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TET1 overexpression attenuates paclitaxel-induced neuropathic pain through rescuing $K_{2p}1.1$ expression in primary sensory neurons of male rats

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Abstract

Aims: Paclitaxel-induced downregulation of two-pore domain K+ channel 1.1 ($K_{2p}1.1$) caused by increasing DNA methylation within its gene promoter in the dorsal root ganglion (DRG) contributes to neuropathic pain. Given that ten-eleven translocation methylcytosine dioxygenase 1 (TET1) promotes DNA demethylation and gene transcription, the present study investigated whether DRG overexpression of TET1 produces an antinociceptive effect on the paclitaxel-induced nociceptive hypersensitivity.

Main methods—TET1 was overexpressed in the DRG through unilateral microinjection of the herpes simplex virus expressing full-length *Tet1* mRNA into the fourth and fifth lumbar DRGs of male rats. Behavioral tests were carried out to examine the effect of this overexpression on the paclitaxel-induced nociceptive hypersensitivity. Western blot analysis, chromatin immunoprecipitation assay and 5-hydroxymethylcytosine detection assay were performed to assess the levels of TET1/$K_{2p}1.1$, 5-methylcytosine and 5-hydroxymethylcytosine, respectively.

Key findings—DRG overexpression of TET1 mitigated the paclitaxel-induced mechanical allodynia, heat hyperalgesia and cold hyperalgesia on the ipsilateral side during the development

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Authors' contributions

Y.X.T. conceived the project and supervised all experiments. S.J. performed the animal model, conducted behavioral experiments, and carried out microinjection. S.J., G.W., J.B., Z. P. carried out Western blot, chromatin immunoprecipitation, PCR experiments and constructed HSV-TET1/EGFP. S.J., G.W., J.B., B.Z., B.W., A.A., C.T., A.B. and Y.X.T. analyzed the data. B.W. and Y.X.T. wrote the manuscript. All of the authors read and discussed the manuscript.

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Declaration of competing interest

All authors declare no conflict of interests.

and maintenance periods. Locomotor function or basal (acute) responses to mechanical, heat or cold stimuli were not affected. Mechanistically, DRG overexpression of TET1 rescued the expression of $K_{2p}1.1$ by blocking the paclitaxel-induced increase in the level of 5-methylcytosine and correspondingly reversing the paclitaxel-induced decreases in the amount of 5-hydroxymethylcytosine within the *$K_{2p}1.1$* promoter region in the microinjected DRGs of male rats.

Significance—Our findings suggest that DRG overexpression of TET1 alleviated chemotherapy-induced neuropathic pain likely through rescuing DRG $K_{2p}1.1$ expression. Our findings may provide a potential avenue for the management of this disorder.

Keywords

TET1; K_{2p} 1.1; DNA methylation; dorsal root ganglion; paclitaxel; chemotherapy-induced neuropathic pain

1. Introduction

Chemotherapy is a prevalent approach in cancer treatment. Chemotherapeutic drugs target cancer cells through a variety of mechanisms, such as affecting cellular metabolism, causing cell cycle arrest and inducing the production of reactive oxygen species [1]. Although these medications effectively treat a variety of cancers, they also produced numerous unwanted side effects, such as chemotherapy-induced peripheral neuropathy (CIPN). These side effects caused cancer patients to reduce the doses of chemotherapeutic drugs and eventually discontinue cancer therapy, resulting in a decrease of survival rates [2,3]. CIPN is a significant adverse consequence seen in cancer survivors, affecting about 30% of patients for 6 months or more after chemotherapy treatment has ended [4]. CIPN is described as pain, dysesthesia, paresthesia, numbness, tingling, burning and weakness [5,6]. Understanding how chemotherapeutic drugs produce CIPN is essential for improving patient care.

DNA methylation represses gene transcription. In the mammalian genome, DNA methylation involves DNA-methyltransferases (DNMTs), which transfer a methyl group to the 5th position of a cytosine residue, converting cytosine to 5-methylcytosine (5-mC) [7]. Three types of functional DNMT members, DNMT1, DNMT3a and DNMT3b, have been identified. DNA methylation can also be removed by ten-eleven translocation methylcytosine dioxygenases (TETs, including TET1–3), which convert 5-mC to 5-hydroxymethylcytosine (5-hmC), promoting DNA demethylation and gene transcription [8,9]. Thus, gene activation is controlled by the balances in the activity and expression between DNMTs and TETs.

Our recent work revealed that DNMT3a-triggered epigenetic silencing of the two-pore domain K+ channel 1.1 (*$K_{2p}1.1$*) gene (encoding K_{2p} 1.1 protein) in the dorsal root ganglion (DRG) contributed to paclitaxel-induced neuropathic pain [10]. Systemic administration of paclitaxel reduced the expression of *$K_{2p}1.1$* mRNA and K_{2p} 1.1 protein in the DRG neurons in a time-dependent manner [10]. Rescuing this reduction attenuated the development and maintenance of paclitaxel-induced nociceptive hypersensitivity [10]. Mimicking this reduction decreased outward potassium current, increased the excitability of

DRG neurons, and produced augmented responses to mechanical and heat stimuli [10]. Further work has demonstrated systemic administration of paclitaxel to upregulate the expression of DNMT3a, but not DNMT1 and DNMT3b, in the DRG [10]. Pharmacological inhibition and genetic knockdown of DNMT3a in the DRG reversed the paclitaxel-induced decrease of DRG K_{2p} 1.1 and mitigated the paclitaxel-induced neuropathic pain development [10]. DRG overexpression of DNMT3a reduced DRG K_{2p} 1.1 expression [10]. More importantly, paclitaxel administration increased the binding of DNMT3a to the *K_{2p} 1.1* gene promoter region and increased the level of DNA methylation within this promotor region in the DRG [10]. Therefore, blocking the paclitaxel-induced increase of DNA methylation within DRG *K_{2p} 1.1* gene promoter may have an antinociceptive effect under the conditions of paclitaxel-induced CIPN.

In the present study, we constructed a HSV expressing the full-length rat TET1 (HSV-TET1) and first examined whether DRG overexpression of TET1 through DRG microinjection of HSV-TET1 affected the development and maintenance of paclitaxel-induced mechanical and heat nociceptive hypersensitivity in male rats. We then observed whether this microinjection rescued the paclitaxel-induced downregulation of DRG K_{2p} 1.1. Finally, we determined whether this microinjection blocked the paclitaxel-induced increase in DNA methylation within *K_{2p} 1.1* gene promoter in the DRG of male rats.

2. Materials and methods

2.1 Animal preparation

Adult male Sprague-Dawley rats (weighing 220–250 g; Charles River Laboratories) housed in a 12-hour light/dark cycle with free access to food and water were used in all experiments. Before the experiments, all the animals were allowed to habituate to the animal facility for at least 3 days. All procedures used were approved by the Animal Care and Use Committee at Rutgers New Jersey Medical School and consistent with the ethical guidelines of the US National Institutes of Health and the International Association for the Study of Pain. The experimenters were blind to viral treatment or drug treatment conditions.

2.2 Paclitaxel-induced CIPN Model

Animals were treated with paclitaxel as previously described [10,11]. Briefly, 6 mg/mL stock pharmaceutical grade paclitaxel (TEVA Pharmaceuticals,Inc, North Wales, PA) was diluted to 1 mg/mL with sterile saline and injected intraperitoneally at a dosage of 4 mg/kg every other day for a total of 4 injections (days 0, 2, 4, and 6). Control animals received an equivalent volume of the vehicle only, which consisted of equal amounts of Cremophor EL (SigmaAldrich, St. Louis, Missouri) and ethanol diluted with saline. Rats were observed carefully for any abnormal behavioral changes every day after treatment.

2.3 DRG microinjection

The DRGs were exposed by laminectomy as previously described [12–14]. Briefly, after the rats were anesthetized with isoflurane, a dorsal midline incision was made in the lower lumbar region. Unilateral L4/5 articular processes were removed. After the exposure of the L4/5 DRGs, the viral solution (1–1.5 μl/DRG; 3–5 × 10^8) or 0.01 M phosphate buffer saline

(PBS; 1–1.5 μl) was injected into the exposed L4 and L5 DRGs, respectively, with a glass micropipette connected to a Hamilton syringe. The pipette was kept in place for 10 min after injection. The surgical field was irrigated with sterile saline and the skin incision closed with wound clips.

2.4 Behavioral tests

Paw withdrawal threshold to mechanical stimulation was examined by using the up-down method as described previously [12–14]. In brief, rats were habituated to the testing environment daily for 3 days before baseline testing. Each rat was placed in a plastic chamber on a metal mesh floor. After 30-minute habituation, the calibrated von Frey filaments (Stoelting Co., Wood Dale, IL, USA) in log increments of force (0.69, 1.20, 2.04, 3.63, 5.50, 8.51, 15.14, and 26 g) were used to stimulate the plantar surface of the ipsilateral and contralateral hind paws. The 3.63-g stimulus was used first. If a negative response occurred, the next larger von Frey hair was applied; if a positive response was seen, the next smaller von Frey hair was applied. The application was terminated when (i) a negative response was seen with the 26-g stimulation or (ii) three stimuli were used after the first positive response. Based on a formula provided by Dixon [15], paw withdrawal threshold was calculated by converting the pattern of positive and negative responses to a 50% threshold value.

Paw withdrawal latency to heat stimulation was measured as described previously [12–14]. Each animal was placed in a plastic chamber on a glass plate (Model 336 Analgesia Meter, IITC Life Science, Inc.). After 30-minitues habituation, a radiant heat stimulus was applied by aiming a beam of light at the heel of each hind paw. When the animal lifted its paw in response to the heat, the light beam was turned off. The time from the start of light beam to the foot lift was defined as paw withdrawal latency. Five-minute interval was allowed between stimulations. Five measurements were averaged for each side. A cut-off time of 20 seconds was used to prevent tissue damage.

Paw withdrawal latency to noxious cold stimulation was measured as previously described [12–14]. Each rat was placed in a Plexiglas chamber on the cold aluminum plate, the temperature of which was set at 0 °C and monitored continuously by a thermometer. The length of time between the placement of the hind paw on the plate and the animal jumping, with or without paw licking and flinching, was defined as the paw withdrawal latency. Ten minutes were allowed between 3 repeated trials. A cut-off time of 60 seconds was used to avoid paw tissue damage.

Locomotor function test was carried out before tissue collection according to the methods described previously [12–14]. For placing reflex, each rat’s hind limbs were placed slightly lower than the forelimbs, and the dorsal surfaces of the hind paws were brought into contact with the edge of a table. Whether or not the hind paws were placed on the table surface reflexively was recorded. For grasping reflex, the rat was placed on a wire grid, and the experimenter recorded whether the hind paws grasped the wire on contact. For righting reflex, the rat was placed on its back on a flat surface, and whether it immediately assumed the normal upright position was recorded. Five trials were conducted for each test, and the scores were derived from counts of normal reflexes for each trial.

2.5 Western blotting analysis

Protein extraction and Western blotting were carried out according to our previously published protocol [12–14,16]. In brief, the ipsilateral L4/5 DRGs were homogenized in chilled lysis buffer (10 mM Tris, 1 mM phenylmethylsulfonyl fluoride, 5 mM MgCl2, 5 mM EGTA, 1 mM EDTA, 1 mM DTT, 40μM leupeptin, and 250 mM sucrose). After centrifugation at 4 °C for 15 min at 1,000g, the supernatant was collected for cytosolic/membrane proteins and the pellet was collected for nuclear proteins. The pellet was dissolved in lysis buffer containing 2% sodium dodecyl sulfate (SDS) and 0.1% Triton X-100. After being heated at 99 °C for 5 min in the loading buffer (containing 4% SDS), the samples (15–20 μg/sample) were loaded onto a 4% stacking/7.5% separating SDS-polyacrylamide gel (Bio-Rad), and then electrophoretically transferred onto a polyvinylidene difluoride membranes. After being blocked with 3% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h, the membrane was incubated with the following primary antibodies, respectively, overnight at 4 °C. The primary antibodies used included rabbit anti-TET1 (1:1,000, Abcam), mouse anti-K_{2p}1.1 (1:500, NeuroMab), rabbit anti-histone H3 (1:1,000; Cell Signaling) and rabbit anti-GAPDH (1:1,000; Santa Cruz). After incubation with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (1:3,000, Jackson ImmunoResearch), the blots were visualized with western peroxide reagent and luminol/enhancer reagent (Clarity Western ECL Substrate, Bio-Rad), and exposed by the ChemiDoc XRS System with Image Lab software (Bio-Rad). The intensities of the blots were quantified with Image J software. The band intensities for cytosol/membrane proteins were normalized to GAPDH and those for nucleus protein were normalized to H3.

2.6. Methylated DNA immunoprecipitation

Genomic DNA was extracted and purified by using DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. After DNA was sonicated to 100–500 bp fragments, immunoprecipitation for 5-mC was carried out by using the Methylated-DNA immunoprecipitation Kit (Zymo Research) as described previously [10]. Briefly, apart from about 15–20% of the genomic DNA fragments used as an input control, the remaining fragmented DNA (160 ng) in 50 μl DNA denaturing buffer was denatured at 98°C for 5 min. The 250 μl of MIP buffer containing 15 μl of ZymoMag Protein A and 1.6 μl of mouse anti-5-mC antibody (Zymo Research) was then added to the denatured DNA for incubation at 37°C for 1 hour on a rotator. After the beads were suspended with a 500 μl MIP buffer, DNA was precipitated and resuspended in 200 μl of water for real-time PCR analysis as described below. The purified normal mouse IgG in the reaction was used as a negative control.

2.7. Detection assay of 5-hmC levels

The enrichment of 5-hmC in the DNA was measured by using Thermo Scientific EpiJET 5-hmC Enrichment Kit (Catalog number: K1491BID, Thermo Fisher Scientific, Waltham, MA) as described previously [16,17]. Briefly, about 15–20% of the genomic DNA fragments were used as an input control. 5-hmC groups in the remaining fragmented genomic DNA were modified with the addition of a linker by the incubation with 5-hmC modifying enzyme

in enzyme reaction buffer at 30° C for 1 hour. For a negative control, no 5-hmC modifying enzyme was added into the reaction buffer. After DNA was purified by washing steps and with DNA purification columns, a biotin conjugation reaction with the linker was carried out by the incubation with biotin reagent at 50° C for 5 min. DNA was then purified again as described before. The modified 5-hmC DNA was separated from the unmodified DNA by the incubation with the streptavidin magnetic beads for 15 min. After being eluted into water, the modified 5-hmC DNA was used for real-time PCR analysis as described below.

2.8. Quantitative real-time PCR

Quantitative real-time PCR was carried out as described previously [12–14,16]. In brief, DNA concentration was measured using the NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE). DNA templates were amplified using real-time PCR with primers derived from the promoter region of rat *$K_{2p}1.1$* gene. Four pairs of the primers listed in Table 1 covered the four regions (R1: −590/−402 bp; R2: −448/−204 bp; R3: −234/−1 bp and R4: −21/+140 bp) of *$K_{2p}1.1$* promoter, respectively (Integrated DNA Technologies). Each sample was run in a 20 μl reaction using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). The reactions were carried out in a BIO-RAD CFX96 real-time PCR system. The PCR amplification consisted of an initial 3-min incubation at 95°C, followed by 40 cycles at 95°C for 10 s, 60°C for 20 s, and 72°C for 20 s. All data were normalized to the corresponding input. Ratios of the remaining treated groups to naive group were calculated using the ΔCt method ($2^{-\Delta\Delta Ct}$).

2.9. Statistical analysis

The data are given as the mean ± standard error (SEM). Analysis of the data was done with one-way or two-way ANOVA. When there was a significant difference in the ANOVA, pairwise comparisons between the mean values were tested by the *post hoc* Tukey method. $P < 0.05$ were considered significant (SigmaStat, San Jose, CA).

3 Results

3.1. Microinjection of HSV-TET1 rescues $K_{2p}1.1$ expression in the microinjected DRGs after paclitaxel injection

Our previous study showed that paclitaxel-induced downregulation of DRG $K_{2p}1.1$ was predominantly attributed to the DNMT3a-triggered increase in DNA methylation within the *$K_{2p}1.1$* gene promoter from mouse DRG [10]. Thus, we first examined if DRG overexpression of TET1 rescued the $K_{2p}1.1$ downregulation in microinjected DRGs after paclitaxel injection in the rats. To this end, we microinjected the HSV expressing full-length *Tet1* mRNA (HSV-TET1) into unilateral L4/5 DRGs. The HSV expressing enhanced GFP (HSV-EGFP) was used as a control. As expected, the level of TET1 in the ipsilateral HSV-TET1-microinjected L4/5 DRGs was increased by 3.57-fold and 3.61-fold on day 10 after the first intraperitoneal (i.p.) injection of vehicle and paclitaxel, respectively, as compared to that in naive rats (Fig. 1A). Repeated i.p. injection of paclitaxel did not alter basal expression of TET1 in the PBS- or HSV-EGFP-microinjected L4/5 DRGs on the ipsilateral side (Fig. 1A). In contrast, the amount of $K_{2p}1.1$ was reduced by 54% and 61% in the PBS- and HSV-EGFP-microinjected L4/5 DRGs, respectively, on the ipsilateral side

on day 10 after first i.p. injection of paclitaxel (Fig. 1B). These reductions were not seen in the HSV-TET1-microinjected L4/5 DRGs on the ipsilateral side on day 10 after first i.p. injection of vehicle or paclitaxel (Fig. 1B).

3.2 DRG microinjection of HSV-TET1 attenuates the paclitaxel-induced nociceptive hypersensitivity during the development period

We next asked whether DRG overexpression of TET1 affected the development of paclitaxel-induced pain hypersensitivity. PBS or HSV was microinjected into unilateral L4/5 DRGs on day 4 after the first i.p. injection of vehicle or paclitaxel. Consistent with previous studies [10,11], the rats microinjected with the PBS- or HSV-EGFP displayed mechanical allodynia as evidenced by significant decreases in paw withdrawal thresholds in response to von Frey filament stimulation and heat and cold hyperalgesia as demonstrated by marked reductions in paw withdrawal latencies to thermal and cold stimuli, respectively, on both ipsilateral and contralateral sides on days 7 and 10 after the first i.p. injection of paclitaxel (Fig. 2A–E). These nociceptive hypersensitivities were markedly attenuated or abolished on the ipsilateral side after microinjection of HSV-TET1 into unilateral L4/5 DRGs in the paclitaxel-injected group (Fig. 2A–C). Paw withdrawal threshold to mechanical stimulation and paw withdrawal latencies to thermal and cold stimuli on the ipsilateral side in the HSV-TET1-microinjected group were higher compared to those in the PBS- or HSV-EGFP-microinjected groups on days 7 and 10 post-paclitaxel injection (Fig. 2A–C). Expectedly, DRG microinjection of HSV-TET1 did not alter the paclitaxel-induced decreases in paw withdrawal thresholds and latencies on the contralateral side during the observation period (Fig. 2D–E). This microinjection also did not affect basal paw withdrawal responses to mechanical, thermal and cold stimuli on both ipsilateral and contralateral sides of the vehicle-injected rats (Fig. 2A–E). As shown in Table 2, rats from four treated groups displayed normal locomotor functions including placing, grasping and righting reflexes.

3.3 DRG microinjection of HSV-TET1 impairs the paclitaxel-induced nociceptive hypersensitivity during the maintenance period

We further examined the role of DRG overexpression of TET1 in the maintenance of paclitaxel-induced nociceptive hypersensitivity. HSV-EGFP or HSV-TET1 was microinjected into unilateral L4/5 DRGs on day 7 after the first injection of paclitaxel, at this time point mechanical allodynia and heat and cold hyperalgesia were completely developed according to previous studies [10,11]. DRG microinjection of HSV-TET1, but not HSV-EGFP, markedly impaired the paclitaxel-induced nociceptive hypersensitivity on the ipsilateral side during the maintenance period (Fig. 3A–C). On days 12 and 16 post-first paclitaxel injection, paw withdrawal thresholds in response to mechanical stimulation increased by 2.0-fold and 3.4-fold, respectively (Fig. 3A), paw withdrawal latencies in response to thermal stimulation increased by 1.2-fold and 1.2-fold, respectively (Fig. 3B), and paw withdrawal latencies in response to cold stimulation increased by 1.3-fold and 1.5-fold, respectively (Fig. 3C), in the HSV-TET1-microinjected group as compared with the corresponding HSV-EGFP-microinjected group. Expectedly, DRG microinjection of neither HSV-EGFP nor HSV-TET1 affected the paclitaxel-induced decreases in paw withdrawal responses to mechanical or thermal stimuli applied to the contralateral hind paw during the observation period (Fig. 3D–E).

3.4 Effect of DRG microinjection of HSV-TET1 on paclitaxel-induced increase in 5-mC and decrease in 5-hmC within the $K_{2p}1.1$ promoter

Finally, we explored whether DRG overexpression of TET1 affected the DNA methylation of *$K_{2p}1.1$* gene promoter under the conditions of paclitaxel-induced neuropathic pain. The ChiP assay showed that 5-mC was enriched in two regions (R1: −590/−402 bp; R3: −234/−1 bp), but not the remaining two regions (R2: −448/−204 bp; R4: −21/+140 bp), of the *$K_{2p}1.1$* gene promoter. This was demonstrated by the amplifications of only R1 and R3 from the complexes immunoprecipitated with anti-5-mC in nuclear fractions from the DRGs of naive rats (Fig. 4A). Paclitaxel injection substantially increased the levels of 5-mC in both R1 and R3 of the *$K_{2p}1.1$* gene promoter in the ipsilateral L4/5 DRGs of the PBS- and HSV-EGFP-microinjected rats on day 10 after the first injection of paclitaxel (Fig. 4B). However, these increases were dramatically blocked in the ipsilateral L4/5 DRGs of the HSV-TET1-microinjected rats (Fig. 4B). Interestingly, DRG microinjection of HSV-TET1 did not alter basal level of 5-mC in R1 and R3 of the *$K_{2p}1.1$* gene promoter in the ipsilateral L4/5 DRGs on day 10 after i.p. vehicle injection.

We also detected 5-hmC level in four regions mentioned before and found that 5-hmC was highly enriched in both R1 and R3 and weakly detected in R2 (but not in R4) within the *$K_{2p}1.1$* gene promoter in the DRGs of naive rats (Fig. 5A). However, paclitaxel injection produced significant decreases in the amounts of 5-hmC in both R1 and R3 of the *$K_{2p}1.1$* gene promoter in the ipsilateral L4/5 DRGs of the PBS- and HSV-EGFP-microinjected rats on day 10 after the first injection of paclitaxel (Fig. 5B). These decreases were completely reversed in the ipsilateral L4/5 DRGs of the HSV-TET1-microinjected rats (Fig. 5B). As expected, basal level of 5-hmC in R1 and R3 of the *$K_{2p}1.1$* gene promoter in the HSV-TET1-microinjected L4/5 DRGs was not altered on day 10 after i.p. vehicle injection (Fig. 5B).

4 Discussion

In the present study, we demonstrated that DRG overexpression of TET1 mitigated nociceptive hypersensitivity during the development and maintenance periods of paclitaxel-induced neuropathic pain. This overexpression also rescued the expression of $K_{2p}1.1$ in the ipsilateral L4/5 DRGs following paclitaxel injection. Mechanistically, this rescue was attributed to the removal of paclitaxel-induced DNA methylation within the *$K_{2p}1.1$* gene promoter in the ipsilateral L4/5 DRGs. Given that DRG $K_{2p}1.1$ downregulation is required for paclitaxel-induced CIPN pain genesis and that DRG overexpression of TET1 did not affect basal (acute) pain or locomotor function, our findings indicate that overexpressing TET1 in the DRG may open a new door for the management of CIPN-induced pain.

The two-pore domain background potassium (K_2P) channel family and the leak currents carried by K_2P channels affect neuronal activity because disrupting these leak currents can result in depolarization and increased neuronal excitability [18]. $K_{2p}1.1$ is one of the K_2P family channels and recently was demonstrated to contribute to chronic neuropathic pain [10,19,20]. $K_{2p}1.1$ is abundant in DRG neurons [10,19,20]. Peripheral nerve injury or systemic paclitaxel injection downregulated the expression of *$K_{2p}1.1$* mRNA and $K_{2p}1.1$ protein in the DRG neurons [10,19,20]. Rescuing this downregulation alleviated the development and maintenance of spinal nerve injury- and paclitaxel-induced neuropathic

pain [10,19,20]. In contrast, mimicking this downregulation produced a decrease in outward potassium current and an increase in DRG neuronal excitability [10]. The evidence suggests that DRG $K_{2p}1.1$ plays a pivotal role in the genesis of neuropathic pain including CIPN-induced pain.

Further studies demonstrated that the paclitaxel-induced downregulation of K2p1.1 expression in DRG neurons may be associated with the DNMT3a-triggered DNA methylation in its gene promoter. DNA methylation represses gene expression primarily through physically blocking the binding of transcription factors and/or functioning as a docking site for transcription repressors/corepressors, such as the family of methyl-CpG-binding domain proteins [21,22]. Systemic injection of paclitaxel time-dependently increased the expression of DNMT3a protein in the DRG neurons [10]. Blocking this increase reversed the paclitaxel-induced downregulation of DRG K2p1.1 and attenuated the paclitaxel-induced nociceptive hypersensitivities [10]. Further study demonstrated that the paclitaxel-induced increase of DRG DNMT3a elevated the binding of DNMT3a with the promoter region of *K2p1.1* gene (−596/−406 bp) and the level of DNA methylation within this region in the DRG [10]. These findings suggest that the elevated DNA methylation within the promoter region of *K2p1.1* gene in the DRG may cause the paclitaxel-induced DRG K2p1.1 downregulation and nociceptive hypersensitivities. Thus, targeting this elevated DNA methylation in the DRG may be a new approach for the management of the paclitaxel-induced neuropathic pain.

Indeed, in the present study, DRG overexpression of TET1 through DRG microinjection of HSV-TET1 attenuated the development and maintenance of paclitaxel-induced nociceptive hypersensitivity. As demonstrated previously [16], exogenous TET1 delivered by DRG microinjection of HSV was expressed predominantly in DRG neurons and their fibers, as the microinjected HSV transduced into the neurons, but not other cells, in the DRG [16,23]. We found that microinjection of HSV-TET1 into unilateral L4/5 DRGs blocked the paclitaxel-induced increase in the level of 5-mC and correspondingly restored the paclitaxel-induced decrease in the amount of 5-hmC in the R1 (−590/−402) and R3 (−234/−1) regions of the $K_{2p}1.1$ promoter from the ipsilateral L4/5 DRGs of male rats. Levels of 5-mC and 5hmC were weakly detected or undetected in both R2 (−448/−204) and R4 (−21/+140) regions of the $K_{2p}1.1$ gene. Interestingly, there were no CpG sites in R2 region. We speculate that these weak signals are most likely non-specific background or DNA methylations occurring in non-CpG siters [24,25]. As discussed above, DNMT3a bound to the −596/−406 region of the $K_{2p}1.1$ gene, in which the level of 5-mC was DNMT3a-dependently increased after systemic injection of paclitaxel in male mice [10]. Given that both DNMT3a and $K_{2p}1.1$ are expressed exclusively in DRG neurons [10,26,27], TET1 overexpression in the DRG neurons likely erased the increased DNA methylation triggered by DNMT3a within the R1 region of the $K_{2p}1.1$ gene promoter in the ipsilateral L4/5 DRGs of male rats after paclitaxel injection. Consistent with this conclusion, our findings showed that DRG overexpression of TET1 rescued the paclitaxel-induced DRG downregulation of $K_{2p}1.1$. The previous study did not examine whether there was an increase in DNA methylation within the R3 region from mouse DRG, because DNMT3a was found to unbind to this region [10]. However, other DNA methyltransferases such as DNMT1 and DNMT3b may bind to the R3 region in rat DRG. The elevation in the level of DNA methylation in this region reported in the

present study is very likely due to an increase in the activities of DNMT1 and DNMT3b following paclitaxel injection, even if paclitaxel injection did not alter their basal levels in rat DRGs, like in mouse DRGs [10]. These possibilities remain to be addressed in our future study. It should be noted that exogenous TET1 through DRG microinjection of HSV-TET1 may also directly regulate the expression of other specific pain-associated genes in DRG neurons. Thus, DRG microinjection of TET1 produces an antinociceptive effect on the paclitaxel-induced CIPN pain likely through regulating multiple pain-associated genes including $K_{2p}1.1$ in the DRG neurons. Additionally, the present work only focused on male rats. Whether DRG overexpression of TET1 has similar or distinct effects on CIPN-induced pain in female rats remains to be determined in our future studies.

In summary, we have demonstrated that DRG overexpression of exogenous TET1 through microinjection of HSV-TET1 into unilateral L4/5 DRGs markedly mitigated the paclitaxel-induced nociceptive hypersensitivity during the development and maintenance periods. This microinjection did not affect acute (basal) pain and locomotor function. Given that DRG microinjection led to local effects on gene expression and that HSV-mediated gene therapy was used in clinical trial [28,29], the present study may open a new door in the management of CIPN pain.

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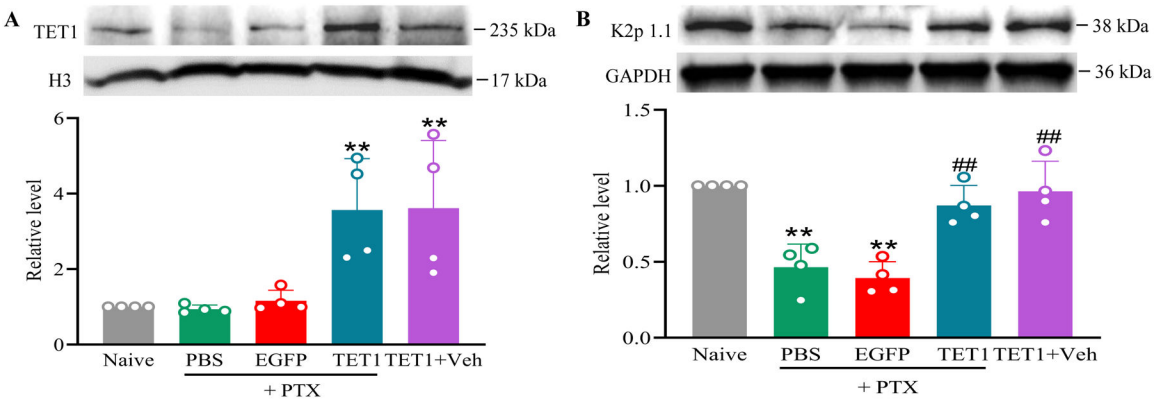

Fig. 1.

Microinjection of HSV-TET1 into unilateral L4/5 DRGs rescued the expression of $K_{2p}1.1$ in the ipsilateral L4/5 DRGs after paclitaxel injection. Expression of TET1 (A) and $K_{2p}1.1$ (B) in the ipsilateral L4/5 DRGs of the rats with pre-microinjection with HSV-TET1 (TET1), HSV-EGFP (EGFP) or PBS 10 days after the first injection of paclitaxel (PTX) or vehicle (Veh). Representative Western blots (top) and summary of densitometric analysis (bottom) are shown. n = 4 biological replicates (2 rats/replicate) per group. $^{**}P < 0.01$ versus the corresponding naïve group. $^{\#\#}P < 0.\ 01$ versus the corresponding PBS plus PTX group. One-way ANOVA with followed by post hoc Tukey test.

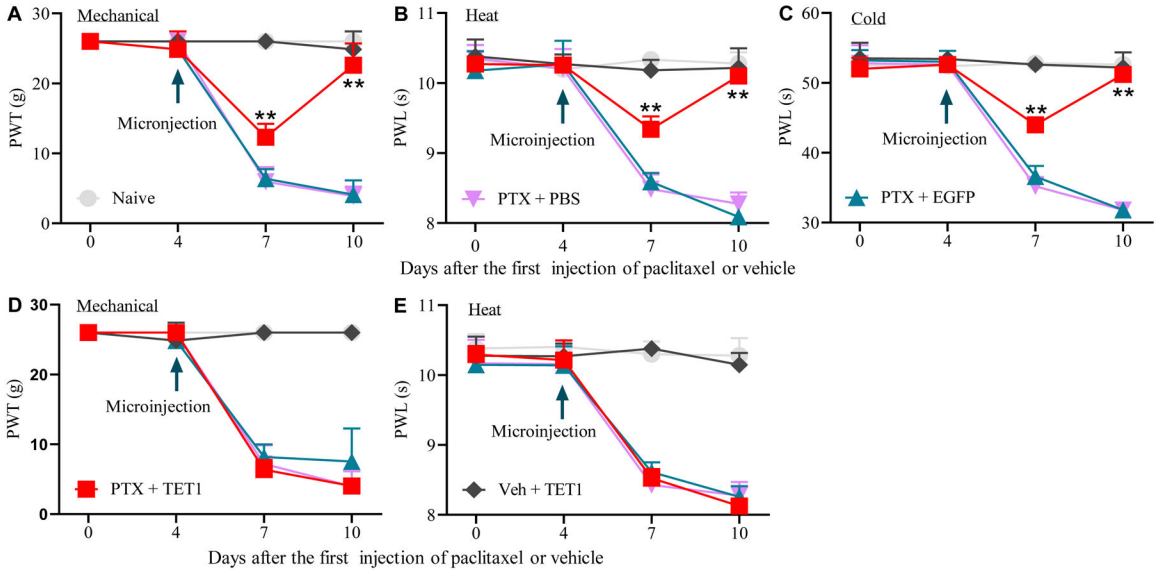

Fig. 2.

Microinjection of HSV-TET1 into unilateral L4/5 DRGs attenuated the paclitaxel-induced pain hypersensitivity during the development period. HSV-TET1 (TET1), HSV-EGFP (EGFP) or PBS was microinjected on day 4 after first injection of paclitaxel (PTX) or vehicle (Veh). Paw withdrawal threshold (PWT) to mechanical stimulation (A, D), paw withdrawal latency (PWL) to heat (B, E) and cold (C) stimuli on the ipsilateral (A-C) and contralateral (D, E) sides. Five groups: naive, PTX + PBS, PTX + EGFP, PTX + TET1 and Veh + TET1. n = 5 rats per group. $^{**}P < 0.01$ versus the PTX plus PBS group at the corresponding time point. Two-way ANOVA with repeated measures followed by post hoc Tukey test.

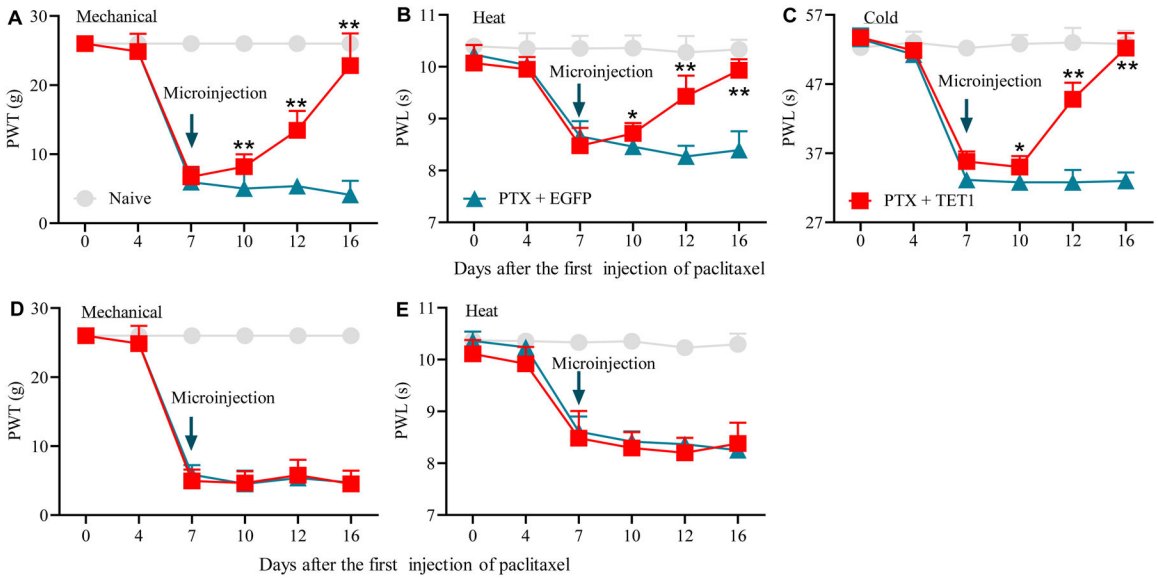

Fig. 3.

Microinjection of HSV-TET1 into unilateral L4/5 DRGs mitigated the paclitaxel-induced pain hypersensitivity during the maintenance period. HSV-TET1 (TET1) or HSV-EGFP (EGFP) was microinjected on day 7 after first injection of paclitaxel (PTX) or vehicle (Veh). Paw withdrawal threshold (PWT) to mechanical stimulation (A, D), paw withdrawal latency (PWL) to heat (B, E) and cold (C) stimuli on the ipsilateral (A-C) and contralateral (D, E) sides. Three groups: naive, PTX + EGFP and PTX + TET1. n = 5 rats19 per group. ***P* < 0.01 versus the PTX plus PBS group at the corresponding time point. Two-way ANOVA with repeated measures followed by post hoc Tukey test.

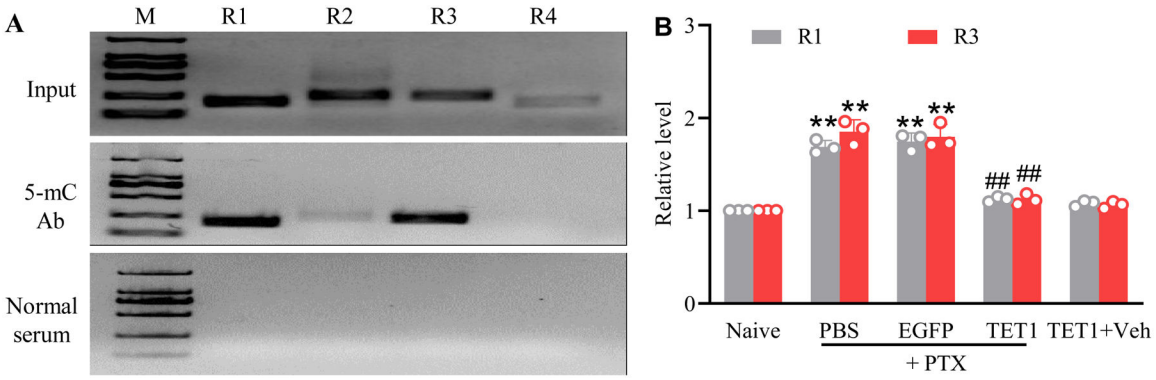

Fig. 4.

Microinjection of HSV-TET1 into unilateral L4/5 DRGs blocked the paclitaxel-induced increase in the level of 5-mC within the $K_{2p}1.1$ gene promotor in the ipsilateral L4/5 DRGs 10 days after the first injection of paclitaxel or vehicle. (A) The fragments from two regions (R1: −590/−402; R3: −234/−1), but not other two regions (R2: −448/−204; R4: −21/+140), of the $K_{2p}1.1$ gene promotor were immunoprecipitated by anti-5-mC antibody (Ab) in the L4/5 DRGs from naive rats. Normal serum was used as a control. Input: total purified fragments. R: region. M: DNA ladder marker. (B) Effects of microinjection of HSV-TET1 (TET1), HSV-EGFP (EGFP), or PBS into unilateral L4/5 DRGs on the level of 5-mC within the regions 1 and 3 of the $K_{2p}1.1$ gene promotor in the ipsilateral L4/5 DRGs 10 days after the first injection of paclitaxel (PTX) or vehicle (Veh). n = 3 biological repeats (6 rats/repeats/group). $^{**}P<0.01$ versus the corresponding naïve. $^{\#\#}P< 0.01$ versus the corresponding PBS plus PTX group. One-way ANOVA followed by post hoc Tukey test.

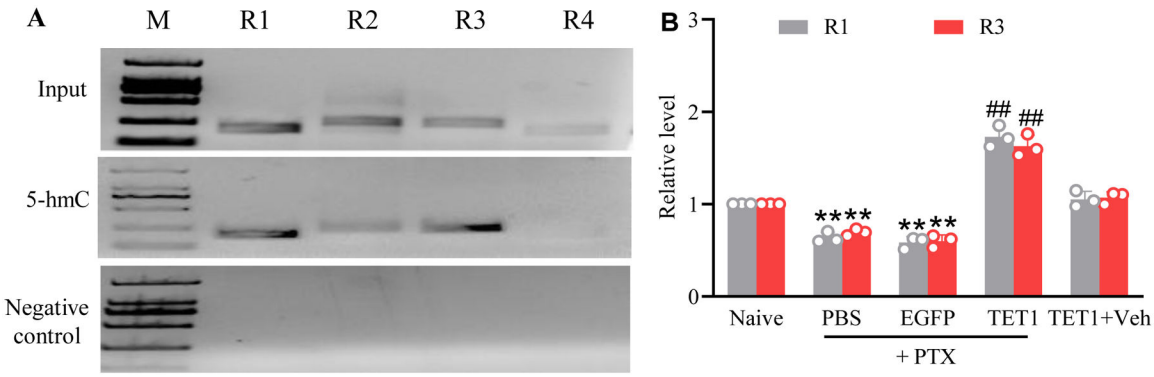

Fig. 5.

Microinjection of HSV-TET1 into unilateral L4/5 DRGs reversed the paclitaxel-induced decrease in the level of 5-hmC within the *$K_{2p}1.1$* gene promotor in the ipsilateral L4/5 DRGs 10 days after the first injection of paclitaxel or vehicle. (A) 5-hmC was highly enriched in both R1 (−590/−402) and R3 (−234/−1) regions, weakly detected in R2 region (−448/−204) and undetected in R4 region (−21/+140) of the *$K_{2p}1.1$* gene promotor in the L4/5 DRGs from naive rats. Negative control: without addition of 5-hmC modifying enzyme. Input: total purified fragments. R: region. M: DNA ladder marker. (B) Effects of microinjection of HSV-TET1 (TET1), HSV-EGFP (EGFP), or PBS into unilateral L4/5 DRGs on the level of 5-hmC within the regions 1 and 3 of the *$K_{2p}1.1$* gene promotor in the ipsilateral L4/5 DRGs 10 days after the first injection of paclitaxel (PTX) or vehicle (Veh). n = 3 biological repeats (6 rats/repeats/group). ***P* <0.01 versus the corresponding naïve. ##*P* < 0.01 versus the corresponding PBS plus PTX group. One-way ANOVA followed by post hoc Tukey test.

Table 1.

The primers used

Regions	Forward primers	Reverse primers
R1 (−590/−402)	5’-CCTCAGACCCTTTTTGTTCCA-3’	5’-CCCCAGGCATTCCTCCTGAT-3’
R2 (−448/−204)	5’-TCTATCTGTGCCCTTGGTAT-3’	5’-TCCCTCTTCCCTTCCTCAGT-3’
R3 (−234/−1)	5’-TGCTGACTGCACTGAGGAAG-3’	5’-CTGGTTCCGTGCAAATACAAG-3’
R4 (−21/+140)	5’-CTTGTATTTGCACGGAACCAG-3’	5’-AGCAGGTAGCCCAGCACCA-3’

Table 2.

Locomotor function.

Treatment groups	Placing	Grasping	Righting
Naïve	5 (0)	5 (0)	5 (0)
PTX + PBS	5 (0)	5 (0)	5 (0)
PTX + EGFP	5 (0)	5 (0)	5 (0)
PTX + TET1	5 (0)	5 (0)	5 (0)
Veh + TET1	5 (0)	5 (0)	5 (0)

n = 5 rats per group; five trials; Mean (SEM). EGFP: enhanced green fluorescent protein. PTX: paclitaxel. TET1: ten-eleven translocation methylcytosine dioxygenase 1. Veh: vehicle.