the CFA group (Fig. 1G). A few METTL3-positive cells (11.8% in saline mice and 25.5% in CFA mice) were labeled with glial fibrillary acidic protein (GFAP) (a specific astrocyte marker) (Fig. 1H), and (20.9% in saline mice and 20% in CFA group) with IBA1 (a specific microglial marker) (Fig. 1I). These data indicate that METTL3 is predominantly expressed in dorsal horn neurons. Spinal METTL3 downregulation in a specific response to inflammation suggests a potential role of METTL3 during the process of inflammatory pain.

3.2. Mettl3 is required for reduction of global spinal m^6^A in inflammatory pain
Next, we tested whether Mettl3 regulated the global spinal m^6^A level in inflammatory pain. We first overexpressed full-length Mettl3 in the spinal cord using lentivirus. We found that Lenti-Mettl3 was expressed mainly in spinal neurons and less in astrocyte and microglia cells on day 3 after virus intrathecal injection (Fig. 2A, and Fig. S3A, available at http://links.lww.com/PAIN/B283). This OE reversed the decreased Mettl3 at both mRNA (Fig. 2B) and protein levels (Fig. 2C), elevated m^6^A content by 40% in control mice, and reversed global m^6^A to the near basal level from 58.2% to 81.6%, compared with an empty vector (Fig. 2D) in the ipsilateral L3/5 spinal cord on day 3 after 2 consecutive days of intrathecal injection in CFA mice. We further evaluated the Mettl3 regulation in spinal m^6^A content by knocking down Mettl3 with siRNA or Lentivirus-shRNA. Mettl3-siRNA (1664) of 2 siRNAs decreased Mettl3 by 52% in mRNA (Fig. 2E) and 41% in protein (Fig. 2F) on day 2 after 2 consecutive days of intrathecal injection in naïve mice, compared with the control scrambled siRNA. However, another Mettl3 siRNA (790) did not reduce spinal Mettl3 expression (Figs. 2E and F). Mettl3-siRNA (1664) did not change the expression level of spinal METTL14 and WTP (Fig. S4A, available at http://links.lww.com/PAIN/B283), demonstrating the specificity and selectivity of siRNA-1664 to Mettl3. Moreover, siRNA-1664-causing loss of Mettl3 was slightly reduced in ipsilateral L3/5 DRG (Fig. S3A), and protein levels (Fig. S3B, available at http://links.lww.com/PAIN/B283). The levels of spinal Mettl3 mRNA (Fig. 2I) and protein (Fig. 2J) were decreased by 33.4% and 37.2%, respectively, the amount of global m^6^A in the spinal cord was reduced by 23.5% after intrathecal Lenti-Mettl3-shRNA (Fig. 2K). By contrast, the levels of spinal METTL14 and WTP protein expression were not affected by Lenti-Mettl3-shRNA (Fig. S4B, available at http://links.lww.com/PAIN/B283). These data indicate the specificity of Lenti-Mettl3-shRNA to METTL3. Our findings reveal Mettl3 is required for the modulation of spinal m^6^A
inflammatory pain and that RNA m⁶A modification may be involved in the process of chronic inflammatory pain.

3.3. Mettl3 contributes to initiation and maintenance of inflammatory pain

We therefore set about examining the functional role of spinal Mettl3 in inflammatory pain. Complete Freund’s Adjuvant (CFA)-induced nociceptive hypersensitivities assessed by thermal hyperalgesia (upper in Fig. 3A), mechanical allodynia (middle in Fig. 3A), and cold hyperalgesia (lower in Fig. 3A), were markedly attenuated from day 2 to 4 after 2 consecutive days of intrathecal injection of Lenti-Mettl3. To determine the contribution of Mettl3 to initiation of inflammatory pain, we pretreated animals with Lenti-Mettl3 for 2 days before CFA injection. This pretreatment inhibited the CFA-induced thermal hyperalgesia (upper in Fig. 3B), mechanical allodynia (middle in Fig. 3B), and cold hyperalgesia (lower in Fig. 3B). However, locomotor impairment, measured by reflex tests including placing, grasping, and righting as previously described, was not observed (Table S2, available at http://links.lww.com/PAIN/B283). The evidence suggests that decreased METTL3 in the spinal cord is required for the initiation and maintenance of inflammatory pain.

Conversely, intrathecal injection of Mettl3-siRNA-1664 but not scrambled siRNA for 2 consecutive days produced the enhanced responses to thermal, mechanical, and cold stimuli (Fig. 3C). Similar reductions in nociceptive thresholds were observed after intrathecal Lenti-Mettl3-shRNA, but not negative control, in naive mice (Fig. 3D). Moreover, spontaneous pain behaviors were measured using CPP paradigm. Compared with the saline-treated group, the time spent in a lidocaine-paired chamber was longer than that in a saline-paired chamber in the Lenti-Mettl3-shRNA-treated group. As expected, there was no an obvious difference in time spent between the lidocaine-paired and saline-paired chambers after intrathecal Lenti-scrambled shRNA (Figs. 3E and F). The reflex tests confirmed...
no impairment of locomotor function after downregulating Mettl3 with siRNA (Table S2, available at http://links.lww.com/PAIN/B283). Our results indicate a production of spontaneous pain sensitivity after spinal Mettl3 KD.

Given that siRNA or Lenti-shRNA may have potential off-target effects, we further generated Mettl3fl/fl mice and examined the effect of genetic knockout spinal Mettl3 on pain thresholds after intrathecal injection of rAAV-Cre with hSyn promoter, a specific gene expressing in the neurons. The rAAV-hSyn-Gfp was used to a control. As expected, on week 5 after intrathecally injecting rAAV-hSyn-Cre, the levels of spinal Mettl3 mRNA and protein were reduced by 65% (Fig. 3G) and protein by 63.8% (Fig. 3H), respectively, as compared with the rAAV-hSyn-Gfp-treated mice, indicating the efficiency of conditional knockout of Mettl3 gene. Such as siRNA- or Lenti-shRNA-treated mice, Mettl3fl/fl mice injected with rAAV-hSyn-Cre, but not rAAV-hSyn-Gfp, exhibited both evoked mechanical, thermal, and cold sensitivities (Fig. 3I) and spontaneous ongoing pain sensitivity (Figs. 3J and K) on week 5 to 7 after injection. However, no changes in locomotor were observed in virus-injected Mettl3fl/fl mice (Table S2, available at http://links.lww.com/PAIN/B283). Collectively, our data strongly suggest that spinal Mettl3 plays an essential role during the process of inflammatory pain.

3.4. Mettl3 is essential for spinal global de novo protein synthesis in inflammatory pain

RNA m^6^A can affect mRNA stability or protein translation efficiency, leading to the alteration of the protein level. To
Figure 3. Mettl3 regulates inflammatory pain. (A) Intrathecal injection of Lenti-Mettl3 (Lenti-M3) for 2 consecutive days reversed CFA-induced hypersensitivity to thermal, mechanical, and cold stimulus. n = 6. *P < 0.05; **P < 0.01 vs CFA + Lenti-GFP. (B) Intrathecal preinjection of Lenti-M3 for 2 consecutive days prevented CFA-induced pain. n = 6. *P < 0.05 vs Lenti-GFP + CFA. (C and D) Intrathecal injection of Mettl3-siRNA (M3-siRNA) (C) or LV-M3-shRNA (D) for 2 consecutive days enhanced the responses to thermal, mechanical, and cold stimuli in naïve mice. n = 6. *P < 0.05; **P < 0.01 vs Scr or LV-Scr. (E and F) Mettl3 knockdown with LV-M3-shRNA evoked spontaneous pain measured by conditional place preference (CPP) on day 5 after intrathecal injection in naïve mice. Lido, lidocaine. n = 6. *P < 0.05 vs LV-Scr. (G and H) Mettl3 mRNA (G) and protein (H) expression on week 5 after intrathecally injecting rAAV-hSyn-Cre (AAV-Cre) or rAAV-hSyn-Gfp (AAV-Gfp) in Mettl3fl/fl mice. n = 5. **P < 0.01 vs AAV-Gfp. (I) Conditional knockout (cKO) of Mettl3 caused the hypersensitivity by intrathecal injection of rAAV-hSyn-Cre on week 5 after injection in Mettl3fl/fl mice. n = 6. *P < 0.05, **P < 0.01 vs Mettl3fl/fl + rAAV-hSyn-Gfp. (J and K) Mettl3 cKO evoked the spontaneous pain after same treatment as (F). n = 6. *P < 0.05 vs Mettl3fl/fl + rAAV-hSyn-Gfp. CFA, Complete Freund’s Adjuvant; Mettl3, methyltransferase-like 3.
examine the potential impact of m^6^A on the global protein translation level in the spinal cord, we used SUnSET assay in vivo to label nascent proteins with puromycin by intraperitoneal injection 1 hour before spinal tissues collection for Western blot analysis. Global new protein synthesis in the ipsilateral L3/5 spinal cord was increased on day 3 after CFA injection. Overexpressing spinal Mettl3 reduced the basal level of new protein synthesis in the control saline group and blocked the CFA-induced increase in the spinal cord new protein synthesis on day 2 after CFA (Fig. 4A). By contrast, knocking down Mettl3 with siRNA elevated the new protein synthesis content in naive mice (Fig. 4B), suggesting CFA-induced de novo protein synthesis increase is attributed to spinal Mettl3 decrease.

3.5. Decreased Mettl3 stabilizes Tet1 upregulation in inflammatory pain

What is the downstream target regulated by Mettl3? Recent growing evidence has demonstrated that DNA (de)methylation-triggered dysregulation of multiple genes involved in the pathogenesis of chronic pain. We next examined what DNA (de)methylation-related genes are regulated by Mettl3. According to previous reports, a total of 5 genes are differentially expressed in the spinal cord after CFA-induced inflammatory pain: 3 DNA methyltransferases—DNMT1, DNMT3a, and DNMT3b, and 2 DNA demethylase—TET1 and TET3. We found that spinal Mettl3 OE reversed the increase of TET1 protein from 115% to 73% and enhanced the increase of DNMT3b from 62%
to 85%, but did not affect DNMT1, DNMT3a, and TET3 in the ipsilateral L3/5 spinal cord from CFA mice (Fig. 4C). Furthermore, double labeling revealed that TET1 and METTL3 coexpressed in spinal cellular nuclei (Fig. 4D), indicating they share similar subcellular distribution in the individual cells. Therefore, Tet1 was chosen as a possible downstream target for Mettl3 in the following test. To this end, we further examined whether Mettl3 KD could increase the TET1 expression. As expected, Mettl3 KD by intrathecal siRNA (Fig. 4E) or Lentivirus-shRNA for Mettl3 (Fig. 4F) increased TET1 content by 24.7% on day 1 (Fig. 4E) or 21.4% on day 2 (Fig. 4F) after 2-day consecutive injection in naïve mice. Their respective controls did not have these effects. Finally, we examined the effect of deleting spinal METTL3 on TET1 expression using intrathecal injection of rAAV-hSyn-Cre in Mettl3fl/fl mice. We found that knockout of Mettl3 increased the spinal TET1 level by 36.4% on week 5 after injection of rAAV-hSyn-Cre, as compared with the rAAV-hSyn-Gfp-treated group (Fig. 4G). These findings indicate METTL3 can regulate the level of TET1 expression in the spinal cord.

3.6. Methyltransferase-like 3-mediated loss of m6A in Tet1 mRNA is responsible for the increase of the spinal cord TET1 protein in inflammatory pain

Next, we explored how decreased Mettl3 contributed to the increase of spinal TET1 after CFA. Given that METTL3 functions as a "writer" to add m6A on mRNAs’ motif regions characterized by “GGACW” (W presents T or A) sequence, m6A modification frequently located in the coding domain sequence (CDS) and in 3′untranslation region (3′UTR) near termination codon,3,35 enhances or inhibits mRNA post-transcription or translation.68 We hypothesized that m6A in “GGACW” motif in CDS and 3′UTR of Tet1 mRNA may contribute to Tet1 translation under inflammatory pain conditions. First, we analyzed the sequence and found 9 “GGACW” motifs in CDS (6119 bp in length) and 17 “GGACW” motifs in 3′UTR (7962 bp in length). Considering m6A prefers to express near termination codon, thereafter, we designed 8 pairs of primers covering all motif sites in CDS, and 6 pairs covering 11 motifs in 3′UTR near termination codons (from +1 to +4000, the first base of 3′UTR as +1 to quantitatively evaluate m6A change in the inflammatory pain model (Fig. 5A). RNA immunoprecipitation showed that m6A was detectable in 3 CDS sites (second (1020-1098), seventh (4359-4544) and eighth (4868-4990), the first base in CDS as +1) (Fig. 5B) and 3′UTR sites (first (922-1232), second (1455-1615), fifth (3548-3777), the first base of 3′UTR as +1) (Fig. 5C). To further determine whether m6A in 3′UTR sites was regulated by Mettl3, we evaluated the effect of Mettl3 on the m6A level in Tet1 mRNA. With comparison with control Lenti-vector, Mettl3 OE with Lenti-Mettl3 rescued the loss in m6A of 3′UTR fifth, but not first and second site in the spinal cord of CFA mice. By contrast, spinal Mettl3 KD with LV-Mettl3-shRNA, but not LV-Scramble shRNA, decreased m6A in fifth sites by 38.5% in naïve mice. m6A levels in first and second sites were unaffected by spinal Mettl3 OE (Fig. 5D). Together, the results indicate that m6A in fifth sites of Tet1 3′UTR may be a potential regulated mediator by Mettl3.

To investigate whether fifth m6A of 3′UTR is required for the process of Mettl3-mediated Tet1 regulation, we constructed a dual-luciferase reporter inserted by Tet1 3′UTRs including fifth wild “GGACA” or mutated “GGTCA” fifth motif (Fig. 5E). Mettl3 OE by Lenti-Mettl3, but not control, decreased the activity of the wild reporter; however, the same treatment did not change the activity of the mutated reporter (Fig. 5E). Overall, it is demonstrated that Mettl3 can regulate gene expression through m6A in fifth motif in Tet1 3′UTR.

Because the reduction of m6A decreases mRNA decay, causing the elevation of mRNA and the translation level,9,47 we then asked whether Mettl3 modulated the translation level of Tet1. Here, we used polysome fractionation assay9,47 to investigate this conclusion. Spinal cord Mettl3 OE blocked the CFA-induced increase in Tet1 transcripts in fractions of heavier polysomes, compared with the control GFP group (Fig. 5F). By contrast, spinal Mettl3 KD with LV-M3-shRNA, but not its Scr control shRNA, significantly increased Tet1 transcripts in fractions of heavier polysomes (Fig. 5G). These findings suggest Mettl3 not only is involved in the level of Tet1 translation but also is responsible for a loss of m6A in Tet1 mRNA, resulting in stabilizing the TET1 protein upregulation under the condition of inflammatory pain.

3.7. Mettl3 regulates chronic inflammatory pain by targeting Tet1

Does blocking Tet1 rescue the pain behaviors induced by the decreased Mettl3? Our previous study evidenced that Tet1 was preferentially expressed in spinal neurons, and played an essential role in chronic inflammatory pain by the use of Tet1-siRNA or Lentivirus-Tet1-shRNA strategies.30 Considering the potential off-target effects of siRNA or shRNA,67 we further generated a genetic Tet1fl/fl mice, then intrathecally injected rAAV-hSyn-Cre with GFP to Tet1fl/fl mice, and finally evaluated its effect on pain sensitivity. The expression of rAAV-hSyn-Cre with GFP label was observed in the spinal cord 3 weeks after intrathecal injection (Fig. 6A). Spinal cord TET1 was decreased by 51.7% in Tet1fl/fl mice as compared with that in rAAV-Hsyn-Gfp-injected mice (Fig. 6B). Such as Lenti-Tet1-shRNA-treated mice, CFA injection did not cause thermal hyperalgesia and mechanical allodynia in Tet1fl/fl mice pretreated with rAAV-hSyn-Cre, but not with rAAV-Hsyn-Gfp (Fig. 6C). Neither rAAV-Hsyn-Cre nor rAAV-hSyn-Gfp impaired the locomotor function (Table S2, available at http://links.lww.com/PAIN/B283). These results verified the regulatory function of Tet1 in inflammatory pain. To study whether the decreased spinal Mettl3 regulated pain sensitivity by Tet1 mediation, we intrathecally preinjected rAAV-hSyn-Cre or -Gfp in Tet1fl/fl mice 4 weeks before Mettl3-siRNA intrathecal injection. Intrathecal injection of rAAV-Hsyn-Cre, but not of rAAV-Hsyn-Gfp, markedly inhibited nociception-like behavior caused by spinal Mettl3 KD (Fig. 6D). Likewise, intrathecal Tet1-siRNA, but not its scrambled siRNA, reduced thermal and mechanical sensitivities induced by intrathecal LV-Mettl3-shRNA (Fig. 6E). The intrathecal injection of Tet1-siRNA, not of scrambled siRNA, 5 weeks after rAAV injection also impaired the pain hypersensitivity in Mettl3fl/fl mice intrathecally preinjected with rAAV-Hsyn-Cre (Fig. 6F). As expected, intrathecal Tet1-siRNA significantly reduced spinal TET1 expression (Fig. 6G). Collectively, these findings suggest that Mettl3 participates in inflammatory pain by mediation of Tet1 in the spinal cord.

3.8. YTHDF2 modulates inflammatory pain through post-transcriptionally regulating Tet1 mRNA in an m6A-dependent manner

We further ask how a loss of m6A in Tet1 mRNA leads to the increase of TET1 protein in the spinal cord after inflammation. YT521-B homology domain family protein (Ythdf1, 2, and 3) is cytoplasmic m6A reader that selectively recognizes and binds to m6A. Therefore, we determined which Ythdf member could participate in Tet1 regulation. First, we measured their expression in the spinal cord after CFA. Ythdf1 and Ythdf2 were significantly downregulated, but Ythdf3 unchanged on day 3 after CFA injection (Fig. 7A). Given that Ythdf1 and 3 positively regulate gene expression by m6A-controlled translation efficiency of mRNA and that Ythdf2 negatively gene
expression by m^6^A-controlled stability of mRNA,5,52 we supposed that Ythdf2 was a potential binding protein of Tet1 mRNA through m^6^A-dependent mRNA decay mechanism. Spinal Ythdf2 mRNA was decreased in a time-dependent manner from hour 2 to day 7 after CFA injection, and reached the valley on day 3 post-CFA (Fig. 7B), and YTHDF2 protein significantly decreased by 25.6% on day 3 post-CFA (Fig. 7C). Next, we used single-cell RT-PCR assay to determine their coexistence in spinal neurons. Among 5 spinal neurons expressed Mettl3, 3 neurons coexpressed with both Tet1 and Ythdf2 (Fig. 7D). Thus, we chose Ythdf2 as a possible binding

Figure 5. Spinal Mettl3 regulates Tet1 expression by m^6^A methylation. (A) The location of m^6^A motif in Tet1 mRNA: 8 covering 9 “GGACW” in CDS, 6 covering 11 “GGACW” in 3’ UTR. (B and C) Spinal m^6^A content in 8 regions of CDS (B) and in 6 regions of 3’ UTR (C) on day 3 after CFA. n = 5. *P < 0.05 vs Sal groups (2-tailed paired Student’s t-test). (D) The effect of spinal Mettl3 overexpression with Lenti-Mettl3 in CFA mice or of spinal Mettl3 knockdown with Mettl3-siRNA in naive mice on m^6^A of 3’ UTR first, second, and fifth region on day 2 after 2 consecutive days injection. n = 5. *P < 0.05 vs LV-Scr or CFA 1GFP. (E) Luciferase activities of Tet1 3’ UTR wild or mutated fifth region (A-to-T mutation) reporter at 48 hours after cotransfection of reporter and Lenti-M3 or control virus in HEK293T. n = 5. *P < 0.05 vs Lenti-GFP or PBS groups. (F and G) Change of Tet1 translation after spinal Mettl3 overexpression in CFA mice (F) or spinal Mettl3 knockdown in naive mice (G) on day 2 after injection for 2 consecutive days. n = 5. *P < 0.05; **P < 0.01 vs Sal + GFP or LV-Scr groups, #P < 0.05; ##P < 0.01 vs CFA + GFP, CFA, Complete Freund’s Adjuvant; Mettl3, methyltransferase-like 3.
Figure 6. Mettl3 modulates chronic inflammatory pain through mediation of Tet1. (A) GFP and NeuN expression on 4 weeks after intrathecal injection of AAV-hSyn-GFP in Tet1 fl/fl mice. (B) Verification of Tet1 cKO on week 4 after intrathecal injection of rAAV-hSyn-Cre or rAAV-hSyn-Gfp in Tet1 fl/fl mice. n = 5. *P < 0.05 vs AAV-GFP. (C and D) Tet1 cKO prevented the production of CFA- (C) or Mettl3-siRNA- (D) induced pain sensitivity on week 4 after rAAV-hSyn-Cre injection in Tet1 fl/fl mice. n = 6. *P < 0.05, **P < 0.01 vs Tet1 fl/fl + Sal or Tet1 fl/fl + AAV-Gfp. (E and F) Downregulating Tet1 inhibited pain hypersensitivity induced by intrathecal injection of LV-M3-shRNA in naïve mice (E) or by intrathecal injection of rAAV-hSyn-Cre in Mettl3 fl/fl mice (F). n = 7. *P < 0.05, **P < 0.01 vs LV-M3-shRNA + Scr or M3 fl/fl + AAV-Gfp + Scr. (G) TET1 expression on day 2 after intrathecal injection of Tet1-siRNA or Scr for 2 consecutive days. n = 5. *P < 0.05 vs Scr group. CFA, Complete Freund’s Adjuvant; Mettl3, methyltransferase-like 3.
Figure 7. YTHDF2 mediates the decay of Tet1 mRNA by an m6A-dependent manner and regulates pain behavior. (A) Three m6A binding proteins levels on day 3 after CFA. (B and C) Inflammatory pain decreased spinal Ythdf2 mRNA (B) and protein (C) on day 3 after CFA. n = 5. **P < 0.01 vs the Sal. (D) Gene coexpression analysis in spinal neurons by the use of single-cell RT-PCR assay. 1 to 6, different neurons. H2O, negative control. (E) Upregulating YTHDF2 with intrathecal injection of Lenti-Ythdf2 (YF2) for 2 consecutive days reversed the decrease of YTHDF2 and the increase of TET1 in the ipsilateral L3/S spinal cord of CFA mice. n = 6. *P < 0.05; **P < 0.01 vs corresponding groups. NC, IgG control. (F and G) Inhibiting YTHDF2 by intrathecal injection of siRNA for 2 consecutive days decreased the binding amount of YTHDF2 to m6A of Tet1 3’UTR, but reduced m6A-labelled Tet1 mRNA in the ipsilateral L3/S spinal cord of CFA mice. n = 6. **P < 0.01 vs the Sal. (H) Tet1 mRNA stability in naive mice after Ythdf2-siRNA or Scr for 2 consecutive days, and after one-time intrathecal injection of actinomycin D (Act-D, 10 μg). n = 5. *P < 0.05 vs Scr. (I and J) Knockdown of Ythdf2 induced the thermal and mechanical hypersensitivity after 2 consecutive days injections of Ythdf2-siRNA-573 and -801 in naive mice. n = 6. **P < 0.01 vs Scr. (O) Tet1 cKO with rAAV-hSyn-Cre prevented the thermal and mechanical hypersensitivity by Ythdf2 knockdown in Tet1fl/fl mice. n = 6. *P < 0.05; **P < 0.01 vs Tet1fl/fl AAV-Cre+Scr. CFA, Complete Freund’s Adjuvant; Mettl3, methyltransferase-like 3.
protein in mRNA of Tet1 mRNA. We hypothesized that Ythdf2 may be involved in Tet1 expression by negatively regulating stability of Tet mRNA mediated by 3′UTR in inflammatory pain.

To this end, we determined whether YTHDF2 could regulate Tet1 expression. Here, we used Lentivirus-Ythdf2 to upregulate Ythdf2 and siRNA to downregulate it. First, the efficiency of Lentivirus-Ythdf2 was validated. YTHDF2 was increased by 28% in the spinal cord on day 3 after 2-day consecutive injection of Lentivirus-Ythdf2 in naive mice, whereas the increased spinal Tet1 was reduced by 48.9% after Lentivirus-Ythdf2 injection, in CFA mice compared with Lentivirus-GFP (Fig. 7E). As expected, the binding number of YTHDF2 to Tet1 mRNA was increased by 66.5% in naive mice, and the decreased binding of YTHDF2 was reversed by 86% day 3 after injection of Lentivirus-Ythdf2 in CFA mice (Fig. 7F). However, YTHDF2 OE with Lentivirus-Ythdf2, but not control Lentivirus-GFP, reduced Tet1 mRNA with m6A tag in CFA mice (Fig. 7G). As expected, siRNA-573 and siRNA-801 knocked down YTHDF2 by 86% day 3 after injection of Lentivirus-Ythdf2 in CFA mice (Fig. 7F). However, YTHDF2 OE with Lentivirus-Ythdf2, but not control Lentivirus-GFP, reduced Tet1 mRNA with m6A tag in CFA mice (Fig. 7G).

To further verify the mechanism of YTHDF2 regulation of Tet1 by decaying Tet1 mRNA, we tested the effect of Ythdf2 KD on Tet1 mRNA lifetime. Measuring the decay of Tet1 mRNA after blocking new RNA synthesis with actinomycin-D showed that silencing YTHDF2 elevated total Tet1 mRNA (Fig. 7K), suggesting that YTHDF2 destabilized Tet1 mRNA. Collectively, these data suggested that YTHDF2 modulated Tet1 mRNA in an m6A-dependent manner.

Next, we examined whether spinal Ythdf2 participates in inflammatory pain and whether this process was mediated by Tet1 in the spinal cord. First, we found that CFA-induced pain hypersensitivity was markedly attenuated on day 3 postinjection of Lentivirus-Ythdf2 once daily for 2 days starting from day 1 after CFA injection, compared with that in the Lentivirus-GFP-treated CFA mice (Fig. 7L). Repeated intrathecal injection of Lentivirus-Ythdf2, but not Lentivirus-GFP, once daily for 2 days after CFA injection significantly prevented the development of pain hypersensitivity (Fig. 7M). By contrast, spinal Ythdf2 KD by intrathecal injection of its siRNA, but not control scrambled siRNA, once daily for 2 days induced the production of pain-like behavior as evidenced by the decreases in thermal and mechanical pain threshold on day 1 post-treatment in naive mice (Fig. 7N).

The evidence suggests that the decreased Ythdf2 contributed to pain hypersensitivity. Then, we further evaluated whether the blockade of Tet1 could prevent pain hypersensitivity induced by the decrease of Ythdf2. The intrathecal preinjection of rAAV-hSyn-Cre 4 weeks before Ythdf2 siRNA in Tet1fl/fl mice prevented the generation of pain-like behaviors induced by Ythdf2 KD with siRNA (Fig. 7O), suggesting that blocking Tet1 can inhibit the pain hypersensitivity induced by Ythdf2. The treated mice displayed normal locomotor activity (Table S2, available at http://links.lww.com/PAIN/B283). Taken together, the findings suggest that spinal Ythdf2 participates in the regulation of inflammatory pain by negatively targeting Tet1 mRNA in an m6A-dependent manner.

3.9. Methyltransferase-like 3 coordinates with Ythdf2 to regulate spinal neuron activation by Tet1/Stat3 axis in inflammatory pain

STAT3 plays a critical role in neuroinflammation and immunity by promoting pro-oncogenic inflammatory pathways including nuclear factor-kappa B (NF-kappa B) and interleukin-6 (IL-6)-Janus kinase pathways. As Tet1 regulates nociceptive sensitization in a Stat3-dependent manner, we further explored whether Mettl3 together with Ythdf2 co-ordinately regulated spinal neuronal activation by mediation of Tet1/Stat3 axis. Overexpressing Mettl3 inhibited reversed the increase of spinal STAT3 and C-FOS (a neuron activation marker) on day 3 postinjection of Lentivirus-Mettl3, but not control Lentivirus-GFP, in CFA mice (Fig. 8A). Conversely, KD of Mettl3 increased the levels of STAT3 and C-FOS expression in naive mice on day 2 after intrathecal injection of Mettl3-siRNA once daily for 2 days. This increase could be abolished by downregulation of Tet1 by Tet1-siRNA injection, but not the control scramble siRNA injection, once daily for 2 days postinjection of Mettl3-siRNA (Fig. 8B). Our results suggest that Mettl3 regulates spinal neuronal activation by Tet1/Stat3.

We further examined whether Ythdf2 was involved in spinal neuronal activation in Stat3-dependent manner in the chronic inflammatory pain model. Spinal Ythdf2 OE blocked CFA-evoked the increase in spinal STAT3 and C-FOS on day 3 after intrathecal injection of Lentivirus-Ythdf2 in CFA mice compared with that in control Lentivirus-GFP (Fig. 8C); this treatment did not change the decrease of spinal Mettl3 in CFA mice (data not shown). This result excluded the possibility of Mettl3 affecting STAT3 and C-FOS expression, indicating the specificity of Ythdf2 the regulation of STAT3 and C-FOS. As expected, spinal Ythdf2 KD increased STAT3 and C-FOS expression in the spinal cord once daily for 2 days postinjection of Ythdf2-siRNA but not control siRNA in naive mice (Fig. 8D). The alteration of METTL3 expression was not observed by the treated (data not shown). Finally, we further examined whether deleting spinal Tet1 blocked the increased STAT3 and C-Fos expression triggered by Ythdf2 KD. Ythdf2-siRNA was intrathecally injected once daily for 2 days on 4 weeks after rAAV-hSyn-Cre or rAAV-hSyn-Gfp was intrathecally preinjected in Tet1fl/fl mice. Neither Ythdf2-siRNA nor its control scrambled changed STAT3 and c-Fos/Fos expression in the spinal cord (Fig. 8E), suggesting Ythdf2 can modulate neuron activation by Tet1/Stat3 axis. Together, our findings indicate that Mettl3 coordinates with Ythdf2 to negatively regulate inflammatory pain through Tet1/Stat3 axis in the spinal neuronal in RNA m6A-dependent manner (Fig. 9).

4. Discussion

Chronic pain is a debilitating public health concern, and has been fully studied for several decades, but how pain hypersensitivities are caused is still incompletely understood. Central sensitization is recognized to a critical cause in formation of pain hypersensitivities. Abrupt pain-related gene expression is the molecular basis for central sensitization. Therefore, uncovering the mechanisms of gene regulation underlying central sensitization will improve our understanding of chronic pain and may provide potential targets for developing new therapeutic strategies. Multiple layers of epigenetic modifications, such as DNA methylation, histone modification, and noncoding RNA, play essential roles in chronic pain. m6A modification of RNA, an additional layer of gene expression, has shown its potential role in various biological processes through regulating chromatin state, transcription, RNA splicing, post-transcription, and translation. In this study, we unveiled that peripheral inflammation led to a decrease in spinal METTL3. This decrease correlated with a loss of m6A sites in Tet1 mRNA and an elevation of Tet1 protein in the spinal cord, resulting in pain hypersensitivity. Blocking this decrease reversed the loss of Tet1 mRNA m6A and alleviated pain sensitivity induced by Tet1. The downregulated METTL3 likely contributes to chronic inflammatory pain.

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Recent studies have confirmed the involvement of RNA m^6A in dysfunction of the central nerve system. m^6A is markedly elevated in the brain during development, and affects such neuronal development as proliferation and differentiation in axon regeneration, adult neurogenesis, and synapse function. Therefore, m^6A participates in physiological and pathological by YTHDF2-dependent post-transcriptional stabilization of TET1 in spinal neurons.

Figure 8. Mettl3 coordinates with Ythdf2 to regulate spinal neuron activation by Tet1/Stat3 axis in inflammatory pain. (A) Upregulating Mettl3 reversed the increased STAT3 and C-FOS by intrathecal injection of Lenti-Mettl3 for 2 consecutive days in the spinal cord of CFA mice. n = 6. *P < 0.05 vs the corresponding groups. (B) Downregulating Tet1 with siRNA reversed the increased STAT3 and C-FOS by Mettl3-siRNA by intrathecal injection of Tet1-siRNA for 2 consecutive days in the spinal cord of naive mice. n = 6. *P < 0.05; **P < 0.01 vs the corresponding groups. (C) Overexpressing YTHDF2 with Lenti-Ythdf2 blocked the increase of STAT3 and C-FOS in the spinal cord of CFA mice. n = 6. *P < 0.05; **P < 0.01 vs the corresponding groups. (D) Downregulation of YTHDF2 with intrathecal injection of siRNA increase STAT3 and C-FOS in the spinal cord of naive mice. n = 6. *P < 0.05; **P < 0.01 vs Scr groups. (E) Knock out Tet1 with rAAV-hSyn-Cre in Tet1^fl/fl mice blocked the increase of STAT3 and C-FOS by Ythdf2-siRNA. n = 6. rAAV-hSyn-Cre or rAAV-hSyn-Gfp was intrathecally injected 5 weeks before Ythdf2-siRNA or Scr administration. CFA, Complete Freund’s Adjuvant; Mettl3, methyltransferase-like 3.

Figure 9. The schematic of spinal METTL3 regulating inflammatory pain through stabilizing upregulation of TET1 in a YTHDF2-dependent manner. Mettl3, methyltransferase-like 3.
process such as brain dopaminergic circuitry and memory formation and consolidation. In adult DRG neurons of mouse on day 3 after sciatic nerve lesion, the gain of m6A-tagged transcripts is induced, and KD of m6A-tagged transcripts leads to DRG axons regeneration. However, it is only beginning to explore how m6A is involved in neuronal dysfunction or CNS diseases. In this study, we found a marked decrease of spinal m6A content in chronic inflammatory pain mice. Our data suggest spinal m6A is an important player in the development of inflammatory pain.

Methyltransferase-like 3 is a key component of methyltransferase complex responsible for RNA m6A and highly conserved in mammals. METTL3 is originally reported predominantly localized to the nucleus of human HeLa and 293FT. The following studies focus on the regulatory function of METTL3 in the nucleus of central nerve system tissues. Interestingly, recent report shows that METTL3 in gastric cancer cell cytoplasm transcripts is induced, and KD of m6A-tagged transcripts leads to formation and consolidation. In adult DRG neurons of mouse, we explore how m6A is involved in neuronal dysfunction or CNS system tissues such as hippocampus, prefrontal cortex, and cerebellum. METTL3 mRNA and its protein were decreased in the spinal cord of the inflammatory pain model. This reduction was not observed in DRG of CFA-injected mice. Therefore, we speculate that Mettl3 in the spinal cord, but not in DRG, possibly contributes to the development of inflammation pain. This is the rationale why we investigated spinal Mettl3 mechanism underlying inflammation pain. We intrathecally injected Lent-Mettl3 into overexpress Mettl3 and found, although Mettl3 was increased not only in the spinal cord (Fig. 2B-C), but also in DRG (Fig. S5, available at http://links.lww.com/PAIN/B283) in saline mice, the baseline of pain threshold was not changed (Fig. 3A-B) in saline mice. However, rescuing the decrease of spinal Mettl3 in inflammatory pain mice significantly increased pain threshold (Fig. 2B-C and Fig. 3A-B). These data confirmed our speculation.

Interestingly, in preparation of the CCI model, to reduce the ligation-causing inflammation level as far as possible, we chose silk sutures, which could cause a lower level of inflammation than chromic gut suture. We found that spinal Mettl3 was decreased only on day 7 after CCI. This alteration seems to be a transient response to peripheral nerve injury, suggesting that Mettl3 plays an important role in inflammatory pain, but not in neuropathic pain. Likely in DRG postnerve injury, most of RNA m6A “writer” or “eraser” were not altered. Only Flo was increased in injured DRG, although inflammation pain did not alter Flo expression in DRG on either ipsilateral or contralateral side. We found no changes in spinal Wtap, Flo, and Alkbh5 except for Mettl4 in inflammatory pain. Our findings, together with previous reports, suggest that m6A “writer” or “eraser” enzymes display distinct changes in different nervous tissues from different types of pain models. Given that only Mettl3 was decreased in the spinal cord of inflammatory pain mice, it cannot completely explain how methylated mRNA is reversed. Therefore, it is worth considering whether other undiscovered “writer” or “eraser” exists.

Functionally, Mettl3 was involved in brain development, neurogenesis and neuronal development, synaptic formation, learning and memory, and traumatic brain injury. Alzheimers disease. Mettl3 depletion in mouse hippocampus reduces memory consolidation ability. By contrast, OE of Mettl3 enhances long-term memory consolidation. Traumatic brain injury leads to the decreased of Mettl3 in hippocampus. In this study, overexpressing Mettl3 rescued the dysregulation of spinal m6A and alleviated inflammatory pain. Spinal Mettl3 downregulation-induced pain hypersensitivity symptom. Notably, our results are not consistent with a recent describe, in which spinal Mettl3 was increased in the inflammatory pain model and links positively to CFA-induced pain behaviors. To verify whether distinct mouse strain may lead to the difference in spinal Mettl3 expression after CFA, we used C57BL/6 mice described in a previous study and showed that spinal Mettl3 mRNA and protein were decreased on day 3 after CFA (Fig. S6A-B, available at http://links.lww.com/PAIN/B283), which is consistent with our findings in this study.

Mechanically, we found Mettl3 may be responsible for the initiation and maintenance of inflammatory pain by upregulating Tet1 expression. Although Mettl3 may target many mRNAs, this work focused on Tet1 because (1) Tet1 could be regulated by Mettl3 among the given 5 DNA (de)methylation enzymes. (2) Tet1 is a key player in regulation of pain-related genes such as receptors, enzymes, and ion channels. (3) Through bioinformatics prediction and experimental verification, the decreased spinal Mettl3 led to a loss of Tet1 mRNA m6A. Rescuing this decrease reversed the decreased Tet1 mRNA m6A, inhibited the increase of Tet1 protein, and blocked the expression of Stat3 and c-Fos, a marker for dorsol horn central sensitization in inflammation and neuropathic pain. Mimicking this decrease of spinal Mettl3 by pharmacological inhibition or genetic depletion/ko reduced m6A in Tet1 mRNA, causing the production of pain-like behaviors. Interestingly, Tet1 is a DNA demethylation enzyme in the DNA layer, whereas Mettl3 is an RNA methylation enzyme in the RNA layer. Our study provides the first evidence that their crossing talk contributes to nociceptive information processing. A large number of transcripts m6A loss or gain on knockout Mettl3 was reported in mouse cortex. We speculate that Mettl3 may regulate many transcripts m6A in the spinal cord after peripheral inflammation. What other transcripts are regulated by Mettl3 needs further investigation in future.

RNA m6A “reader” Ythdf2 is able to destabilize or degrade mRNA. Our work provides in vivo evidence for a loss of m6A in Tet1 mRNA in stabilizing the upregulation of Tet1 and its downstream Stat3 protein in a Ythdf2-dependent manner in inflammatory pain. Different from previous findings on the in vivo role of m6A-dependent promotion of protein translation in regulating DRG nerve regeneration, m6A seems a great impact on protein translation level, but not or less on mRNA levels. Our study demonstrated that RNA m6A plays a critical role in peripheral inflammation-induced global protein translation in the mouse spinal cord. We have identified RNA m6A promotes Tet1 protein upregulation by decreasing the decay of Tet1 mRNA by Ythdf2 in the peripheral inflammation spinal cord. Blocking Ythdf2 increased Tet1 mRNA because of the degeneration reduction, resulting in the elevation of Tet1 protein and a marked pain hypersensitivity. These findings may at least partly explain why peripheral inflammation-induced or nerve injury-induced increase of spinal Tet1 protein. Indeed, Ythdf2 is the first discovered m6A binding protein and acts as a regulator of mRNA stability by promoting degradation of m6A-containing mRNAs. Ythdh2-deficient neuron could not produce normal synapses; therefore, Ythdf2-mediated m6A mRNA clearance has a regulatory effect on neurodevelopment in mice. Limited studies suggest a potential critical role
of Ythdf2 in the nervous system. Given m6A-containing mRNAs undergo deamination mediated by YTHDF2 and CCR4-NOT complex, whether other complex contents together YTHDF2 are involved in inflammatory pain need to be addressed in future.

In summary, our study demonstrated that METTL3 coordinates with YTHDF2 to negatively regulate inflammatory pain by modulating Tef1/Stat3 axis in an m6A-dependent manner. These findings shed light on new RNA layer of mechanism in inflammatory pain and may provide the rationale for future development of potential targeted interventions by RNA m6A modification for chronic pain.

Conflict of interest statement
The authors have no conflicts of interest to declare.

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Appendix A. Supplemental digital content
Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/B283.

Supplemental video content
A video abstract associated with this article can be found at http://links.lww.com/PAIN/B284.

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