Dorsal root ganglia coactivator-associated arginine methyltransferase 1 contributes to peripheral nerve injury-induced pain hypersensitivities.

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Abstract

Aims: Neuropathic pain is associated with gene expression changes within the dorsal root ganglia (DRG), which are involved in epigenetic mechanisms. However, whether protein arginine methylation plays an epigenetic role is unknown. In this study, we aimed to determine how peripheral nerve injury changes the transcriptional expression of the protein arginine methylation enzyme family genes in injured DRG and further to investigate their roles in neuropathic pain after nerve injury.

Methods: RNA-Seq was used to comprehensively analyze changes in the protein expression of arginine methyltransferases and its demethylase in the injured DRG neuronal system following spinal nerve ligation (SNL) and to screen differentially expressed genes in injured DRGs following SNL. After qPCR verification of these differentially expressed genes at corresponding timepoints after SNL and sciatic nerve chronic constriction injury (CCI), CCI-induced arginine methyltransferase coactivator-associated arginine methyltransferase 1 (CARM1) increases were blocked by microinjection of its corresponding siRNA in injured DRG, and observations were made to determine whether it attenuated the behaviors of CCI-induced pain hypersensitivities.

Key findings: Peripheral nerve injury induced the upregulation of the protein expression of CARM1 in the injured DRG and blocking its expression in the injured DRG attenuated the development and maintenance of neuropathic pain after peripheral nerve injury.

Significance: Given that CARM1 siRNA attenuated the development and maintenance of neuropathic pain after peripheral nerve injury, our findings suggest that CARM1 in clinical applications may be a good target for neuropathic pain treatment.

Keywords: Coactivator-associated arginine methyltransferase 1, Peripheral nerve injury, Dorsal root ganglia, Neuropathic pain.

Introduction

Neuropathic pain induced by peripheral nerve injury, which is characterized by distressing and debilitating conditions, including spontaneous ongoing pain or intermittent burning pain, allodynia and thermal hypersensitivities, is a worldwide healthcare problem [1]. These symptoms affect approximately 1/10 of the population in the United States and Europe. Among this population, most of the patients achieve unsatisfactory pain control with current medications because most of these medications are nonspecific, based on the causal mechanism of neuropathic pain [2,3]. Thus, understanding the mechanisms of neuropathic pain genesis could provide new and highly efficient strategies of neuropathic pain management.

Abnormal ectopic firing and hyperexcitability in the corresponding Dorsal Root Ganglion (DRG) and neuromas after peripheral nerve injury are generally considered to be peripheral mechanisms of neuropathic pain genesis [2,4,5]. This unusual spontaneous activity in injured DRG neurons, as well as the subsequent enhanced neurotransmitter release from their primary afferents, may result from maladaptive changes in gene transcription and the translation of enzymes, receptors, and voltage-dependent ion channels in the DRG [4]. This maladaptive gene transcription and translation changes would involve protein methylation modifications [6].

Protein methylation has primarily been detected on lysine and arginine residues of histone or non-histone proteins [7]. N-ε-lysine methylation is one of the most abundant histone marks in euchromatic chromatin, and the process of methylation is catalyzed by an enzyme family of lysine methyltransferases, such as EHMT2 (also known as G9a) and SUV39h1; this modification results in condensed chromatin and gene transcriptional repression [8]. Recent studies showed that G9a and SUV39h1 are involved in nerve injury-induced downregulation of DRG potassium channels and opioid receptor genes, leading to abnormal ectopic firing and hyperexcitability, as well as opioid drug analgesic inefficiency [9-14]. These lines of evidence suggest that lysine methyltransferases induce histone methylation and, as endogenous instigators, participate in neuropathic pain genesis. Arginine methylation, as another important component
of protein posttranslational modifications, is catalyzed by a group of nine protein arginine methyltransferases (PRMT1-3, CARM1, PRMT5-8, and PRMT10) [15], which have been found in histone and non-histone proteins and are involved in functions such as transcriptional regulation, RNA processing, DNA repair, and signal transduction in the processes of cancer, cardiovascular diseases, viral pathogenesis, multiple sclerosis, spinal muscular atrophy and other disease genes [16,17]. For example, coactivator-associated arginine methyltransferase 1 (CARM1), a member of the PRMT family, induces methylation of histones and is also thought to promote active gene transcriptional expression through methylating H3R26 and H3R26 sites on histone substrates and by other mechanisms [18]. However, which protein arginine methyltransferase contributes to neuropathic pain genesis is still unknown.

In the present study, we performed strand-specific next-generation RNA sequencing (RNA-Seq) to comprehensively analyze the changes in transcriptional expression of protein arginine methyltransferase and arginine demethylase genes and to screen differentially expressed genes in injured dorsal root ganglia following peripheral nerve injury caused by Spinal Nerve Ligation (SNL); we further verified the expression of these differentially expressed genes in injured DRGs in different neuropathic pain models by RT-qPCR. Finally, we observed whether knockdown of upregulated CARM1 gene expression in the DRG affects nerve injury-induced pain hypersensitivities.

Materials and Methods

Animals

Adult male C57BL/6J wild-type mice weighing 25 to 30 g were used in this study. All animals were purchased from the center of laboratory animal science of Southern Medical University and allowed to adapt to the animal facilities for at least 2 days before the experiments. They were kept in a standard 12 h light/dark cycle, with water and food pellets available ad libitum. Three-week-old mice were used for primary neuron culture. All procedures used were approved by the animal care and use committee at the southern medical university and were consistent with the ethical guidelines of the Southern Medical University 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. Mice were randomly assigned to the treated groups, and the group sizes were based on previous experience. All experimenters were blind to the treatment conditions.

Neuropathic pain model

Unilateral or bilateral fourth lumbar (L4) spinal nerve ligation (SNL) and chronic constriction injury (CCI), as neuropathic pain models, were carried out as described in previously published methods [12,19,20]. Briefly, for the SNL model, an incision on the lower back was made after the mice were anesthetized with isoflurane. The L4 spinal nerve on one side was tightly ligated with 3-0 silk thread and transected distal to the ligature after it was exposed and isolated from the adjacent nerves. For harvesting DRG tissues to detect the RNA-Seq test, the L4 spinal nerve was ligated bilaterally using the same method as for the unilateral L4 spinal nerve ligation [19]. Next, the skin and muscles were closed in layers. For the CCI model, we used 3-0 silk thread to loosely ligate the exposed sciatic nerve at four sites at a position approximately 1 mm proximal to the trifurcation of the sciatic nerve. The sham-operated mice underwent the same procedures as the SNL or CCI mice, but without the ligature or transection of the respective nerves.

RNA sequencing and bioinformatics analysis

As in our previously published study [19], extracted RNA (1.2 µg/sample) was subjected to rRNA depletion by a Ribo-Zero rRNA Removal (Human/Mouse/Rat) Kit (Illumina, San Diego, CA) after total RNA extraction. A TruSeq Stranded Total RNA Sample Preparation Kit (Illumina) without poly-A selection was used to prepare Strand-specific RNA libraries. Sequencing was performed using an Illumina HiSeq2500 platform in High Output Mode, with a 2 × 100 base pairs in a paired-end configuration, with a total of more than 190 M reads per lane (at least 60 M reads per sample). The sequences were first quality trimmed using trimmomatic 0.32 (minimal length of 50 base pairs, leading and trailing PhredQ30). The results of the sequencing data were then mapped to the musculus genome, sequence version GRCm38.72, from ENSEMBLE. Gene hit counts and reads per kilobase per million (RPKM) mapped reads were calculated for each gene to determine expression levels [21]. Comparisons of gene expression between groups were conducted using Student’s t-test. Mapped reads were visualized on the UCSC browser using bigwig files converted from bam files.

Behavioral analysis

Mice were habituated 1 to 2 hours each day for 2 to 3 days before basal behavioral testing. Mechanical and thermal tests, as well as locomotor functional performance tests, were carried out prior to surgery or siRNA microinjection and at different time points after surgery. Each of the behavioral tests was carried out at 1-hour intervals.

The mechanical stimulus responses (calibrated von Frey filaments) for paw withdrawal frequency were first measured, as previously described [9,10,12]. Briefly, the mouse was placed on an elevated mesh screen within an individual Plexiglas chamber. A 0.4 g von Frey filament was used to stimulate the hind paw for one second, and each stimulation was repeated 10 times to both hind paws, with 5 min intervals. Paw withdrawal responses in each of these 10 stimulations are represented as a percent response frequency (number of paw withdrawal responses/10 trials) × 100%=Paw withdrawal frequency), and this obtained percentage was an indication of the amount of paw withdrawal. The noxious thermal stimulation for paw withdrawal latency response was then examined as previously [10,12]. In brief, the mouse was placed within an individual Plexiglas chamber on a glass plate. A beam of light was shone through a hole in the light box of a Model 336 Analogesic Meter (IITC Inc. Life Science Instruments, Woodland Hills, CA) to stimulate the middle of the plantar surface of each hind paw through a glass plate. When the mouse withdrew its foot, the light beam was automatically turned off. The length of time between the start of the light beam and the foot withdrawal was recorded as the paw withdrawal latency. This was repeated five times at 5 min intervals for the


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paw on each side in each test. A cut-off time of 20 s was set to avoid hind paw tissue damage.

Locomotor functional performance (1) Placing reflex: the placed locations of the hind limbs were lower than those of the forelimbs, and the dorsal surfaces of the hind paws were brought into contact with the edge of a table. Whether the hind paws were reflexively placed on the table surface was recorded; (2) Grasping reflex: After the animal was placed on a wire grid, whether the hind paws grasped the wire on contact was recorded; (3) Righting reflex: when the animal was placed on its back on a flat surface, whether it immediately assumed a normal upright position was recorded. Each trial was repeated five times with a 5-min interval, and scores for each reflex were recorded based on counts of each normal reflex.

**Dorsal root ganglia neuronal culture**

Primary dorsal root ganglia neuronal cultures and siRNA transfections were performed as described [20]. In brief, after three-week-old C57BL6 mice were euthanized with isoflurane, all DRG tissues were harvested in cold Neurobasal Medium (Gibco/Thermo Fisher Scientific) containing 10% fetal bovine serum (JR Scientific, Woodland, CA), 100 units/ml Penicillin, and 100 mg/ml Streptomycin (Quality Biological, Gaithersburg, MD). DRG tissues were then treated with enzyme solution (5 mg/ml dispase, 1 mg/ml collagenase type I, in Hanks’ balanced salt solution (HBSS) without Ca²⁺ and Mg²⁺ (Gibco/ThermoFisher Scientific)). After trituration and centrifugation, the dissociated neurons were resuspended in mixed Neurobasal Medium and plated in a six-well plate coated with 50 mg/ml poly-D-lysine (Sigma). Cells were incubated at 37°C in a humidified incubator with 95% O₂ and 5% CO₂. After 24 hours, siRNAs were transfected into the DRG neuronal cells with Lipofectamine 2000 (Invitrogen, USA) at a concentration of 100 nM, according to the manufacturer’s protocol. Forty-eight hours after transfection, cells were harvested for reverse transcription PCR.

**DRG microinjection**

Dorsal root ganglion microinjection was performed as described previously [11,12]. Briefly, after the animal was anesthetized with isoflurane, a midline incision was made in the lower lumbar back region, and the L4 DRG was exposed. The exposed DRG was injected with *Carm1* siRNA (catalog no. sc-37730, 1 μl, 20 uM) and its negative control siRNA (catalog no. sc-44230, 1 μl, Santa Cruz Biotechnology), through a glass micropipette connected to a Hamilton syringe. To improve delivery efficiency of 100 nM, according to the manufacturer’s protocol. Forty-eight hours after transfection, cells were harvested for reverse transcription PCR.

**Quantitative real-time PCR**

Quantitative real-time PCR was performed as described previously [12,20]. Briefly, both L4 DRGs of the SNL and the L3-4 DRGs of the CCI, or cultured DRG neuronal cells, were harvested at corresponding timepoints after SNL/CCI or sham surgery or 48 hours after culture. Total RNA from tissues or cultured cells was extracted using TRIzol® Reagent (Invitrogen), and its quality and concentration were measured using a NanoDrop800 Spectrophotometer and 1% agarose gel electrophoresis. First strand synthesis was performed using 1 μg of total RNA, following the manufacturer’s instructions of the PrimeScript™ RT reagent Kit (TaKaRa). Next, quantitative real-time PCR (qRT-PCR) was performed using a SYBR® Premix Ex Taq M II kit on a BIO-RAD CFX96 real-time PCR system (Bio-Rad) with a 10 μl reaction volume. A two-step RT-PCR reaction and melt curve analysis were chosen. The cycle parameters were set as follows: 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 mRNA levels from ipsilateral sides to mRNA levels from contralateral sides were calculated using the ΔCt method (2-ΔΔCt). Primers were designed using Primer 3 software on the internet and are listed in Table 1. *Tubala* was used as an internal control.

**Western blotting**

Western Blotting was performed as described previously [12,20]. Briefly, the SNL-injured L4 and CCI-injured L3-4 DRGs were harvested at corresponding timepoints after SNL/CCI or sham surgery. The total amount of protein samples was extracted using RIPA lysis buffer (Thermo Fisher Scientific), and concentration of the proteins was measured by a BCA Kit (Solarbio). Protein samples with loading buffer were boiled for 5 min and loaded on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 40 μg/lane; then, the proteins were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane. After 5% skimmed milk blocking, PVDF membranes were incubated overnight with specific antibodies against *CARM1* (#12495, Cell signaling technology) (1:1000), and β-Actin (Mouse monoclonal IgG, Santa Cruz) (1:500). Next, membranes were washed with Tris-buffered saline containing 0.05% Tween 20 (TBST) and incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (1:2,000 Jackson Immuno Research). Finally, protein bands were revealed using chemiluminescent substrates (ThermoScientific) and quantified by ImageJ software. All target protein bands were normalized to β-Actin.

**Statistical analysis**

All results were presented as the means ± S.E.M. The data were statistically analyzed with a two-tailed, independent Student’s

<table>
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<td>Reverse CTGTCAGAAGAGCACTTGGC</td>
<td></td>
<td></td>
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<tr>
<td>Prmt8</td>
<td>Forward TGGTGACCAATGCGCTGTTTA</td>
<td>166 bp</td>
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<tr>
<td>Reverse CCAATTTTTCTGTTGGACCTT</td>
<td></td>
<td></td>
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<tr>
<td>Jmjd6</td>
<td>Forward GGCATGTTGTCCTCAACCTT</td>
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<tr>
<td>Reverse AGGGTGCTCCTGTTCAAGA</td>
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Results

Protein arginine methyltransferase and demethylase genes are differentially expressed in the injured drug following peripheral nerve injury

Recently, nine protein arginine methyltransferases have been found, including PRMT1-3, CARM1 (also known as PRMT4), PRMT5-8, and PRMT10 (also known as PRMT9), which are encoded by Prmt1-3, Carm1, Prmt5-8 and Prmt10 genes, respectively [15]. In contrast, so far, just two enzymes, the Jumonji domain containing protein 6 (JMJD6, encoded by Jmjd6 gene) and peptidyl arginine deiminase 4 (PADI4, encoded by the Padi4 gene), have been shown to have a potential arginine demethylation activity in vivo [22,23]. Just like in our previous study [19], the transcriptional analysis of injured DRG at six days after SNL showed that all of the above genes were expressed in DRG tissue, the higher basal expression was in the Prmt2 and Prmt3 genes, and the lowest basal expression was the Padi4 gene. Interestingly, the expression level of the Carm1 gene was significantly increased in the L4 injured DRG. Compared to the sham group, the transcriptional levels of the Carm1 gene of the SNL group increased 2.06-fold (P<0.001) (Figure 1A). Conversely, the Prmt8 gene was downregulated and remarkably decreased 16.3-fold (P<0.001) (Figure 1A). However, the transcriptional level of the protein arginine methyltransferase genes Prmt1-3 and Prmt5, 6, 7, and 10 and the arginine demethylase genes Jmjd6 and Padi4 did not significantly change in the ipsilateral L4 DRG after peripheral nerve injury (Figure 1A). Indeed, the stacked reads in the RNA-seq data demonstrated that the Carm1 gene was markedly increased (Figure 1B), and the Prmt8 gene was significantly decreased (Figure 1C) in the SNL samples compared with the sham samples.

Figure 1. Protein arginine methyltransferase and arginine demethylase genes are differentially expressed mRNAs in the injured DRG following peripheral nerve injury: Differentially expressed mRNAs of protein arginine methyltransferase and arginine demethylase genes in the ipsilateral fourth lumbar dorsal root ganglia after SNL are represented in the form of heat maps obtained using RNA-seq (1A). P<0.001 compared to the sham group following t-test. RNA-seq: RNA sequencing. RPKM, reads per kilobase per million. A representative differentially expressed gene of stacked reads remarkably changed in the NL sample compared with the sham sample in the genomic region of the Carm1 (1B) and Prmt8 (1C) genes in visualized reads mapping. The open box indicates that the typical changes of reads were aligned to the unique exon region of Carm1 and Prmt8. SNL: spinal nerve ligation.
RT-qPCR validation of differentially expressed protein arginine methyltransferase and arginine demethylase genes in the injured DRG following SNL injury

For validation experiments, an RT-qPCR analysis was used to further confirm the differential expression of selected genes, including Carm1, Prmt5, and Prmt8 as well as Jmjd6 genes on 0, 3, 7, and 14 days after SNL. To make sure that all SNL mice used in the RT-qPCR experiments showed pain hypersensitivity, tests of the mouse paw withdrawal frequency in response to mechanical stimulation and paw withdrawal latency to heat stimulation were carried out. Consistent with our previous studies [9,20], SNL produced long term mechanical allodynia Figure 2A and thermal hyperalgesia Figure 2B on the ipsilateral side in SNL mice. As expected, sham surgery did not produce a marked change in paw withdrawal frequency and paw withdrawal latency in either hind paw Figure 2A and 2B. Consistent with the RNA-Seq results, SNL time-dependently increased the ratios of the ipsilateral side to the contralateral side of Carm1 mRNA abundance, which increased 1.62-fold on day 3, 2.07-fold on day 7, and 1.84-fold on day 14 after SNL, compared to naïve mice (0 day) Figure 2C. In addition, the ratios of the ipsilateral side to the contralateral side of Prmt8 mRNA abundance time-dependently decreased 2.67-fold on day 3, 4.97-fold on day 7, and 3.91-fold on day 14 after SNL, compared to naïve mice Figure 2D. However, Prmt1 and Jmjd6 gene expression was inconsistent with the RNA-Seq data, and there was no significant change on days 3, 7 and 14 after SNL compared to naïve mice Figure 2E and 2F. As expected, sham surgery did not cause significant changes in the basal abundance of all the above gene mRNAs in the ipsilateral L4 DRGs Figure 2C-2F. The significant change in the transcriptional expression of Carm1 and Prmt8 mRNA in the ipsilateral DRG after SNL suggests a potentially possible role of Carm1 and Prmt8 in neuropathic pain after peripheral nerve injury.

Chronic constriction injury (CCI) of the sciatic nerve transcriptionally upregulated CARM1 expression in the ipsilateral DRG, but not in the spinal cord dorsal horn

CARM1 methylates several histone and non-histone substrates and impacts many cellular processes, including transcriptional

Figure 2. Validation of differentially expressed protein arginine methyltransferase and arginine demethylase genes in the injured DRG following SNL injury by RT-qPCR. Painful behavioral changes in paw withdrawal frequencies to mechanical stimuli (2A) and paw withdrawal latency to thermal stimulation (2B) at different days after SNL or sham surgery; n=12 mice/group. Two-way ANOVA followed by Tukey’s post hoc test, **P<0.01 compared to the contralateral side of sham group in baseline (day 0). PWF, paw withdrawal frequencies; PWL, paw withdrawal latency. Con, contralateral side; Ips, ipsilateral side. (2C-2H) The ratios of mRNA levels from the ipsilateral side to mRNA levels from the contralateral side of Carm1 (2C), Prmt8 (2D), Prmt1 (2E), and Jmjd6 (2F) genes at corresponding timepoints after SNL. Unilateral L4 DRGs from 4 mice were pooled together. N=3-4 repeated/group. One-way ANOVA followed by Tukey’s post hoc test, **P<0.01 compared to sham group baseline (day 0).
coactivation, RNA splicing and processing, control of the cell cycle, and cellular differentiation [18]. However, whether CARM1 plays a role in the neuropathic pain induced by peripheral nerve injury was still unclear. Therefore, we next examined whether CARM1 is altered in two pain-related regions (DRG and spinal cord dorsal horn) after CCI, another preclinical animal model of neuropathic pain that simulates nerve injury-induced neuropathic pain in the clinic. CCI time-dependently increased the expression of Carm1 mRNA and protein in the L3/4 DRGs on the ipsilateral side but not the contralateral side (Figure 3A-3E). The ratio of the ipsilateral side to the contralateral side of Carm1 mRNA abundance in the L3/4 DRGs was time-dependently upregulated 1.52-fold on day 3, 1.59-fold on day 7, and 1.62-fold on day 14 after CCI, compared to naïve mice (0 day) (Figure 3A). CARM1 protein abundance in the ipsilateral L3/4 DRGs was consistently increased 2.22-fold on day 3, 2.67-fold on day 7, and 2.26-fold on day 14 after CCI, compared to naïve mice (Figure 3C). However, Carm1 mRNA abundance did not significantly change in the ipsilateral L3 spinal cord dorsal horn (Figure 3B). Consistent with our previous reports [12], CCI led to mechanical allodynia and heat hyperalgesia, as demonstrated by a dramatic increase in paw withdrawal frequency and a decrease in paw withdrawal latency for the ipsilateral hind paws on days 3, 7, and 14 post CCI surgery, but not on the contralateral hind paws. As expected, sham surgery did not present a remarkable change in mechanical stimulus responses to paw withdrawal frequency and heat stimulus responses to paw withdrawal latency in either hind paw (Figure 3D and 3E).

Blocking CCI-induced CARM1 increase in the injured DRGs attenuated development of CCI-induced pain hypersensitivities

To further confirm the role of CARM1 in neuropathic pain, we determined if blocking-increased CARM1 in the DRG through microinjection of its small interfering RNA (siRNA) into the

![Figure 3](image-url)
ipsilateral DRGs affects CCI-induced pain hypersensitivity. We first examined whether Carm1 siRNA had a knockdown effect on DRG Carm1 expression. In in vitro cultured DRG primary neurons, transfection of Carm1 siRNA decreased the abundance of Carm1 gene and protein expression by 45.2% and 51.3%, respectively, compared to transfection with its negative control siRNA (Figure 4A and 4B). Next, we pre-microinjected with vehicle or negative control siRNA (1 µM/1 µl each DRG body) 4 days before CCI and found that the abundance of Carm1 mRNA and protein were remarkably increased in the ipsilateral L3/4 DRGs on day 5 after CCI compared to sham mice pre-microinjected with vehicle (Figures 4C and 4D). These increases were not obvious changes in the CCI mice pre-microinjected with Carm1 siRNA (1 µM/1 µl each DRG body). No significant decreases in the basal abundance of Carm1 mRNA and protein were present in the ipsilateral L3/4 DRGs of the sham mice pre-microinjected with Carm1 siRNA (Figure 4C and 4D). This evidence demonstrates that pre-microinjection of Carm1 siRNA can block in vivo the CCI-induced increase in CARM1 in the ipsilateral DRG.

We examined whether blocking the CCI-induced Carm1 increase in the injured DRG would attenuate the development of hypersensitivity. The abundance of Carm1 in mRNA (4A) and protein (4B) after transfection of Carm1 siRNA (siRNA) or a negative control siRNA (siNC) into cultured DRG neurons. Representative Western blotting and a summary of densitometric analysis are shown in (4B). n=3-4 biological replicates/treatment. *P<0.05 or **P<0.01 compared to siNC group by two-tailed, independent Student’s t-test. (4C-4D) Effect of pre-microinjection of Carm1 siRNA, negative control siRNA, or vehicle into the ipsilateral L3/4 DRGs on basal or CCI-induced increases in Carm1 mRNA expression (4C) and Carm1 protein abundance (4D) on day 5 after CCI or post-sham surgery in the ipsilateral L3/4 DRGs. Unilateral L3/4 DRGs from two mice were pooled together. N=6 to 8 mice per group. One-way ANOVA followed by Tukey’s post hoc test. **P<0.01 compared to the vehicle (V) plus sham group. #P<0.05 compared to the vehicle plus CCI group. (4E-4H) Effect of pre-microinjection of Carm1 siRNA, negative control siRNA, or vehicle into the ipsilateral L3/4 DRGs on paw withdrawal frequencies to mechanical stimuli (4E) and paw withdrawal latency to thermal stimulation (4F) on the ipsilateral side and on basal paw withdrawal responses to mechanical (4G) and thermal (4H) stimuli on the contralateral side at different timepoints after CCI or sham surgery. n=10-12 mice/group. **P<0.01 compared to the corresponding baseline (day -4) by two-way ANOVA followed by Tukey’s post hoc test.
period of CCI-induced pain hypersensitivity behaviors. Consistent with previous studies [12,24], CCI induced by peripheral nerve injury produced long-term mechanical allodynia and thermal hyperalgesia on the ipsilateral side in the vehicle-injected mice (Figures 4E-4H). The ipsilateral hind paw withdrawal frequency in response to mechanical stimuli was markedly increased on days 3 and 5 after CCI compared to the corresponding baseline (Figure 4D). In addition, the ipsilateral hind paw withdrawal latency in response to heat stimuli was significantly reduced on days 3 and 5 after CCI compared with preinjury baseline values (Figure 4E). Pre-injection of Carm1 siRNA did not alter basal paw responses to mechanical or heat stimulation on the ipsilateral side of sham mice (Figures 4G and 4H) but mitigated CCI-induced mechanical allodynia and thermal hyperalgesia (Figures 4E and 4F). Compared with basal values, there were no remarkable alterations in paw withdrawal frequencies and latencies on the ipsilateral side of mice injected with Carm1 siRNA (Figures 4E-4H). As expected, pre-injection of a negative control siRNA did not affect CCI-induced allodynia in response to mechanical stimuli and hyperalgesia in response to heat stimuli on the ipsilateral side. There were no significant differences in paw withdrawal responses both in the negative control siRNA injected and vehicle injected groups (Figures 4E-4F). Baseline values of paw withdrawal responses on the contralateral side and locomotor function Table 2 were not changed by pre-injection of either vehicle, negative control siRNA, or siRNA.

Post-blocking the CCI-induced DRG Carm1 increase in the injured DRG attenuated CCI-induced maintenance of pain hypersensitivity

Next, to further check the role of Carm1 in the injured DRG in the maintenance period of CCI-induced pain hypersensitivities, we conducted behavioral tests in mice subjected to CCI and injected with siRNA, negative control siRNA, or vehicle. Post-injection of Carm1 siRNA, but not vehicle or negative control siRNA, alleviated mechanical allodynia and thermal hyperalgesia induced by CCI on the ipsilateral side during the maintenance period (Figures 5A-5D). At 10 and 14 days after CCI surgery, the hind paw withdrawal frequencies in response to mechanical stimulation were significantly decreased (Figure 5A) and paw withdrawal latency in response to heat stimulation

Table 2. Locomotor function change after DRG microinjection.

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All values are mean (SEM). n=6 rats/group. 5 trials. siNC: Small Interference Negative Control RNA. siRNA: Small Interference RNA. CCI: Chronic Construction Injury

**Figure 5.** Effect of DRG post-microinjection of Carm1 siRNA into the ipsilateral L3/4 DRGs on CCI-induced maintenance of pain hypersensitivity. (5A-5D) Effect of vehicle, negative control siRNA, or Carm1 siRNA by DRG microinjection starting on day 6 after CCI on paw withdrawal responses to mechanical stimulation (5A) and thermal stimuli (5B) on the ipsilateral side and on basal paw withdrawal frequencies to mechanical stimuli (5C) and paw withdrawal latencies to thermal stimulation (5D) on the contralateral side on days 10 and 13 after CCI. n=10 mice/group. Two-way ANOVA followed by Tukey’s post hoc test, **P<0.01 compared to the vehicle plus CCI group at the different time-points.
was markedly increased (Figure 5B) compared with the corresponding vehicle-injected group. As we expected, post-injection of vehicle, negative control siRNA, or Carm1 siRNA did not change basal values of the hind paw withdrawal responses to mechanical and heat stimulation applied to the corresponding contralateral hind paw during the maintenance period (Figures 5C and 5D). Together, these data provide functional evidence for the essential role of CARM1 in the dorsal root ganglia in development and maintenance of neuropathic pain. Thus, CARM1 may be a potential target in the prevention and treatment of neuropathic pain.

Discussion

Peripheral nerve injury caused by SNL or CCI leads to long time pain hypersensitivity in a mouse model, which well mimics nerve injury-induced mechanical allodynia and thermal hyperalgesia in patients with neuropathic pain symptoms. Understanding the mechanisms that underlie peripheral nerve injury-induced pain hypersensitivity may open a new avenue to prevention and/or treatment of neuropathic pain for therapeutic strategies. In this study, we report that peripheral nerve injury induced an increase in injured DRG protein arginine methyltransferase CARM1, blocking this increase through its small interfere RNA mitigated development and maintenance of nerve injury-induced pain hypersensitivities. Thus, CARM1 may be a potential target for prevention and/or therapy of peripheral neuropathic pain.

RNA-seq is a highly sensitive method for analyzing mRNAs, ncRNAs and splice variants with differential expression. Unlike gene microarrays, RNA-seq not only analyzes a subset of known genes but can also map reads to the tissues and organs of the entire genome, providing more information on possible functions of unknown genes [19,25]. This study provided the first evidence to our knowledge that protein arginine methyltransferase and arginine demethylase genes exhibit differentially expressed mRNAs in the injured DRG following peripheral nerve injury through a RNA-Seq assay, which provides a thorough and in-depth analyses of the transcriptome changes associated with peripheral nerve injury [19]. The RNA-Seq results demonstrated that all the 9 protein arginine methyltransferase genes and 2 arginine demethylase genes are expressed in DRG tissue, and the highest expression amount is Prmt2 and Prmt3. Moreover, Carm1 gene expression was significantly upregulated, but the Prmt8 gene was downregulated in the injured DRG after SNL compared with the sham group. Consistent with the RNA-Seq results, Carm1 gene expression was time-dependently increased, and in contrast, Prmt8 was time-dependently reduced in injured DRG after SNL during the observation period of the validation experiments by RT-qPCR analysis. Interestingly, no significant changes were seen in the arginine demethylase genes JmjD6 and Padi4 after SNL based on RNA-Seq and RT-qPCR validation analysis. It appears that peripheral nerve injury mainly involves protein arginine methylation (induced by Carm1 and Prmt8) changes and likely does not involve arginine demethylation activities.

We further demonstrated that Carm1 gene and protein expression were increased in L3 and L4 DRGs, but not in the corresponding dorsal horn, on the ipsilateral side following CCI, in another neuropathic pain animal model. This increase in DRG, which has important roles in the transmission and modulation of noxious information [26], was closely related to pain hypersensitivity for mechanical allodynia and thermal hyperalgesia after CCI and may trigger the development and maintenance of neuropathic pain after peripheral nerve injury. Knockdown of Carm1 gene expression through DRG microinjection of siRNA mitigated CCI-induced mechanical allodynia and thermal hyperalgesia. Increased CARM1 in the injured DRG was required for CCI-induced pain hypersensitivity.

Unexpectedly, Carm1 siRNA administration did not affect the basal amount of Carm1 gene and protein expression in sham surgery, although this siRNA significantly and specifically knocked down Carm1 gene and protein expression in the in vitro primary DRG neurons. We still do not know the detailed reasons why Carm1 siRNA had no effect on basal Carm1 expression; it may be associated with the low level of Carm1 expression in the DRG under normal conditions. Despite the fact that the dose of Carm1 siRNA used completely blocked CCI-induced Carm1 increase in the DRG, this dose of Carm1 siRNA used could not further reduce basal expression of Carm1 in sham mice. Given that Carm1 siRNA attenuated the development and maintenance of neuropathic pain after peripheral nerve injury but did not affect basal (or acute) behavioral responses and locomotor functions, our findings suggest that CARM1, in clinical implications, may be a good target for neuropathic pain treatment.

Peripheral nerve injury leads to alterations in genes encoding ion channels, receptors, and enzymes in the injured DRG, and this contributes to neuropathic pain development and maintenance [19,27,28]. Histone arginine methylation is one mechanism of epigenetic regulation in eukaryotic cells and catalyzes the addition of methyl groups onto arginine of histone and non-histone proteins, resulting in either mono- or dimethylated-arginine residues by an enzyme family of the above nine protein arginine methyltransferases [17]. Different protein arginine methyltransferases induce different functions in transcriptional regulation, RNA processing, signal transduction, and DNA repair [16]. CARM1, a protein arginine methyltransferase, is known to enhance transcriptional activation through methylation of histone H3 at arginine 17 and 26 (H3-R17/26) [18] and activate the JAK/STAT pathway in signal transduction [29]. However, it is still unknown whether CARM1 was a key player in these alterations on injured DRG under neuropathic pain conditions. We demonstrated here that blocking CCI-induced increases in Carm1 attenuated neuropathic pain development and maintenance after peripheral nerve injury. The evidence suggests that increased Carm1 in the injured DRG is required for peripheral nerve injury-induced pain hypersensitivity. Mechanistically, whether Carm1 in DRG regulated some pain-related factors expression contributed to neuropathic pain is still elusive and remains to be further studied.

This study also demonstrated that Prmt8 was notably reduced in injured DRG after peripheral nerve injury. Prmt8 has important roles in neuronal stem cell development and differentiation, altering synapse composition and functions, and tumorigenesis
however, whether Prmt8 is involved in neuropathic pain, synaptic plasticity, neurodegeneration, or nerve damage and repair after nerve injury is still elusive, and we will continue to further study the question.

**Conclusion**

In summary, we demonstrated for the first time that blocking the CCI-induced increase in CARM1 expression through microinjection of its siRNA into the DRG impaired CCI-induced pain-like hypersensitivities without affecting basal nociceptive responses or locomotor functional activities. Thus, CARM1 may be a potential target for prevention and/or therapy of peripheral neuropathic pain.

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