Research Paper

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Epigenetic reduction of miR-214-3p upregulates astrocytic colony-stimulating factor-1 and contributes to neuropathic pain induced by nerve injury

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Abstract

Emerging evidence has indicated that colony-stimulating factor-1 (CSF1) modulates neuroinflammation in the central nervous system and the development of neuropathic pain, while the underlying mechanism remains unknown. Here, we identified the increased expression of CSF1 derived from activated astrocytes in the ipsilateral dorsal horn in rats with spinal nerve ligation (SNL). Suppression of CSF1 expression alleviated neuroinflammation, neuronal hyperexcitability, and glutamatergic receptor subunit upregulation in the dorsal horn and improved SNL-induced pain behavior. We also found reduced miR-214-3p expression in the ipsilateral dorsal horn following an SNL procedure; miR-214-3p directly bound to the 3′-UTR of CSF1 mRNA and negatively regulated CSF1 expression. Intrathecal delivery of miR-214-3p mimic reversed the enhanced expression of CSF1 and astrocyte overactivity and alleviated the IL-6 upregulation and pain behavior induced by SNL. Moreover, suppression of spinal miR-214-3p increased astrocyte reactivity, promoted CSF1 and IL-6 production, and induced pain hypersensitivity in naive animals. Furthermore, SNL induced the expression of DNA methyltransferase 3a (DNMT3a) that was associated with the hypermethylation of the miR-214-3p promoter, leading to reduced miR-214-3p expression in the model rodents. Treatment with the DNMT inhibitor zebularine significantly reduced cytosine methylation in the miR-214-3p promoter; this reduced methylation consequently increased the expression of miR-214-3p and decreased the content of CSF1 in the ipsilateral dorsal horn and, further, attenuated IL-6 production and pain behavior in rats with SNL. Together, our data indicate that the DNMT3a-mediated epigenetic suppression of miR-214-3p enhanced CSF1 production in astrocytes, which subsequently induced neuroinflammation and pain behavior in SNL model rats.

Keywords: miRNA, Cytosine methylation, Colony-stimulating factor-1, Astrocyte

1. Introduction

Over the past decades, extensive evidence has described the active involvement of microglia and astrocytes in the development and maintenance of chronic pain. Peripheral nerve injury activated spinal microglia and astrocytes to produce proinflammatory cytokines, which enhance neuronal excitability and contribute to central sensitization.[4,23] Recently, colony-stimulating factor-1 (CSF1) and its endogenous receptor CSF1R have emerged as key inflammatory molecules mediating crosstalk between glia and neurons. CSF1, a hematopoietic growth factor, regulates innate immunity by binding to CSF1R on macrophage-lineage cells.[20,44] CSF1R is primarily distributed on the surface of microglia in the central nervous system (CNS).[7] CSF1 activates microglia to exhibit remarkable immune potencies, including proliferation,[12,46] migration,[9,46] phagocytosis,[17] and immunity polarization.[10] Accumulating studies have confirmed the critical role of the CSF1-CSF1R signaling pathway in chronic pain.[8,16,55] Our previous study illustrated the contribution of the interaction between astrocytic CSF1 and microglial CSF1R to neuronal hyperexcitability and behavioral hypersensitivity in chronic ischemic pain[46]; yet, the evidence for whether and how astrocytic CSF1 regulates neuroinflammation in neuropathic pain is still lacking.

Epigenetic modifications are inherited, and the mechanisms cause reversible changes in gene expression or activity in response to environmental stimuli.[11,28] Examples of epigenetic modifications include DNA methylation, histone modifications, and noncoding RNA expression.[11,28] MicroRNAs (miRNAs) are noncoding, single-stranded RNAs that bind to their target mRNA and exert their functions of either mRNA degradation or translational repression.[2,5] Recent genome or microarray research has identified numerous miRNAs that are overexpressed or

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downregulated in pain-related CNS regions, including the dorsal horn. Some of these miRNAs have been suggested to be pathogenic factors in chronic pain.[1,32,41] Here, we explored the potential miRNA targeting CSF1 expression and the functional adaptation of target miRNAs in neuropathic pain.

As gene expression regulators, miRNA expression is tightly controlled by epigenetic modifications.[34] DNA methylation, mostly occurring within the gene promoter and exons, is a leading candidate for the epigenetic regulation of miRNA expression.[34] The DNA methylation process is catalyzed by DNA methyltransferases (DNMTs), such as DNMT1, DNMT3a, and DNMT3b. DNA methyltransferases add methyl groups to the fifth carbon of cytosine residues, thus blocking the binding of transcription enzymes and silencing gene expression.[43] Recent studies have discovered that DNMT3a-triggered DNA methylation caused neuronal hyperexcitability and contributed to neuropathic pain.[33,45] DNMT3a also modulated the expression of some astrocytic genes.[37] Conditional knockout of DNMT3a in nestin-positive cells reduced postnatal remethylation of the glial fibrillary acidic protein (GFAP) promoter in the cortex and resulted in neuromuscular defects.[38] Moreover, chemical exposure to ethanol decreased the levels of DNMT3a, thereby inducing demethylation of the tissue plasminogen activator promoter and increasing the expression of these proteins in astrocytes.[58] Given that DNMT3a was tightly linked with the development and function of astrocytes, we sought to examine whether DNMT3a altered the cytosine methylation of miRNAs targeting astrocytic CSF1 in neuropathic pain.

2. Methods

2.1. Animal model

All animal procedures were conducted after receiving ethical approval from the Institutional Animal Care and Use Committee of Sichuan University. Healthy adult male Sprague-Dawley rats weighing 250 to 300 g were housed in a certified specific pathogen-free rodent unit. After anesthetization with pentobarbital (50 mg/kg), a spinal nerve ligation (SNL) model was established by tightly ligating the L5 and L6 spinal nerves with 4-0 silk suture distal to the dorsal root ganglia (DRG).[27] Sham rats underwent the same operation procedure to expose nerves but without ligation. Naive animals received no operation. A PE-10 tube (BD) for drug injection was implanted into the lumbar enlargement (L4-6) as we previously described.[46]

2.2. Pain behavioral testing

Animals were allowed to habituate to the environment for at least 30 minutes before the behavioral experiments were performed. To evaluate mechanical allodynia, a series of von Frey filaments calibrated from 1 to 26 g of bending force were sequentially applied to the plantar surface.[46] Six consecutive paw withdrawal responses (including the first positive response and its previous negative response) were calculated with the "up-down" method and deemed the mechanical withdrawal threshold.[6] Thermal hyperalgesia was assessed by applying radiant heat stimulation to the hind paw.[18,46] Each paw withdrawal time was recorded, and the average of 3 measurements was calculated as paw withdrawal latency. Pain behavioral measurements were performed in a blinded fashion.

2.3. Immunohistochemistry

Immunofluorescence double staining was performed as previously described.[46] Briefly, after transcardial perfusion with heparinized saline and 4% formaldehyde, the enlarged lumbar segment was harvested, dehydrated with sucrose, and then transversely cryosectioned into 25-μm-thick sections for further immunostaining. The primary antibodies used were mouse anti-CSF1 (1:200, Santa Cruz, sc-365779, Dallas, TX), rabbit anti-GFAP (1:500, Abcam, ab7260, Cambridge, United Kingdom), mouse anti–IL-6 (1:500, Abcam, ab9324), rabbit anti-CD11b (1:500, Abcam, ab187537), mouse anti-NeuN (1:500, Abcam, ab104224), and rabbit anti–c-Fos (1:250, Novus, NB110-75039, Littleton, CO). The secondary antibodies were conjugated to Cy3 (1:500, Abcam, ab97035) or FITC (1:500, Abcam, ab97050). All images were acquired with a Zeiss Axio Imager Z2 microscope. The quantification of immunofluorescence staining and colocalization was analyzed with Image Pro Plus and CoLocalizer Pro software.[46]

Table 1

Specific primer sequences.

Gene	Primer	Sequence
CSF1	Forward	5′-TGACTAGGGGAAGGGAGAG-3′
	Reverse	5′-CTGGCAAGGGACTGGAG-3′
GAPDH	Forward	5′-ATGGCTACAGCAACAGGGT-3′
	Reverse	5′-TTATGGGGTCTGGGATGG-3′
rno-miR-214-3p	Loop primer	5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTGCCTGT-3′
	F primer	5′-TGCGCACAGCAGGCACAGAC-3′
rno-miR-184-3p	Loop primer	5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATAAGGGT-3′
	F primer	5′-TGCGCTGGACGGAGAACTGATA-3′
rno-miR-9a-3p	Loop primer	5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTTTCGG-3′
	F primer	5′-TGCGCATAAAGCTAGATAACCG-3′
rno-miR-448-3p	Loop primer	5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC TGGGACAT-3′
	F primer	5′-TGCGCTTGCATATGTAGGATG-3′
rno-miR-145-5p	Loop primer	5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGGGATTC-3′
	F primer	5′-TGCGCGTCCAGTTTTCCCAGGAA-3′
U6	Forward	5′-CGCTTCGGCAGCACATATAC-3′
	Reverse	5′-AAATATGGAACGCTTCACGA-3′

CSF1, colony-stimulating factor-1.

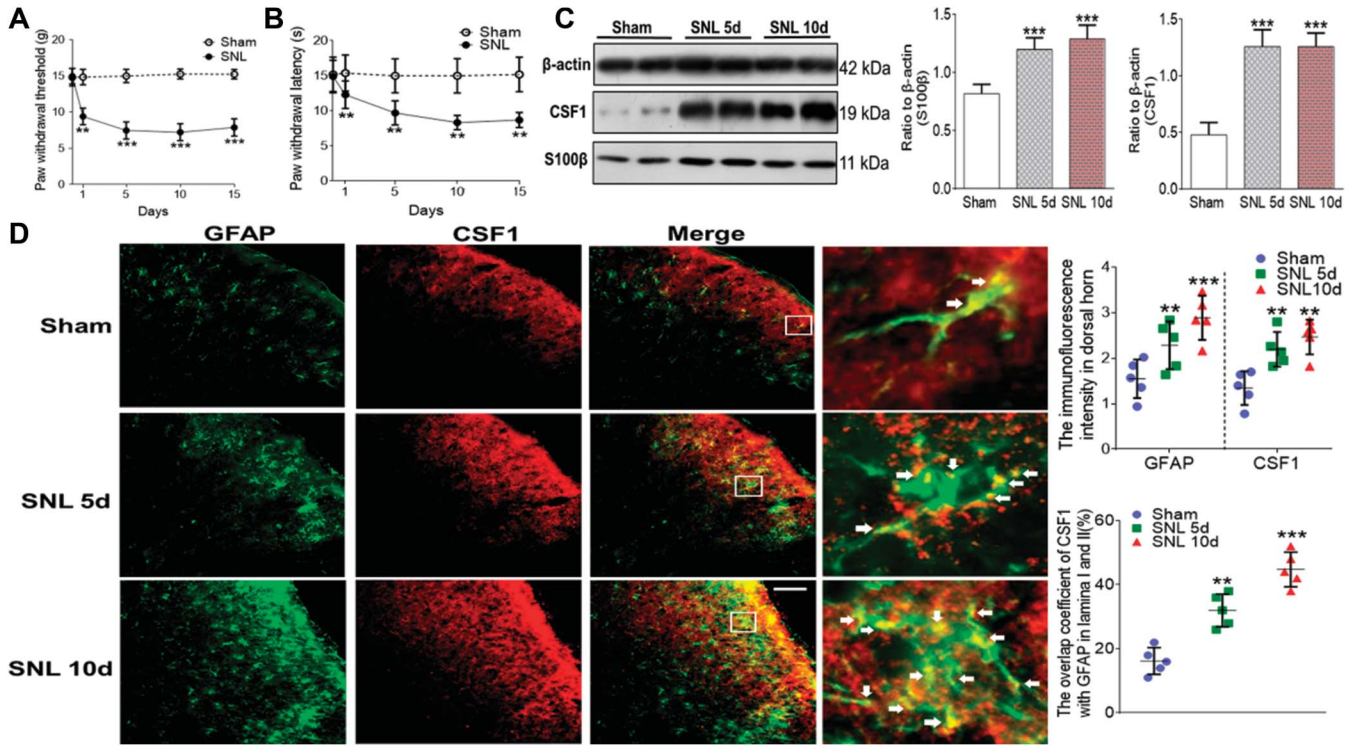

Figure 1. SNL increased CSF1 production from reactive astrocytes in the ipsilateral dorsal horn. (A) Significant decreases in the ipsilateral paw withdrawal threshold were observed 15 days after the SNL surgery (n = 10 rats in each group, effect of group [F 1, 9 = 272.6, $P < 0.0001$], effect of time [F 4, 36 = 48.39, $P < 0.0001$], interaction between group and time [$P < 0.0001$]). (B) Significant decreases in the ipsilateral paw withdrawal latency were induced in SNL model rats (n = 10 rats in each group, effect of group [F 1, 9 = 20.57, $P = 0.0014$], effect of time [F 4, 36 = 48.33, $P < 0.0001$], interaction between group and time [$P < 0.0001$]). (C) Significantly increased expression of s100β and CSF1 was detected in the ipsilateral dorsal horn on days 5 and 10 after the SNL surgery (n = 5 rats in each group, F 2, 12 = 20.17, $P = 0.0001$ for s100β; and F 2, 12 = 56.24, $P < 0.0001$ for CSF1). (D) The immunosignals of GFAP and CSF1 in the ipsilateral dorsal horn were significantly increased on days 5 and 10 (n = 5 rats in each group, F 2, 12 = 9.868, $P = 0.0029$ for GFAP; and F 2, 12 = 11.94, $P = 0.0014$ for CSF1). The overlap of GFAP and CSF1 (arrowheads) was also significantly enhanced in SNL model rats (n = 5 rats in each group, F 2, 12 = 42.52, $P < 0.001$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, compared with the sham group. Scale bars represent 25 μm. CSF1, colony-stimulating factor-1; SNL, spinal nerve ligation.

2.4. Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) analysis was performed to detect the expression of miR-214-3p in the dorsal horn. Fluorescence in situ hybridization Biotin Kit and the rno-miR-214-3p probe were provided by Focofish Biotechnology Company (Guangzhou, China). In brief, 25 μm of frozen sections were dipped with triethanolamine (TEA) buffer containing acetic anhydride for 20 minutes, followed by incubation in pepsin solution for 15 minutes. After rinsing with phosphate buffered saline, the sections were successively immersed in 3% H_2O_2 diluted with methanol for 15 minutes, washed 3 times with 0.1% diethylpyrocarbonate water, and incubated in 4% paraformaldehyde for 15 minutes. After prehybridization with miRNA hybridization buffer for 1 hour at 55°C, these sections were sealed and hybridized with a prepared rno-miR-214-3p probe (1:200) at 37°C for 48 hours. The hybridized sections were then rinsed with 0.1% diethylpyrocarbonate water and incubated with rabbit anti-GFAP (1:500, Abcam, ab7260) or rabbit anti-CSF1 (1:500, Abcam, ab234259) at 4°C overnight. After that, these sections were incubated with TSA-488 (1:500) and goat anti-rabbit IgG H&L (FITC) (1:500, Abcam, ab97050) at 37°C for 30 minutes and were finally counterstained with 4',6-diamidino-2-phenylindole (DAPI). Staining images were obtained with a Nikon confocal microscope (A1RMP^{+}). The immunofluorescence intensity of CSF1 and miR-214-3p was calculated as previously described.[46] The overlap of miR-214-3p with GFAP signals was determined with the percentage of miR-214-3p–positive astrocytes labeled with GFAP signals.

2.5. Western blotting

For Western blotting analysis, the fresh lumbar spinal tissue was flushed out, and the ipsilateral dorsal horn was isolated.[46] Dorsal root ganglion tissue was exposed and dissected from L5 to L6 of the vertebral column.[60] Briefly, after lysis from tissue samples with an ice-cold protease inhibitor cocktail as we previously reported,[46] 10 μg of sample protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis separation and then transferred onto nitrocellulose membranes. The membranes were then blocked with 5% milk buffer and incubated overnight at 4°C with the appropriate primary antibodies, including an anti-CSF1 antibody (1:500, Abcam, ab234259), anti-s100β antibody (1:1000, Abcam, ab868), anti–IL-6 antibody (1:500, Cell Signaling, #12153S, Boston, MA), anti-DNMT3a antibody (1:500, Cell Signaling, #32578), anti-GluR1 antibody (1:1000, Novus, NB110-39033), anti-NR2B antibody (1:1000, Novus, NB100-74476), or anti–β-actin antibody (1:1000; EMD Millipore, Billerica, MA). After washing with tris-buffered saline tween-20 solution extensively, the membranes were probed with horseradish peroxidase–conjugated goat anti-rabbit IgG (1:5000; Jackson Immunoresearch Laboratories, Inc, West Grove, PA) for 60 minutes. The immunosignals of targeted proteins were visualized with enhanced chemiluminescence assay, and the protein band intensities were quantified with ImageJ software. β-actin was used as a loading control to analyze the expression of the targeted protein.

2.6. Microarray analysis

Total RNA was extracted with TRIzol/chloroform reagent (Invitrogen, Carlsbad, CA) and then purified with NucleoSpin miRNA (No 740971.250; Macherey-Nagel, Germany). The purified miRNA was submitted to Shanghai Baygene Biotechnology Company Limited (Shanghai, China) for microarray analysis as previously described.[25]

2.7. Real-time quantitative polymerase chain reaction (qPCR) for mRNA and miRNA

For real-time qPCR analysis, total RNA was obtained with TRIzol Reagent (Aidlab, 252250AX) and reverse-transcribed into cDNA with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). A Roche CFX96 real-time PCR detection system was used to determine the expression of mRNA and miRNAs. For quantification of CSF1 mRNA, the PCR conditions were as follows: 95°C for 10 seconds, 58°C for 20 seconds, and 72°C for 10 seconds for 40 cycles. For quantification of miRNAs, the PCR conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 30 seconds, and 60°C for 30 seconds for 40 cycles. The relative expression ratio of CSF1 mRNA was normalized to GAPDH gene expression, and miRNA was normalized to U6 gene expression using the ΔCt method ($2^{-\Delta\Delta Ct}$). The primers for CSF1 mRNA and miRNAs are presented in **Table 1**.

2.8. Bisulfite sequencing PCR

For bisulfite sequencing PCR, total DNA was isolated from sample tissue using a DNA extraction kit from Aidlab Biotechnologies Co, Ltd (DN0702). The extracted DNA sample was then bisulfite treated with the ZYMO EZ DNA Methylation-Gold Kit. Methylation-specific PCR primers targeting putative fragment sequences in the miRNA-214-3p promoter were designed based on MethPrimer software (http://www.urogene.org/methprimer/). A touchdown PCR protocol was used to evaluate the DNA methylation status of the miRNA-214-3p gene, which involved an initial 95°C for 10 minutes, then 15 cycles of 95°C for 30 seconds, 65°C-50°C (decreased 1°C per cycle) for 30 seconds, and 72°C for 30 seconds, followed by 30 cycles of 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds, and finally 72°C for 7 minutes, and the reaction was stopped at 4°C. The methylation status was determined by the positive methylation sites within the amplified miRNA-214-3p promoter region.

2.9. Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) process was conducted based on previous research.[52] In short, after microdissection on ice, the sample tissue was transferred into fixation solution containing 1% formaldehyde in phosphate buffered saline and a protease inhibitor cocktail. The incubation reaction was stopped with glycine solution,

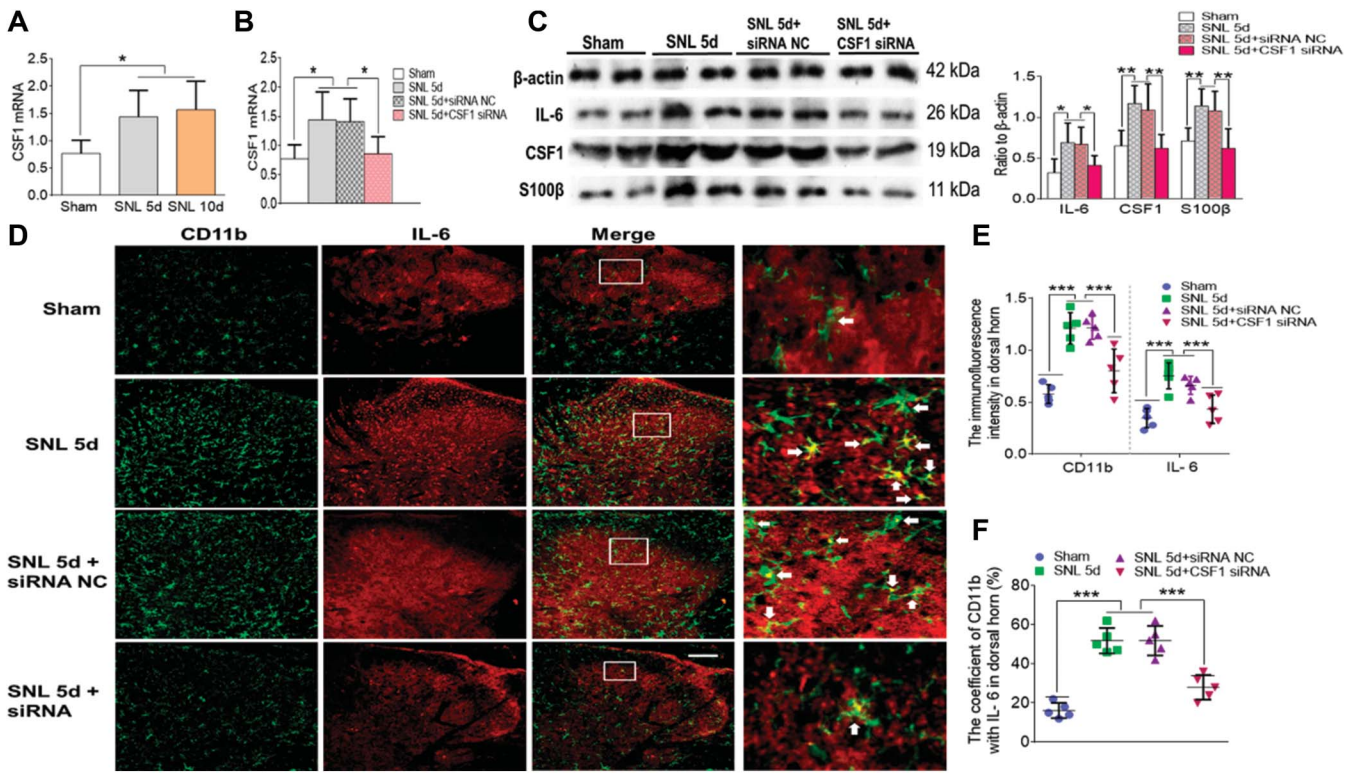

Figure 2. Increased expression of CSF1 contributed to SNL-induced neuroinflammation. (A) CSF1 mRNA expression in the ipsilateral dorsal horn was significantly upregulated on days 5 and 10 after SNL surgery (n = 5 rats in each group, $F_{2, 12} = 4.918$, $P = 0.027$). (B) CSF1 siRNA reversed the enhanced expression of CSF1 mRNA induced by SNL surgery (n = 5 rats in each group, $F_{3, 16} = 4.737$, $P = 0.015$). No significant changes in the expression of CSF1 mRNA were observed between SNL and SNL + siRNA NC. (C) SNL-induced upregulated protein levels of CSF1 and S100β in the ipsilateral dorsal horn were significantly reduced with CSF1 siRNA I.T. administration (n = 5 rats in each group, $F_{3, 16} = 7.765$, $P = 0.002$ for CSF1; $F_{3, 16} = 4.513$, $P = 0.0178$ for S100β), but not with siRNA NC treatment. The SNL-induced increase in the expression of IL6 was also attenuated with CSF1 siRNA (n = 5 rats in each group, $F_{3, 16} = 3.413$, $P = 0.0431$). (D) A micrograph was presented to show the colocalization of CD11b-labeled microglia (green) and IL-6 (red) (arrowheads) in the ipsilateral dorsal horn in SNL model animals that were I.T. injected with CSF1 siRNA or siRNA NC. (E–F) The enhanced immunoactivity and overlap of CD11b and IL-6 induced by SNL injury were significantly reduced by CSF1 siRNA but not siRNA NC (n = 5 rats in each group, $F_{3, 16} = 22.9$, $P < 0.0001$ for CD11b; $F_{3, 16} = 14.43$, $P < 0.0001$ for IL-6; and $F_{3, 16} = 41.31$, $P < 0.0001$ for coefficient of CD11b with IL-6). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Scale bars represent 25 μm. CSF-1, colony-stimulating factor-1; NC, negative control; SNL, spinal nerve ligation.

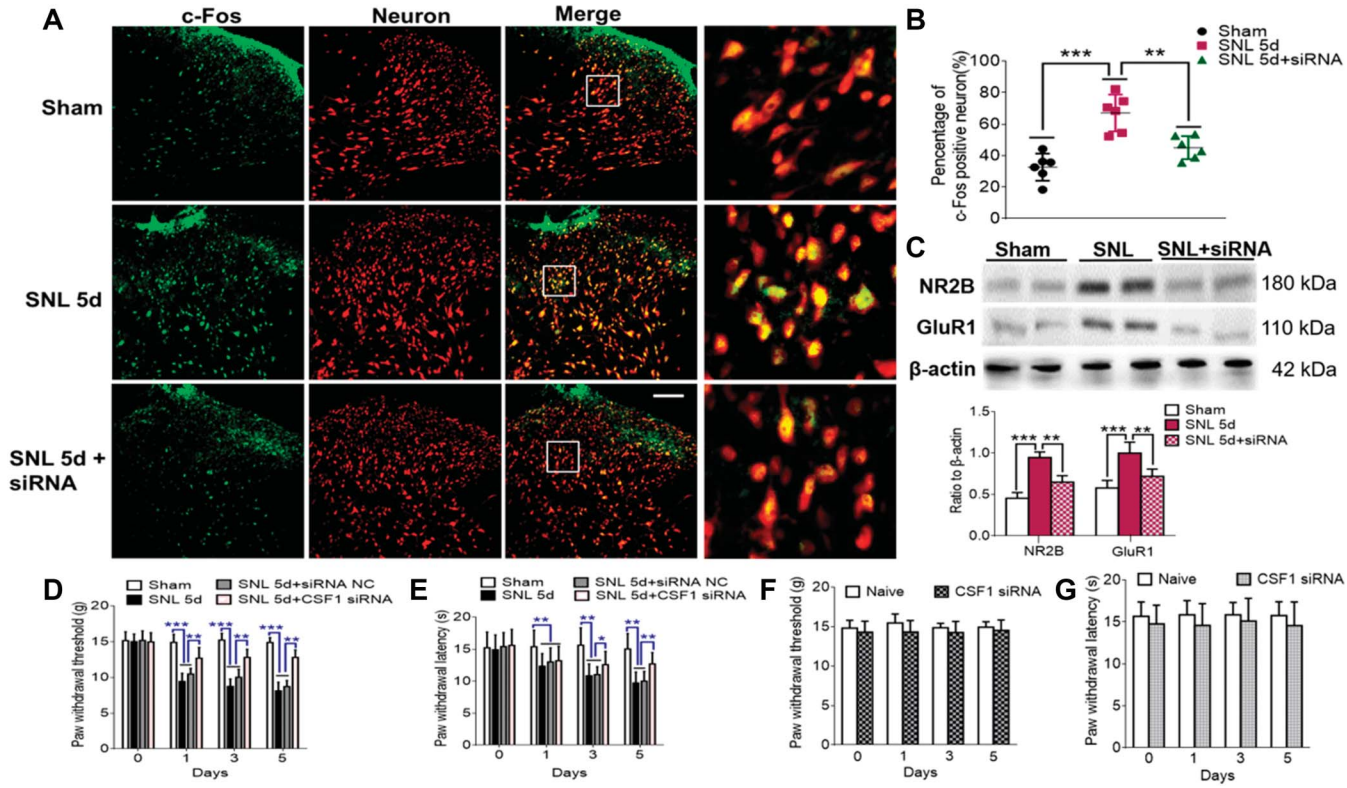

Figure 3. CSF1 enhanced neuronal excitability and glutamatergic receptor expression after SNL injury. (A) Representative immunosignals of c-Fos in the ipsilateral dorsal horn neurons after SNL injury. (B) A significantly increased percentage of c-Fos–labeled neurons was observed in the ipsilateral dorsal horn after SNL injury, which was significantly reversed by the I.T. administration of CSF1 siRNA (n = 6 rats in each group, F 2, 15 = 20.63, $P < 0.0001$). (C) CSF1 siRNA significantly reversed SNL-induced upregulated expression of NR2B (n = 4 rats in each group, F 2, 9 = 16.63, $P = 0.0009$) and GluR1 (n = 4 rats in each group, F 2, 9 = 48.38, $P < 0.0001$) in the ipsilateral dorsal horn; (D) CSF1 siRNA, but not siRNA NC, significantly ameliorated SNL-induced mechanical allodynia (n = 10 rats in each group, effect of group [F 3, 27 = 56.08, $P < 0.0001$], effect of time [F 3, 27 = 161.7, $P < 0.0001$], interaction between group and time [$P < 0.0001$]). (E) CSF1 siRNA, but not siRNA NC, significantly ameliorated SNL-induced thermal hyperalgesia (n = 10 rats in each group, effect of group [F 3, 27 = 5.068, $P = 0.0065$], effect of time [F 3, 27 = 91.26, $P < 0.0001$], interaction between group and time [$P < 0.0001$]). (F–G) CSF1 siRNA had no significant effects on pain-like behaviors in naive animals. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Scale bars represent 25 μm. CSF1, colony-stimulating factor-1; NC, negative control; SNL, spinal nerve ligation.

and the sample tissue was then centrifuged at 4°C. The precipitation was homogenized in SDS lysis buffer and then sonicated on ice to produce fragments. After immunoprecipitation overnight at 4°C with 2 μg of a rabbit anti-DNMT3a antibody (Abcam, ab2850), immunocomplexes were recovered, and the crosslinking between protein and DNA was reversed. The extracted DNA fragments were purified and amplified using primers specific to the rat miR-214-3p promoter (forward primer: 5′-ACATGAGGGCCAGTAACGAT-3′, reverse primer: 5′-TGGTGATTGAAGGAGAGGGG-3′).

2.10. miRNA transfection

Synthesized miRNA-214-3p mimic/inhibitor and mimic negative control (NC)/inhibitor NC were purchased from Gene Pharma, Shanghai, China. The miRNA modulators were transfected into human glioma U251 cells using Lipofectamine 2000 (Invitrogen Life Technologies) at a working concentration of 50 to 100 nM according the manufacturer's instructions. At 24 to 48 hours after transfection, the cells were harvested for RNA and protein extraction.

2.11. Luciferase reporter assay

For dual-luciferase reporter assays, the CSF1 promoter region was amplified by PCR from the genomic DNA, and the resulting fragments were cloned into the PUC57 vector. The mutant CSF1 promoter containing 2 base point mutations in the E-box site was generated and verified by DNA sequencing. The recombinant psiCHECK-2 plasmid containing the wild-type or mutant CSF1 gene promoter was cotransfected into human embryonic kidney 293 (HEK293) cells with the miR-214-3p expression plasmid and pRL-TK containing the Renilla luciferase reporter gene. At 48 hours after the transfection, the luciferase activity was detected with the Dual-luciferase Assay System (Promega, Madison, WI) and normalized to Renilla luciferase activity.

2.11.1. Statistical analysis

Data are presented as the mean ± SD and were analyzed using SPSS version 13.0 software. The difference between the 2 groups was compared with an unpaired Student *t*-test. Differences between more than 3 groups were analyzed with 1-way analysis of variance followed by the Bonferroni test. Pain behavioral comparisons between treatment groups were performed using two-way analysis of variance with repeated-measures followed by Tukey's *post hoc* test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Increased colony-stimulating factor-1 expression derived from reactive astrocytes in the dorsal horn contributed to spinal nerve ligation–induced neuroinflammation and pain behaviors

As previously reported,[27] pain-like behaviors, such as decreases in paw withdrawal thresholds and paw withdrawal latencies, were

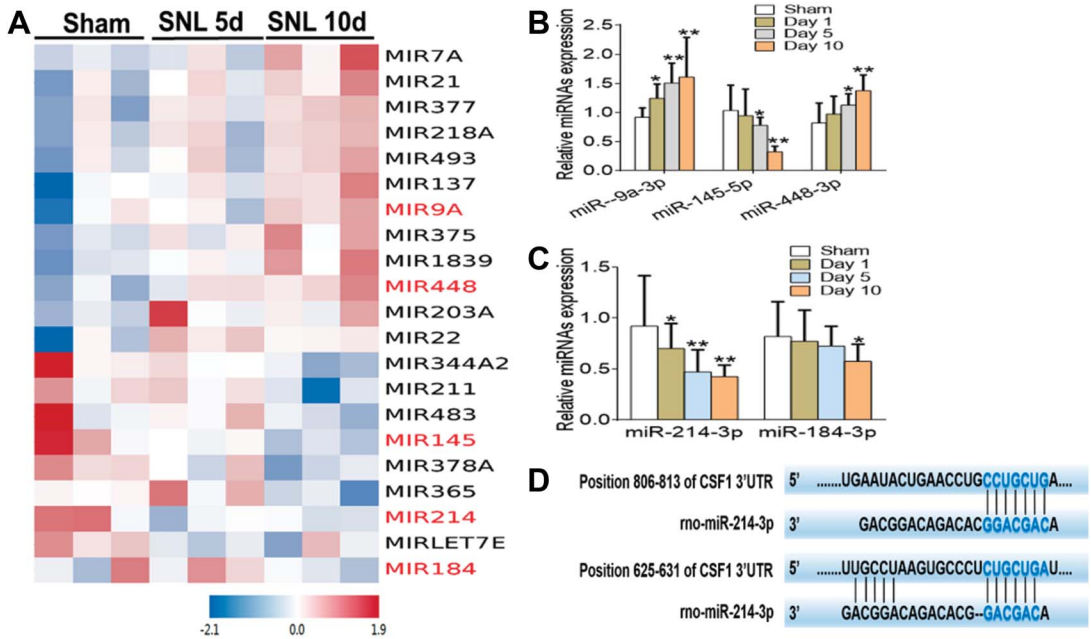

Figure 4. miR-214-3p was a potential target regulating the expression of CSF1. (A) Heat maps of miRNAs found to be significantly changed in the ipsilateral dorsal horn on days 5 and 10 after SNL injury. (B–C) Quantitative PCR measured the time-course changes in the expression of 3 randomly selected miRNAs, as well as 2 potential target miRNAs (miR-214-3p and miR-184-3p). (D) Schematic representation of 2 potential miR-214-3p target sequences within the 3′ UTR of CSF1. $^{*}P < 0.05$; $^{**}P < 0.01$, compared with the sham group. CSF1, colony-stimulating factor-1; SNL, spinal nerve ligation; UTR, untranslated region.

observed within 15 days following the SNL procedure. These pain-like syndromes occurred on day 1, were exacerbated on days 5 and 10, and persisted until the last experimental time point (day 15) (**Figs. 1A and B**). The expression of the S100β protein, a specific astrocyte marker, was robustly increased in the ipsilateral spinal horn on days 5 and 10 after SNL operation (**Fig. 1C**), suggesting that SNL induced astrocyte activation in the dorsal horn. The immunosignal effect of GFAP, another astrocyte

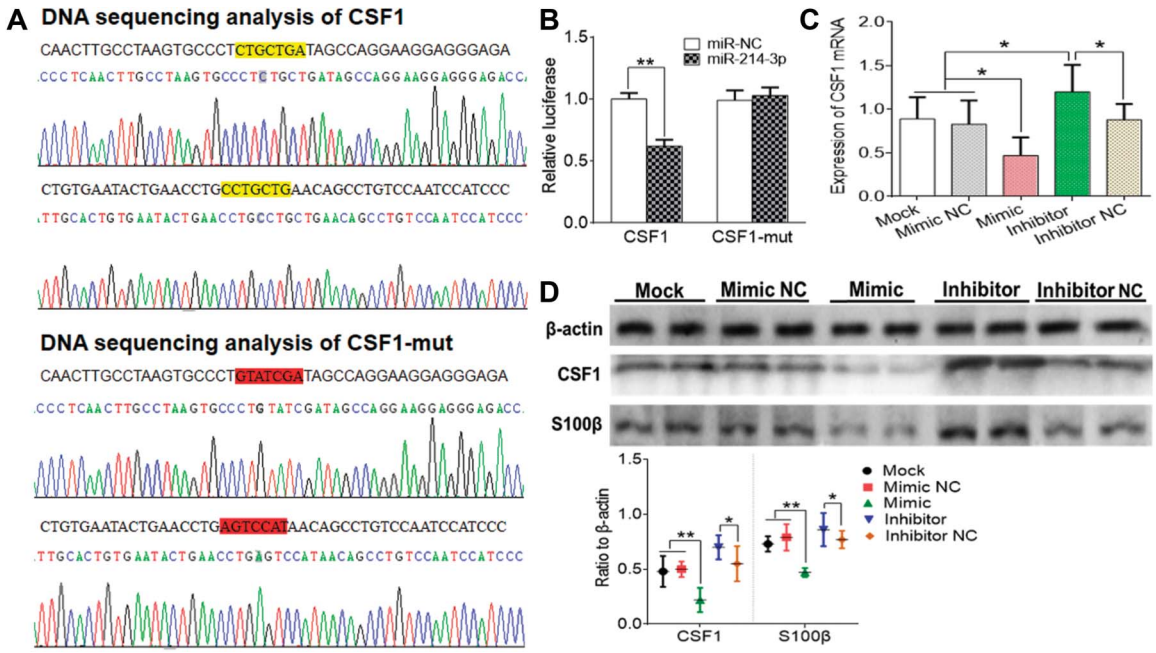

Figure 5. miR-214-3p negatively regulated the expression of CSF1 in vitro. (A) Schematic representation of the mutated binding sites in the 3′-UTR of CSF1 applied in the dual-luciferase reporter assay. (B) Luciferase activity of the reporter constructs that were cotransfected with wild-type miR-214-3p was significantly suppressed when containing the 3′-UTR of CSF1 ($P = 0.0036$) but not with site-mutated CSF1 ($P = 0.5636$). (C) Transfection with the miR-214-3p mimic significantly reduced the expression of CSF1 mRNA in glioma U251 cells (n = 4 samples in each group, $P = 0.043$ for mock vs mimic). Significantly increased expression of CSF1 mRNA was detected in the glioma U251 cells cotransfected with miR-214-3p inhibitor (n = 4 samples in each group, $P = 0.036$ for mock vs inhibitor). No significant changes were observed with the mimic NC or inhibitor NC transfection. (D) Similar changes in the protein expression of CSF1 and S100β in glioma U251 cells cotransfected with the miR-214-3p mimic and inhibitor. $^{*}P < 0.05$; $^{**}P < 0.01$. CSF1, colony-stimulating factor-1; NC, negative control; UTR, untranslated region.

marker, was also steadily increased predominantly in the ipsilateral superficial lamina I-II in SNL model rodents (**Fig. 1D**). Consistent with our previous study,[46] SNL promoted the synthesis and release of CSF1 from reactive astrocytes, as evidenced by the appreciable upregulation of CSF1 (**Figs. 1C and D**) and the increased colocalized immunosignals of CSF1 with GFAP (**Fig. 1D**) in the ipsilateral dorsal horn in model rats.

We then explored the potential functional role of CSF1 in SNL-induced neuropathic pain. To this end, commercialized siRNA targeting CSF1 (Gene Pharma, Shanghai, China) was freshly mixed with Invivofectamine 3.0 Reagent (Ambion, Austin, TX) at a dose of 0.2 μg/μL according to the manufacturer's protocol; the mixture was intrathecally administered at 5 μL for 3 consecutive days following the SNL procedure. As shown in **Figures 2A and B**, CSF1 siRNA, but not scrambled siRNA (siRNA NC), significantly attenuated the SNL-induced upregulation of CSF1 mRNA in the ipsilateral dorsal horn. Furthermore, the upregulated contents of CSF1 and S100β in the ipsilateral dorsal horn in the SNL model rats were dramatically suppressed by CSF1 siRNA (**Fig. 2C**). We also noted that CSF1 siRNA decreased the production of cytokine IL-6 from activated microglia in the SNL model rats (**Figs. 2C and D**). A substantially increased intensity and colocalization of IL-6 and CD11b immune signals were observed in the ipsilateral dorsal horn on postoperative day 5, and the intensity and colocalization were significantly reversed by CSF1 siRNA (**Figs. 2D–F**). This evidence implied that CSF1 siRNA alleviated SNL-induced neuroinflammation.

We then tested whether CSF1 affected spinal neuronal activity in response to peripheral noxious stimulation. As shown in **Figures 3A and B**, SNL injury significantly increased the expression of c-Fos in the ipsilateral dorsal horn neurons, and this expression was notably reversed with intrathecal (I.T.) administration of CSF1 siRNA. Moreover, CSF1 siRNA reduced the SNL-induced upregulation of NR2B and GluR1 in the ipsilateral dorsal horn (**Fig. 3C**). As expected, the mechanical allodynia and thermal hyperalgesia induced by SNL were also significantly ameliorated with CSF1 siRNA (**Figs. 3D and E**). Please note that CSF1 siRNA failed to change the pain threshold in the naive animals (**Figs. 3F and G**). This evidence indicated that CSF1 potentially facilitated spinal neuronal excitability and glutamatergic transmission in SNL model animals. To further determine the function of astrocytic CSF1 in neuropathic pain, fluorocitrate (FC, 10 μL, 1 nmol/L), a specific astrocyte inhibitor, was I.T. administered as we previously described.[46] We found that FC significantly reduced the SNL-induced overexpression of CSF1 in the ipsilateral dorsal horn but not in the ipsilateral DRG (Supplementary Fig. 1A, available at http://links.lww.com/PAIN/A865). Pain behavioral tests also revealed that FC attenuated SNL-induced behavioral hypersensitivity (Supplementary Fig. 1B, available at http://links.lww.com/PAIN/A865). Together, these results demonstrated that astrocytic CSF1 in the dorsal horn contributed to SNL-induced pain hypersensitivity.

3.2. Decreased miR-214-3p regulated spinal nerve ligation–induced colony-stimulating factor-1 overexpression

As miRNA is an important part of epigenetics that destabilizes target mRNA genes or represses protein translation through binding to the 3′ untranslated region (UTR), we wondered whether there are potential miRNAs targeting CSF1 upregulation in the SNL model. To attain this strategy, a miRNA microarray was first performed to screen the changed miRNA in the ipsilateral dorsal horn on days 5 and 10 after the SNL procedure. Compared with the sham group, 21 miRNAs were enriched with more than 2.0-fold changes in L4 to L6 ipsilateral dorsal horn tissue in those SNL model animals (**Fig. 4A**). We

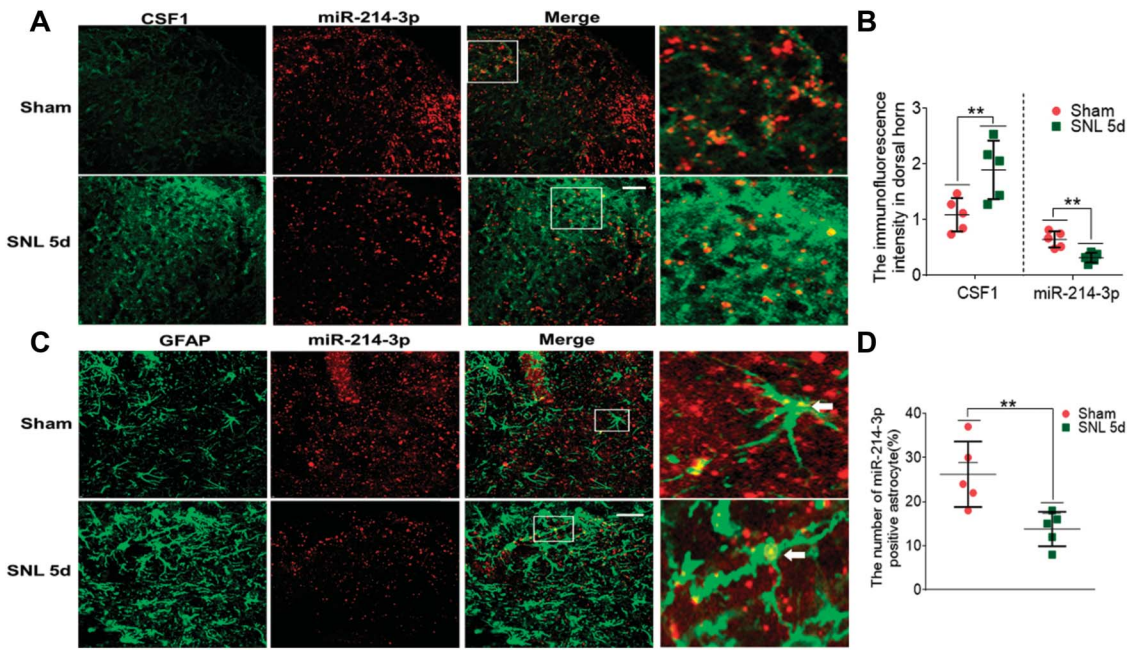

Figure 6. SNL induced distribution changes in miR-214-3p with CSF1 and astrocytes in the ipsilateral dorsal horn. (A) Representative immunosignals of CSF1 (green) and miR-214-3p (red) in the ipsilateral dorsal horn in sham and SNL model animals. (B) The immunoactivity of miR-214-3p in the ipsilateral dorsal horn was significantly weakened in SNL-injured animals (n = 5 rats in each group, $P = 0.0025$). (C) Representative co-localization of immunosignals of GFAP (green) and miR-214-3p (red) (arrowheads) in the ipsilateral dorsal horn in sham and SNL model animals. (D) The percentage of miR-214-3p–positive astrocytes (labeled with GFAP) was significantly decreased in SNL model animals (n = 5 rats in each group, $P = 0.0098$). *$P < 0.05$; **$P < 0.01$. Scale bars represent 50 μm. CSF1, colony-stimulating factor-1; SNL, spinal nerve ligation.

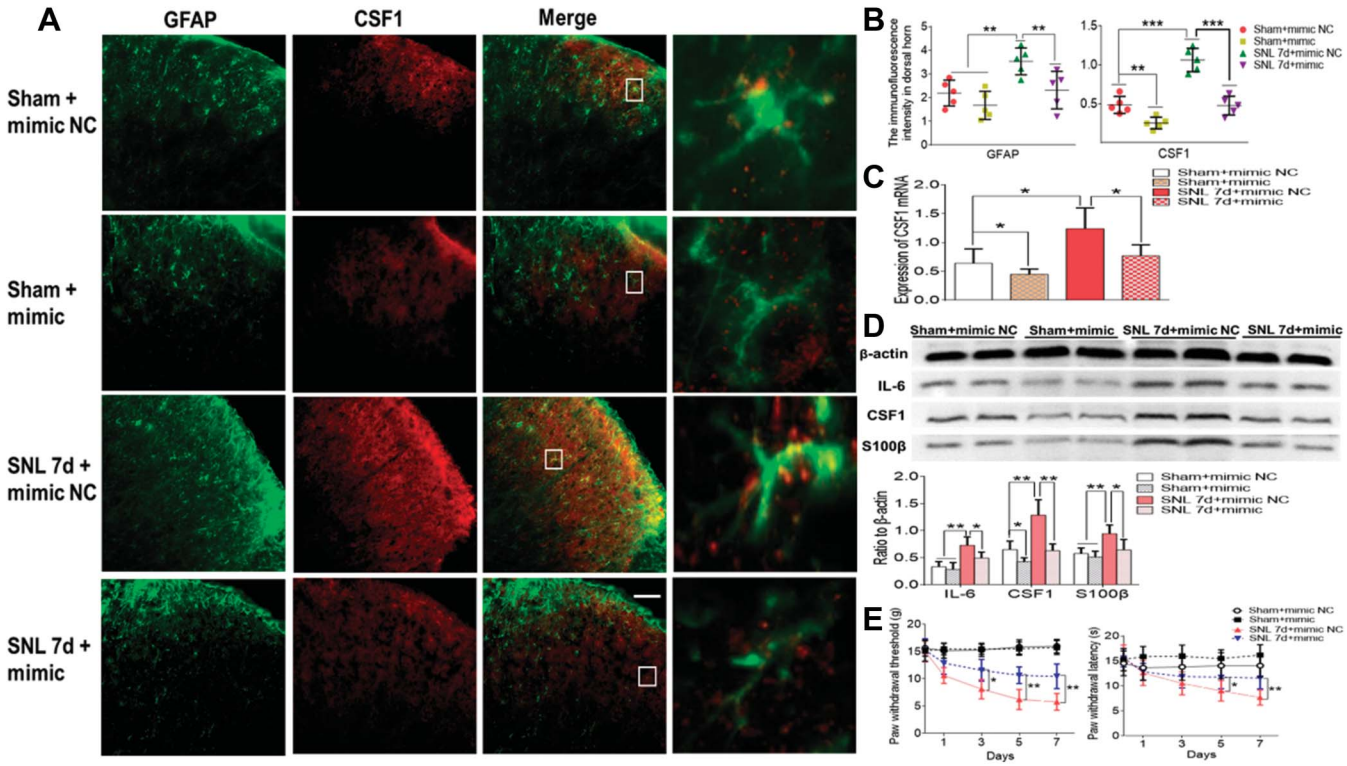

Figure 7. Supplementation with the miR-214-3p mimic substantially ameliorated CSF1-related neuroinflammation and pain-like behaviors induced by SNL. (A) Representative immunosignals of GFAP (green) and CSF1 (red) in the ipsilateral dorsal horn in sham and SNL model animals with the miR-214-3p mimic or mimic NC treatment. (B) The miR-214-3p mimic significantly depressed the immunofluorescence intensity of GFAP and CSF1 induced by SNL (n = 5 rats in each group, F 3, 16 = 7.718, $P = 0.0021$ for GFAP; and F 3, 16 = 44.09, $P < 0.0001$ for CSF1). The miR-214-3p mimic also decreased the immunofluorescence intensity of CSF1 in sham animals (n = 5 rats in each group, $P = 0.0044$). No significant difference was observed in the expression of GFAP between the sham and sham + miR-214-3p mimic. (C) miR-214-3p mimic reduced the expression of CSF1 mRNA in sham and SNL model animals. (D) SNL-induced overexpression of the CSF1, S100β, and IL-6 proteins in the ipsilateral dorsal horn was significantly reduced with I.T. administration of the miR-214-3p mimic (n = 5 rats in each group, F 3, 16 = 22.08, $P < 0.0001$ for CSF1; F 3, 16 = 8.627, $P = 0.0012$ for S100β; F 3, 16 = 12.92, $P = 0.0002$ for IL-6). The miR-214-3p mimic also decreased the content of CSF1 in the dorsal horn of sham animals ($P = 0.0199$). (E) While producing no effect on the pain-like behaviors in sham animals, I.T. administration of the miR-214-3p mimic for 3 consecutive days significantly prevented SNL-induced mechanical allodynia and thermal hyperalgesia (n = 10 rats in each group, effect of group [F 3, 27 = 27.94, $P < 0.0001$], effect of time [F 4, 36 = 61.15, $P < 0.0001$], interaction between group and time [$P < 0.0001$] for paw withdrawal threshold; and effect of group [F 3, 27 = 10.43, $P < 0.0001$], effect of time [F 4, 36 = 58.9, $P < 0.0001$], interaction between group and time [$P < 0.0001$] for paw withdrawal latency). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Scale bars represent 25 μm. CSF1, colony-stimulating factor-1; SNL, spinal nerve ligation; NC, negative control.

then searched 2 computational algorithms (TargetScan and miRanda) and identified that miR-214-3p and miR-184-3p had potential binding sequences with the CSF1 mRNA. Quantitative PCR was further performed to measure the dynamic changes in miR-214-3p and miR-184-3p expression. The reliability of the miRNA microarray was also evaluated by analyzing the expression levels of the 3 other randomly selected miRNAs (**Fig. 4B**). For PCR results, miR-184-3p expression was down-regulated on day 10 after SNL operation but not on days 1 and 5. Instead, miR-214-3p was decreased stepwise in a time-dependent manner postoperatively, roughly consistent with the sustainable increase in CSF1 expression following the SNL procedure. A slightly decreased expression of miR-214-3p in the ipsilateral dorsal horn was found on day 1 after the SNL procedure, and this reduction achieved a maximum on days 5 and 10 (**Fig. 4C**). Two predicted miR-214-3p target sites within the CSF1 3′-UTR (3′-UTR) are also presented in **Figure 4D**. Therefore, miR-214-3p was chosen as the miRNA targeting CSF1 overexpression in the ipsilateral dorsal horn.

We then examined the direct regulatory effects of miR-214-3p on CSF1 expression in vitro. A dual-luciferase reporter assay was first performed to investigate whether CSF1 mRNA was a direct target of miR-214-3p. HEK293 cells were transfected with a recombinant plasmid containing the luciferase gene and 3′-UTR sequences of rat CSF1 cDNA. To validate target specificity, sited-directed mutagenesis was conducted to destroy the potential miR-214-3p binding sites (**Fig. 5A**). The results showed that the luciferase activity was significantly repressed by the constructs harboring the wild-type miR-214-3p target sequence, with an approximately 40% reduction compared with the constructs harboring negative miR-NC (**Fig. 5B**). No obvious luciferase activity change was induced between the miR-214-3p and miR-NC constructs with the mutated forms of CSF1 3′-UTR (**Fig. 5B**). These findings indicated that miR-214-3p directly targeted the 3′-UTR region of CSF1 mRNA and negatively modulated CSF1 expression. We further evaluated the effects of miR-214-3p on the mRNA and protein expression of CSF1. First, CSF1 mRNA and protein were identified to be expressed in glioma U251 cells. Subsequently, the miR-214-3p mimic and inhibitor were separately transfected into glioma U251 cells. The miR-214-3p mimic, but not the mimic NC, markedly decreased CSF1 mRNA and protein expression in glioma U251 cells 24 to 48 hours after transfection (**Figs. 5C and D**). We also observed a prominent increase in the contents of CSF1 mRNA and protein in the miR-214-3p inhibitor–transfected cells (**Figs. 5C and D**). Taken together, these findings support the hypothesis that miR-214-3p negatively regulates CSF1 mRNA and protein expression in vitro.

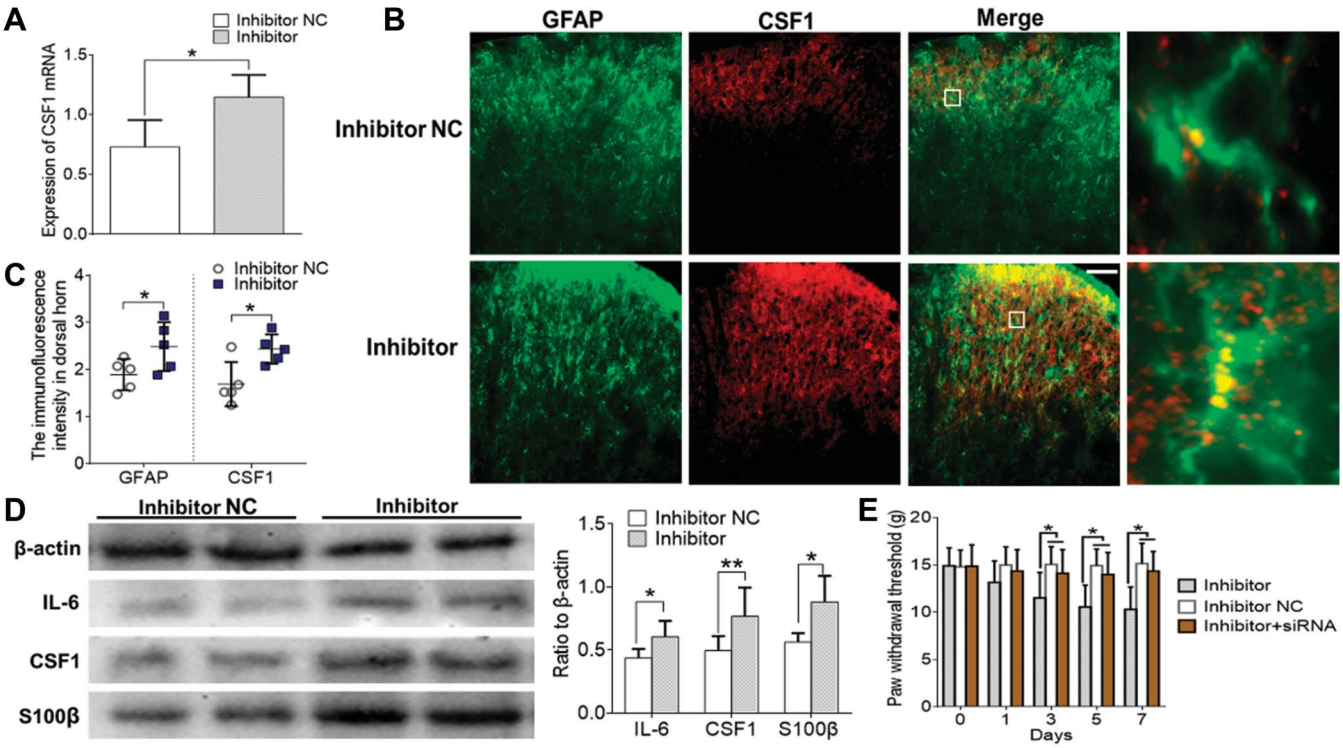

Figure 8. Artificial suppression of miR-214-3p mimicked CSF1-related neuroinflammation and pain-like behaviors induced by SNL. (A) miR-214-3p inhibitor significantly increased the expression of CSF1 mRNA in the dorsal horn (n = 5 rats in each group, $P = 0.0296$). (B) Representative immunosignals of GFAP (green) and CSF1 (red) in the dorsal horn of rats receiving I.T. injection of the miR-214 inhibitor or inhibitor NC. (C) Significantly increased immunofluorescence intensities of GFAP and CSF1 were observed in those animals receiving the miR-214-3p inhibitor (n = 5 rats in each group, $P = 0.0433$ for GFAP; and $P = 0.018$ for CSF1). (D) The miR-214-3p inhibitor significantly increased the contents of the CSF1, S100β and IL-6 proteins in the dorsal horn (n = 5 rats in each group, $P = 0.042$ for IL-6, $P = 0.0078$ for CSF1, and $P = 0.0236$ for S100β). (E) miR-214-3p inhibitor decreased the threshold for mechanism stimulus, which was significantly reversed by CSF1 siRNA (n = 10 rats in each group, effect of group [$F 2, 18 = 6.311$, $P = 0.0084$], effect of time [$F 4, 36 = 11.73$, $P < 0.0001$], interaction between group and time [$P < 0.0001$]). *$P < 0.05$; **$P < 0.01$. Scale bars represent 25 μm. CSF1, colony-stimulating factor-1; NC, negative control; SNL, spinal nerve ligation.

3.3. miR-214-3p contributed to colony-stimulating factor-1–related neuroinflammation and neuropathic pain

Having determined the suppression effect of miR-214-3p on CSF1 expression in vitro, we then investigated the functional profile of miR-214-3p in CSF1-related neuroinflammation and pain behaviors in the SNL model. To this end, FISH analysis was first performed to examine the colocalization of miR-214-3p with CSF1 or GFAP. As **Figures 6A and B** show, animals with SNL injury displayed significantly stronger CSF1 immunoactivity and markedly weakened miR-214-3p immunoactivity in the ipsilateral dorsal horn. We also noted that although the distribution pattern of miR-214-3p partially overlapped with that of the CSF1 protein, most miR-214-3p–positive cells expressed low amounts of CSF1 protein in either sham or SNL model animals (**Figs. 6A and B**). Consistently, the immunosignal effect of miR-214-3p was also partly colocalized with GFAP in the ipsilateral dorsal horn (**Fig. 6C**). Although SNL induced upregulated GFAP expression in the ipsilateral dorsal horn, approximately only 14% of GFAP-positive astrocytes overlapped with miR-214-3p, nearly 2-fold less than sham animals (**Fig. 6D**). All these findings indicated the coexistence of miR-214-3p with CSF1 in astrocytes and further confirmed the negative modulation of miR-214-3p on CSF1 expression.

To further assess the functional involvement of miR-214-3p in CSF1-related neuroinflammation and pain behaviors, we investigated whether artificial increases in miR-214-3p expression would attenuate the SNL-induced astrocyte overactivity and reduce overexpression of CSF1 and IL-6. For this purpose, 5 μL of the miR-214-3p mimic or mimic NC (1 μg/μL, prepared with Invivofectamine 3.0 Reagent) was introduced in sham or SNL model animals intrathecally for 3 consecutive days following the SNL procedure. We observed a significant suppression of the enhanced expression of GFAP and CSF1 in the ipsilateral dorsal horn with miR-214-3p mimic administration after SNL injury (**Figs. 7A and B**). No obvious changes in the expression of GFAP were noted in the sham animals receiving the miR-214-3p mimic or mimic NC treatment. Interestingly, the miR-214-3p mimic slightly decreased the staining intensity of CSF1 in sham rats (**Figs. 7A and B**). We also found that the miR-214-3p mimic had a remarkable inhibitory effect on the expression of CSF1 mRNA and protein in SNL model animals, further indicating the negative interaction between miR-214-3p and CSF1 (**Figs. 7C and D**). The increased expression of s100β and IL-6 in SNL model animals was also abolished with the miR-214-3p mimic treatment (**Fig. 7D**). Notably, SNL model rats exhibited a significant increase in the paw withdrawal threshold and paw withdrawal latency after miR-214-3p mimic treatment (**Fig. 7E**). Taken together, miR-214-3p prevented pain-like syndromes by decreasing CSF1 production and alleviating CSF1-related neuroinflammation.

The critical role of miR-214-3p in CSF1 expression and function was further tested in naive animals. To this end, the miR-214-3p inhibitor or inhibitor NC was I.T. administered in naive rats at a dose of 10 μL (1 μg/μL, prepared with Invivofectamine 3.0 Reagent) for 3 consecutive days. The behaviors were measured for 7 days since the inhibitor/inhibitor NC was first injected, and the spinal samples were then collected. The miR-214-3p inhibitor significantly increased the expression levels of CSF1 mRNA (**Fig. 8A**) and upregulated the contents of GFAP, CSF1, s100β, and IL-6 in the dorsal horn (**Figs. 8B–D**). The miR-214-3p inhibitor also significantly decreased the paw

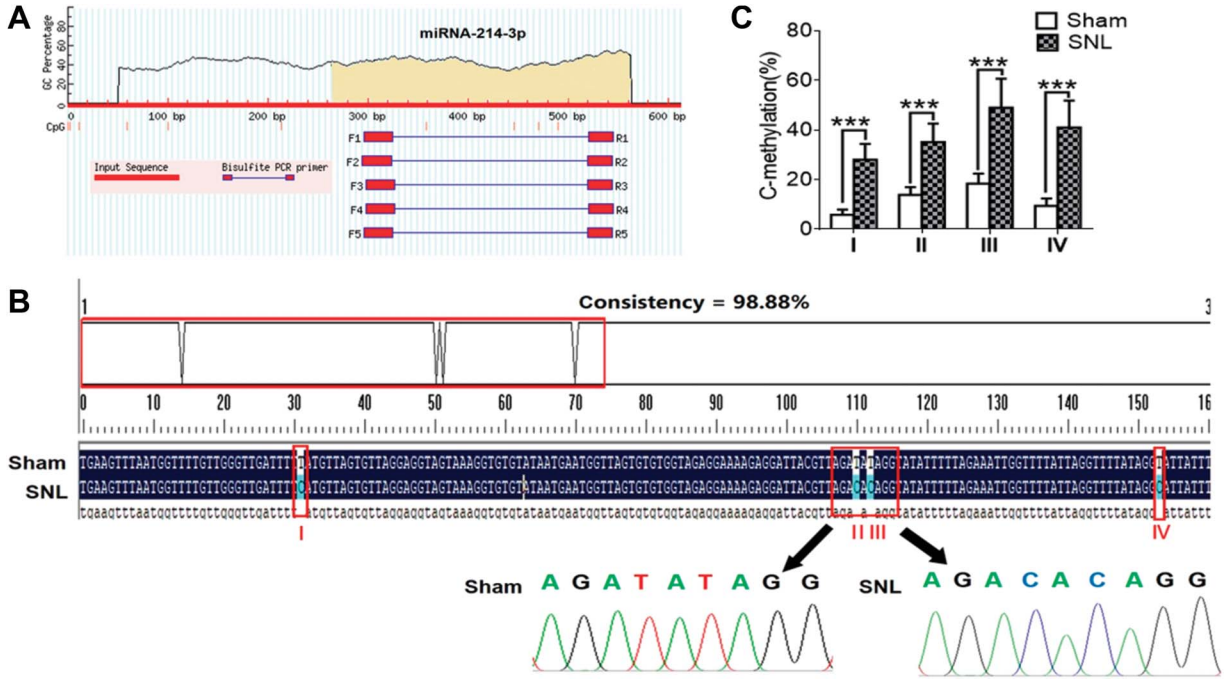

Figure 9. SNL induced DNA methylation within the promoter of miR-214-3p. (A) Specific methylation primers were designed to amplify the DNA fragments (darkened with brown) from the ipsilateral dorsal horn tissue. (B) Bisulfite sequencing analysis found that SNL induced 4 methylated sites within the amplified miR-214-3p promoter (from **I** to **IV**). (C) The frequency of these 4 methylated sites between sham and SNL model animals. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. SNL, spinal nerve ligation.

withdrawal threshold to a mechanical stimulus, which was reversed by CSF1 siRNA (**Fig. 8E**). These results indicated that the inhibition of miR-214-3p substantially mimicked CSF1-related neuroinflammation and pain-like behaviors.

Consistent with a previous study,[16] we also observed an upregulation of CSF1 in the ipsilateral DRG after SNL injury (Supplementary Fig. 2A, available at http://links.lww.com/PAIN/A865). Furthermore, miR-214-3p was also expressed in the ipsilateral DRG and was significantly reduced after SNL injury (Supplementary Fig. 2B, available at http://links.lww.com/PAIN/A865). I.T. administration of CSF1 siRNA or the miR-214-3p mimic or inhibitor also produced marginal effects on CSF1 expression in DRG (Supplementary Fig. 2A and 2C-D, available at http://links.lww.com/PAIN/A865).

3.4. Spinal nerve ligation induced hypermethylation in the miR-214-3p promoter

Because epigenetic modifications regulate the transcription levels of miRNAs,[34] we explored whether DNA methylation controlled SNL-induced decreased expression of miR-214-3p. *In silico* analysis revealed the percentage of CG clusters located within promoter regions of the miR-214-3p gene. Methylation-specific primers (forward primer: 5′-TGAAGTT-TAATGGTTTTGTTGGGTTGATTT-3′, reverse primer: 5′-CACA-TAACAACCCAACCTAAAT-3′) were selected based on the proposed primer and used to amplify the promoter region of bisulfited samples from +257 to +589 (**Fig. 9A**). Bisulfite sequencing PCR resulted in a 356-bp PCR product. As **Figure 9B** demonstrates, the bisulfite treatment of genomic DNA converted unmethylated cytosine into thymine (sham group), while methylated cytosine remained unchanged (SNL group). Four methylated sites (I-IV) were observed in the amplified miR-214-3p promoter in the SNL group (**Figs. 9B–C**), suggesting that DNA methylation was involved in SNL-induced decreased expression of miR-214-3p. As DNMT3a is a pivotal enzyme triggering DNA methylation and has been reported to modulate neuropathic pain,[42] we then explored whether DNMT3a mediated the reduced expression of miR-214-3p. The expression of DNMT3a in the ipsilateral dorsal horn was first measured, and a significantly enhanced amount of DNMT3a was observed in SNL model animals (**Fig. 10A**). We next determined the interaction between DNMT3a and the miR-214-3p gene promoter. The ChIP results showed an increased occupancy of DNMT3a in the promoter region of the miR-214-3p gene, as demonstrated by the amplification of the complexes immunoprecipitated with the DNMT3a antibody (**Fig. 10B**). DNMT3a-triggered DNA methylation was further evaluated with 5 μL of the DNMT inhibitor zebularine (100 ng/μL, Selleck) following the SNL procedure. Bisulfite sequencing PCR revealed that zebularine reversed the increases in the methylation levels at the miR-214-3p gene promoter on day 5 following the SNL procedure (**Fig. 10C**). Concurrently, the reduction in miR-214-3p expression in the rats with SNL was restored after zebularine I.T. administration (**Fig. 10D**). Note that zebularine did not obviously change the expression level of miR-124-3p in the sham animals (**Fig. 10D**). Furthermore, zebularine also attenuated the SNL-induced increased expression of CSF1 and IL-6 (**Fig. 10E**). As expected, zebularine had pronounced effects in mitigating SNL-induced pain-like behaviors (**Figs. 10F and G**). These results indicated that SNL-induced aggravated neuroinflammation and impaired pain behaviors were partly due to the promoted DNA methylation of the miR-214-3p gene.

4. Discussion

This study provided evidence to support the critical involvement of the epigenetic regulation of astrocytic CSF1 in the development of neuropathic pain. First, SNL injury enhanced CSF1 release from reactive astrocytes in the dorsal horn, which promoted the

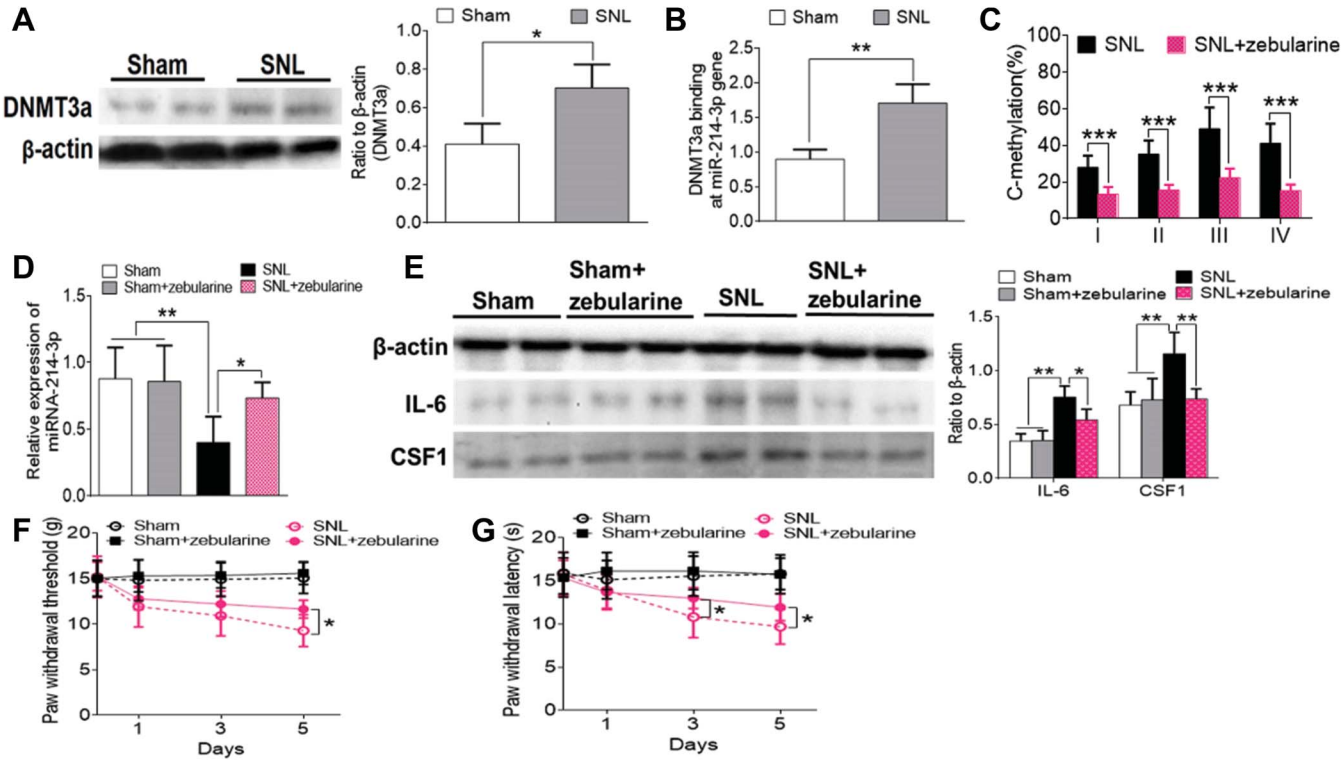

Figure 10. DNMT3a triggered methylation in the miR-214-3p promoter and upregulated astrocytic CSF1 expression in SNL-induced neuropathic pain. (A) SNL injury significantly increased the content of DNMT3a in the ipsilateral dorsal horn (n = 4 rats in each group, $P = 0.011$). (B) A ChIP study showed a significantly increased occupancy of DNMT3a within the miR-214-3p promoter region in SNL model rats (n = 4 rats in each group, $P = 0.002$). (C) Administration of the DNMT3a inhibitor zebularine abolished SNL-induced hypermethylation in the miR-214-3p promoter. (D) SNL-induced downregulation of miR-214-3p was recovered with I.T. administration of zebularine (n = 4 rats in each group, $P = 0.0253$ for SNL vs SNL + zebularine). (E) SNL-induced increases in the amount of IL-6 and CSF1 in the ipsilateral dorsal horn were reversed with zebularine treatment. (F–G) Zebularine prevented SNL-induced mechanical allodynia and thermal hyperalgesia (n = 8 rats in each group, effect of group [F 3, 21 = 8.108, $P = 0.0009$], effect of time [F 3, 21 = 78.79, $P < 0.0001$], interaction between group and time [$P < 0.0001$] for paw withdrawal threshold; effect of group [F 3, 21 = 4.368, $P = 0.0154$], effect of time [F 3, 21 = 67.47, $P < 0.0001$], interaction between group and time [$P < 0.0001$] for paw withdrawal latency). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. ChIP, chromatin immunoprecipitation; CSF1, colony-stimulating factor-1; SNL, spinal nerve ligation.

production of IL-6 from microglia and induced sensory hypersensitivity. Second, SNL injury decreased the expression of miR-214-3p, which directly increased the mRNA and protein expression of CSF1 in reactive astrocytes. Finally, SNL injury induced DNMT3a-associated hypermethylation within the miR-214-3p promoter, which decreased miR-214-3p expression and produced CSF1 overexpression and pain-like behaviors.

Neuroinflammation plays an important role in the development and maintenance of chronic pain.[24] Activated glia (eg, microglia and astrocytes) are a vital hallmark of neuroinflammation.[24] Both microglia and astrocytes release numerous proinflammatory cytokines and chemokines that facilitate synaptic transmission in the dorsal horn and enhance nociceptive transduction to the brain.[24] IL-6 is one of these critical inflammatory cytokines that facilitate neuronal hyperexcitability.[62] The promoted IL-6 production from activated astrocytes and microglia was identified in the dorsal horn in chronic pain.[29,51] Similar to previous studies that microglia were the potent producer of IL-6 in the CNS,[14,53] a significantly enhanced secretion of IL-6 from activated microglia in the dorsal horn was observed in SNL model rats. IL-6 has wide-ranging biological activities in the immune and nervous system. In contrast with its anti-inflammatory effects, IL-6 is always characterized as a proinflammatory cytokine in pathological states by binding to the soluble protein sIL-6R that is ubiquitously distributed on all body cells, including microglia, astrocytes, and neurons.[40] IL-6 overexpression was sufficient to provoke reactive astrocytosis and increase the number of ramified microglia in the CNS.[13] Furthermore, the positive feedback loop between IL-6 and activated glia would sustain neuroinflammation and neuronal hyperexcitability in chronic pain. In this study, we found that IL-6 was involved in CSF1-associated neuroinflammation and pain behaviors.

The CSF1-CSF1R signaling pathway forms a fundamental cytokine network that allows for the proliferation, differentiation, and survival of microglia to be governed. Our previous study reported that the enhanced expression of astrocytic CSF1 promoted the release of the neuropeptide BDNF from activated microglia in the dorsal horn in a chronic ischemic pain model.[46] This study further elucidated the proinflammatory effects of astrocytic CSF1 on microglial IL-6 release, with the evidence that SNL injury increased the expression of astrocytic CSF1 and microglial IL-6, and the enhanced IL-6 production was significantly reduced with CSF1 siRNA. This was consistent with previous studies showing that the expression of CSF1 and IL-6 increased simultaneously in the pathogenesis of malignant tumors[39] and endometriosis.[50] Moreover, CSF1 augmented IL-6 production in microglia,[35] monocytes,[36] and macrophages.[26] CSF1-CSF1R signaling activated several intracellular tyrosine kinase transduction pathways, such as PI3K, JNK, and ERK1/2.[7,44] As a crucial intracellular pathway of CSF1R, PI3K activated NF-κB and initiated IL-6 expression.[21,56] JNK and ERK1/2 were also found to promote IL-6 production in microglia.[22,63] These findings strongly support the hypothesis that CSF1 activates microglia to produce IL-6.

miRNAs are post-transcriptional regulators that bind to mRNAs with complementary sequences.[2] Although many different miRNAs have been detected to regulate aberrant neuronal excitability in chronic pain,[30] their effects on spinal astrocyte function in pain disorders remain to be determined. Previous studies have reported that miRNA-21 ameliorated astrocyte hypertrophy after spinal cord injury.[3] miRNA-146a-5p attenuated neuropathic pain by inhibiting TRAF6 and its downstream signaling molecules JNK/CCL2 in astrocytes.[31] Herein, miR-214-3p was identified as another miRNA influencing astrocyte function. miR-214-3p is widely expressed in the body and targets different genes to regulate multiple human disorders, including cancer,[19] immune disorders,[57] and cardiovascular diseases.[61] Colony-stimulating factor-1 is one of the major targets of miR-214-3p. Consistent with the results of our study, CSF1-induced osteoclastogenesis was inhibited by miR-214-3p.[59] miR-214-3p also decreased CSF1 expression and inhibited the proliferation, invasion, and migration of gastric cancer cells.[49] Our data further supported the negative modulation of miR-214-3p on spinal astrocytic CSF1 expression in neuropathic pain. Moreover, considering the miR-214-3p reduction and its potential regulation of CSF1 expression in DRG after nerve injury, miR-214-3p-CSF1 signaling in DRG might also be involved in the development of neuropathic pain.

Thus far, the potential mechanisms regulating miRNA expression in neuropathic pain are poorly understood. Recently, global DNA methylation in DRG[15,45] and the dorsal horn[33,47] has been increasingly recognized in neuropathic pain. The upregulated levels of DNMT3a in DRG and the dorsal horn were found to facilitate neuronal excitability by repressing the gene expression of the opioid receptor and voltage-dependent potassium channel subunit Kcna2.[33,42,45,60] Our data revealed for the first time that DNMT3a promoted DNA hypermethylation within the miRNA-214-3p promoter and silenced miR-214-3p genes, which contributed to astrocyte-related neuroinflammation and pain hypersensitivity. Indeed, DNMT3a is capable of regulating astrocyte differentiation and function. It appeared when neural precursor cells were not ready to differentiate into astrocytes, and some specific astrocytic gene promoters were heavily methylated to prevent transcription-related protein accessibility.[37] Inhibition or knockout of DNMT3a demethylated some astrocyte genes, thereby urging astrogliogenesis and ultimately resulting in neuroinflammation and neurodegeneration.[37,38,48,54,58] In this study, the DNMT3a inhibitor zebularine reversed the decreased miR-214-3p induced by SNL injury, which consequently restored astrocytic CSF1 expression and alleviated pain behaviors. Therefore, CSF1, as well as its upstream mediator DNMT3a, and miR-214-3p are all promising novel treatment targets for neuropathic pain.

In summary, our data indicated that nerve injury decreased spinal miR-214-3p expression, which led to astrocytic CSF1 and microglial IL-6 overexpression in the dorsal horn and pain hypersensitivity. The reduced miR-214-3p expression was mediated by DNMT3a-triggered DNA methylation.

Conflict of interest statement

The authors have no conflicts of interest to declare.

Acknowledgments

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Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/A865.

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