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Intraperitoneal 5-Azacytidine Alleviates Nerve Injury-Induced Pain in Rats by Modulating DNA Methylation

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Abstract

To investigate the role of DNA methylation in modulating chronic neuropathic pain (NPP), identify possible target genes of DNA methylation involved in this process, and preliminarily confirm the medicinal value of the DNA methyltransferases (DNMTs) inhibitor 5-azacytidine (5-AZA) in NPP by targeting gene methylation. Two rat NPP models, chronic constriction injury (CCI) and spinal nerve ligation (SNL), were used. The DNA methylation profiles in the lumbar spinal cord were assayed using an Arraystar Rat RefSeq Promoter Array. The underlying genes with differential methylation were then identified and submitted to Gene Ontology and pathway analysis. Methyl-DNA immunoprecipitation quantitative PCR (MeDIP-qPCR) and quantitative reverse transcription-PCR (RT-qPCR) were used to confirm gene methylation and expression. The protective function of 5-AZA in NPP and gene expression were evaluated via behavioral assays and RT-qPCR, respectively. Analysis of the DNA methylation patterns in the lumbar spinal cord indicated that 1205 differentially methylated fragments in CCI rats were located within DNA promoter regions, including 638 hypermethylated fragments and 567 hypomethylated fragments. The methylation levels of Grm4, Htr4, Adrb2, Kcnf1, Gad2, and Pparg, which are associated with long-term potentiation (LTP) and glutamatergic synapse pathways, were increased with a corresponding decrease in their mRNA expression, in the spinal cords of CCI rats. Moreover, we found that the intraperitoneal injection of 5-AZA (4 mg/kg) attenuated CCI- or SNL-induced mechanical allodynia and thermal hyperalgesia. Finally, the mRNA expression of hypermethylated genes such as Grm4, Htr4, Adrb2, Kcnf1, and Gad2 was reversed after 5-AZA treatment. CCI induced widespread methylation changes in the DNA promoter regions in the lumbar spinal cord. Intraperitoneal 5-AZA alleviated hyperalgesia in CCI and SNL rats, an effect accompanied by the reversed expression of hypermethylated genes. Thus, DNA methylation inhibition represents a promising epigenetic strategy for protection against chronic NPP following nerve injury. Our study lays a theoretical foundation for 5-AZA to become a clinical targeted drug.

Keywords Chronic neuropathic pain · Nerve injury · Lumbar spinal cord · 5-Azacytidine · Epigenetic · DNA methylation

Introduction

Neuropathic pain (NPP) is the most common category of pathological pain [1] and is defined as pain brought on by a lesion or illness that affects the nervous system at either the peripheral or central level [2]. It is estimated that NPP has a prevalence of 6–10% in the general population, and it causes difficulties in daily activities and decreases productivity at work [3, 4], resulting in a poorer quality of life. However, current treatments for NPP are poorly effective because of our limited understanding of the molecular mechanisms involved. It has been established that central sensitization in the spinal cord plays a crucial role in the NPP condition [5, 6], and altered gene expression contributes critically to the central sensitization and the initiation and maintenance of chronic NPP [7–9]. Besides, long-term NPP in animals also led to alterations in gene expression across the genome, indicating that gene expression alteration in the spinal cord should be noticeable in response to chronic NPP [10–12]. Interestingly, therapies that alleviate hyperalgesia are associated with the reversal of NPP-induced regulation of pain-related mediators in the spinal cord [13–15].

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Without changing the underlying genetic sequence, epigenetic regulation enables the steady control of gene expression in response to experiential and environmental inputs [16]. DNA methylation is an epigenetic process that can be involved in the long-term control of gene expression [17]. DNA methyltransferases (DNMTs), which transfer a methyl group from the methyl donor s-adenosyl methionine to cytosine bases in the DNA, catalyze the methylation of DNA [17]. Typically, DNA methylation in a gene's promoter region causes epigenetic inhibition of gene expression [18]. Numerous studies have shown that chronic pain is associated with changes in DNA methylation throughout cells and tissues involved in pain pathways, including the dorsal root ganglion (DRG) [19, 20], spinal cord [21, 22], and brain [23, 24]. Additionally, artificial enrichment by reducing DNA methylation levels in the spinal cord successfully attenuated pain [25, 26]. As DNMTs inhibitors and regulators, 5-azacytidine (5-AZA) and 5-aza-2′-deoxycytidine (5-AZA-dC) are used to treat a variety of pain-related or pain-comorbid disorders, such as cognitive impairment, depression, osteoarthritis, and cancer pain [27–30]. However, the specific genes and biological systems associated with effective treatment of NPP have not been well explored. Little is known about the methylated genes that 5-AZA targets in the spinal cord during chronic NPP. The discovery of these systems will shed light on the processes by which the epigenome is altered by chronic NPP and how the epigenome might be therapeutically addressed.

Our previous study found that abnormal DNA methylation is involved in chronic constriction injury (CCI)-induced NPP [21]. In this study, we use an Arraystar Rat RefSeq Promoter Array to analyze changes in DNA methylation at the genome-wide level in the spinal cord caused by peripheral nerve injury. We show that CCI induces widespread lumbar spinal cord methylation changes, and more than 1200 genes are differentially methylated after CCI. Furthermore, the specific hypermethylated genes are located mainly in long-term potentiation (LTP) and glutamatergic synapse pathways. Moreover, intraperitoneal injection of 5-AZA alleviates mechanical allodynia and thermal hyperalgesia and counteracts changes in hypermethylated gene mRNA expression induced by chronic NPP following CCI or spinal nerve ligation (SNL).

Materials and Methods

Animals

IN this study, male Sprague−Dawley (SD) rats weighing 220–250 g were employed. All animals were kept in a setting with a temperature of 22 ± 1 °C, a relative humidity of 50 ± 1%, and a cycle of light and dark of 12 h. They had access to food and water at all times in their separate cages. All protocols were approved by the ethics committee of the Fujian Cancer Hospital and adhered to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Experimental Paradigm

Behavioral testing was performed before surgery and at 3, 5, 7, 10, and 14 days after surgery in each rat. Under deep anesthesia with intraperitoneal injection (i.p.) of pentobarbital sodium (40 mg/kg), all the rats were killed and their ipsilateral lumbar spinal cords were immediately dissected 14 days after behavioral testing. The first six rats were randomly divided into two groups and subjected to CCI or sham surgery (n = 3 for each group). On day 14 after surgery, ipsilateral lumbar spinal cords in CCI or sham groups were dissected for methyl-DNA immunoprecipitation (MeDIP)-on-chip and microarray data analysis. Then, to verify the chip results and assess the methylation of target genes by MeDIP-qPCR, six rats were randomly divided into CCI and sham groups ($n = 3$), and their ipsilateral lumbar spinal cords were dissected on day 14 after surgery. Next, twelve rats were randomly divided into CCI and sham groups ($n = 6$), and their ipsilateral lumbar spinal cords were used to detect the mRNA levels of target genes. Furthermore, to study the effects of 5-AZA on CCI-induced NPP, eighteen rats were randomly divided into three groups ($n = 6$ for each group): the sham + NS group, CCI + NS group, and CCI + AZA group. CCI + AZA rats were treated with 4 mg/kg (i.p.) 5-AZA at a concentration of 1 mg/ml (Sigma, USA) once a day from days 3 to 14 after CCI. Meanwhile, rats in the sham + NS group and CCI + NS group received the same volume of 0.9% saline. Moreover, to demonstrate the effects of 5-AZA on SNL-induced NPP, eighteen rats were randomly divided into three groups to receive SNL surgery ($n = 6$ for each group): the Sham + NS group, SNL + NS group, and SNL + AZA group. From days 3 to 14, the SNL + 5-AZA group rats were treated with 4 mg/kg 5-AZA (1 mg/ml, i.p., Sigma, USA) once daily. Meanwhile, sham + NS and SNL + NS rats received the same volume of 0.9% saline. All intraperitoneal injections were administered between 9:00 am and 12:00 am. On day 14 after surgery, the ipsilateral lumbar spinal cords of the last thirty-six rats were dissected to detect the mRNA levels of target genes after behavioral testing.

NPP Model

Chronic Constriction Injury

The CCI procedure was performed as previously described [31]. Briefly, rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.). The trunk of the right sciatic nerve was freed

before it branched. Next, 4–0 chromic catgut was used to mildly ligate the trunk of the nerve with appropriate strength to slightly compress the epineuria and cause a gentle tremor in the associated muscles. Four ligatures were made at intervals of 1 mm in the sciatic nerve trunk. Sham rats underwent the same surgical procedures without ligatures placement.

Spinal nerve ligation

The SNL procedure was performed on the right L5 spinal nerve as previously described [32]. In short, the dorsal vertebral column of L4–L6 was surgically exposed under pentobarbital sodium anesthesia (40 mg/kg, i.p.). At L4–L6, the paraspinal muscles were dissociated from the spinal processes, and the L5 transverse process was delicately removed. The right L5 spinal nerve was exposed and firmly ligated distal to the DRG using 4–0 silk sutures. In the sham-operated group, the same procedure was followed, except that the L5 spinal nerve was not ligated.

Behavioral Testing

The 2390 Electronic von Frey Anesthesiometer (IITC Life Science, USA) and the 37370 Plantar Test Instrument (Ugo Basile, Italy) were used to measure the mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) in all rats before and 3, 5, 7, 10, and 14 days after surgery, respectively, as previously described [25, 26]. Both of the two tests ended with paw removal followed by distinct flinching movements. The intensity was automatically recorded after paw withdrawal. At five-minute intervals, each rat was measured five times. The maximum and minimum values were subtracted before averaging the MWT and TWL. All behavioral measurements were performed between 2:00 pm and 5:00 pm by a researcher who was blinded to the group assignment.

Methyl-DNA Immunoprecipitation-on-Chip

Genomic DNA was extracted from lumbar spinal cords using a DNeasy Blood & Tissue Kit (Qiagen, Germany). Using a Bioruptor sonicator (Diagenode, Belgium), genomic DNA from each sample was sonicated to around 200–1000-bp fragments. One microgram of fragmented genomic DNA was immunoprecipitated with a mouse monoclonal anti-5-methylcytosine antibody (Diagenode, Belgium). MeDIP DNA was purified using Qiagen MinElute columns (Qiagen, Germany). The NimbleGen Dual-Color DNA Labeling Kit (NimbleGen Systems, USA) was used for DNA labeling. One microgram of DNA from each sample was incubated for 10 min at 98 °C with 1 OD of Cy5-9mer primer (IP sample) or Cy3-9mer primer (input sample). The Arraystar Rat RefSeq Promoter Array (Arraystar, USA) was hybridized with labeled DNA. Finally, an Agilent G2505C Scanner (Agilent Technologies, Germany) was used to wash and scan the arrays. The microarray data have been deposited in NCBI Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE219038.

Microarray Data Analysis

From the normalized log2-ratio data, a sliding-window (1500 bp) peak-finding algorithm provided by NimbleScan v2.5 (Roche-NimbleGen, Sweden) was applied to analyze the MeDIP-chip data. A one-sided Kolmogorov−Smirnov (KS) test was used to determine whether the probes were drawn from a significantly more positive distribution of intensity log2-ratios than the rest of the array. The region was assigned to an enrichment peak (EP), if several adjacent probes were significantly increased above a set threshold. The NimbleScan sliding-window peak-finding algorithm was rerun to identify the differentially enriched peaks (DEPs). The log2-ratio value of all probes in the DEP region was used to make a cluster analysis to directly show the methylation of DEP probes in each sample. Genes containing DEPs in the promoter were categorized as differentially methylated genes (DMGs). Then, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was used to analyze these genes. The significance of the pathway correlation to the conditions was denoted by the p value with a cutoff of 0.05. The Gene Ontology project offers a controlled vocabulary to describe gene and gene product properties in any organism (http://www.geneontology.org). Three domains of biological process, cellular component, and molecular function are all covered by Gene Ontology terms. To determine whether there was a greater overlap between the DE list and the GO annotation list than would be predicted by chance, Fisher's exact test was utilized. The significance of GO term enrichment in the DE genes was denoted by the p value with a cutoff of 0.05.

Real-time PCR on MeDIP-Enriched DNA

The MeDIP assay combined with real-time PCR was used to quantitatively assess the methylation status of target genes to verify MeDIP-chip results. DNA extraction and MeDIP were performed as described above. We carried out real-time PCR with immunoprecipitated methylated DNA and input DNA by using the ViiA 7 Real-time PCR System (Applied Biosystems, USA), and 2 × PCR master mix (Arraystar, USA). The methylation levels of Grm4, Htr4, Adrb2, Kcnf1, Gad2, and Pparg were evaluated. The primers used for MeDIP-qPCR are shown in Table 1. $2^{-\Delta\Delta CT}$ values were used, and the relative enrichment of methylation in each sample was calculated using the signal ratios of immunoprecipitated DNA to input DNA: %MeDIP/Input = 2

Table 1 Primer sequences for MeDIP-qPCR analysis

Gene	Primer	Sequence (5′–3′)	bp
Grm4	Forward	CCTGCGGCATACTCATTCG	80
	Reverse	AGGTGGATTCGCCTTTCG	
Htr4	Forward	CCAGAGCTGGACGCTAACG	134
	Reverse	TGAGGATTCAGGGACCGAG	
Adrb2	Forward	CGCACAGCAGTCCCAGATT	160
	Reverse	GGAGGGGCGAGGCACTAA	
Kcnf1	Forward	CAGTGCTCGGGAGTGTCAGA	169
	Reverse	GGGTTCAGGCTGCCAAGTC	
Gad2	Forward	GGTTTGGAGGAAGGAGGGTG	85
	Reverse	CAGCGATTGGCTCATTGTTTG	
Pparg	Forward	ACGGAGCGTGACAGACAAAGT	132
	Reverse	CTTCTCCTCAGACCGCATCC	

Table 2 Primer sequences for RT-qPCR analysis

Gene	Primer	Sequence (5′–3′)	bp
Grm4	Forward	AGTGACAACAGCCGCTATGAC	188
	Reverse	CACACACCTCCGTTCTCTCG	
Htr4	Forward	TTTCAGACGTGCCTTCCTTAT	143
	Reverse	CACCACATTCCACTGTATCCC	
Adrb2	Forward	CACTCAGGAACGGGACGAA	147
	Reverse	GTTATGAAGTAGTTGGTGACGGT	
Kcnf1	Forward	CTCATCCTTGTCTCCTCCGTG	283
	Reverse	CGTTGGTCAGTTCCATCATTC	
Gad2	Forward	CGCACTGCCAAACAACTCTAA	101
	Reverse	ATCTGCTGCTAATCCAACCAT	
Pparg	Forward	TGCGGAAGCCCTTTGGT	177
	Reverse	GCAGCAGGTTGTCTTGGATGT	
GAPDH	Forward	GCTCTCTGCTCCTCCCTGTTCTA	124
	Reverse	TGGTAACCAGGCGTCCGATA	

$(Ct^{Input} - Ct^{ChIP}) \times Fd \times 100\%$, Fold Enrichment = [% (MeDIP/Input)]/[% (Negative control/Input)].

Quantitative Reverse Transcription-PCR

The mRNA expression of the genes was determined by quantitative reverse transcription-PCR (RT-qPCR). TRIzol Reagent (Invitrogen, USA) was used to extract total RNA. The cDNA templates were synthesized by reverse transcription using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, USA) and then saved at − 20 °C. The ViiA 7 Real-time PCR System (Applied Biosystems, USA) and 2 × PCR master mix (Arraystar, USA) were used to perform real-time PCR. GAPDH was used as an internal standard, and the $2^{-\Delta\Delta CT}$ method was used to determine the relative expression levels of the target genes. Primers were produced by Shanghai Lifei Biotechnology Co., Ltd., in China, and are listed in Table 2.

Statistical Analyses

The GraphPad Prism 5.0 software (GraphPad Software, CA) was used to conduct all statistical analyses. Data are presented as the mean ± SD. The unpaired Student's *t*-test was used for comparisons of PCR data between two groups. Differences between sham, CCI, or SNL and 5-AZA treatment conditions were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. Behavioral test data were analyzed using two-way repeated-measurement ANOVA followed by Bonferroni's post hoc test for multiple comparisons. $P < 0.05$ was considered significant.

Results

DNA Methylation Status in Response to CCI in the Lumbar Spinal Cord in Rats

MWT and TWL in rats were measured using behavioral tests. Our results showed that MWT and TWL significantly decreased in the CCI rats than in the sham rats from 3 to 14 days after surgery (all $P < 0.001$, Fig. 1A), indicating mechanical allodynia and thermal hyperalgesia in CCI rats were successfully established.

To study whether CCI led to abnormal methylation changes in DNA promoter regions at the genome-wide level, the Arraystar Rat RefSeq Promoter Arrays covering 15,987 gene promoter regions (between approximately − 1300 and + 500 bp of the TSSs) with ~ 180,000 probes separated by around 158 bp were used to detect differentially methylated regions in the DNAs that were extracted from the lumbar spinal cord of the CCI and sham rats on day 14 (Supplementary Table 1). The cluster diagram directly showed the methylation of DEP probes in each sample (Fig. 1B). There were 1205 differentially methylated fragments within DNA promoter regions, including 638 hypermethylated fragments and 567 hypomethylated fragments in CCI rats (Fig. 1C). And the hypermethylated fragments contained 292 (45.77%) high CpG-density promoters (HCP), 169 (26.49%) intermediate CpG-density promoters (ICP), and 177 (27.74%) low CpG-density promoters (LCP). While the hypomethylated fragments contained 267 (47.09%) HCP, 163 (28.75%) ICP, and 137 (24.16%) LCP (Fig. 1D).

Differentially Methylated Functional Domains and Signaling Pathways in CCI Rats

Gene Ontology (GO) analysis was used to identify ontology terms enriched among differentially methylated genes (DMGs) in the lumbar spinal cord of CCI rats. DMGs between hyper- and hypomethylated regions in response to

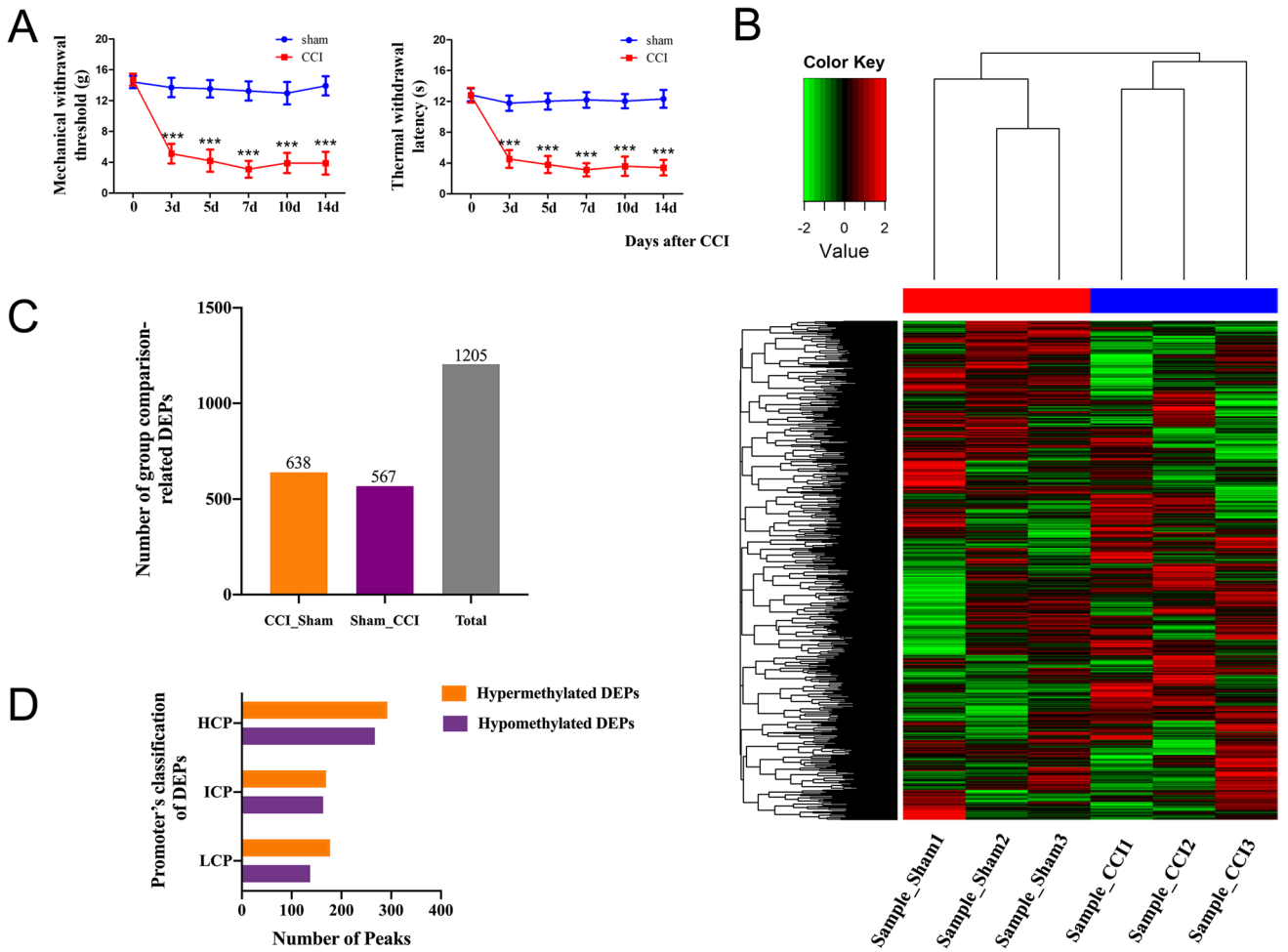

Fig. 1 Differential methylation of promoter regions in the lumbar spinal cord of Sham and CCI rats. **A** Ipsilateral mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) were monitored to identify CCI-induced neuropathic pain ($n=3$), values represented as mean ± SD, two-way repeated-measurement ANOVA followed by Bonferroni's test. $^{*}P<0.05$ vs. the Sham group, $^{**}P<0.01$ vs. the Sham group, $^{***}P<0.001$ vs. the Sham group. **B** Clustering of differentially enriched peaks (DEPs) of methylation in the DNA promoter region in the Sham group and CCI group (red: hypermethylation; green: hypomethylation). **C** Numbers of DEPs of methylation between groups. CCI_Sham represents the DEPs that were hypermethylated in the CCI group compared with the sham group, and Sham_CCI represents the DEPs that were hypermethylated in the sham group compared with the CCI group. **D** The distribution of the total number of DEPs of methylation in the DNA promoter regions enriched in the microarray analysis in each category. Note: HCP, high CpG-density promoter; ICP, intermediate CpG-density promoter; LCP, low CpG-density promoter

peripheral nerve injury were identified. The top 10 most significant enrichment terms among hyper- and hypomethylated DMGs after CCI are shown in Fig. 2A and B. Organism process, developmental process, anatomical structure development, and ion transport were the top GO terms enriched among hypermethylated genes (Fig. 2A), while cellular component organization or biogenesis, establishment of localization, and transport were the top GO terms enriched among hypomethylated genes (Fig. 2B).

KEGG pathway analysis was used to identify the signaling pathways among DMGs. The top 10 most significant enrichment pathways among hyper- and hypomethylated DMGs after CCI are shown in Fig. 2C and D. The hypermethylated genes in the CCI group were mainly enriched in long-term potentiation (LTP), glutamatergic synapse, calcium signaling, and cAMP signaling pathways (Fig. 2C). While hypomethylated genes were mainly enriched in the cell cycle, peroxisome, oocyte meiosis, and gastric acid secretion pathways (Fig. 2D). The full list of GO terms and KEGG pathways enriched in the DMGs can be found in Supplementary Tables 2 and 3.

Screening and Verification of DMGs in CCI Rats

In accordance with the specified conditions, some hypermethylated DMGs were identified from the above microarray findings. Table 3 summarizes the six selected hypermethylated

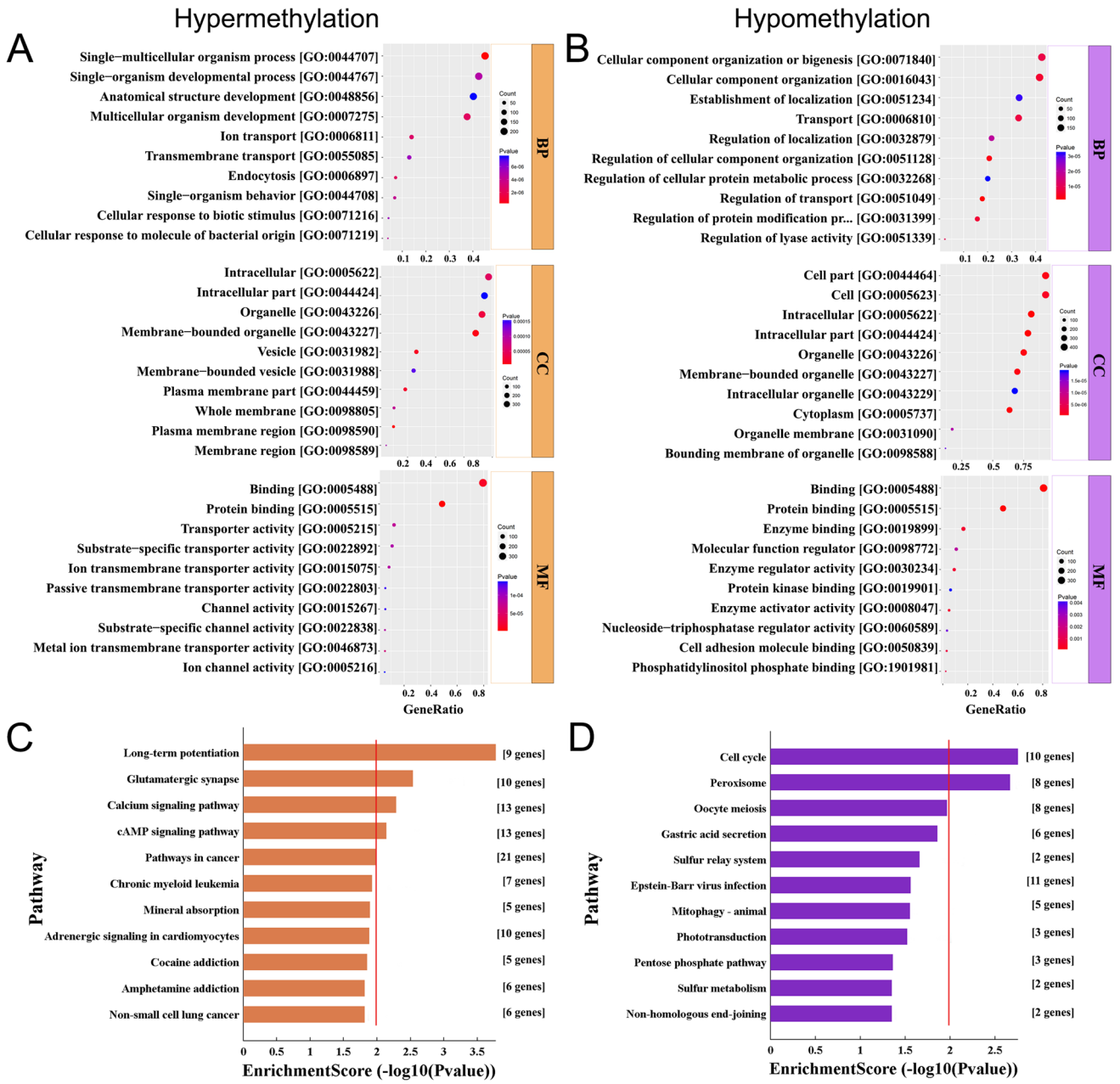

Fig. 2 GO and KEGG pathway analysis of differentially methylated genes (DMGs) between the hyper- and hypo-methylation groups in the lumbar spinal cord of CCI rats. **A**, **B** Dot graphs of the top ten GO terms based on hypermethylated DMGs (**A**) and hypomethylated DMGs (**B**) profiling. The dot plot shows the gene ratio values of the top ten most significant enrichment terms. The gene ratio value equals (the number of DE genes associated with the listed GOID/the total number of DE genes). GO, Gene Ontology; BP, biological process; CC, cellular component; MF, molecular function; GOID, the ID of gene ontology term used in the Gene Ontology Project. **C**, **D** Enrichment analysis of KEGG pathways and the top ten activated pathways identified among hypermethylated DMGs (**C**) and hypomethylated DMGs (**D**) are shown. The bar plot shows the top ten Enrichment score (-log10(P value)) values of the significant enrichment pathways. The red lines in **C** and **D** depict the level of statistical significance ($P = 0.01$). KEGG, Kyoto Encyclopedia of Genes and Genomes

genes that predict the chronic NPP-related pathological signature in CCI rats, in order of PeakScore from high to low.

We further validated the methylation status of the six chronic NPP-related genes by MeDIP-qPCR. As shown in Fig. 3, we observed that the methylation levels of Grm4, Htr4, and Kcnf1 were significantly increased in the CCI group compared to the sham group ($P < 0.05$, $n = 3$). Meanwhile, CCI-induced gene (Adrb2, Gad2, and Pparg) methylation was increased in comparison with the sham group, albeit without statistical significance

Table 3 The detailed information ofselected hypermethylated genes for chronic neuropathic pain-related pathologicalsignature

Gene Name	LocusLink ID	Location	Distance From TSS	PeakScore	PeakDMValue
Grm4	24417	chr20:5727530-5728514	-748	3.6	0.1079867
Htr4	25324	chr18:58415861-58417339	-560	2.76	0.0754781
Adrb2	24176	chr18:58176502-58176863	317	2.47	0.0806944
Kcnf1	298908	chr6:40912729-40913426	-9	2.46	0.0475188
Gad2	24380	chr17:96258807-96259009	-521	2.43	0.1376718
Pparg	25664	chr4:151491931-151493082	-939	2.41	0.0887667

"PeakScore" the average -log10 (P-value) from probes within the peak; "PeakDMvalue" the median M' values from probes within the differential methylation peak.

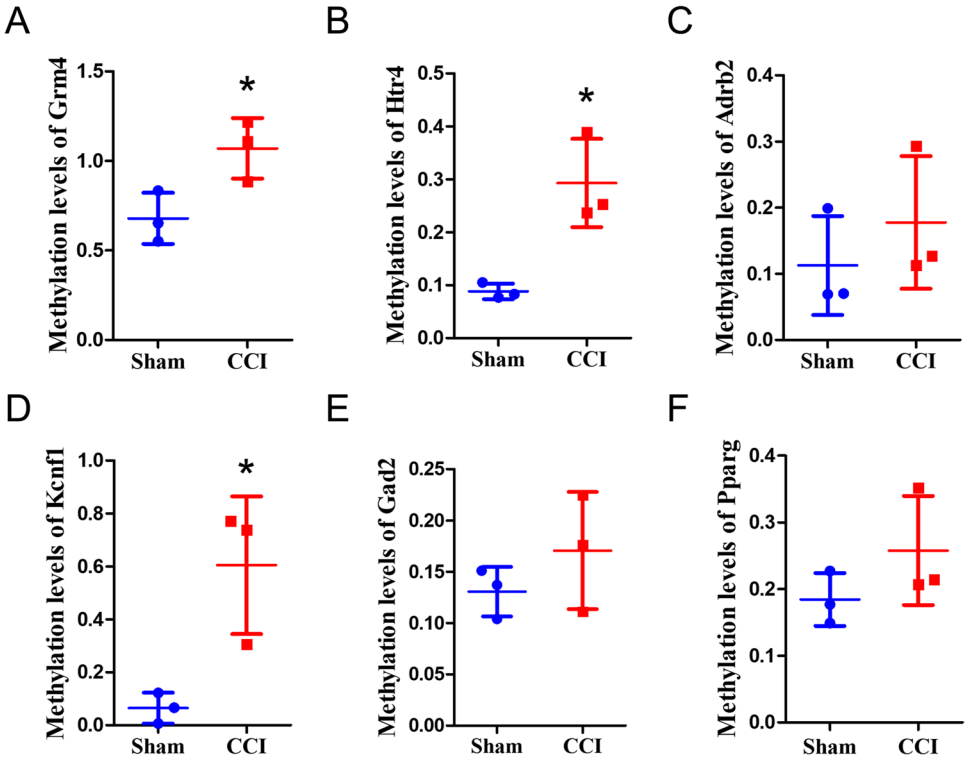

Fig. 3 MeDIP-qPCR results of 6 target hypermethylated genes in CCI rats. **A** DNA methylation of Grm4. **B** DNA methylation of Htr4. **C** DNA methylation of Adrb2. **D** DNA methylation of Kcnf1. **E** DNA methylation of Gad2. **F** DNA methylation of Pparg. Note: $n = 3$; values represented as mean ± SD, unpaired Student's *t*-test. $^{*}P < 0.05$ vs. the sham group

($n = 3$). These results indicated that the methylation levels of various genes, including Grm4, Htr4, Kcnf1, Adrb2, Gad2, and Pparg, might be enhanced by peripheral nerve injury.

Expression Level of Pain-Related Genes in the Lumbar Spinal Cord After CCI

In line with the alterations in DNA methylation, gene expression detection by RT-qPCR showed a decrease in the mRNA expression of Grm4, Htr4, Adrb2, Kcnf1, and Pparg in the CCI group compared with the sham group ($P < 0.05$, Fig. 4). And Gad2 mRNA expression was reduced without statistical significance. Based on these findings, peripheral nerve injury could alter the expression and methylation of Grm4, Htr4, Adrb2, Kcnf1, and Pparg genes in rats following the CCI procedure.

5-AZA Alleviated Hyperalgesia and Induced Pain-Related Gene Expression Changes in the Lumbar Spinal Cord After CCI

We examined the MWT and TWL after the intraperitoneal injection of 5-AZA in the CCI-induced NPP model. The MWT and TWL were recorded before CCI (baseline) and on days 3, 5, 7, 10, and 14 after CCI (Fig. 5A). The baseline MWT and TWL in each group showed no significant differences. Compared to those in the sham + NS group, the MWT and TWL were

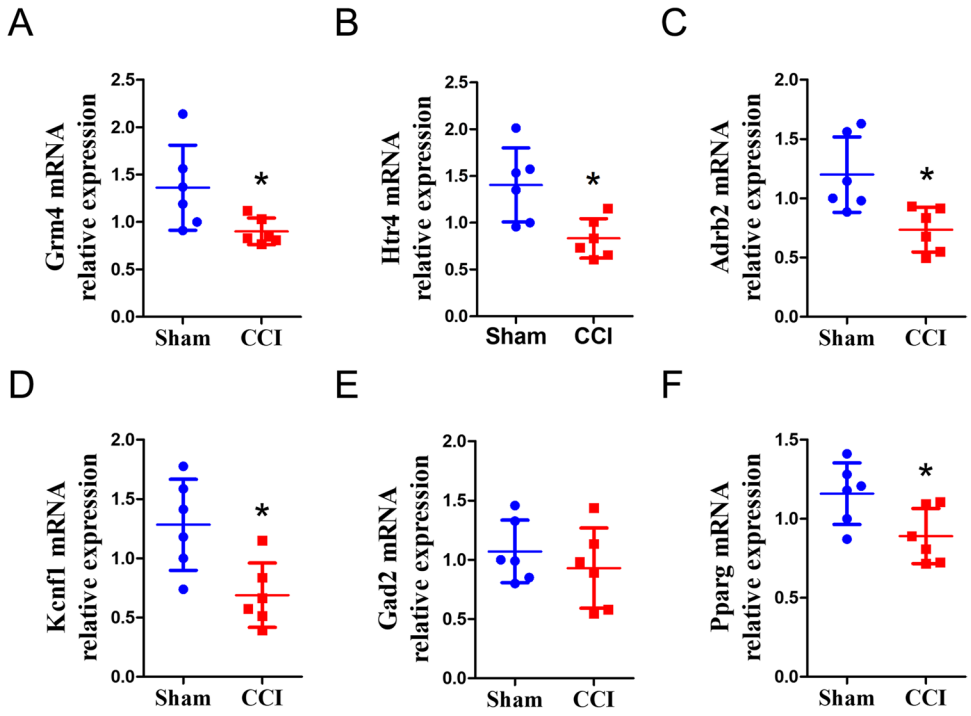

Fig. 4 The RT-qPCR results of 6 target genes in CCI rats. **A** RT-qPCR of Grm4. **B** RT-qPCR of Htr4. **C.** RT-qPCR of Adrb2. **D** RT-qPCR of Kcnf1. **E** RT-qPCR of Gad2. **F** RT-qPCR of Pparg. Note: $n=6$; values represented as mean ± SD, unpaired Student's *t*-test. $^{*}P<0.05$ vs. the sham group

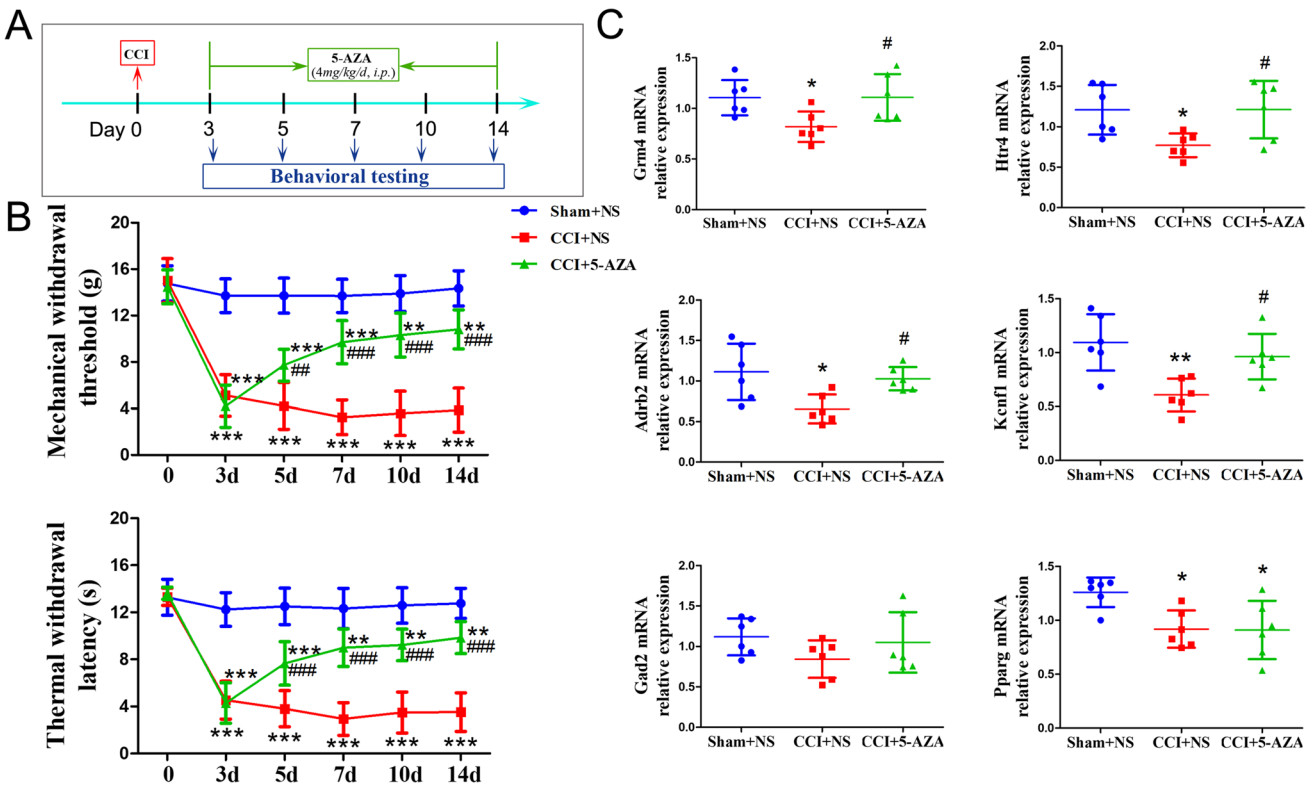

Fig. 5 Intraperitoneal injection of 5-AZA alleviated CCI-induced neuropathic pain. **A** Timescale of the CCI experiment and behavioral test. "i.p." intraperitoneal injection. **B** After 5-AZA treatment, mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) were monitored to identify CCI-induced neuropathic pain ($n=6$). Data were shown as mean ± SD, two-way repeated-measurement ANOVA followed by Bonferroni's test. **C** RT-qPCR of Grm4, Htr4, Adrb2, Kcnf1, Gad2, and Pparg ($n=6$). Data were shown as mean ± SD, one-way ANOVA followed by Tukey's test. Note: $^{*}P<0.05$ vs. the sham + NS group, $^{**}P<0.01$ vs. the sham + NS group, $^{***}P<0.001$ vs. the sham + NS group, $^{\#}P<0.05$ vs. the CCI + NS group, $^{\#\#}P<0.01$ vs. the CCI + NS group, $^{\#\#\#}P<0.001$ vs. the CCI + NS group

significantly reduced in the CCI + NS and CCI + AZA groups after CCI from day 3 (all $P < 0.01$, Fig. 5B), indicating the successful establishment of the CCI-induced NPP model. Mechanical allodynia and thermal hyperalgesia were partially improved on day 5 in the CCI + AZA group compared to the CCI + NS group, and this improvement was maintained throughout the study (all $P < 0.01$, Fig. 5B). These results indicated that the intraperitoneal injection of 5-AZA alleviates the mechanical allodynia and thermal hyperalgesia induced by CCI.

Based on behavioral experiments, we explored the relationship between 5-AZA and pain-related gene expression using RT-qPCR analysis. All pain-related genes (Grm4, Htr4, Adrb2, Kcnf1, Gad2, and Pparg) were evaluated on day 14 after 5-AZA injection. The results showed that the mRNA expression of Grm4, Htr4, Adrb2, and Kcnf1 was significantly increased in the CCI + 5-AZA group compared to the CCI + NS group ($P < 0.05$, Fig. 5C).

5-AZA Alleviated Hyperalgesia and Induced Pain-Related Gene Expression Changes in the Lumbar Spinal Cord After SNL

We further examined the MWT and TWL after intraperitoneal injection of 5-AZA in the SNL-induced NPP model. MWT and TWL were recorded before SNL (baseline) and on days 3, 5, 7, 10, and 14 (Fig. 6A). The baselines of MWT and TWL in each group showed no significant differences. Compared to the sham + NS group, the MWT and TWL were significantly reduced in the SNL + NS and SNL + AZA groups from day 3 after SNL (all $P < 0.05$, Fig. 6B), indicating the successful establishment of the SNL-induced NPP model. Mechanical allodynia and thermal hyperalgesia were partially improved on day 5 in the SNL + AZA group compared to the SNL + NS group and maintained throughout the study (all $P < 0.001$, Fig. 6B). These results indicated that the intraperitoneal injection of 5-AZA alleviated the mechanical allodynia and thermal hyperalgesia induced by SNL.

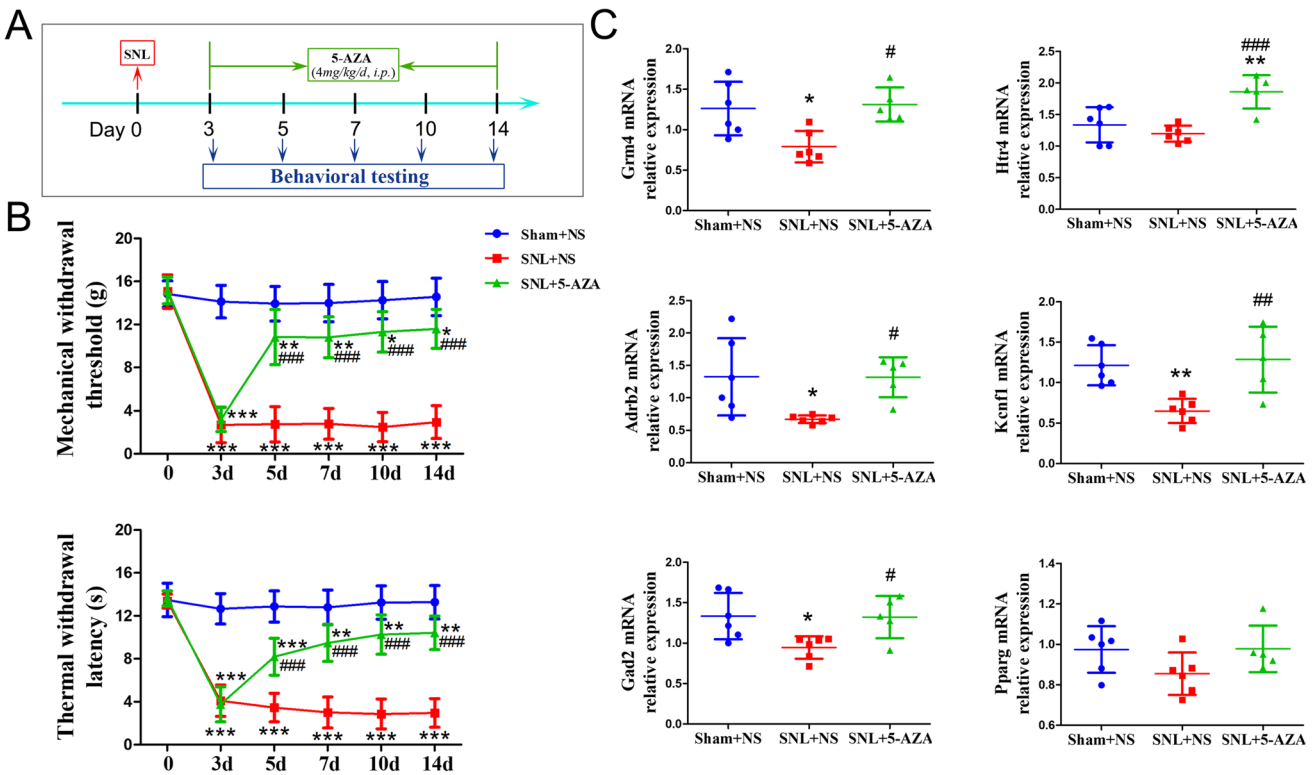

Fig. 6 Intraperitoneal injection of 5-AZA alleviated SNL-induced neuropathic pain. **A** Timescale of SNL experiment and behavioral test, "i.p." intraperitoneal injection. **B** After 5-AZA treatment, mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) were monitored to identify SNL-induced neuropathic pain ($n = 6$). Data were shown as mean ± SD, two-way repeated-measurement ANOVA followed by Bonferroni's test. **C** RT-qPCR of Grm4, Htr4, Adrb2, Kcnf1, Gad2 and Pparg ($n = 5$–6). Data were shown as mean ± SD, one-way ANOVA followed by Tukey's test. Note: $^{*}P < 0.05$ vs. the sham + NS group, $^{**}P < 0.01$ vs. the sham + NS group, $^{***}P < 0.001$ vs. the sham + NS group, $^{\#}P < 0.05$ vs. the SNL + NS group, $^{\#\#}P < 0.01$ vs. the SNL + NS group, $^{\#\#\#}P < 0.001$ vs. the SNL + NS group

Based on behavioral experiments, we explored the relationship between 5-AZA and pain-related gene expression by RT-qPCR analysis. All pain-related genes (Grm4, Htr4, Adrb2, Kcnf1, Gad2, and Pparg) were evaluated on day 14 after 5-AZA injection. The results showed that the mRNA expression of Grm4, Htr4, Adrb2, Kcnf1, and Gad2 was significantly increased in the SNL + 5-AZA group compared to the SNL + NS group ($P < 0.05$, Fig. 6C).

Discussion

Chronic NPP is associated with multiple dimensions, including nociception, emotional distress, and cognitive dysfunction. Epigenetic mechanisms are implicated in these conditions. Our results showed that NPP altered DNA methylation profiles and induced a decrease in the mRNA expression of some pain-related hypermethylated genes. Methylation inhibition improved nociception associated with NPP and reversed the decrease in the hypermethylated gene mRNA expression followed by nerve injury.

Over the past few decades, many animal models for nerve injury-induced NPP have been developed. CCI is a widely used model, especially for behavioral studies. CCI simulates post-traumatic peripheral painful neuropathic states in humans and is reliable and simple to create [33]. However, the degree of damage stability in CCI is lower compared with some other models. SNL possesses less experimental variability and is regarded as an excellent model for mechanical allodynia assessment [33]. But the surgical procedure of the SNL model is more challenging, and animals suffer more trauma. Here, CCI and SNL models were both employed in the present study to ensure the reliability of the research.

DNA methylation is critical in pain memory and synaptic plasticity [34]. Previous studies reported abnormal changes in global DNA methylation and the methylation of specific promoters in rodent lumbar spinal cords 1–21 days following CCI/SNL surgery [22, 25, 35]. However, the methylation profiles of the DNA promoter region in the lumbar spinal cord in nerve injury-induced NPP rats have not been well explored. Here, we used the Arraystar Rat DNA Promoter Array, a reliable and efficient platform [36], to identify the methylation profiles at the genome-wide level in the DNA promoter regions of the lumbar spinal cord in rats 14 days following CCI surgery. In this study, more than 1200 fragments were differentially methylated after CCI treatment, including 638 hypermethylated fragments and 567 hypomethylated fragments, which indicated that CCI could be capable of inducing widespread lumbar spinal cord methylation changes.

After identifying differentially methylated genes associated with CCI-induced NPP, GO analysis and KEGG pathway analysis were used to reveal genome-wide methylation patterns. The top GO terms among hypomethylated genes were cellular component organization or biogenesis, establishment of localization, and transport, while the top KEGG pathways were cell cycle, peroxisome, oocyte meiosis, and gastric acid secretion, which seem to be little related to pain. Although studies reported that CCI and SNL induced demethylation and increased expression of receptor tyrosine kinase-like orphan receptor 2 (ROR2) [37] and chemokine receptor CXCR3 [38], the two genes were not observed in our chip results. Besides, our previous study showed that the overall DNA methylation level in the spinal cord increased significantly in CCI rats [25]. Here, we focused on genes whose methylation increased and whose encoded mRNAs were expressed at a lower level in CCI rats.

In the present study, the top GO terms among hypermethylated genes were organism process, developmental process, anatomical structure development, and ion transport, while the top KEGG pathways were long-term potentiation (LTP), glutamatergic synapse, calcium signaling, and cAMP signaling pathways. Among the genes altered by NPP in the promoter region, Grm4, Htr4, Adrb2, Kcnf1, Gad2, and Pparg belong to development processes and ion transport and are involved in LTP and glutamatergic synapse pathways [39–43]. Previous studies have shown that DNA methylation changes in certain genes (such as Htr4, Gad2, Adrb2, and Pparg) could interfere with the expression of their encoded mRNA in some pathological states [30, 44–47]. Here, our results showed that Grm4, Htr4, and Kcnf1 were significantly hypermethylated and that Adrb2, Gad2, and Pparg were insignificantly hypermethylated in CCI rats. Correspondingly, the mRNA levels of these genes decreased to various degrees, suggesting the negative regulation of DNA methylation in its encoded targets under NPP conditions.

Aberrant DNA methylation in the nervous system is involved in pain conditions. Numerous studies have demonstrated the possibility of attenuating chronic pain by modulating DNA methylation [22, 25, 48, 49]. For example, restoring DNA methylation through the methyl donor s-adenosyl methionine improved cognitive and emotional functions damaged by spared nerve injury [50]. Increased DNMT3a in the DRG led to undesirable opioid analgesic effects, and blocking this increase restored the analgesic effects of morphine or loperamide and attenuated the development of their analgesic tolerance under NPP [51]. Moreover, intrathecal administration of 5-AZA or 5-AZA-dC reversed methylation levels corresponding to the amelioration of pain [25, 26]. The US Food and Drug Administration (FDA) has approved 5-AZA as a chemotherapy agent in the treatment of malignant tumors and myelodysplastic syndromes [52]. However, the operation of intrathecal administration of 5-AZA is complex, so its clinical application is limited. A previous study showed that intraperitoneal administration of 5-AZA-dC provided relief from incisional

nociceptive sensitization [49]. Thus, we examined the effect of 5-AZA on CCI/SNL-induced NPP via intraperitoneal injection for the first time.

An increase in DNA methylation in response to nerve injury is well established, and inhibiting this increase resulting in pain relief has been reported. Sun et al. found that DNMT1 expression was upregulated after peripheral nerve injury and that blocking DNMT1 could reverse the abnormal excitability and sensitivity in injured DRG neurons [53]. Liu et al. revealed that blocking the upregulation of DNMTs with RG108 alleviated spared nerve injury-induced pain [54]. Furthermore, nerve injury or incision-related hyperalgesia can be attenuated by 5-AZA-dC, which also induces α5-$GABA_A$ receptor or Oprm1 reversion [49, 55]. DNA demethylation in the DRG was proven to alleviate SNL-induced pain hypersensitivities coinciding with rescued expression of μ-opioid receptors and the potassium voltage-gated channel Kv1.2 [56]. Moreover, 5-AZA was reported to reverse the hypermethylation and upregulate the expression of Adrb2 in PM2.5-induced cardiomyocyte apoptosis [47]. Consistent with these studies, our results showed that 5-AZA treatment obviously attenuated CCI/SNL-induced mechanical allodynia and thermal hyperalgesia, coinciding with reversed mRNA expression of the hypermethylated genes; Grm4, Htr4, Adrb2, and Kcnf1 expression was observed in both CCI and SNL rats, and Gad2 expression was observed in SNL rats. We infer that the modulation of DNA methylation in these genes in the spinal cord through 5-AZA treatment might be a promising epigenetic mechanism and therapeutic target for the relief of chronic NPP.

Specifically, Grm4 encodes metabotropic glutamate receptor 4 (mGluR4), which contributes to pain modulation by suppressing glutamatergic hyperactivation [57, 58]; its exogenous activation reversed the sensitivity imbalances of chronic NPP in the dorsal spinal region [59, 60]. Adrb2 encodes the β2-adrenergic receptor (β2-AR) [61], which is widely expressed in the normal and injured central nervous system [41]. Adrb2 activation could alleviate multiple chronic NPPs [62, 63]. Consistent with these studies, our results showed that intraperitoneal 5-AZA reversed Grm4 and Adrb2 mRNA expression, coinciding with attenuated hyperalgesia in both CCI and SNL rats. These results further demonstrate the pivotal role of Grm4 and Adrb2 in NPP. Htr4 encodes 5-hydroxytryptamine receptor 4 (5-HT4R), which has been found to produce antinociceptive or pronociceptive effects in the spinal cord [64–66]. We observed that Htr4 mRNA levels recovered after 5-AZA treatment in parallel with pain relief, which indicates that Htr4 may produce antinociceptive effects as an inhibitory receptor subtype of 5-HT in the lumbar spinal cord after nerve injury. Moreover, KCNF1 is a potassium channel gene and encodes Kv5.1, which can form a functional complex with Kv2.1, resulting in physiological effects [67, 68]. The contribution of the Kv5.1 channel to the regulation of NPP has not been investigated, and our results first showed that the decrease in Kv5.1 mRNA was reversed after 5-AZA treatment along with pain relief in CCI/SNL rats, suggesting that Kv5.1 may be involved in the occurrence and development of NPP. Finally, although Gad2 and Pparg have been reported to be downregulated in CCI rats [69, 70], we observed the reduction of Gad2 only after SNL, and that of Pparg only after CCI. More experiments are needed to further investigate the correlations of Gad2 and Pparg with NPP. It is crucial to note that the correlation of Grm4 and Kcnf1 between DNA methylation and NPP was reported here for the first time and might become a new promising direction for pain treatment.

There are several limitations in this study. First, due to the small sample size of 3 to 6 animals, these results should be considered exploratory. Second, DNA methylation assessment was performed at only one time point and therefore cannot depict the temporal dynamics of DNA methylation in response to pain during the acute-to-chronic process. Third, the impact of 5-AZA on the target gene methylation level was not examined. As stated above, chronic systemic administration of 5-AZA relieves NPP along with the restoration of hypermethylated gene expression. Future studies incorporating the modulation of target gene methylation and protein levels are needed to characterize the explicit change during NPP. Finally, the phenomena described here are correlative; further investigation is needed to explore the causal and detailed relationships between spinal cord methylation and chronic NPP. Despite these limitations, these results provide an important insight into the DNA methylation of novel spinal cord target genes associated with the successful treatment of chronic NPP.

Conclusions

In summary, our findings reveal the DNA methylation profiles at the genome-wide level in the DNA promoter regions of the spinal cord in CCI rats and offer important evidence for the reversal of NPP and this decrease in target gene expression following nerve injury by employing an epigenetic regulator to enhance DNA methylation in CCI and SNL rats. The hypermethylation occurred mainly in genes which are associated with development, structure, and ion transport and are involved in LTP and glutamatergic synapse pathways. The methylation and mRNA levels of these target genes (Grm4, Htr4, Adrb2, Kcnf1, Gad2, and Pparg) may contribute to NPP persistence or recovery in the spinal cord after therapeutic interventions with intraperitoneal 5-AZA. These findings provide crucial insight into the relationship and call for increased exploration between chronic NPP and epigenetic interventions, such as intraperitoneal 5-AZA, for the treatment of chronic NPP.

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Author Contribution Ying Wang and Xuan Li designed the study, collated the data, carried out data analyses, and produced the initial draft of the manuscript. DeZhao Liu and ZhiSen Dai provided critical advice and contributed to drafting the manuscript. YiSheng You, Yan Chen, ChenXing Lei, and YouYou Lv performed the experiments. All authors have read and approved the final submitted manuscript.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics Approval All protocols were approved by the ethics committee of the Fujian Cancer Hospital and adhered to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Consent to Participate Not applicable.

Consent for Publication All authors have read and approved the submission of the manuscript.

Conflict of Interest The authors declare no competing interests.

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