Published in final edited form as:

Anesthesiology. 2016 October ; 125(4): 765–778. doi:10.1097/ALN.0000000000001261.

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

Jun Zhang, M.D.^{a,b}, Lingli Liang, M.D., Ph.D.^a, Xuerong Miao, M.D., Ph.D.^{a,c}, Shaogen Wu, M.D., Ph.D.^a, Jing Cao, M.D.^a, Bo Tao, B.S.^d, Qingxiang Mao, M.D.^{a,e}, Kai Mo, M.D., Ph.D.^a, Ming Xiong, M.D., Ph.D.^a, Brianna Marie Lutz, B.S.^a, Alex Bekker, M.D., Ph.D.^a, and Yuan-Xiang Tao, M.D., Ph.D.^{a,f}

^aDepartment of Anesthesiology, New Jersey Medical School, Rutgers, The State University of New Jersey, Newark, NJ07103, USA

^bDepartment of Anesthesiology, Union Medical Center, Nankai University, Tianjin 300122, China

^cDepartment of Anesthesiology and Intensive Care, Eastern Hepatobiliary Surgical Hospital, The Second Military Medical University, Shanghai200438, China

^dRowan University School of Osteopathic Medicine, Stratford, NJ08084, USA

^eDepartment of Anesthesiology, Institute of Surgery Research, Daping Hospital, The Third Military Medical University, Chongqing 400042, China

^fDepartments of Cell Biology & Molecular Medicine and Physiology, Pharmacology & Neuroscience, New Jersey Medical School, Rutgers, The State University of New Jersey, Newark, NJ 07103, USA

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 RT-PCR 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 co-localization with DRG mu opioid receptor (MOR). The effects of a SUV39H1 inhibitor (chaetocin) or SUV39H1 siRNA on fifth lumbar spinal nerve ligation (SNL)-induced DRG MOR downregulation and nociceptive hypersensitivity were examined.

Corresponding author: Dr. Yuan-Xiang Tao, Department of Anesthesiology, New Jersey Medical School, Rutgers, The State University of New Jersey, 185 S. Orange Ave., MSB, F-548, Newark, NJ 07103. Tel: +1-973-972-9812; Fax: +1-973-972-1644. yuanxiang.tao@njms.rutgers.edu.

The first three authors equally contributed to this work.

Competing Interests

The authors declare no competing interests.

Results—SUV39H1 was detected in neuronal nuclei of the DRG and dorsal horn. It was distributed predominantly in small DRG neurons, in which it co-expressed 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 downregulation (n = 6/group).

Conclusion—Our findings 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.

Neuropathic pain resulting from neurological disorders such as peripheral nerve and spinal cord injury is a distressing condition affecting approximately 7% of the American population¹. Of that percentage, only 30% of patients achieve efficient pain control with existing medications². This public health problem translates into billions of US dollars in health care expenses and lost productivity in the USA alone. Neuropathic pain is characterized by spontaneous ongoing pain or intermittent burning pain, allodynia, and hyperalgesia³. 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 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 DRG gene expression may provide novel therapeutic strategies in the prevention and/or treatment of nerve injury-induced nociceptive hypersensitivity.

Post-translational 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 3D chromatin structure and gene expression⁸. This process is catalyzed by *S*-adenosylmethionine-dependent histone lysine methyltransferases and protein arginine methyltransferases⁸. 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) or histone H4 at Lys20 (H4K20) leads to transcriptional repression, while methylation of H3K4, H3K36 and H3K79 produces transcriptional activation⁹. 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 downregulation of several genes including mu opioid receptor (MOR) 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 DRG gene downregulation.

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 following 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 downregulation.

Materials and Methods

Animals

Adult male Sprague-Dawley rats weighing 250g – 300g were used. They were purchased from a commercial supplier (Charles River Lab., Wilmington, MA) 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, USA) and consistent with the ethical guidelines of the US National Institutes of Health and the International Association for the Study of Pain. 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 (L₅) spinal nerve ligation was used as a neuropathic pain model in this study. The procedure was carried out as described previously^{14;16}. Briefly, the L₅ 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 L₅ 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 centimeter 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 neurological deficits (e.g., paralysis) or poor grooming habits after catheter insertion surgery.

Chaetocin (Cayman, Ann Arbor, Michigan) dissolved in 30% DMSO or vehicle (30% DMSO) alone was intrathecally administered in a 10 µl volume followed by a 10 µl saline flush before SNL/sham surgery and once daily for 3 days after SNL/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. (Dallas, TX). TurboFect *in vivo* transfection reagent (Thermo Scientific Inc., Pittsburgh PA) was used as a delivery vehicle for siRNA as described¹⁹. After the siRNA (10 μ M/10 μ l) or vehicle (10 μ l) 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 L₅ DRG was exposed. The siRNA (1 μ l, 20 μ M) or vehicle (1 μ l) was injected into the DRG with a glass micropipette (tip diameter 20 – 40 μ m) 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 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, under constant anesthesia with isoflurane, unilateral laminectomy of thoracic vertebra 12 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 μ m lateral from the posterior median sulcus and 200 μ m below the dorsal surface of the spinal cord at the level of L₅ 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. Paw withdrawal threshold 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 Dixon²³.

Thermal test

Paw withdrawal latencies (PWLs) to noxious heat were measured with Model 336 Analgesia Meter (IITC Inc. Life Science Instruments, Woodland Hills, CA, 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. Paw withdrawal latency was defined as the number of seconds between the start

of the light beam and the foot response. The cut-off time is 20 sec to avoid tissue damage. Each trial was repeated five times at 5-min intervals for each side.

Cold plate test

Paw withdrawal latencies (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 paw withdrawal latency. Each trial was repeated three times at 10-min intervals for the paw on the ipsilateral side. A cut-off time of 60 sec was used to avoid tissue damage.

Conditioned place preference test

Conditioned place preference (CPP) test was carried out as described²⁴. Briefly, the CPP apparatus (MED Associates Inc., St. Albans, VT) consists of 2 Plexiglas chambers connected with an internal door. Movement of the rats and time spent in each chamber is monitored by photobeam detectors installed along the chamber walls and automatically recorded in MED-PC IV CPP software. 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 4th day, the rats were placed into one chamber with full access to both chambers for 15 min (900 sec). The duration of time spent in each chamber was recorded. Animals spending more than 720 sec or less than 180 sec in any chamber were excluded from further testing. The conditioning protocol was performed on days 5 and 6 post-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 hour later, lidocaine (0.8 % in 10µL saline) was given intrathecally paired with the opposite conditioning chamber in the afternoon. On day 7 (test day) post-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-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, Grand Island, NY) medium containing 5% FBS, 5% horse serum (Gibco/Thermo Fisher Scientific) and 1% antibiotics. The cells were incubated at 37 °C in a humidified incubator with 5% CO₂. The siRNAs were transfected into the PC-12 cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) 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 RT-PCR were performed as described^{14;16;20}. Briefly, the bilateral L₅ DRGs and dorsal parts of L₅ spinal cord were collected. To obtain enough RNA, L₅ 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, Valencia, CA) according to manufacturer's instructions. RNA was then reverse-transcribed using the ThermoScript reverse transcriptase (Invitrogen) using oligo (dT) primers. 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'-TCGGTGTGAACGGATTGTC-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, Hercules, CA). 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 of 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 Ct method (2^{-Ct}) at a threshold of 0.02. All data were normalized to glyceraldehyde 3-phosphate dehydrogenase (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). L₅ DRGs and L₅ spinal cord were removed, post-fixed in the same fixative for 2–4 h, and dehydrated in 30% sucrose in 0.01M PBS. The DRG was sectioned at 20 µm and spinal cord at 25 µm. For single labeling, every fourth section was collected (at least 3–4 sections/DRG and 6–7 sections/spinal cord). For double labeling, six sets of sections (at least 2–3 sections/set) were collected from each tissue by grouping every sixth serial section.

Single-label immunofluorescence histochemistry was carried out as described previously²⁰. 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, Cambridge, MA) overnight at 4 °C. The sections were then incubated with goat anti-rabbit IgG conjugated to Cy3 (1:200, Jackson ImmunoResearch, West Grove, PA) 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 previously²⁰. Six sets of sections from naïve 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-NF200 (1:500, Sigma-Aldrich, St. Louis, MO), biotinylated isolectin B4 (IB4, 1:100, Sigma-Aldrich), mouse anti- calcitonin gene-related peptide (CGRP, 1:50, Abcam), mouse anti-NeuN (1:50, EMD Millipore, Germany), mouse anti-Glutamine synthetase (GS, 1:500, EMD Millipore), and guinea pig anti-MOR (1:1000, EMD Millipore) overnight at 4 °C. Four sets of sections from naïve 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 anti- glial fibrillary acidic protein (GFAP, 1:600; Cell signaling, Danvers, MA), mouse anti-OX42 (1:600; EMD Millipore), and mouse anti-NG2 (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 anti-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 FITC-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 spinal cord dorsal horn was quantified with NIH Image J Software.

Western blotting analysis

Protein extraction and Western blot analysis were carried out as described¹⁴. Briefly, the bilateral L₅ DRGs and dorsal parts of L₅ spinal cord were collected. To obtain enough protein, L₅ 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 MgCl₂, 5 mM EGTA, 1 mM EDTA, 1mM DTT, 40 μM leupeptin, 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 4–15% stacking/7.5% separating SDS-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 anti-phosphorylated extracellular signal-regulated kinase (p-ERK1/2, 1:1,000, Cell Signaling), rabbit anti- extracellular signal-regulated kinase (ERK1/2, 1:1,000, Cell Signaling), rabbit anti-glial fibrillary acidic protein (GFAP, 1:1,000, Cell Signaling), rabbit anti-MOR (1:500, Neuromics, Edina, MN), and rabbit anti-GAPDH (1:1000; Santa Cruz, Dallas, Texas). The proteins were detected by horseradish peroxidase–conjugated anti-

rabbit secondary antibody (1:3,000, Bio-Rad) and visualized by western peroxide reagent and luminol/enhancer reagent (Clarity Western ECL Substrate, Bio-Rad) and exposure by ChemiDoc XRS and System with Image Lab software (Bio-Rad). The intensity of blots was quantified with densitometry by System with Image Lab software (Bio-Rad). The bands of nucleus proteins were normalized to H3 and those of cytosol proteins to GAPDH.

Statistical analysis

The data are presented as means $\pm$ SD. The results from the behavioral tests, RT-PCR, Western blot, and immunohistochemistry were statistically analyzed with a one-way or two-way analysis of variance (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 (San Jose, CA). All probability values were two tailed, and a *P* value less than 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 co-expressed with NeuN in cellular nuclei and was not detected in GS-positive cells (Figure 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 NF200 (a marker for medium/large neurons and myelinated A-fibers) (Figure 1A). In the dorsal horn of spinal cord, the immunohistochemical staining of SUV39H1 was distributed throughout whole laminae of dorsal horn (Figure 1B). Under high magnification, many cellular nuclei positive for SUV39H1 were seen. These nuclei were also positive for NeuN (Figure 1B). SUV39H1 was not detected in the cells positive for GFAP (a marker for astrocyte), OX42 (a marker for microglia), or NG2 (a marker for oligodendrocyte precursor cells) (Figure 1B). The evidence indicates that SUV39H1 is expressed predominantly in the neurons of the DRG and spinal cord dorsal horn.

Time-dependent increase in SUV39H1 in the ipsilateral L₅ DRG and L₅ dorsal horn after nerve injury

Next, we examined whether SUV39H1 activity and expression were altered in DRG and spinal cord dorsal horn following SNL. SNL activated SUV39H1 as indicated by time-dependent increases in expression of SUV39H1 in L₅ DRG and L₅ 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 (Figure 2A) and day 7 in the dorsal horn (Figure 2B). These increases were absent in the contralateral L₅ DRG (Figure 2A) and L₅ dorsal horn (Figure 2B) and the ipsilateral intact L₄ DRG (Figure 2C). As expected, sham surgery did not lead to a marked change in the level of SUV39H1 protein in L_{4/5} DRGs and

L₅ 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 L₅ DRG (Figure 2D). The staining densities in the ipsilateral L₅ dorsal horn laminae I–II, III–IV, and V–VI on day 3 after SNL increased by 1.4-fold, 1.5-fold, and 1.2-fold, respectively, compared to the staining densities at the corresponding laminae on day 3 after sham surgery (Figure 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 sham surgery, data not shown) in the injured L₅ DRG compared to that in naïve rats (0 day, Figure 2F). Unexpectedly, the ratios of ipsilateral to contralateral SUV39H1 mRNAs were not altered significantly in the ipsilateral L₅ dorsal horn during the observation period after SNL (Figure 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 L₅ DRG and L₅ dorsal horn involved in nerve injury-induced nociceptive hypersensitivity? To address this question, we first intrathecally administered a specific SUV39H1 inhibitor chaetocin²⁵ 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 (Figures 3A–3C). 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 (Figures 3A–3C). 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 (Figures 3A–3C). Chaetocin at 0.25 µg did not significantly affect SNL-induced decreases in PWTs and PWLs on the ipsilateral side during the observation period (Figures 3A–3C). Chaetocin at the doses used did not affect basal paw withdrawal responses on the contralateral side of SNL rats (Figures 3D and 3E) and on either side of sham rats (Figures 3A–3E).

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 (Figures 3F–3H). Consistently, basal paw withdrawal responses on the contralateral side were not altered in either treatment group (Figures 3F and 3G).

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 previous studies²⁴, SNL rats from the vehicle-treated group exhibited obvious preference (i.e. spent more time) toward the lidocaine-paired chamber (Figures 3I and 4J), 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 (Figures 3I and 3J),

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 di-methylation 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 (Figure 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

Non-specific pharmacological 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 in 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 (Figure 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 L₅ DRG and L₅ dorsal horn (Figure 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 (Figure 4B). Neither SUV39H1 siRNA nor its negative control siRNA altered the basal expression of SUV39H1 protein in the contralateral L₅ DRG and L₅ 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 (Figure 4C–4E). Its negative control siRNA had no effect on SNL-induced mechanical allodynia and thermal or cold hyperalgesia (Figure 4C–4E). 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 following intrathecal injection of either siRNA (Figure 4C–4G).

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 following SNL^{28,29}. In agreement with the previous study³⁰, the levels of p-ERK1/2 (not total ERK1/2) and GFAP were markedly increased on day 3 post-SNL compared to those post-sham surgery in the ipsilateral L₄ (data not shown) and L₅ dorsal horn of vehicle-microinjected rats (Figure 4H). These increases were not detected in SUV39H1 siRNA-microinjected rats (Figure 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 L₅ DRG or L₅ 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 L₅ DRG (Figure 5) or L₅ dorsal horn (Figure 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 from SNL rats and on both ipsilateral and contralateral sides from sham rats in the L₅ DRG (Figure 5) or L₅ dorsal horn (Figure 6). Additionally, microinjection of siRNA into the ipsilateral L₅ DRG did not change the basal expression of SUV39H1 in the ipsilateral L₅ spinal cord and vice versa (data not shown). Like intrathecal injection of SUV39H1 siRNA, its microinjection into the ipsilateral L₅ 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 (Figures 5B, 5D, 5F). 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 following DRG microinjection of SUV39H1 siRNA or its control negative siRNA (Figures 5B–5F). Similar behavioral responses were seen after microinjection of SUV39H1 siRNA or its control siRNA into the ipsilateral L₅ dorsal horn (Figures 6B–6F).

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 DRG⁸. 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 downregulation 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 L₅ DRG on day 3 post-SNL in the rats treated with vehicle or negative control siRNA (Figure 7A). Microinjection of SUV39H1 siRNA into the ipsilateral L₅ DRG completely reversed this decrease, although it did not alter the basal level of MOR in the ipsilateral L₅ DRG from sham rats (Figure 7A). Double-label immunofluorescence histochemistry revealed that SUV39H1 was detected in the nuclei of MOR-positive neurons in naïve DRG (Figure 7B). Approximately 40.4% of SUV39H1-labeled neurons were positive for MOR and about 93.8% of MOR-labeled neurons for SUV39H1. The results suggest the involvement of DRG SUV39H1 in nerve injury-induced DRG MOR downregulation.

To further confirm this conclusion, we subcutaneously injected morphine (a MOR agonist) on day 3 post-SNL or -sham surgery in DRG microinjected rats. Consistent with previous studies^{37;39;40}, the analgesic effect of morphine on day 3 post-SNL significantly declined compared to that on day 3 post-sham surgery on the ipsilateral side of vehicle-microinjected rats (Figure 7C). This reduction was reversed markedly on the ipsilateral side of SUV39H1 siRNA microinjected rats (Figure 7C). As expected, morphine produced robust analgesia on the contralateral side of all treated groups (Figure 7C). In addition, we intraperitoneally injected methyl naltrexone bromide (Medchemexpress USA, Monmouth Junction, NJ; a peripheral MOR antagonist, 5 mg/kg, dissolved in saline) or saline on day 3 post-SNL or

sham surgery in DRG microinjected rats (Figures 7D–7G). 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 following intraperitoneal saline injection (Figures 7D and 7F). However, these effects were absent after intraperitoneal methylnaltrexone administration (Figures 7D and 7F). Methylnaltrexone at the dose used did not affect basal behavioral responses on the contralateral side (Figures 7E and 7G). 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 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 present 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 L₅ DRG and L₅ dorsal horn on the ipsilateral side following SNL, although the durations of its increases in these two regions were different. In the ipsilateral L₅ 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 L₅ 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 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 pharmacological 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 downregulation of several K⁺ channel genes in the injured DRG⁴². 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 co-expresses with MOR in the DRG neurons, SUV39H1's production of H3K9me3 may be required for SNL-induced downregulation 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 downregulation 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 the line with a previous report⁴⁵. 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 L4–6 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.

Acknowledgments

This work was supported by the grants (NS072206, NS094664, HL117684, and DA033390) from the National Institutes of Health (Bethesda, Maryland, USA).

Reference List

1. van HO, Austin SK, Khan RA, Smith BH, Torrance N. Neuropathic pain in the general population: a systematic review of epidemiological studies. *Pain*. 2014; 155:654–662. [PubMed: 24291734]
2. O'Connor AB. Neuropathic pain: quality-of-life impact, costs and cost effectiveness of therapy. *Pharmacoeconomics*. 2009; 27:95–112. [PubMed: 19254044]
3. Campbell JN, Meyer RA. Mechanisms of neuropathic pain. *Neuron*. 2006; 52:77–92. [PubMed: 17015228]
4. Chung JM, Chung K. Importance of hyperexcitability of DRG neurons in neuropathic pain. *Pain Pract*. 2002; 2:87–97. [PubMed: 17147683]

5. Devor M. Ectopic discharge in Abeta afferents as a source of neuropathic pain. *Exp. Brain Res.* 2009; 196:115–128. [PubMed: 19242687]
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. [PubMed: 19400724]
7. Lutz BM, Bekker A, Tao YX. Noncoding RNAs: new players in chronic pain. *Anesthesiology.* 2014; 121:409–417. [PubMed: 24739997]
8. Liang L, Lutz BM, Bekker A, Tao YX. Epigenetic regulation of chronic pain. *Epigenomics.* 2015; 7:235–245. [PubMed: 25942533]
9. Kouzarides T. Chromatin modifications and their function. *Cell.* 2007; 128:693–705. [PubMed: 17320507]
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; 406:593–599. [PubMed: 10949293]
11. Moss TJ, Wallrath LL. Connections between epigenetic gene silencing and human disease. *Mutat. Res.* 2007; 618:163–174. [PubMed: 17306846]
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; 6:1095–1104. [PubMed: 21814037]
13. Sen N, Snyder SH. Neurotrophin-mediated degradation of histone methyltransferase by S-nitrosylation cascade regulates neuronal differentiation. *Proc. Natl. Acad. Sci. U. S. A.* 2011; 108:20178–20183. [PubMed: 22123949]
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; 156:711–721. [PubMed: 25630025]
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; 100:912–921. [PubMed: 15087627]
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; 16:1024–1031. [PubMed: 23792947]
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. [PubMed: 26626404]
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; 16:1186–1199. [PubMed: 26342649]
19. Xu JT, Zhou X, Zhao X, Ligons D, Tiwari V, Lee CY, Atianjoh FE, 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. [PubMed: 24382350]
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. [PubMed: 24472174]
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; 26:2923–2932. [PubMed: 16540569]
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; 167:148–159. [PubMed: 17949823]
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. [PubMed: 7990513]

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; 12:1364–1366. [PubMed: 19783992]
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; 1:143–145. [PubMed: 16408017]
26. Shinkai Y, Tachibana M. H3K9 methyltransferase G9a and the related molecule GLP. *Genes Dev.* 2011; 25:781–788. [PubMed: 21498567]
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; 19:815–826. [PubMed: 15774718]
28. Latremoliere A, Woolf CJ. Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *J Pain.* 2009; 10:895–926. [PubMed: 19712899]
29. Ji RR, Gereau RW, Malcangio M, Strichartz GR. MAP kinase and pain. *Brain Res. Rev.* 2009; 60:135–148. [PubMed: 19150373]
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; 114:149–159. [PubMed: 15733640]
31. Collin E, Frechilla D, Pohl M, Bourgoin S, Le BD, 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; 609:211–222. [PubMed: 8389648]
32. Heinke B, Gingl E, Sandkuhler J. Multiple targets of mu-opioid receptor-mediated presynaptic inhibition at primary afferent Adelta- and C-fibers. *J Neurosci.* 2011; 31:1313–1322. [PubMed: 21273416]
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; 14:151–159. [PubMed: 2559354]
34. Taddese A, Nah SY, McCleskey EW. Selective opioid inhibition of small nociceptive neurons. *Science.* 1995; 270:1366–1369. [PubMed: 7481826]
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; 15:669–675. [PubMed: 21310637]
36. Obara I, Makuch W, Spetea M, Schutz 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; 558:60–67. [PubMed: 17204264]
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; 309:380–387. [PubMed: 14718584]
38. Uchida H, Ma L, Ueda H. Epigenetic gene silencing underlies C-fiber dysfunctions in neuropathic pain. *J. Neurosci.* 2010; 30:4806–4814. [PubMed: 20357131]
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. [PubMed: 25118039]
40. Obara I, Przewlocki R, Przewlocka B. Local peripheral effects of mu-opioid receptor agonists in neuropathic pain in rats. *Neurosci. Lett.* 2004; 360:85–89. [PubMed: 15082185]
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. [PubMed: 21345196]
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; 18:1746–1755. [PubMed: 26551542]
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; 128:208–211. [PubMed: 26318673]

44. Zhang Y, Chen SR, Laumet G, Chen H, Pan HL. Nerve Injury Diminishes Opioid Analgesia through Lysine Methyltransferase-Mediated Transcriptional Repression of mu-Opioid Receptors in Primary Sensory Neurons. *J Biol. Chem.* 2016; 291:8475–8485. [PubMed: 26917724]
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; 141:283–291. [PubMed: 19147290]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Summary statement

The level of the histone methyltransferase SUV39H1 increases in the ipsilateral dorsal root ganglion and spinal cord dorsal horn following peripheral nerve injury. Blocking this increase restores mu opioid receptor expression in the injured DRG and impairs nerve injury-induced allodynia and hyperalgesia.

Fig. 1.

The expression and distribution of 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 panel) 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 in A and 25 μm in B.

Fig. 2.

Expression changes in SUV39H1 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), L₅ DRG on days 3, 7, and 14 after SNL. *n* = 3 repeats (6 rats)/time point. Two-way ANOVA (expression *vs* side $\times$ time interaction) followed by post hoc Tukey test, $F_{\text{time}}(3, 27) = 5.5$, $*P < 0.05$ or $**P < 0.01$ *vs* the corresponding naïve rats (day 0). (B) SUV39H1 protein increased in the ipsilateral (Ipsi), but not contralateral (Cont), L₅ 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 $\times$ time interaction) followed by post hoc Tukey test, $F_{\text{time}}(3, 23) = 29.8$, $**P < 0.01$ *vs* the corresponding naïve rats (day 0). (C) No significant changes in SUV39H1 protein were seen in the ipsilateral L₄ DRG after SNL. *n* = 3 repeats (6 rats)/time point. One-way ANOVA (expression *vs* time points) followed by post hoc Tukey test, $F_{\text{time}}(3, 11) = 0.4$. (D, E) The number of SUV39H1-labeled neurons in the ipsilateral L₅ DRG (D) and the densities of SUV39H1-positive neurons in the ipsilateral L₅ dorsal horn laminae (E) increased on days 3 after SNL. *n* = 3 rats in the sham group and 4 rats in the SNL group. $**P < 0.01$ *vs* the corresponding sham group by two-tailed unpaired *t*-test. Scale bars: 50 μm in D and 25 μm in E. (F) The amounts of SUV39H1 mRNA increased in L₅ DRG, but not in L₅ dorsal horn, on the ipsilateral side. *n* = 3 repeats (6 rats)/time point. One-way ANOVA (relative level *vs* time points) followed

by post hoc Tukey test, $F_{\text{time}}(3, 11) = 7.4$, $**P < 0.01$ vs the corresponding naïve rats (day 0) for the DRG and $F(3, 11) = 0.8$ for dorsal horn.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 3.

Effect of intrathecal SUV39H1 inhibitor chaetocin on spinal nerve ligation (SNL)-induced nociceptive hypersensitivity. (A–E). Pre-treatment 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 side and had no effects on basal paw withdrawal responses to mechanical (D) and thermal (E) stimuli on the contralateral side. $n = 5$ rats/group. Two-way ANOVA (effect *vs* group $\times$ time interaction) followed by post hoc Tukey test, $F_{\text{group}}(12, 99) = 16.3$ in A, $F_{\text{group}}(12, 99) = 28.8$ in B, $F_{\text{group}}(12, 99) = 158.7$ in C, $F_{\text{group}}(12, 99) = 0.0004$ in D, and $F_{\text{group}}(12, 99) = 0.6$ in E. $*P < 0.05$ or $**P < 0.01$ *vs* the corresponding time point in the vehicle (Veh) plus SNL group. (F–H) Post-treatment 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 ipsilateral side (Ipsi). Behavioral tests were carried out one day before surgery, before drug injection on day 7 post-surgery, and on day 9 post-surgery. Cont, contralateral. $n = 5$ rats/group. Two-way ANOVA (effect *vs* group $\times$ time interaction) followed by post hoc Tukey test, $F_{\text{group}}(6, 59) = 205.4$ in F, $F_{\text{group}}(6, 59) = 87.9$ in G, and $F_{\text{group}}(2, 29) = 17.5$ in H. $**P < 0.01$ *vs* the corresponding time point in the vehicle (Veh) plus SNL group. (I and J) Pre-treatment of chaetocin at 2 µg blocked the SNL-induced spontaneous ongoing nociceptive responses. Post, post-preconditioning. $n = 5$ rats/group, $*P < 0.05$ or $**P < 0.01$ *vs* the corresponding preconditioning (Pre) (I) or the vehicle plus SNL group (J) by two-tailed paired *t*-test. (K) Intrathecal chaetocin at 2 µg did not affect the expression of G9a's two protein isoforms,

SUV39H1 protein (SUV), and dimethylation of Lys9 on histone H3 (H3K9me2) in the DRGs of sham rats. Veh, vehicle; L, long isoform; S, short isoform. n = 3 repeats (6 rats)/group.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 4.

Effect of intrathecal administration of SUV39H1 siRNA on spinal nerve ligation (SNL)-induced nociceptive hypersensitivity and dorsal horn central sensitization. V: Vehicle. NC: negative control siRNA. Si: SUV39H1 siRNA. SUV: SUV39H1. (A) The level of SUV39H1 protein was markedly reduced in *in vitro* PC12 cells treated with SUV39H1 siRNA compared to that treated with the negative control siRNA. (B) Intrathecal SUV39H1 siRNA blocked the SNL-induced increase in the level of SUV38H1 on day 7 post-SNL and did not affect its basal expression on day 7 post-sham surgery in the ipsilateral L₅ dorsal root ganglion (DRG) and L₅ dorsal horn (DH). $n = 3$ repeats (6 rats)/time point. One-way ANOVA (effect *vs* the treated groups) followed by post hoc Tukey test, $F_{\text{group}}(4, 14) = 45.8$ for DRG and $F_{\text{group}}(4, 14) = 102.3$ for dorsal horn, $^{**}P < 0.01$ *vs* the corresponding vehicle plus sham group. $^{##}P < 0.01$ *vs* the corresponding vehicle plus SNL group. (C–G) Intrathecal SUV39H1 siRNA 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 $\times$ time interaction) followed by post hoc Tukey test, $F_{\text{group}}(6, 59) = 56.2$ in C, $F_{\text{group}}(6, 59) = 34.8$ in D, $F_{\text{group}}(6, 59) = 36.8$ in E, $F_{\text{group}}(6, 59) = 0.5$ in F, and $F_{\text{group}}(6, 59) = 0.3$ in G. $^{**}P < 0.01$ *vs* the corresponding time point in the vehicle plus SNL group. (H) Intrathecal SUV39H1 siRNA blocked the increases

in the levels of phosphorylated extracellular signal-regulated kinases1/2 (p-ERK1/2) and glial fibrillary acidic protein (GFAP) in the ipsilateral L5 dorsal horn on day 3 post-SNL. n = 3 rats/group. One-way ANOVA (expression *vs* the treated groups) followed by post hoc Tukey test, $F_{\text{group}}(2, 8) = 12.8$ for p-ERK1, $F_{\text{group}}(2,8) = 20.1$ for p-ERK2, $F_{\text{group}}(2,8) = 0.3$ for total extracellular signal-regulated kinase 1 (ERK1), $F_{\text{group}}(2,8) = 0.4$ for total extracellular signal-regulated kinase 2 (ERK2), and $F_{\text{group}}(2,8) = 20.1$ for GFAP. $**P < 0.01$ *vs* the corresponding vehicle plus sham group. $\#P < 0.05$, $\##P < 0.01$ *vs* the vehicle plus SNL group.

Fig. 5.

Effect of dorsal root ganglion (DRG) microinjection of SUV39H1 siRNA on spinal nerve ligation (SNL)-induced nociceptive hypersensitivity. V: Vehicle. NC: negative control siRNA. Si: SUV39H1 siRNA. SUV: SUV39H1. (A) Microinjection of SUV39H1 siRNA into the ipsilateral L₅ DRG blocked the SNL-induced increase in the level of SUV38H1 on day 7 post-SNL and did not affect its basal expression on day 7 post-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, $F_{\text{group}}(4, 14) = 13.5$, $*P < 0.05$ vs the

corresponding vehicle plus sham group. $\#P < 0.05$ vs the corresponding vehicle plus SNL group. (*B–F*) Microinjection of SUV39H1 siRNA into the ipsilateral L₅ 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 $\times$ time interaction) followed by post hoc Tukey test, $F_{\text{group}}(12, 119) = 4.9$ in *B*, $F_{\text{group}}(12, 119) = 3.8$ in *D*, $F_{\text{group}}(12, 119) = 8.9$ in *F*, $F_{\text{group}}(12, 119) = 0.4$ in *C*, and $F_{\text{group}}(12, 119) = 0.2$ in *E*. $**P < 0.01$ vs the corresponding time point in the vehicle plus SNL group.

Fig. 6. Effect of intraspinal microinjection of SUV39H1 siRNA on spinal nerve ligation (SNL)-induced nociceptive hypersensitivity. V: Vehicle. NC: negative control siRNA. Si: SUV39H1 siRNA. SUV: SUV39H1. (A) Intraspinal microinjection of SUV39H1 siRNA attenuated the SNL-induced increase in the level of SUV39H1 on day 7 post-SNL and did not affect its basal expression on day 7 post-sham surgery in the ipsilateral L5 dorsal horn. $n = 3$ rats/time point. One-way ANOVA (expression vs the treated groups) followed by post hoc Tukey test, $F_{\text{group}}(4, 14) = 14.4$, $*P < 0.05$ vs the corresponding vehicle plus sham group. $\#P < 0.05$ vs

the corresponding vehicle plus SNL group. (*B–F*) Intraspinal microinjection of SUV39H1 siRNA 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 $\times$ time interaction) followed by post hoc Tukey test, $F_{\text{group}}(12, 99) = 19.8$ in *B*, $F_{\text{group}}(12, 99) = 17.9$ in *D*, $F_{\text{group}}(12, 99) = 28.5$ in *F*, $F_{\text{group}}(12, 99) = 0.7$ in *C*, and $F_{\text{group}}(12, 99) = 1.4$ in *E*. $**P < 0.01$ *vs* the corresponding time point in the vehicle plus SNL group.

Fig. 7.

Participation of SUV39H1 in spinal nerve ligation (SNL)-induced downregulation of mu 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 SUV39H1 siRNA, but not negative control siRNA, into the injured DRG restored the decrease of MOR in the injured DRG on day 3 post-SNL. $n = 3$ repeats (6 rats)/group. One-way ANOVA (expression *vs* the treated groups) followed by post hoc Tukey test, $F_{\text{group}}(4, 14) = 24.9$, $*P < 0.05$ *vs* the vehicle plus sham group. $##P < 0.01$ *vs* the vehicle plus SNL group. (B) Double immunofluorescent staining showed that SUV39H1 (SUV, red) was detected in the nuclei of many MOR (green)-positive neurons in naïve DRG. Scale bar: 50 μ m. $n = 3$ rats. (C) Microinjection of SUV39H1 siRNA ($n = 6$ 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, $F_{\text{group}}(2, 15) = 13.2$ on the ipsilateral side and $F_{\text{group}}(1, 15) = 2.8$ on the contralateral side. $**P < 0.01$ *vs* the vehicle plus sham group ($n = 5$ rats). $##P < 0.01$ *vs* the vehicle plus SNL group ($n = 5$ rats). (D–G) Intraperitoneal injection with methylnatrexone (MNTX, 5 mg/kg) blocked the SUV39H1 siRNA-produced antinociceptive effect on day 3 post-SNL in the SUV39H1 siRNA-treated group ($n = 6$ rats). MNTX was administered on day 3 post-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 $\times$ time interaction) followed by post hoc Tukey test, $F_{\text{group}}(6, 65) = 15.9$ in D, $F_{\text{group}}(6, 65) = 1.7$ in E, $F_{\text{group}}(6, 65) = 6.3$ in F, and $F_{\text{group}}(6, 65) = 0.6$ in G. $**P < 0.01$ *vs* the vehicle plus saline group 3 days post-sham surgery ($n = 5$ rats). $##P < 0.01$ *vs* the vehicle plus saline group 3 days post-SNL ($n = 5$ rats). $$$P < 0.01$ *vs* the SUV39H1 siRNA plus saline group 3 days post-SNL ($n = 6$ rats).