Contribution of the Suppressor of Variegation 3-9 Homolog 1 in Dorsal Root Ganglia and Spinal Cord Dorsal Horn to Nerve Injury–induced Nociceptive Hypersensitivity

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ABSTRACT

Background: Peripheral nerve injury–induced gene alterations in the dorsal root ganglion (DRG) and spinal cord likely participate in neuropathic pain genesis. Histone methylation gates gene expression. Whether the suppressor of variegation 3-9 homolog 1 (SUV39H1), a histone methyltransferase, contributes to nerve injury–induced nociceptive hypersensitivity is unknown.

Methods: Quantitative real-time reverse transcription polymerase chain reaction analysis, Western blot analysis, or immunohistochemistry were carried out to examine the expression of SUV39H1 mRNA and protein in rat DRG and dorsal horn and its colocalization with DRG μ-opioid receptor (MOR). The effects of a SUV39H1 inhibitor (chaetocin) or SUV39H1 siRNA on fifth lumbar spinal nerve ligation (SNL)–induced DRG MOR down-regulation and nociceptive hypersensitivity were examined.

Results: SUV39H1 was detected in neuronal nuclei of the DRG and dorsal horn. It was distributed predominantly in small DRG neurons, in which it coexpressed with MOR. The level of SUV39H1 protein in both injured DRG and ipsilateral fifth lumbar dorsal horn was time dependently increased after SNL. SNL also produced an increase in the amount of SUV39H1 mRNA in the injured DRG (n = 6/time point). Intrathecal chaetocin or SUV39H1 siRNA as well as DRG or intraspinal microinjection of SUV39H1 siRNA impaired SNL-induced allodynia and hyperalgesia (n = 5/group/treatment). DRG microinjection of SUV39H1 siRNA also restored SNL-induced DRG MOR down-regulation (n = 6/group).

Conclusions: The findings of this study suggest that SUV39H1 contributes to nerve injury–induced allodynia and hyperalgesia through gating MOR expression in the injured DRG. SUV39H1 may be a potential target for the therapeutic treatment of nerve injury–induced nociceptive hypersensitivity. **(Anesthesiology 2016; 125:765-78)**

NEUROPATHIC pain resulting from neurologic disorders such as peripheral nerve and spinal cord injury is a distressing condition affecting approximately 7% of the American population.[1] Of that percentage, only 30% of patients achieve efficient pain control with existing medications.[2] This public health problem translates into billions of U.S. dollars in healthcare expenses and lost productivity in the United States alone. Neuropathic pain is characterized by spontaneous ongoing pain or intermittent burning pain, allodynia, and hyperalgesia.[3] It is hypothesized that neuropathic pain is triggered by abnormal ectopic firing occurring in the neuroma at the site of injury and in primary sensory neurons of the dorsal root ganglion (DRG).[4,5] Such abnormal hyperexcitability may be due to nerve injury–induced

What We Already Know about This Topic

- Epigenetic mechanisms regulating gene expression influence many processes including those underlying pain
- Expression of the μ-opioid receptor on nociceptive neurons helps to control nociceptive input

What This Article Tells Us That Is New

- Using the spinal nerve ligation model of neuropathic pain, the authors observed down-regulation of μ-opioid receptor in conjunction with allodynia and hyperalgesia
- Reducing the activity or expression of the histone methyltransferase SUV39H1 using chaetocin or siRNA, μ-opioid receptor expression was increased in the spinal nerve ligation animals, and nociceptive sensitization was reduced

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changes in gene transcription and translation of receptors, voltage-dependent channels, and enzymes in the DRG.[6,7] Therefore, understanding how nerve injury leads to changes in gene expression of the DRG may provide novel therapeutic strategies in the prevention and/or treatment of nerve injury–induced nociceptive hypersensitivity.

Posttranslational histone modifications participate in the epigenetic regulation of gene expression and genome organization. Histone methylation is one process of histone modifications by which methyl groups are transferred onto amino acids of histone proteins in chromosomes, resulting in changes in the three-dimensional chromatin structure and gene expression.[8] This process is catalyzed by *S*-adenosylmethionine–dependent histone lysine methyltransferases and protein arginine methyltransferases.[8] Histone methylation can repress or activate gene transcription depending on the sites and content being methylated. Generally, methylation of histone H3 at Lys9 or Lys27 (H3K9 or H3K27, respectively) or histone H4 at Lys20 (H4K20) leads to transcriptional repression, while methylation of H3K4, H3K36, and H3K79 produces transcriptional activation.[9] The suppressor of variegation 3-9 homolog 1 (SUV39H1), first identified as a histone lysine methyltransferase, is primarily responsible for trimethylation of H3K9 (H3K9me3).[10] Previous reports showed that SUV39H1 played a critical role in chromosome dynamics and development, neurite outgrowth, and ethanol-induced neuroadaptation through gene-specific silencing.[11–13] Given that nerve injury–induced down-regulation of several genes including μ-opioid receptor (MOR) gene in the injured DRG contributes to the genesis and maintenance of nerve injury–induced nociceptive hypersensitivity,[14–16] we hypothesized that SUV39H1 likely acts as a gene repressor and is a key player in nerve injury–induced allodynia and hyperalgesia by gating nerve injury–induced gene down-regulation in the DRG.

In this study, we first characterized cellular expression and distribution patterns of SUV39H1 in rat DRG and spinal cord dorsal horn, two key regions in noxious information transmission and modulation. We then examined whether the activity and expression of SUV39H1 changed in these two regions after peripheral injury. We also investigated whether the inhibition or knockdown of SUV39H1 in the DRG or dorsal horn affected nerve injury–induced nociceptive hypersensitivities. Finally, we observed whether this effect was mediated through DRG MOR down-regulation.

Materials and Methods

Animals

Adult male Sprague-Dawley rats weighing 250 to 300*g* were used. They were purchased from a commercial supplier (Charles River Laboratories, Inc., USA) and allowed to habituate to the animal facility for at least 2 days before the experiments. Rats were housed in groups of two on a standard 12-h light/dark cycle, with water and food pellets available *ad libitum*. The group sizes were based on previous experience, and no *a priori* statistical power calculation was conducted. Rats were randomly assigned to treatment groups. All procedures used were approved by the Animal Care and Use Committee at the Rutgers New Jersey Medical School (Newark, New Jersey) and consistent with the ethical guidelines of the U.S. National Institutes of Health (Bethesda, Maryland) and the International Association for the Study of Pain (Washington, D.C.). All efforts were made to minimize animal suffering and to reduce the number of animals used. All of the experimenters were blind to treatment condition.

Neuropathic Pain Model

The fifth lumbar (L_5) spinal nerve ligation (SNL) was used as a neuropathic pain model in this study. The procedure was carried out as described previously.[14,16] Briefly, the L_5 spinal nerve was tightly ligated with 3-0 silk thread and transected distal to the ligature after it was exposed and isolated from the adjacent nerves. The skin and muscles were then closed in layers. In sham-operated rats, the L_5 spinal nerve was isolated, but was neither ligated nor transected.

Intrathecal Catheter Implantation and Drug Administration

A polyethylene 10 catheter was inserted into the subarachnoid space between the L4 and L5 vertebrae and advanced 2 to 2.5 cm into the lumbar enlargement of the spinal cord as previously described.[17,18] The residual catheter was tunneled under the skin to the neck area, and the outer part of the catheter was exposed, carefully plugged, and fixed onto the skin. Briefly, after 7 days of recovery, the drugs or siRNAs were injected intrathecally before SNL or sham surgery. None of the animals exhibited postoperative neurologic deficits (*e.g.*, paralysis) or poor grooming habits after catheter insertion surgery.

Chaetocin (Cayman, USA) dissolved in 30% dimethyl sulfoxide (DMSO) or vehicle (30% DMSO) alone was intrathecally administered in a 10-μl volume followed by a 10-μl saline flush before SNL or sham surgery and once daily for 3 days after SNL or sham surgery or once daily for 3 days starting on day 7 after SNL. Rat SUV39H1 siRNA (catalog number: sc-38464B) and its negative control siRNA (catalog number: sc-37007) were purchased from Santa Cruz Biotechnology, Inc. (USA). TurboFect *in vivo* transfection reagent (Thermo Scientific Inc., USA) was used as a delivery vehicle for siRNA as described.[19] After the siRNA (10 μM/10 μl) or vehicle (10 μl) was intrathecally injected twice daily for 3 days, SNL or sham surgery was carried out 6 days after injection.

DRG Microinjection

DRG microinjection was carried out as described in detail in our previous publication.[14,16,20] In brief, a midline incision was made in the lower lumbar back region, and the unilateral L_5 DRG was exposed. The siRNA (1 μl, 20 μM) or

vehicle (1 μl) was injected into the DRG with a glass micropipette (tip diameter, 20 to 40 μm) connected to a Hamilton syringe. The pipette was removed 10 min later after injection. The surgical field was irrigated with sterile saline, and the skin incision was closed with wound clips. Six days later, SNL or sham surgery on the injected side was carried out.

Intraspinal Microinjection

Spinal cord microinjection was carried out as described with minor modification.[21,22] Briefly, during constant anesthesia with isoflurane, unilateral laminectomy of the twelfth thoracic vertebra was carried out. After the spinal cord was exposed, the rat was placed in the stereotaxic frame and the vertebral column was immobilized. The glass micropipette was positioned 200 μm lateral from the posterior median sulcus and 200 μm below the dorsal surface of the spinal cord at the level of L_5 spinal cord. The remaining procedure of microinjection and the dosage and volume of siRNA or vehicle injected were similar to those in DRG microinjection. No impairment of motor function after intraspinal microinjection was observed. Six days later, SNL or sham surgery on the injected side was carried out.

Mechanical Test

Paw withdrawal thresholds (PWTs) in response to mechanical stimuli were measured with the up–down testing paradigm described previously.[16,20] Briefly, von Frey filaments in log increments of force (0.69, 1.20, 2.04, 3.63, 5.50, 8.51, 15.14, and 26 g) were applied to the plantar surface of the rats' left and right hind paws. The 3.63-g stimulus was applied first. If a positive response occurred, the next smaller von Frey hair was used; if a negative response was observed, the next larger von Frey hair was used. The test was terminated when (i) a negative response was obtained with the 26-g hair or (ii) three stimuli were applied after the first positive response. PWT was determined by converting the pattern of positive and negative responses to the von Frey filament stimulation to a 50% threshold value with a formula provided by Dixon.[23]

Thermal Test

Paw withdrawal latencies (PWLs) to noxious heat were measured with Model 336 Analgesia Meter (IITC Inc./Life Science Instruments, USA) as described previously.[16,20] Briefly, a beam of light that provided radiant heat was aimed at the middle of the plantar surface of each hind paw. When the animal lifted its foot, the light beam turned off. PWL was defined as the number of seconds between the start of the light beam and the foot response. The cutoff time is 20 s to avoid tissue damage. Each trial was repeated five times at 5-min intervals for each side.

Cold Plate Test

PWLs to noxious cold (0°C) were measured with a cold aluminum plate, the temperature of which was monitored continuously by a thermometer as described.[16,20] Each rat was placed in a Plexiglas chamber on the cold aluminum plate, which was set at 0°C. The length of time between the placement of the hind paw on the plate and a flinching of the paw was defined as the PWL. Each trial was repeated three times at 10-min intervals for the paw on the ipsilateral side. A cutoff time of 60 s was used to avoid tissue damage.

Conditioned Place Preference Test

Conditioned place preference (CPP) test was carried out as described.[24] Briefly, the CPP apparatus (MED Associates Inc., USA) consists of two Plexiglas chambers connected with an internal door. Movement of the rats and time spent in each chamber are monitored by photo beam detectors installed along the chamber walls and automatically recorded using MED-PC IV CPP software (MED Associates Inc.). Preconditioning was performed 3 days after SNL or sham surgery. The rats were allowed to explore both chambers for 30 min with the internal door opened. On the fourth day, the rats were placed into one chamber with full access to both chambers for 15 min (900 s). The duration of time spent in each chamber was recorded. Animals spending more than 720 s or less than 180 s in any chamber were excluded from further testing. The conditioning protocol was performed on days 5 and 6 after SNL or sham surgery when the internal door was closed. The rats first received intrathecal injection of saline (10 μl) paired with one conditioning chamber in the morning. Six hours later, lidocaine (0.8% in 10 μl saline) was given intrathecally paired with the opposite conditioning chamber in the afternoon. On day 7 (test day) after SNL or sham surgery, the rats were placed in one chamber with free access to both chambers. The duration of time spent in each chamber was recorded for 15 min. Difference scores were calculated as test time minus preconditioning time spent in the lidocaine chamber.

Locomotor Functions

The following locomotor function tests were performed: (1) placing reflex: the rat was held with the hind limbs slightly lower than the forelimbs, and the dorsal surfaces of the hind paws were brought into contact with the edge of a table. The experimenter recorded whether the hind paws were placed on the table surface reflexively; (2) grasping reflex: the rat was placed on a wire grid, and the experimenter recorded whether the hind paws grasped the wire on contact; (3) righting reflex: The rat was placed on its back on a flat surface, and the experimenter noted whether it immediately assumed the normal upright position. Scores for placing, grasping, and righting reflexes were based on counts of each normal reflex exhibited in five trials.

Cell Culture and siRNA Transfection

Rat PC-12 cells were cultured in Dulbecco's Modified Eagle's Medium/high glucose (Gibco/Thermo Fisher Scientific, USA) containing 5% fetal bovine serum, 5% horse serum (Gibco/Thermo Fisher Scientific), and 1% antibiotics. The

cells were incubated at 37°C in a humidified incubator with 5% CO_2. The siRNAs were transfected into the PC-12 cells with Lipofectamine 2000 (Invitrogen, USA) at the concentration of 100 nM according to the manufacturer's protocol. Three days after transfection, the cells were collected for Western blot analysis.

RNA Extraction and Quantitative Real-time RT-PCR

RNA extraction and real-time reverse transcription polymerase chain reaction (RT-PCR) were performed as described.[14,16,20] Briefly, the bilateral L_5 DRGs and dorsal parts of L_5 spinal cord were collected. To obtain enough RNA, L_5 DRGs from one side of two rats per time point were pooled. The tissues were first subjected to total RNA extraction using the miRNeasy kit with on-column digestion of genomic DNA (QIAGEN, USA) according to manufacturer's instructions. RNA was then reverse transcribed using the ThermoScript reverse transcriptase (Invitrogen) using oligo(dT) primers. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control for normalization, which has been demonstrated to be stable after peripheral nerve injury insult.[14,16,20] The SUV39H1 PCR primer sequences were 5′-CAGGTAGCTGTTGGCTGTGA-3′ (forward) and 5′-GACTACACGGTTTGGGCAGT-3′ (reverse). The GAPDH PCR primer sequences were 5′-TCGGTGTGAACGGATTTGGC-3′ (forward) and 5′-CCTTCAGGTGAGCCCCAGC-3′ (reverse). Each cDNA was run in triplicate in a 20-μl reaction with 250 nM forward and reverse primers and 10 μl of Advanced Universal SYBR Green Supermix (Bio-Rad Laboratories, USA). All real-time PCR reactions were performed in a BIO-RAD CFX96 real-time PCR system (Bio-Rad Laboratories) with an initial 3-min incubation at 95°C, followed by 40 cycles at 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. Ratios of ipsilateral-side mRNA levels to contralateral-side mRNA levels were calculated by using the ΔCt method ($2^{-\Delta\Delta Ct}$) at a threshold of 0.02. All data were normalized to GAPDH.

Immunohistochemistry

After being deeply anesthetized with isoflurane, the rats were perfused through the ascending aorta with 100 ml of 0.01 M phosphate-buffered saline (PBS; pH 7.4) followed by 400 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). L_5 DRGs and L_5 spinal cord were removed, postfixed in the same fixative for 2 to 4 h, and dehydrated in 30% sucrose in 0.01 M PBS. The DRG was sectioned at 20 μm and spinal cord at 25 μm. For single labeling, every fourth section was collected (at least three to four sections/DRG and six to seven sections/spinal cord). For double labeling, six sets of sections (at least two to three sections/set) were collected from each tissue by grouping every sixth serial section.

Single-label immunofluorescence histochemistry was carried out as described previously.[20] After being blocked for 1 h at 37°C in PBS containing 10% goat serum and 0.3% TritonX-100, the sections were incubated alone with rabbit anti-SUV39H1 (1:20; Abcam, USA) overnight at 4°C. The sections were then incubated with goat anti-rabbit IgG conjugated to Cy3 (1:200; Jackson ImmunoResearch, USA) for 2 h at room temperature. Control experiments included substitution of normal mouse serum for the primary antiserum and omission of the primary antiserum. Finally, the sections were rinsed in 0.01 M PBS and mounted onto gelatin-coated glass slides. Cover slips were applied with a mixture of 50% glycerin and 2.5% triethylene diamine in 0.01 M PBS.

Double-label immunofluorescence histochemistry was carried out as described previously.[20] Six sets of sections from naive DRGs were incubated overnight at 4°C with primary rabbit anti-SUV39H1 and one each of the following primary antibodies or the reagents: mouse anti-neurofilament 200 (1:500; Sigma-Aldrich, USA), biotinylated isolectin B4 (IB4; 1:100; Sigma-Aldrich), mouse anticalcitonin gene–related peptide (CGRP; 1:50; Abcam), mouse anti-NeuN (1:50, EMD Millipore, Germany), mouse antiglutamine synthetase (GS; 1:500; EMD Millipore), and guinea pig anti-MOR (1:1,000; EMD Millipore) overnight at 4°C. Four sets of sections from naive spinal cord were incubated overnight at 4°C with primary rabbit anti-SUV39H1 and one each of the following primary antibodies: mouse anti-NeuN, mouse antiglial fibrillary acidic protein (GFAP; 1:600; Cell signaling, USA), mouse anti-OX42 (1:600; EMD Millipore), and mouse anti–neural/glial antigen 2 (1:500; EMD Millipore) overnight at 4°C. The sections were then incubated for 1 h at 37°C with a mixture of goat anti-rabbit IgG conjugated with Cy3 (1:200, Jackson ImmunoResearch) and donkey anti-mouse IgG conjugated with Cy2 (1:400, Jackson ImmunoResearch), with a mixture of goat ant-rabbit IgG conjugated with Cy3 and donkey anti-guinea pig IgG conjugated to Cy2 (1:200; Jackson ImmunoResearch) or with a mixture of goat anti-rabbit IgG conjugated with Cy3 and fluorescein isothiocyanate–labeled avidin D (1:200; Sigma-Aldrich). Control experiments were performed in parallel as described in a single labeling. After the sections were rinsed in 0.01 M PBS, cover slips were applied. The SUV39H1 positive area in the spinal cord dorsal horn was quantified with National Institutes of Health Image J Software (NIH, USA).

Western Blotting Analysis

Protein extraction and Western blot analysis were carried out as described.[14] Briefly, the bilateral L_5 DRGs and dorsal parts of L_5 spinal cord were collected. To obtain enough protein, L_5 DRGs from one side of two rats per time point were pooled. The tissues were homogenized in chilled lysis buffer (10 mM Tris, 1 mM phenylmethylsulfonyl fluoride, 5 mM $MgCl_2$, 5 mM EGTA, 1 mM EDTA, 1 mM DTT, 40 μM leupeptin, and 250 mM sucrose). After centrifugation at 4°C for 15 min at 1,000*g*, the supernatant was collected for cytosolic proteins and the pellet for nuclear proteins. After protein concentration was measured, the samples (20 μg/sample) were heated at 99°C for 5 min and loaded

onto a 4 to 15% stacking/7.5% separating sodium dodecyl sulfate polyacrylamide gel (Bio-Rad Laboratories). The proteins were then electrophoretically transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories). The membranes were then blocked with 3% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h. The following primary antibodies were used: rabbit anti-SUV39H1 (1:1,000; Cell signaling), rabbit anti–histone H3 (1:1,000; Cell Signaling), rabbit antiphosphorylated extracellular signal–regulated kinase (p-ERK1/2, 1:1,000; Cell Signaling), rabbit anti–ERK1/2 (1:1,000; Cell Signaling), rabbit anti-GFAP (1:1,000; Cell Signaling), rabbit anti-MOR (1:500, Neuromics, USA), and rabbit anti-GAPDH (1:1000; Santa Cruz Biotechnology, Inc.). The proteins were detected using horseradish peroxidase–conjugated anti-rabbit secondary antibody (1:3,000, Bio-Rad Laboratories), visualized using western peroxide reagent and luminol/enhancer reagent (Clarity Western ECL Substrate; Bio-Rad Laboratories), and exposed using ChemiDoc XRS and System with Image Lab software (Bio-Rad Laboratories). The intensity of blots was quantified with densitometry using System with Image Lab software (Bio-Rad Laboratories). The bands of nucleus proteins were normalized to H3 and those of cytosol proteins to GAPDH.

Statistical Analysis

The data are presented as means ± SD. The results from the behavioral tests, RT-PCR, Western blot, and immunohistochemistry were statistically analyzed with a one-way or two-way ANOVA or paired or unpaired Student's *t* test. When ANOVA showed a significant difference, pairwise comparisons between means were tested by the *post hoc* Tukey method. The data were analyzed by SigmaPlot 12.5 (Systat Software Inc., USA). All probability values were two tailed, and a $P < 0.05$ was considered statistically significant.

Results

Neuronal Expression and Distribution of SUV39H1 in the DRG and Spinal Cord

To define the role of SUV39H1 in nerve injury–induced nociceptive hypersensitivity, we first examined its cellular distribution pattern in DRG and spinal cord dorsal horn. In the DRG, the double immunohistochemistry for SUV39H1 and NeuN (a specific neuronal marker) or GS (a marker for satellite glial cells) was carried out. SUV39H1 coexpressed with NeuN in cellular nuclei and was not detected in GS-positive cells (fig. 1A). In neuronal profiles, approximately 53.3% of DRG neurons were positive for SUV39H1, of which about 31.7% were labeled by CGRP (a marker for small DRG peptidergic neurons), 52% by IB4 (a marker for small nonpeptidergic neurons), and 16.7% by neurofilament 200 (a marker for medium/large neurons and myelinated A-fibers) (fig. 1A). In the dorsal horn of spinal cord, the immunohistochemical staining of SUV39H1 was distributed throughout whole laminae of the dorsal horn (fig. 1B). Under high magnification, many cellular nuclei positive for SUV39H1 were seen. These nuclei were also positive for NeuN (fig. 1B). SUV39H1 was not detected in the cells positive for GFAP (a marker for astrocyte), OX42 (a marker for microglia), or neural/glial antigen 2 (a marker for oligodendrocyte precursor cells; fig. 1B). The evidence indicates that SUV39H1 is expressed predominantly in the neurons of the DRG and spinal cord dorsal horn.

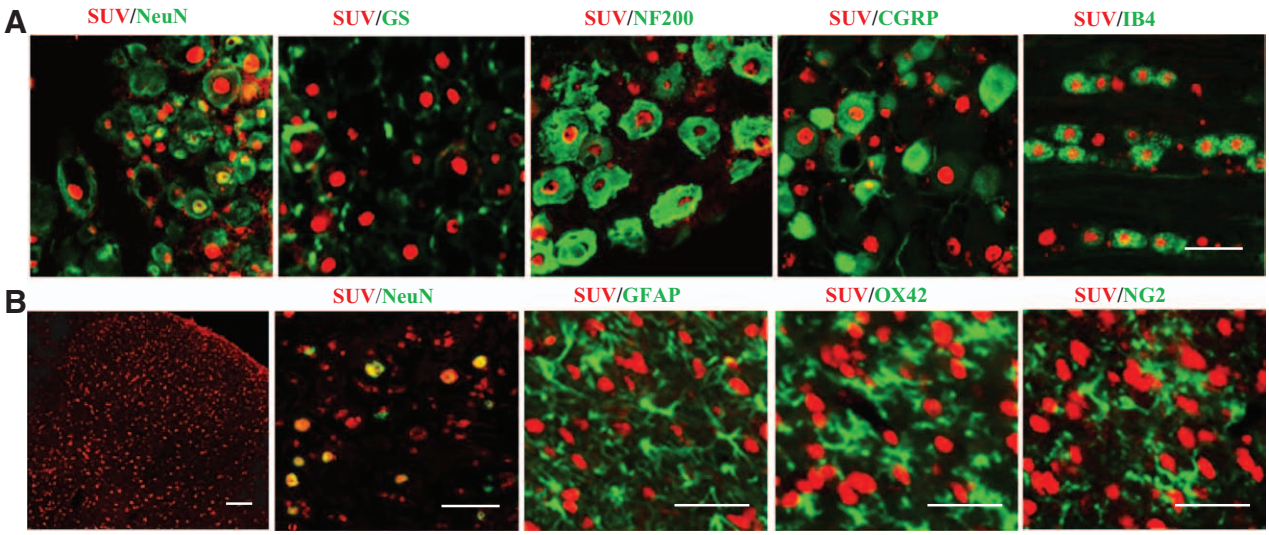

Fig. 1. The expression and distribution of suppressor of variegation 3-9 homolog 1 (SUV39H1 [SUV]) in the dorsal root ganglion (DRG) and spinal cord dorsal horn. n = 3 rats. (*A*) Double-label immunofluorescent staining of SUV39H1 with NeuN, glutamine synthetase (GS), neurofilament 200 (NF200), calcitonin gene–related peptide (CGRP), or isolectin B4 (IB4) in the DRG. (*B*) Single-label immunofluorescent staining of SUV39H1 (*left*) and double-label immunofluorescent staining of SUV39H1 with NeuN, glial fibrillary acidic protein (GFAP), OX42, or neural/glial antigen 2 (NG2) in dorsal horn (remaining panels). *Scale bars*: 50 μm (*A*) and 25 μm (*B*).

Time-dependent Increase in SUV39H1 in the Ipsilateral L_5 DRG and L_5 Dorsal Horn after Nerve Injury

Next, we examined whether SUV39H1 activity and expression were altered in DRG and spinal cord dorsal horn after SNL. SNL activated SUV39H1 as indicated by time-dependent increases in expression of SUV39H1 in L_5 DRG and L_5 dorsal horn on the ipsilateral side. A significant increase in the amounts of SUV39H1 started at day 3 post-SNL and was maintained until at least day 14 in the DRG (fig. 2A) and day 7 in the dorsal horn (fig. 2B). These increases were absent in the contralateral L_5 DRG (fig. 2A) and L_5 dorsal horn (fig. 2B) and the ipsilateral intact L_4 DRG (fig. 2C). As expected, sham surgery did not lead to a marked change in the level of SUV39H1 protein in $L_{4/5}$ DRGs and L_5 dorsal horn on both sides (data not shown). Furthermore, the number of SUV38H1-positive neuronal nuclei on day 3 after SNL was 1.4-fold greater than that on day 3 after sham surgery in the ipsilateral L_5 DRG (fig. 2D). The staining densities in the ipsilateral L_5 dorsal horn laminae I to II, III to IV, and V to VI on day 3 after SNL increased by 1.4-, 1.5-, and 1.2-fold, respectively, compared to the staining densities at the corresponding laminae on day 3 after sham surgery (fig. 2E). We also examined SUV39H1 expression at the transcription level in the DRG and dorsal horn after SNL. The ratios of ipsilateral to contralateral SUV39H1 mRNAs increased on days 3, 7, and 14 after SNL (but not

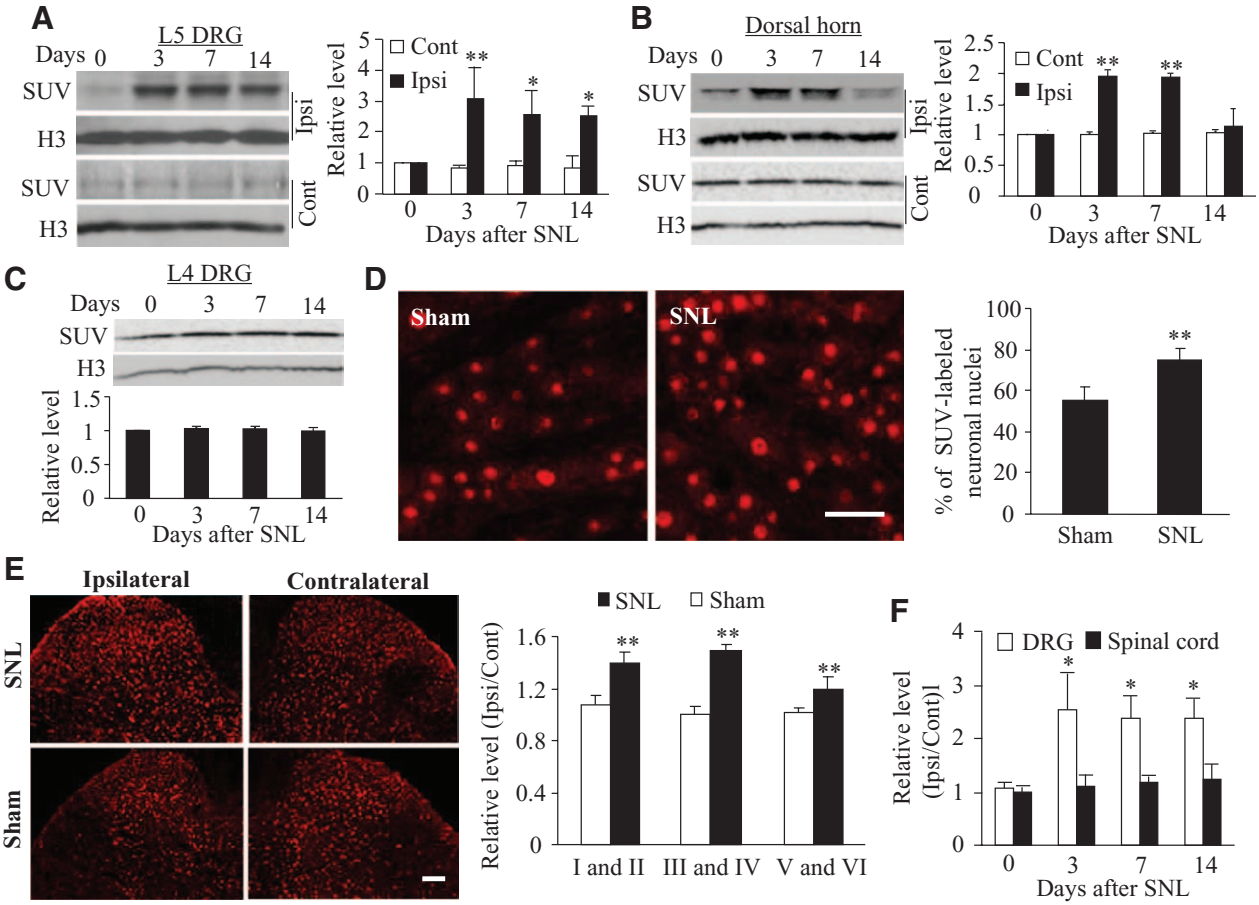

Fig. 2. Expression changes in suppressor of variegation 3-9 homolog 1 (SUV39H1 [SUV]) mRNA and protein in the dorsal root ganglion (DRG) and dorsal horn after fifth lumbar spinal nerve ligation (SNL). (*A*) SUV39H1 (SUV) protein increased in the ipsilateral (Ipsi), but not contralateral (Cont), L_5 DRG on days 3, 7, and 14 after SNL. n = 3 repeats (6 rats)/time point. Two-way ANOVA (expression *vs.* side × time interaction) followed by *post hoc* Tukey test, F_{time} (3, 27) = 5.5, $^{*}P < 0.05$ or $^{**}P < 0.01$ *versus* the corresponding naive rats (day 0). (*B*) SUV39H1 protein increased in the Ipsi, but not Cont, L_5 dorsal horn on days 3 and 7 (but not on days 14) after SNL. n = 3 rats/time point. Two-way ANOVA (expression *vs.* side × time interaction) followed by *post hoc* Tukey test, F_{time} (3, 23) = 29.8, $^{**}P < 0.01$ *versus* the corresponding naive rats (day 0). (*C*) No significant changes in SUV39H1 protein were seen in the Ipsi L_4 DRG after SNL. n = 3 repeats (6 rats)/time point. One-way ANOVA (expression *vs.* time points) followed by *post hoc* Tukey test, F_{time} (3, 11) = 0.4. (*D*, *E*) The number of SUV39H1-labeled neurons in the Ipsi L_5 DRG (*D*) and the densities of SUV39H1-positive neurons in the Ipsi L_5 dorsal horn laminae (*E*) increased on days 3 after SNL. n = 3 rats in the sham group and 4 rats in the SNL group. $^{**}P < 0.01$ *versus* the corresponding sham group by two-tailed unpaired Student's *t* test. *Scale bars*: 50 μm (*D*) and 25 μm (*E*). (*F*) The amounts of SUV39H1 mRNA increased in L_5 DRG, but not in L_5 dorsal horn, on the Ipsi side. n = 3 repeats (6 rats)/time point. One-way ANOVA (relative level *vs.* time points) followed by *post hoc* Tukey test, F_{time} (3, 11) = 7.4, $^{**}P < 0.01$ *versus* the corresponding naive rats (day 0) for the DRG and F(3, 11) = 0.8 for dorsal horn.

sham surgery; data not shown) in the injured L_5 DRG compared to that in naive rats (0 day; fig. 2F). Unexpectedly, the ratios of ipsilateral to contralateral SUV39H1 mRNAs were not altered significantly in the ipsilateral L_5 dorsal horn during the observation period after SNL (fig. 2F) or sham surgery (data not shown).

Effect of SUV39H1 Inhibition on the Development and Maintenance of Nerve Injury–induced Nociceptive Hypersensitivity

Is the increased SUV39H1 in the ipsilateral L_5 DRG and L_5 dorsal horn involved in nerve injury–induced nociceptive hypersensitivity? To address this question, we first intrathecally administered a specific SUV39H1 inhibitor chaetocin[25] 1 h before SNL or sham surgery and once daily for 6 days after SNL or sham surgery. SNL-induced mechanical allodynia and thermal or cold hyperalgesia were attenuated dose-dependently (fig. 3, A–C). PWTs to mechanical stimulation and PWLs to thermal or cold stimulation were significantly higher in the 2-μg chaetocin-treated group than in the vehicle-treated group on the ipsilateral side from days 3 to 7 post-SNL (fig. 3, A–C). Compared to the vehicle treatment, chaetocin at 1 μg also markedly increased PWTs and PWLs on the ipsilateral side at days 5 and 7 post-SNL (fig. 3, A–C). Chaetocin at 0.25 μg did not significantly affect SNL-induced decreases in PWTs and PWLs on the ipsilateral side during the observation period (fig. 3, A–C). Chaetocin at the doses used did not affect basal paw withdrawal responses

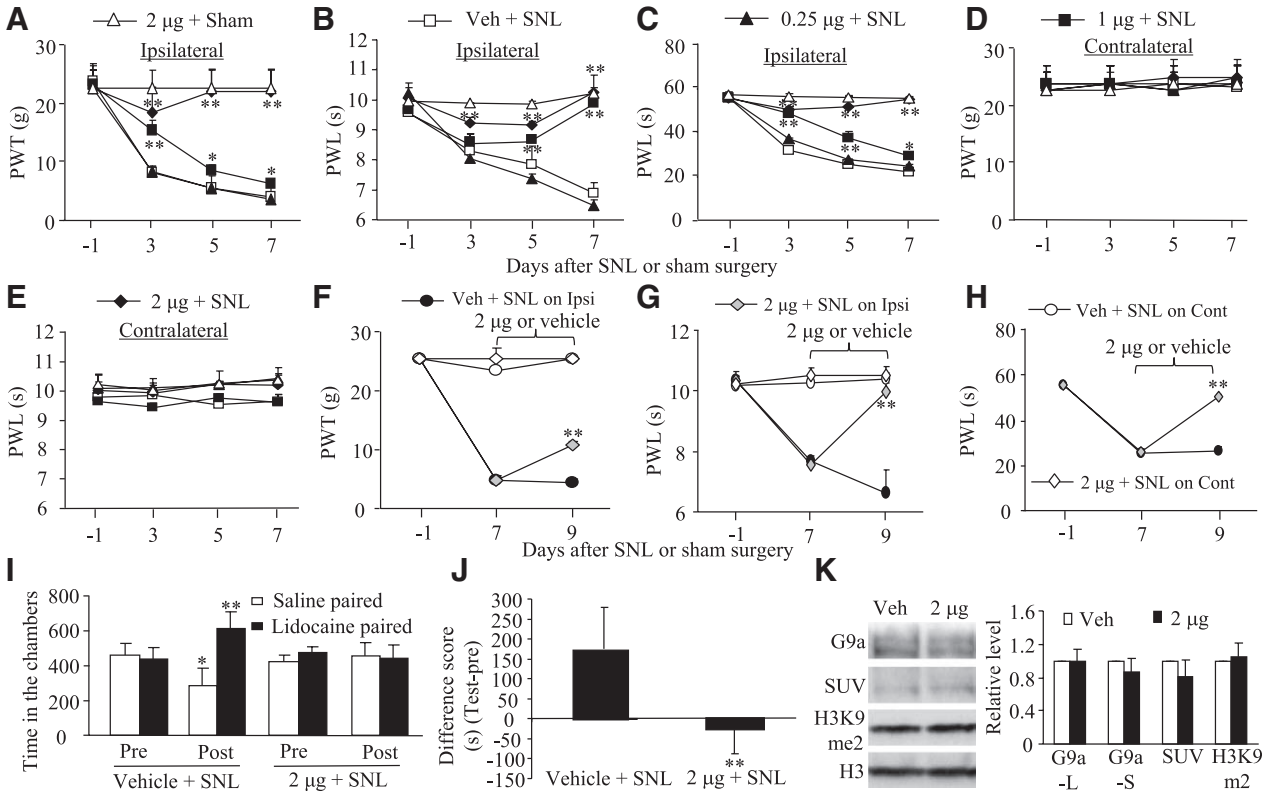

Fig. 3. Effect of intrathecal suppressor of variegation 3-9 homolog 1 (SUV39H1 [SUV]) inhibitor chaetocin on spinal nerve ligation (SNL)–induced nociceptive hypersensitivity. (*A–E*). Pretreatment of chaetocin dose-dependently blocked the decreases in paw withdrawal thresholds (PWTs) to mechanical stimulation (*A*) and in paw withdrawal latencies (PWLs) to thermal (*B*) or cold (*C*) stimulation on the ipsilateral (Ipsi) side and had no effects on basal paw withdrawal responses to mechanical (*D*) and thermal (*E*) stimuli on the contralateral (Cont) side. n = 5 rats/group. Two-way ANOVA (effect *vs.* group × time interaction) followed by *post hoc* Tukey test, F_{group} (12, 99) = 16.3 (*A*), F_{group} (12, 99) = 28.8 (*B*), F_{group} (12, 99) = 158.7 (*C*), F_{group} (12, 99) = 0.0004 (*D*), and F_{group} (12, 99) = 0.6 (*E*). $^{*}P < 0.05$ or $^{**}P < 0.01$ *versus* the corresponding time point in the vehicle (Veh) plus SNL group. (*F–H*) Posttreatment of chaetocin at 2 μg attenuated the decreases in paw withdrawal thresholds to mechanical stimulation (*F*) and in paw withdrawal latencies to thermal (*G*) or cold (*H*) stimulation on day 9 post-SNL on the Ipsi side. Behavioral tests were carried out 1 day before surgery, before drug injection on day 7 postsurgery, and on day 9 postsurgery. n = 5 rats/group. Two-way ANOVA (effect *vs.* group × time interaction) followed by *post hoc* Tukey test, F_{group} (6, 59) = 205.4 (*F*), F_{group} (6, 59) = 87.9 (*G*), and F_{group} (2, 29) = 17.5 (*H*). $^{**}P < 0.01$ *versus* the corresponding time point in the Veh plus SNL group. (*I*, *J*) Pretreatment of chaetocin at 2 μg blocked the SNL-induced spontaneous ongoing nociceptive responses. n = 5 rats/group, $^{*}P < 0.05$ or $^{**}P < 0.01$ *versus* the corresponding preconditioning (Pre; *I*) or the Veh plus SNL group (*J*) by two-tailed paired Student's *t* test. (*K*) Intrathecal chaetocin at 2 μg did not affect the expression of G9a's two protein isoforms, SUV protein, and dimethylation of Lys9 on histone H3 (H3K9me2) in the DRGs of sham rats. L = long isoform; Post = postconditioning; S = short isoform. n = 3 repeats (6 rats)/group.

on the contralateral side of SNL rats (fig. 3, D and 3E) and on either side of sham rats (fig. 3, A–E).

To test the effect of chaetocin on the maintenance of nerve injury–induced nociceptive hypersensitivity, we intrathecally administered chaetocin or vehicle starting on day 7 post-SNL when SNL-induced nociceptive hypersensitivity reaches a peak level. Chaetocin at 2 μg significantly reversed decreases in PWTs to mechanical stimulation and PWLs to thermal or cold stimulation compared to the vehicle-treated group on the ipsilateral side on day 9 post-SNL (fig. 3, F–H). Consistently, basal paw withdrawal responses on the contralateral side were not altered in either treatment group (fig. 3, F and G).

In addition to SNL-induced evoked nociceptive hypersensitivities, the effect of chaetocin on SNL-induced spontaneous ongoing nociceptive responses was examined using a CPP paradigm. Consistent with the previous studies,[24] SNL rats from the vehicle-treated group exhibited obvious preference (*i.e.*, spent more time) toward the lidocaine-paired chamber (fig. 3, I and J), indicating stimulation-independent spontaneous nociceptive responses. In contrast, SNL rats from the 2-μg chaetocin-treated group did not display marked preference toward either saline- or lidocaine-paired chamber (fig. 3, I and J), demonstrating no marked spontaneous nociceptive responses. As expected, sham rats from either treatment groups did not show any preference to the saline- or lidocaine-paired chamber (data not shown).

Finally, we examined whether chaetocin affected the activity and expression of another histone methyltransferase, G9a, which produces mono- and dimethylation of H3K9.[26,27] As shown in figure 3K, the levels of G9a's two protein isoforms and its catalyzed H3K9me2 in the DRGs of the 2 μg chaetocin-treated sham group were similar to those in the DRGs of the vehicle-treated sham group. The chaetocin at 2 μg also did not affect basal expression of DRG SUV39H1 (fig. 3K). The evidence indicates that chaetocin at the doses used may predominantly inhibit SUV39H1 activity.

Effect of SUV39H1 siRNA on the Development of Nerve Injury–induced Nociceptive Hypersensitivity

Nonspecific pharmacologic effects of chaetocin still cannot be excluded from our observations. To further confirm the role of SUV39H1 in nerve injury–induced nociceptive hypersensitivity, we examined whether blocking the increase of SUV39H1 in the DRG and dorsal horn *via* intrathecal injection of its siRNA affected the induction of SNL-induced nociceptive hypersensitivity. We first checked whether the SUV39H1 siRNA used had an effect on SUV39H1 expression. In *in vitro* cultured rat PC12 cells, transfection of SUV39H1 siRNA significantly reduced SUV39H1 expression compared to transfection with negative control siRNA (fig. 4A). We then intrathecally injected siRNAs 6 days before SNL or sham surgery and found that SUV39H1 siRNA (10 μM/10 μl), but not its negative control siRNA (10 μM/10 μl), diminished the SNL-induced increase in the level of SUV39H1 protein in the ipsilateral L_5 DRG and L_5 dorsal horn (fig. 4B). No significant changes in the basal level of SUV39H1 protein were seen in these two regions from the SUV39H1 siRNA plus sham surgery group (fig. 4B). Neither SUV39H1 siRNA nor its negative control siRNA altered the basal expression of SUV39H1 protein in the contralateral L_5 DRG and L_5 dorsal horn (data not shown). Like chaetocin, SUV39H1 siRNA dramatically blocked SNL-induced decreases in PWTs to mechanical stimulation and PWLs to thermal or cold stimulation on the ipsilateral side (fig. 4, C–E). Its negative control siRNA had no effect on SNL-induced mechanical allodynia and thermal or cold hyperalgesia (fig. 4, C–E). Basal mechanical, thermal, or cold responses on the contralateral side of SNL rats and on both ipsilateral and contralateral sides of sham rats were not affected after intrathecal injection of either siRNA (fig. 4, C–G).

We also examined whether intrathecal SUV39H1 siRNA affected nerve injury–induced dorsal horn central sensitization indicated by the increases in p-ERK1/2 and GFAP in dorsal horn after SNL.[28,29] In agreement with the previous study,[30] the levels of p-ERK1/2 (not total ERK1/2) and GFAP were markedly increased on day 3 after SNL compared to those after sham surgery in the ipsilateral L4 (data not shown) and L5 dorsal horn of vehicle-microinjected rats (fig. 4H). These increases were not detected in SUV38H1 siRNA-microinjected rats (fig. 4H).

To further define if SUV39H1 expressed in the DRG, dorsal horn, or both regions has a function in the development of nerve injury–induced nociceptive hypersensitivity, we microinjected SUV39H1 siRNA into the L_5 DRG or L_5 dorsal horn on the ipsilateral side 6 days before SNL or sham surgery. As expected, the SNL-induced increase in the level of SUV39H1 protein was not seen in the ipsilateral L_5 DRG (fig. 5) or L_5 dorsal horn (fig. 6) from the SUV39H1 siRNA plus SNL group compared to the vehicle plus SNL group or the negative control siRNA plus SNL group. Microinjection of SUV39H1 siRNA did not alter the basal expression of SUV39H1 on the contralateral side in SNL rats and on both ipsilateral and contralateral sides in sham rats in the L_5 DRG (fig. 5) or L_5 dorsal horn (fig. 6). Additionally, microinjection of siRNA into the ipsilateral L_5 DRG did not change the basal expression of SUV39H1 in the ipsilateral L_5 spinal cord and *vice versa* (data not shown). Like intrathecal injection of SUV39H1 siRNA, its microinjection into the ipsilateral L_5 DRG blocked SNL-induced decreases in PWTs to mechanical stimulation and PWLs to thermal or cold stimulation on the ipsilateral sides on days 3 and 5 post-SNL (fig. 5, B, D, and F). No changes were observed in basal mechanical, thermal, or cold responses on the contralateral side of SNL rats and on both ipsilateral and contralateral sides of sham rats after DRG microinjection of SUV39H1 siRNA or its negative control siRNA (fig. 5, B–F). Similar behavioral responses were seen after microinjection of SUV39H1 siRNA or its control siRNA into the ipsilateral L_5 dorsal horn (fig. 6, B–F).

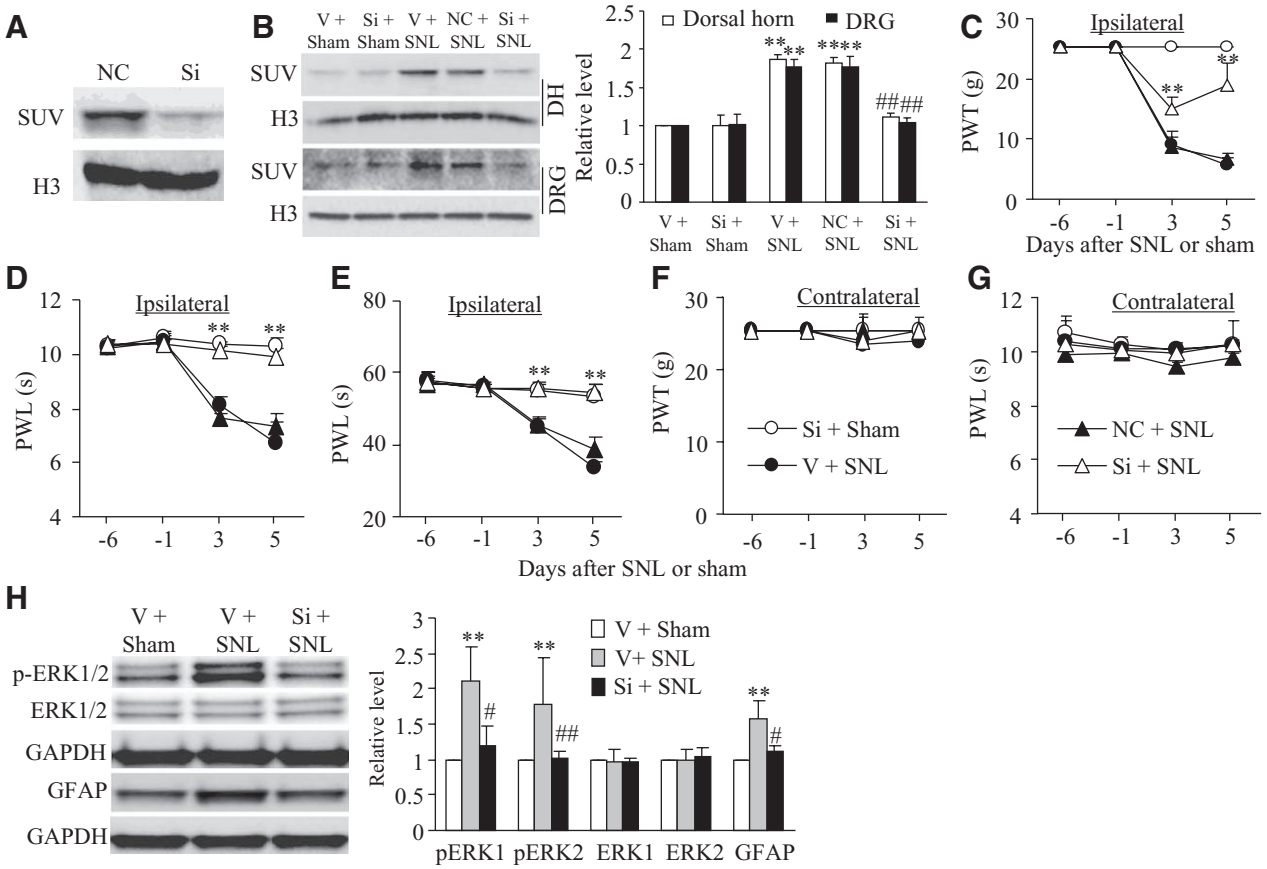

Fig. 4. Effect of intrathecal administration of suppressor of variegation 3-9 homolog 1 (SUV39H1 [SUV]) siRNA (Si) on spinal nerve ligation (SNL)–induced nociceptive hypersensitivity and dorsal horn (DH) central sensitization. (*A*) The level of SUV protein was markedly reduced in *in vitro* PC12 cells treated with Si compared to that treated with the negative control siRNA (NC). (*B*) Intrathecal Si blocked the SNL-induced increase in the level of SUV38H1 on day 7 after SNL and did not affect its basal expression on day 7 after sham surgery in the ipsilateral L_5 dorsal root ganglion (DRG) and L_5 DH. n = 3 repeats (6 rats)/time point. One-way ANOVA (effect *vs.* the treated groups) followed by *post hoc* Tukey test, F_{group} (4, 14) = 45.8 for DRG and F_{group} (4, 14) = 102.3 for DH, $^{**}P < 0.01$ *versus* the corresponding vehicle (V) + sham group. $\#\#P < 0.01$ *versus* the corresponding V + SNL group. (*C–G*) Intrathecal Si attenuated the SNL-induced decreases in paw withdrawal thresholds (PWTs) to mechanical stimulation (*C*) and in paw withdrawal latencies (PWLs) to thermal (*D*) or cold (*E*) stimulation on the ipsilateral side and had no effects on basal paw withdrawal responses to mechanical (*F*) and thermal (*G*) stimuli on the contralateral side. n = 5 rats/group. Two-way ANOVA (effect *vs*. group × time interaction) followed by *post hoc* Tukey test, F_{group} (6, 59) = 56.2 (*C*), F_{group} (6, 59) = 34.8 (*D*), F_{group} (6, 59) = 36.8 (*E*), F_{group} (6, 59) = 0.5 (*F*), and F_{group} (6, 59) = 0.3 (*G*). $^{**}P < 0.01$ *versus* the corresponding time point in the V + SNL group. (*H*) Intrathecal Si blocked the increases in the levels of phosphorylated extracellular signal–regulated kinases 1/2 (p-ERK1/2) and glial fibrillary acidic protein (GFAP) in the ipsilateral L5 DH on day 3 post-SNL. n = 3 rats/group. One-way ANOVA (expression *vs.* the treated groups) followed by *post hoc* Tukey test, F_{group} (2, 8) = 12.8 for p-ERK1, F_{group} (2, 8) = 20.1 for p-ERK2, F_{group} (2, 8) = 0.3 for total ERK1, F_{group} (2, 8) = 0.4 for total ERK2, and F_{group} (2, 8) = 20.1 for GFAP. $^{**}P < 0.01$ *versus* the corresponding V + sham group. $\#P < 0.05$, $\#\#P < 0.01$ *versus* the V + SNL group.

Effects of SUV39H1 siRNA on DRG MOR Expression and Its Analgesia after SNL

Peripheral nerve injury led to a transcriptional change in the expression of numerous genes including *MOR* in the DRG.[8] These changes are considered to be associated with the development and maintenance of nerve injury–induced nociceptive hypersensitivity. Given that endogenous opioids acting at MOR exert tonic inhibitory effects on nociceptive information transmission[31–34] and that SUV39H1 is a gene repressor, we proposed that the effects of SUV39H1 observed might be due to its participation in MOR down-regulation in the injured DRG after SNL. Consistent with the previous reports,[35–39] SNL led to a dramatic decrease in MOR expression in the injured L_5 DRG on day 3 post-SNL in the rats treated with vehicle or negative control siRNA (fig. 7A). Microinjection of SUV39H1 siRNA into the ipsilateral L_5 DRG completely reversed this decrease, although it did not alter the basal level of MOR in the ipsilateral L_5 DRG from sham rats (fig. 7A). Double-label immunofluorescence histochemistry revealed that SUV39H1 was detected in the nuclei of MOR-positive

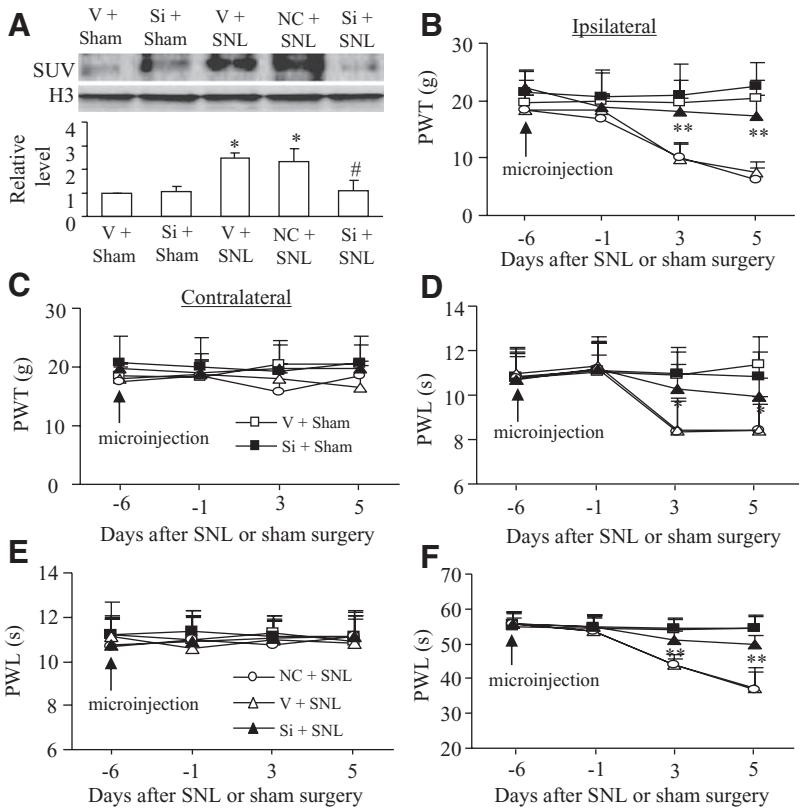

Fig. 5. Effect of dorsal root ganglion (DRG) microinjection of suppressor of variegation 3-9 homolog 1 (SUV39H1 [SUV]) siRNA (Si) on spinal nerve ligation (SNL)–induced nociceptive hypersensitivity. (*A*) Microinjection of Si into the ipsilateral L_5 DRG blocked the SNL-induced increase in the level of SUV38H1 on day 7 after SNL and did not affect its basal expression on day 7 after sham surgery in the injured DRG. n = 3 repeats (6 rats)/time point. One-way ANOVA (expression *vs.* the treated groups) followed by *post hoc* Tukey test, F_{group} (4, 14) = 13.5, **P* < 0.05 *versus* the corresponding vehicle (V) + sham group. #*P* < 0.05 *versus* the corresponding V + SNL group. (*B–F*) Microinjection of Si into the ipsilateral L_5 DRG blocked the SNL-induced decreases in paw withdrawal thresholds (PWTs) to mechanical stimulation (*B*) and in paw withdrawal latencies (PWLs) to thermal (*D*) or cold (*F*) stimulation on the ipsilateral side and had no effects on basal paw withdrawal responses to mechanical (*C*) and thermal (*E*) stimuli on the contralateral side. n = 5 rats/group. Two-way ANOVA (effect *vs.* group × time interaction) followed by *post hoc* Tukey test, F_{group} (12, 119) = 4.9 (*B*), F_{group} (12, 119) = 3.8 (*D*), F_{group} (12, 119) = 8.9 (*F*), F_{group} (12, 119) = 0.4 (*C*), and F_{group} (12, 119) = 0.2 (*E*). ***P* < 0.01 *versus* the corresponding time point in the V + SNL group. NC = negative control siRNA.

neurons in naive DRG (fig. 7B). Approximately 40.4% of SUV39H1-labeled neurons were positive for MOR and about 93.8% of MOR-labeled neurons were positive for SUV39H1. The results suggest the involvement of DRG SUV39H1 in nerve injury–induced DRG MOR down-regulation.

To further confirm this conclusion, we subcutaneously injected morphine (a MOR agonist) on day 3 after SNL or sham surgery in DRG microinjected rats. Consistent with the previous studies,[37,39,40] the analgesic effect of morphine on day 3 after SNL significantly declined compared to that on day 3 after sham surgery on the ipsilateral side of vehicle-microinjected rats (fig. 7C). This reduction was reversed markedly on the ipsilateral side of SUV39H1 siRNA microinjected rats (fig. 7C). As expected, morphine produced robust analgesia on the contralateral side of all treated groups (fig. 7C). In addition, we intraperitoneally injected methylnaltrexone bromide (Medchemexpress USA, USA; a peripheral MOR antagonist, 5 mg/kg, dissolved in saline) or saline on day 3 after SNL or sham surgery in DRG microinjected rats (fig. 7, D–G). In line with our observations in figure 5, SUV39H1 siRNA microinjected rats exhibited the attenuation of SNL-induced decreases in PWTs to mechanical stimulation and PWLs to thermal stimulation on the ipsilateral side after intraperitoneal saline injection (fig. 7, D and F). However, these effects were absent after intraperitoneal methylnaltrexone administration (fig. 7, D and F). Methylnaltrexone at the dose used did not affect basal behavioral responses on the contralateral side (fig. 7, E and G). Taken together, these findings indicate the implication of DRG MOR in antinociceptive effects of SUV39H1 inhibition or knockdown under the conditions of nerve injury–induced nociceptive hypersensitivity.

Locomotor Activities in the Experimental Animals

To exclude the possibility that the observed effects were produced by impaired locomotor activities, we examined the locomotor functions of experimental rats. None of the treatments led to any effects on locomotor activities, including

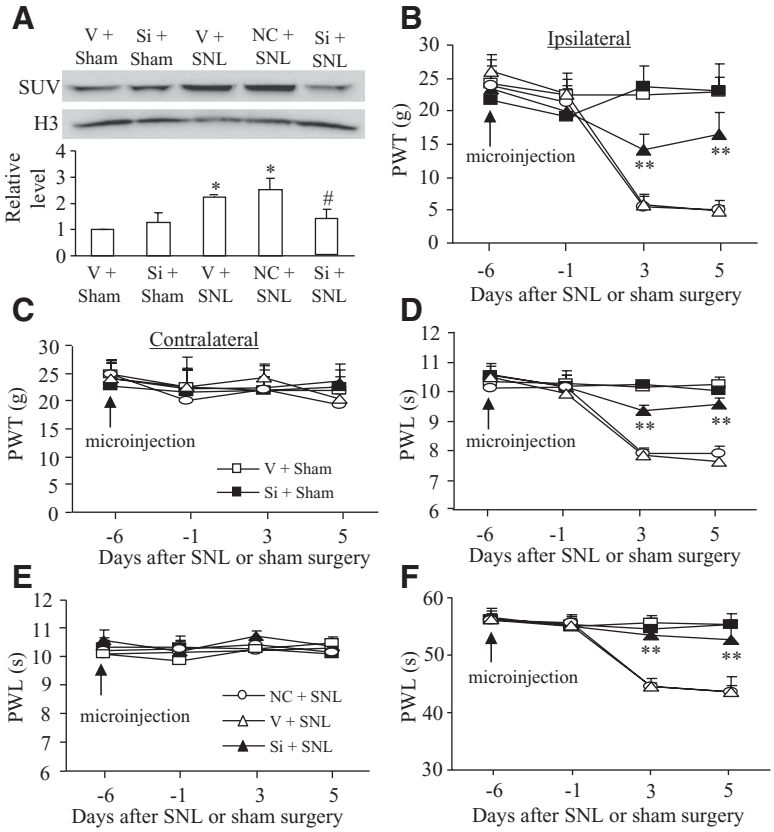

Fig. 6. Effect of intraspinal microinjection of suppressor of variegation 3-9 homolog 1 (SUV39H1 [SUV]) siRNA (Si) on spinal nerve ligation (SNL)-induced nociceptive hypersensitivity. (*A*) Intraspinal microinjection of Si attenuated the SNL-induced increase in the level of SUV38H1 on day 7 after SNL and did not affect its basal expression on day 7 after sham surgery in the ipsilateral L_5 dorsal horn. n = 3 rats/time point. One-way ANOVA (expression *vs.* the treated groups) followed by *post hoc* Tukey test, F_{group} (4, 14) = 14.4, **P* < 0.05 *versus* the corresponding vehicle (V) + sham group. #*P* < 0.05 *versus* the corresponding V + SNL group. (*B–F*) Intraspinal microinjection of Si blocked the SNL-induced decreases in paw withdrawal thresholds (PWTs) to mechanical stimulation (*B*) and in paw withdrawal latencies (PWLs) to thermal (*D*) or cold (*F*) stimulation on the ipsilateral side and had no effects on basal paw withdrawal responses to mechanical (*C*) and thermal (*E*) stimuli on the contralateral side. n = 5 rats/group. Two-way ANOVA (effect *vs.* group × time interaction) followed by *post hoc* Tukey test, F_{group} (12, 99) = 19.8 (*B*), F_{group} (12, 99) = 17.9 (*D*), F_{group} (12, 99) = 28.5 (*F*), F_{group} (12, 99) = 0.7 (*C*), and F_{group} (12, 99) = 1.4 (*E*). ***P* < 0.01 *versus* the corresponding time point in the V + SNL group. NC = negative control siRNA.

placing, grasping, and righting reflexes (data not shown). Hypermobility and convulsions were not seen in any of the treated groups. No marked differences in general behaviors, including the gait and spontaneous activity, were observed among the treatment groups.

Discussion

Peripheral nerve injury caused by SNL leads to long-term spontaneous nociceptive responses, allodynia, and thermal and cold hyperalgesia in a rat model, which mimics trauma-induced pain hypersensitivity in neuropathic pain patients. Understanding the mechanisms that underlie SNL-induced nociceptive hypersensitivity may develop a new therapeutic strategy for prevention and/or treatment of neuropathic pain. Despite intensive research into nerve injury–induced nociceptive hypersensitivity in the past decades, how nociceptive hypersensitivity develops after peripheral nerve injury is still incompletely understood. Here, we report that H3K9 methyltransferase SUV39H1 in both DRG and spinal cord dorsal horn is required for the development and maintenance of SNL-induced nociceptive hypersensitivity. SUV39H1 may be a potential target for therapeutic treatment of peripheral nerve injury–induced nociceptive hypersensitivity.

The current study provided the first evidence to our knowledge that SUV39H1 was expressed predominantly in the neuronal nuclei of both DRG and spinal cord dorsal horn, two key regions in the transmission and modulation of noxious information.[3,6] In the DRG, the majority of SUV39H1-labeled neurons were positive for small DRG neuronal markers, CGRP and IB4. The evidence indicates that SUV39H1 may be involved in nociceptive information conduction and processing. It has been observed that some SUV39H1-positive cells were negative for NeuN in the DRG and spinal cord. These cells likely express lower levels of NeuN that cannot be detected by the antibody used. We further demonstrated that SUV39H1 protein expression was increased in both L_5

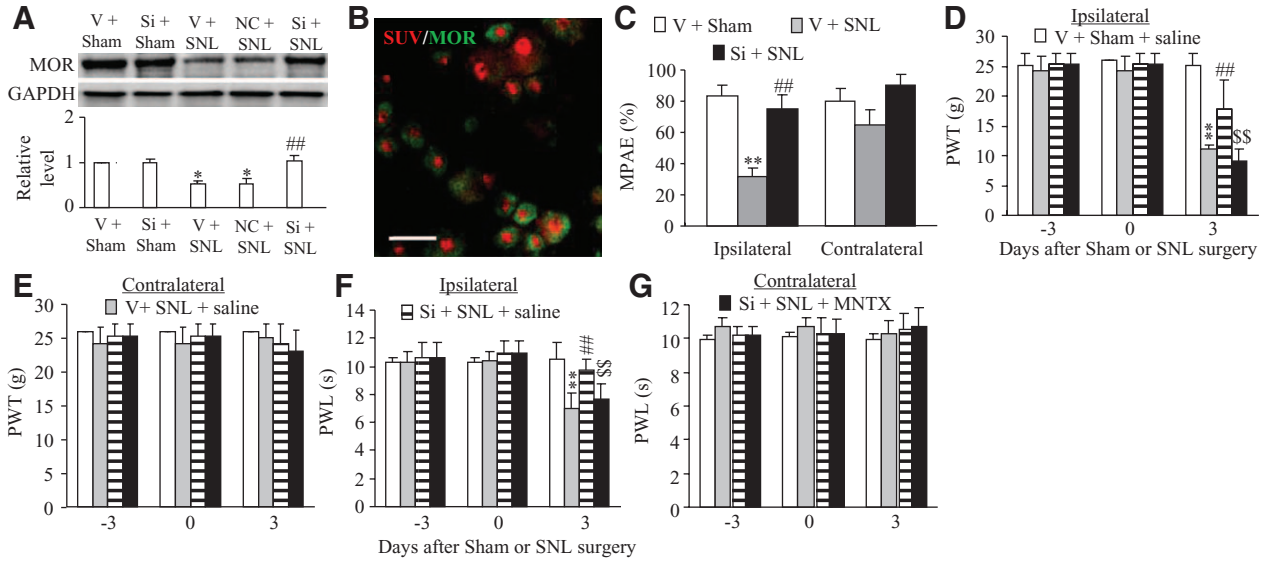

Fig. 7. Participation of suppressor of variegation 3-9 homolog 1 (SUV39H1 [SUV]) in spinal nerve ligation (SNL)–induced down-regulation of μ-opioid receptor (MOR) in the injured dorsal root ganglion (DRG). SUV39H1 siRNA (Si), negative control siRNA (NC), or vehicle (V) was microinjected into the injured DRG 3 days before SNL or sham surgery. (*A*) Microinjection of Si, but not NC, into the injured DRG restored the decrease of MOR in the injured DRG on day 3 after SNL. n = 3 repeats (6 rats)/group. One-way ANOVA (expression *vs.* the treated groups) followed by *post hoc* Tukey test, F_{group} (4, 14) = 24.9, **P* < 0.05 *versus* the V + sham group. ##*P* < 0.01 *versus* the V + SNL group. (*B*) Double immunofluorescent staining showed that SUV (*red*) was detected in the nuclei of many MOR (*green*)-positive neurons in naive DRG. *Scale bar*: 50 μm. n = 3 rats. (*C*) Microinjection of Si (n = 6 rats), but not vehicle, into the injured DRG reversed the decrease in morphine (1.5 mg/kg, subcutaneous injection) analgesia on the ipsilateral side 3 days after SNL. One-way ANOVA (effect *vs.* the treated groups) followed by *post hoc* Tukey test, F_{group} (2, 15) = 13.2 on the ipsilateral side and F_{group} (1, 15) = 2.8 on the contralateral side. ***P* < 0.01 *versus* the V + sham group (n = 5 rats). ##*P* < 0.01 *versus* the V + SNL group (n = 5 rats). (*D–G*) Intraperitoneal injection with methylnatrexone (MNTX; 5 mg/kg) blocked the Si-produced antinociceptive effect on day 3 after SNL in the Si-treated group (n = 6 rats). MNTX was administered on day 3 after SNL or sham surgery. Paw withdrawal threshold (PWT) to mechanical stimuli (*D*, *E*) and paw withdrawal latency (PWL) to thermal stimulation (*F*, *G*) were measured 30 min after drug administration. Two-way ANOVA (effect *vs.* group × time interaction) followed by *post hoc* Tukey test, F_{group} (6, 65) = 15.9 (*D*), F_{group} (6, 65) = 1.7 (*E*), F_{group} (6, 65) = 6.3 (*F*), and F_{group} (6,65) = 0.6 (*G*). ***P* < 0.01 *versus* the V + saline group 3 days after sham surgery (n = 5 rats). ##*P* < 0.01 *versus* the V + saline group 3 days after SNL (n = 5 rats). $$*P* < 0.01 *versus* the Si plus saline group 3 days after SNL (n = 6 rats).

DRG and L_5 dorsal horn on the ipsilateral side after SNL, although the durations of its increases in these two regions were different. In the ipsilateral L_5 DRG, SUV39H1 mRNA was also time dependently upregulated after SNL. This suggests that the *SUV39H1* gene in the injured DRG is activated at both transcription and translation levels after peripheral nerve injury. The nerve injury–induced increase in SUV39H1 mRNA may be triggered by transcription factors and/or caused by epigenetic modifications or an increase in RNA stability. These expectations will be investigated in our future studies. Interestingly, no significant changes in SUV39H1 mRNA were seen in the ipsilateral L_5 dorsal horn after SNL during the observation period. It appears that, unlike in the injured DRG, peripheral nerve injury produces only translational activation of SUV39H1 in the dorsal horn. The mechanisms that underlie the activation of the *SUV39H1* gene between DRG and dorsal horn are likely distinct under the conditions of nerve injury–induced nociceptive hypersensitivity.

The increased SUV39H1 in the injured DRG and ipsilateral dorsal horn is required for SNL-induced nociceptive hypersensitivity. Our pharmacologic study showed that intrathecal administration of the SUV39H1 inhibitor chaetocin significantly attenuated SNL-induced spontaneous nociceptive responses, mechanical allodynia, and thermal or cold hyperalgesia during the development and maintenance periods. As intrathecal chaetocin lacks anatomical and biochemical specificity, we further carried out a siRNA knockdown strategy and found that intrathecal injection, DRG microinjection, or intraspinal microinjection of SUV39H1 siRNA mitigated SNL-induced mechanical allodynia and thermal or cold hyperalgesia. Moreover, the role of SUV39H1 in the injured DRG in nerve injury–induced nociceptive hypersensitivity could not be compensated by dorsal horn SUV39H1 and *vice versa*, because SNL-induced nociceptive hypersensitivity could be attenuated by microinjection of SUV39H1 siRNA into the injured DRG and ipsilateral dorsal horn, respectively. Unexpectedly, the administration of SUV39H1 siRNA did not affect the basal level of SUV39H1 protein in the sham group although this siRNA specifically and markedly knocked down SUV39H1 expression in an *in vitro* rat cell line. No effect of SUV39H1 siRNA on *in vivo* basal SUV39H1 expression may be related

to low levels of SUV39H1 expression in the DRG and dorsal horn under normal conditions. SUV39H1 siRNA at the dose used could not further decrease the basal expression of SUV39H1 in sham rats despite the finding that SUV39H1 siRNA at this dose completely blocked the SNL-induced increase in SUV39H1 in the DRG or dorsal horn.

Peripheral nerve injury leads to changes in genes encoding ion channels, receptors, and enzymes in the injured DRG.[6,7,41] The histone methyltransferases may be key contributors to these changes. G9a methylates H3K9 to produce monomethylation (H2K9me1) and dimethylation (H3K9me2).[26] Previous reports revealed that G9a was essential for nerve injury–induced down-regulation of several K^+ channel genes in the injured DRG.[42] We showed here that blocking the SNL-induced increase in SUV39H1 restored the loss of MOR expression in the injured DRG and morphine analgesia. The antinociceptive effect caused by blocking increased DRG SUV39H1 in nerve injury–induced nociceptive hypersensitivity may be mediated by DRG MOR, because antagonizing peripheral MOR blocks this antinociception. Given that SUV39H1 coexpresses with MOR in the DRG neurons, SUV39H1's production of H3K9me3 may be required for SNL-induced down-regulation of DRG MOR. SUV39H1 likely contributes to nerve injury–induced nociceptive hypersensitivity through epigenetic silencing of MOR in the injured DRG. Nerve injury–induced DRG MOR down-regulation is also restored by the inhibition of DRG histone deacetylase or G9a,[38,43,44] suggesting multiple epigenetic mechanisms by which the *MOR* gene is silenced in the injured DRG after peripheral nerve injury. Compensation by other mechanisms may not occur, as blocking each mechanism can restore MOR expression in the injured DRG. It should be noted that SUV39H1 may also participate in nerve injury–induced changes in other genes in the injured DRG. Other potential mechanisms by which DRG SUV39H1 participates in nerve injury–induced nociceptive hypersensitivity cannot be ruled out. Interestingly, we found that the level of MOR in the dorsal horn was unchanged after siRNA or vehicle treatment in SNL or sham rats (data not shown), which is in line with a previous report.[45] How SUV39H1 in dorsal horn is involved in nerve injury–induced nociceptive hypersensitivity is still elusive and remains to be further studied.

Increased SUV39H1 in the injured DRG participates in the induction and maintenance of nerve injury–induced nociceptive hypersensitivity likely by gating peripheral MOR expression. The activation of MOR expressed on the central terminals of primary afferents inhibits the release of nociceptive neurotransmitters on these terminals.[31–34] The SNL-induced increase in SUV39H1 in the injured L5 DRG may down-regulate MOR expression in the ipsilateral primary afferents, resulting in enhanced release of neurotransmitters from the central terminals of these afferents and subsequent central sensitization in the ipsilateral L4 to L6 dorsal horn. This conclusion is supported by the fact that blocking the SNL-evoked increase of SUV39H1 in the injured L5 DRG not only restored the reduction of DRG MOR expression but also attenuated SNL-induced nociceptive hypersensitivity and the increases of p-ERK1/2 and GFAP (the markers for central sensitization) in the ipsilateral L4 and L5 dorsal horn. Thus, SUV39H1 may be an endogenous trigger in the development and maintenance of nerve injury–induced nociceptive hypersensitivity.

In conclusion, we, for the first time, demonstrated that blocking the SNL-induced increase in SUV39H1 activity and/or expression through intrathecal injection of its specific inhibitor or siRNA and microinjection of its siRNA into the DRG or dorsal horn impaired SNL-induced nociceptive hypersensitivity, without affecting basal or acute nociceptive responses and locomotor functions. Given that SUV39H1 is expressed mainly in small DRG neurons and that blocking its SNL-induced increase restored MOR expression in the injured DRG, SUV39H1 inhibitors may serve as promising medications for use as adjuvants with opioids in the treatment of nerve injury–induced nociceptive hypersensitivity.

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Competing Interests

The authors declare no competing interests.

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References

1. van Hecke O, Austin SK, Khan RA, Smith BH, Torrance N: Neuropathic pain in the general population: A systematic review of epidemiological studies. Pain 2014; 155:654–62
2. O'Connor AB: Neuropathic pain: Quality-of-life impact, costs and cost effectiveness of therapy. Pharmacoeconomics 2009; 27:95–112
3. Campbell JN, Meyer RA: Mechanisms of neuropathic pain. Neuron 2006; 52:77–92
4. Chung JM, Chung K: Importance of hyperexcitability of DRG neurons in neuropathic pain. Pain Pract 2002; 2:87–97
5. Devor M: Ectopic discharge in Abeta afferents as a source of neuropathic pain. Exp Brain Res 2009; 196:115–28
6. Costigan M, Scholz J, Woolf CJ: Neuropathic pain: A maladaptive response of the nervous system to damage. Annu Rev Neurosci 2009; 32:1–32
7. Lutz BM, Bekker A, Tao YX: Noncoding RNAs: New players in chronic pain. Anesthesiology 2014; 121:409–17
8. Liang L, Lutz BM, Bekker A, Tao YX: Epigenetic regulation of chronic pain. Epigenomics 2015; 7:235–45
9. Kouzarides T: Chromatin modifications and their function. Cell 2007; 128:693–705

10. Rea S, Eisenhaber F, O'Carroll D, Strahl BD, Sun ZW, Schmid M, Opravil S, Mechtler K, Ponting CP, Allis CD, Jenuwein T: Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature 2000; 406:593–9
11. Moss TJ, Wallrath LL: Connections between epigenetic gene silencing and human disease. Mutat Res 2007; 618:163–74
12. Qiang M, Denny A, Lieu M, Carreon S, Li J: Histone H3K9 modifications are a local chromatin event involved in ethanol-induced neuroadaptation of the NR2B gene. Epigenetics 2011; 6:1095–104
13. Sen N, Snyder SH: Neurotrophin-mediated degradation of histone methyltransferase by S-nitrosylation cascade regulates neuronal differentiation. Proc Natl Acad Sci USA 2011; 108:20178–83
14. Li Z, Gu X, Sun L, Wu S, Liang L, Cao J, Lutz BM, Bekker A, Zhang W, Tao YX: Dorsal root ganglion myeloid zinc finger protein 1 contributes to neuropathic pain after peripheral nerve trauma. Pain 2015; 156:711–21
15. Mansikka H, Zhao C, Sheth RN, Sora I, Uhl G, Raja SN: Nerve injury induces a tonic bilateral mu-opioid receptor-mediated inhibitory effect on mechanical allodynia in mice. Anesthesiology 2004; 100:912–21
16. Zhao X, Tang Z, Zhang H, Atianjoh FE, Zhao JY, Liang L, Wang W, Guan X, Kao SC, Tiwari V, Gao YJ, Hoffman PN, Cui H, Li M, Dong X, Tao YX: A long noncoding RNA contributes to neuropathic pain by silencing Kcna2 in primary afferent neurons. Nat Neurosci 2013; 16:1024–31
17. Cao J, Wang PK, Tiwari V, Liang L, Lutz BM, Shieh KR, Zang WD, Kaufman AG, Bekker A, Gao XQ, Tao YX: Short-term pre- and post-operative stress prolongs incision-induced pain hypersensitivity without changing basal pain perception. Mol Pain 2015; 11:73
18. Wang PK, Cao J, Wang H, Liang L, Zhang J, Lutz BM, Shieh KR, Bekker A, Tao YX: Short-term sleep disturbance-induced stress does not affect basal pain perception, but does delay postsurgical pain recovery. J Pain 2015; 16:1186–99
19. Xu JT, Zhao JY, Zhao X, Ligons D, Tiwari V, Atianjoh FE, Lee CY, Liang L, Zang W, Njoku D, Raja SN, Yaster M, Tao YX: Opioid receptor-triggered spinal mTORC1 activation contributes to morphine tolerance and hyperalgesia. J Clin Invest 2014; 124:592–603
20. Fan L, Guan X, Wang W, Zhao JY, Zhang H, Tiwari V, Hoffman PN, Li M, Tao YX: Impaired neuropathic pain and preserved acute pain in rats overexpressing voltage-gated potassium channel subunit Kv1.2 in primary afferent neurons. Mol Pain 2014; 10:8
21. Cameron AA, Smith GM, Randall DC, Brown DR, Rabchevsky AG: Genetic manipulation of intraspinal plasticity after spinal cord injury alters the severity of autonomic dysreflexia. J Neurosci 2006; 26:2923–32
22. Meunier A, Mauborgne A, Masson J, Mallet J, Pohl M: Lentiviral-mediated targeted transgene expression in dorsal spinal cord glia: Tool for the study of glial cell implication in mechanisms underlying chronic pain development. J Neurosci Methods 2008; 167:148–59
23. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL: Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 1994; 53:55–63
24. King T, Vera-Portocarrero L, Gutierrez T, Vanderah TW, Dussor G, Lai J, Fields HL, Porreca F: Unmasking the tonic-aversive state in neuropathic pain. Nat Neurosci 2009; 12:1364–6
25. Greiner D, Bonaldi T, Eskeland R, Roemer E, Imhof A: Identification of a specific inhibitor of the histone methyltransferase SU(VAR)3-9. Nat Chem Biol 2005; 1:143–5
26. Shinkai Y, Tachibana M: H3K9 methyltransferase G9a and the related molecule GLP. Genes Dev 2011; 25:781–8
27. Tachibana M, Ueda J, Fukuda M, Takeda N, Ohta T, Iwanari H, Sakihama T, Kodama T, Hamakubo T, Shinkai Y: Histone methyltransferases G9a and GLP form heteromeric complexes and are both crucial for methylation of euchromatin at H3-K9. Genes Dev 2005; 19:815–26
28. Latremoliere A, Woolf CJ: Central sensitization: A generator of pain hypersensitivity by central neural plasticity. J Pain 2009; 10:895–926
29. Ji RR, Gereau RW IV, Malcangio M, Strichartz GR: MAP kinase and pain. Brain Res Rev 2009; 60:135–48
30. Zhuang ZY, Gerner P, Woolf CJ, Ji RR: ERK is sequentially activated in neurons, microglia, and astrocytes by spinal nerve ligation and contributes to mechanical allodynia in this neuropathic pain model. Pain 2005; 114:149–59
31. Collin E, Frechilla D, Pohl M, Bourgoin S, Le Bars D, Hamon M, Cesselin F: Opioid control of the release of calcitonin gene-related peptide-like material from the rat spinal cord *in vivo*. Brain Res 1993; 609:211–22
32. Heinke B, Gingl E, Sandkühler J: Multiple targets of μ-opioid receptor-mediated presynaptic inhibition at primary afferent Aδ- and C-fibers. J Neurosci 2011; 31:1313–22
33. Pohl M, Lombard MC, Bourgoin S, Carayon A, Benoliel JJ, Mauborgne A, Besson JM, Hamon M, Cesselin F: Opioid control of the *in vitro* release of calcitonin gene-related peptide from primary afferent fibres projecting in the rat cervical cord. Neuropeptides 1989; 14:151–9
34. Taddese A, Nah SY, McCleskey EW: Selective opioid inhibition of small nociceptive neurons. Science 1995; 270:1366–9
35. Lee CY, Perez FM, Wang W, Guan X, Zhao X, Fisher JL, Guan Y, Sweitzer SM, Raja SN, Tao YX: Dynamic temporal and spatial regulation of mu opioid receptor expression in primary afferent neurons following spinal nerve injury. Eur J Pain 2011; 15:669–75
36. Obara I, Makuch W, Spetea M, Schütz J, Schmidhammer H, Przewlocki R, Przewlocka B: Local peripheral antinociceptive effects of 14-O-methyloxymorphone derivatives in inflammatory and neuropathic pain in the rat. Eur J Pharmacol 2007; 558:60–7
37. Rashid MH, Inoue M, Toda K, Ueda H: Loss of peripheral morphine analgesia contributes to the reduced effectiveness of systemic morphine in neuropathic pain. J Pharmacol Exp Ther 2004; 309:380–7
38. Uchida H, Ma L, Ueda H: Epigenetic gene silencing underlies C-fiber dysfunctions in neuropathic pain. J Neurosci 2010; 30:4806–14
39. Zhou XL, Yu LN, Wang Y, Tang LH, Peng YN, Cao JL, Yan M: Increased methylation of the MOR gene proximal promoter in primary sensory neurons plays a crucial role in the decreased analgesic effect of opioids in neuropathic pain. Mol Pain 2014; 10:51
40. Obara I, Przewlocki R, Przewlocka B: Local peripheral effects of mu-opioid receptor agonists in neuropathic pain in rats. Neurosci Lett 2004; 360:85–9
41. Wang W, Gu J, Li YQ, Tao YX: Are voltage-gated sodium channels on the dorsal root ganglion involved in the development of neuropathic pain? Mol Pain 2011; 7:16
42. Laumet G, Garriga J, Chen SR, Zhang Y, Li DP, Smith TM, Dong Y, Jelinek J, Cesaroni M, Issa JP, Pan HL: G9a is essential for epigenetic silencing of K(+) channel genes in acute-to-chronic pain transition. Nat Neurosci 2015; 18:1746–55
43. Uchida H, Matsushita Y, Araki K, Mukae T, Ueda H: Histone deacetylase inhibitors relieve morphine resistance in neuropathic pain after peripheral nerve injury. J Pharmacol Sci 2015; 128:208–11
44. Zhang Y, Chen SR, Laumet G, Chen H, Pan HL: Nerve injury diminishes opioid analgesia through lysine methyltransferase-mediated transcriptional repression of μ-opioid receptors in primary sensory neurons. J Biol Chem 2016; 291:8475–85
45. Obara I, Parkitna JR, Korostynski M, Makuch W, Kaminska D, Przewlocka B, Przewlocki R: Local peripheral opioid effects and expression of opioid genes in the spinal cord and dorsal root ganglia in neuropathic and inflammatory pain. Pain 2009; 141:283–91