MicroRNA-182-5p Regulates Nerve Injury–induced Nociceptive Hypersensitivity by Targeting Ephrin Type-b Receptor 1

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ABSTRACT

Background: The authors and others have previously shown that the up-regulation of spinal ephrin type-b receptor 1 plays an essential role in the pathologic process of nerve injury–induced nociceptive hypersensitivity, but the regulatory mechanism remains unclear.

Methods: Radiant heat and von Frey filaments were applied to assess nociceptive behaviors. Real-time quantitative polymerase chain reaction, Western blotting, fluorescence *in situ* hybridization, immunofluorescence, immunohistochemistry, dual-luciferase reporter gene assays, recombinant lentivirus, and small interfering RNA were used to characterize the likely mechanisms.

Results: Periphery nerve injury induced by chronic constriction injury of the sciatic nerve significantly reduced spinal microRNA-182-5p (miR-182-5p) expression levels, which were inversely correlated with spinal ephrin type-b receptor 1 expression ($R^2 = 0.90$; $P < 0.05$; n = 8). The overexpression of miR-182-5p in the spinal cord prevented and reversed the nociceptive behaviors induced by sciatic nerve injury, accompanied by a decreased expression of spinal ephrin type-b receptor 1 (recombinant lentiviruses containing pre-microRNA-182: 1.91 ± 0.34 *vs.* 1.24 ± 0.31, n = 4; miR-182-5p mimic: 2.90 ± 0.48 *vs.* 1.51 ± 0.25, n = 4). In contrast, the down-regulation of spinal miR-182-5p facilitated the nociceptive behaviors induced by sciatic nerve injury and increased the expression of spinal ephrin type-b receptor 1 (1.0 ± 0.26 *vs.* 1.74 ± 0.31, n = 4). Moreover, the down-regulation of miR-182-5p and up-regulation of ephrin type-b receptor 1 caused by sciatic nerve injury were mediated by the *N*-methyl-D-aspartate receptor.

Conclusions: Collectively, our findings reveal that the spinal ephrin type-b receptor 1 is regulated by miR-182-5p in nerve injury–induced nociceptive hypersensitivity. **(ANESTHESIOLOGY 2017; 126:967-77)**

EPHRIN type-b receptor 1 (ephb1) as well as its ligands are involved in a variety of physiologic and pathologic processes, such as axon guidance, synaptic maintenance, and central nervous system (CNS) diseases.[1,2] Recent advances have indicated that ephb1 is also expressed in the spinal cord and plays an important role in the development and maintenance of nerve injury–induced nociceptive hypersensitivity.[3] The spinal ephb1 receptor is persistently up-regulated and activated after peripheral nerve injury.[4,5] Blocking the spinal ephb1 receptor has been reported to prevent and reverse nerve injury–induced allodynia and hyperalgesia.[6] In addition, the spinal ephb1 receptor is implicated in the modulation of nerve injury–induced nociceptive hypersensitivity through interactions with the *N*-methyl-D-aspartate (NMDA) receptor and subsequent signal transduction pathways.[7,8] Despite the fact that the spinal ephb1 receptor is a key regulator of nerve injury–induced nociceptive hypersensitivity and that this receptor has become a novel target for nerve injury–induced nociceptive hypersensitivity prevention and treatment, the mechanism of its up-regulation in response to peripheral nerve injury is still unclear.

What We Already Know about This Topic

- Ephrin type-b receptor 1 is involved in regulating nociceptive signaling in the central nervous system
- MicroRNA species are endogenous regulators of gene expression after nerve injury

What This Article Tells Us That Is New

- After sciatic nerve injury in rats, microRNA-182-5p expression levels decreased in spinal cord tissue while ephrin type-b receptor 1 increased
- The overexpression of microRNA-182-5p reduced ephrin type-b receptor expression and prevented nociceptive sensitization after nerve injury

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MicroRNAs (miRNAs), which are 18- to 25-nucleotide noncoding RNAs, can bind to the 3′-untranslated regions (UTRs) of messenger RNAs (mRNAs) to regulate gene expression posttranscriptionally.[9] Increasing studies have shown that the miRNA-mediated modulation of gene expression is important for the regulation of nerve injury–induced nociceptive hypersensitivity.[10,11] For example, miR-103 attenuates nerve injury–induced nociceptive behaviors by suppressing the expression of the voltage-gated calcium channels Cav2.1 and Cav2.2 in the dorsal root ganglion.[12] miR-146a-5p reduced nerve injury–induced allodynia and hyperalgesia by inhibiting tumor necrosis factor receptor–associated factor-6 signaling in the spinal cord.[13] The overexpression of spinal miR-186-5p was shown to decrease C-X-C motif chemokine 13 expression and alleviate nerve injury–induced nociceptive hypersensitivity.[14] Because the miRNA-mediated modulation of gene expression contributes to the modulation of nerve injury–induced nociceptive hypersensitivity, we hypothesized that miRNAs may be involved in the regulation of the spinal ephb1 receptor during nerve injury.

Here, we found that miR-182-5p, miR-129-5p, and miR-450b-5p potentially target the 3′-UTR of ephb1 mRNA. However, only miR-182-5p expression was negatively correlated with the expression of ephb1 after chronic constriction injury (CCI). Luciferase reporter assays showed that cotransfecting an miR-182-5p mimic but not miR-129-5p or miR-450b-5p mimics with an ephb1 3′-UTR vector resulted in a significantly reduced luciferase activity compared with negative controls. Therefore, we focused on the potential role of miR-182-5p in ephb1 regulation. The up-regulation of miR-182-5p decreased spinal ephb1 expression and prevented and reversed CCI-induced nociceptive behaviors. In contrast, the down-regulation of miR-182-5p increased spinal ephb1 expression and facilitated CCI-induced nociceptive behaviors. Together, our study revealed that miR-182-5p regulates nerve injury–induced nociceptive hypersensitivity by targeting the spinal ephb1 receptor.

Materials and Methods

Animals

Adult male Institute of Cancer Research mice (8 weeks old) were provided and housed in the Center for Experimental Animals of Nanjing Medical University, Nanjing, China. All experimental procedures were approved by the Animal Care and Use Committee of Nanjing Medical University and adhered to the guidelines of the International Association for the Study of Pain (Washington, D.C.). Mice were randomly allocated to experimental conditions, and the number used in each group was chosen according to our preliminary experiments.

Chronic Constrictive Injury of Sciatic Nerve Model

To produce a CCI-induced nerve injury model, mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg), and their right sciatic nerve was exposed and then tightly ligated with 5-0 silk thread (spaced at 1-mm intervals). For sham-operated mice, the nerve was exposed without ligation. The incision was closed in layers, and the wound was treated with antibiotics (penicillin).

Drugs and Administration

miRNA is first transcribed into primary miRNAs, which are then cleaved into pre-miRNAs in the nucleus. The pre-miRNA is exported from the nucleus and enters the cytoplasm, where it is further processed into its mature form (mature miRNA). Therefore, in the current study, recombinant lentiviruses containing pre-miRNA-182-5p (LV-pre-miR-182-5p) or a miRNA mimic (miR-182-5p mimic) were used to up-regulate the expression of spinal miR-182-5p at different stages. An miR-182-5p mimic (5′-UUUGGCAAUGGUAGAACUCACACCG-3′) and its scrambled negative control (5′-AAGTTUAGAAGA-CATTCCTGUTTGCCAUG-3′), an miR-182-5p inhibitor (5′-CGGTGTGAGTTCTACCATTGCCAAA-3′) and its scrambled negative control (TUGTACGGACGTTGT-TAGACCTCG), and an rno-miR-182 mimic (UUUG-GCAAUGGUAGAACUCACACCG) were synthesized by Shanghai GenePharma (China). The recombinant lentivirus containing pre-mmu-miR-182 (accession: MI0000224) was constructed and packaged by Shanghai GeneChem (China). The noncompetitive NMDA glutamate receptor antagonist MK801 and the agonist NMDA were purchased from Sigma. ephb1-Fc was purchased from R&D Systems Inc. (USA) ephb1 small interfering ribonucleic acid ([siRNA] 5′-GTTCGAAGACGTTGCACACTT-3′) and its scrambled negative control (5′-TAAGATTCC-GTCGTAGGTGATT-3′) were designed and synthesized by Shanghai GenePharma. Intrathecal injections were performed using a 28-gauge stainless steel needle between the L5 and L6 vertebrae to deliver drugs to the subarachnoid space. Three animals exhibited signs of motor dysfunction and were excluded from the experiments. Before intrathecal injection, miRNA mimics or miRNA inhibitors were mixed with the reagent Lipofectamine 2000 (Thermo Fisher, USA). The time points and doses of drug administration were chosen based on the results of our preliminary experiments and related studies.[15]

Behavioral Tests

Behavioral tests were performed in a blinded manner. Paw-withdrawal latency to thermal stimulus and paw-withdrawal threshold to mechanical stimulus were performed as described previously.[15] For paw-withdrawal latency, mice were placed in a plastic box on a glass plate and allowed to adapt for 1 h. The plantar surface of the hind paw was exposed to radiant heat through a transparent glass surface. Baseline values were adjusted to 12 to 15 s, and an automatic 25-s cutoff was used to prevent tissue injury. The values were averaged for three trials at intervals of 5 min. For paw-withdrawal threshold, mice were placed in a plastic box

on a metal mesh floor. The plantar surface was stimulated with an electronic von Frey unit.[8] The force (*g*) that induced paw withdrawal was digitized by the unit and used as the threshold for mechanical nociception.

Real-time Quantitative Polymerase Chain Reaction for mRNA and miRNA

For mRNA analysis, total RNA was extracted and reverse transcribed into complementary DNA using Trizol (Invitrogen, USA) and oligo primers, respectively. For miRNA analysis, small RNAs were extracted and reverse transcribed using the RNAiso Kit (Takara, China) and the One-Step PrimeScript miRNA cDNA Synthesis Kit (Takara), respectively. Real-time quantitative polymerase chain reaction (qPCR) was performed with the Real-Time Detection System by SYBR Green I dye detection (Takara). Data were analyzed using the $2^{-\Delta\Delta}$CT method. Primers for the selected genes were as follows: ephb1, sense 5-CCTCCTCCTATGGACTGCCC-3, antisense 5-AAGGCCGTGAAGTCTGGGATA-3; glyceraldehyde-3-phosphate dehydrogenase, sense 5-AGGTCGGTGTGAACGGATTTG-3, antisense 5-TGTAGACCATGTAGTTGAGGTCA-3; miR-182-5p, 5′-TTTGGCAATGGTAGAACTCACACCG-3′; miR-129-5p, 5′-CTTTTTGCGGTCTGGGCTTGC-3′; miR-450b-5p, 5′-TTTTGCAGTATGTTCCTGAATA-3′; and U6, sense 5′-GCTTCGGCAGCACATATACTAA-3′, antisense 5′-CGAATTTGCGTGTC ATCCTT-3′.

Western Blot Analysis

Protein samples were prepared as described previously.[15] Proteins were separated *via* sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. After the membranes were blocked with milk, they were incubated overnight with primary antibodies against ephb1 (rabbit, 1:1,000; Abcam, United Kingdom; ab129103) and glyceraldehyde phosphate dehydrogenase (glyceraldehyde-3-phosphate dehydrogenase; rabbit, 1:5,000; CST, USA; No. 2118) at 4°C. The blots were washed extensively and further incubated with horseradish peroxidase–conjugated secondary antibody (rabbit immunoglobulin G, 1:5,000; CST; No. 7074) for 2 h at room temperature. The immune complexes were detected using chemiluminescence (Pierce, USA). The intensity of each band was determined using ImageJ software (National Institutes of Health, USA).

Construction of Vectors

The partial 3′-UTR sequences of ephb1 that contain the miR-182-5p, miR-129-5p, and miR-450b-5p target region were cloned into pmiR-GLO dual-luciferase reporter vectors (Promega, USA). The pmiR-GLO luciferase vectors containing the mutant miR-182-5p-, miR-129-5p-, or miR-450b-5p-binding sites were also constructed using the mutated oligonucleotide pairs.[14] The sequences for producing matched and mismatched miRNA-binding site regions are shown as follows: miR-182-5p: sense 5-CTGAGTCTCCAGATGTCGTCTGTCAGTTGCCAAAG-3, antisense 5-CTAGCTTTGGCAACTGACAGACGACATCTGGAG ACTCAGAGCT-3; mutated miR-182-5p: sense 5-CTGAGTCTCCAGATGTCGTCTGTCAGTCACCGGAG-3, antisense 5-CTAGCTCCGGTGACTGACAGACGACATCTGGAGACTCAGAGCT-3; miR-129-5p: sense 5-TTGTTTTTCTT TTGTTTGCATTTTCTGCAAAAAGG-3, antisense 5-CTAGCCTTTTTGCAGAAAATGCAAACAAAAGAAAAAC AAAGCT-3; mutated miR-129-5p: sense 5-TTGTTTTTCTTTTGTTTGCATTTTCTGCAAAAAGG-3, antisense 5-CTAGCCACTTGGCAGAAAATGCAAACAAAAGAAAAACAAAGCT-3; miR-450b-5p: sense 5-CCTTGTTTTTCTTTTGTTTGCATTTTCTGCAAAAA-3, antisense 5-CTAGTTTTTGCAGAAAATGCAAACAAA AGAAAAACAAGGAGCT-3; and mutated miR-450b-5p: sense 5-CCTTGTTTTTCTTTTGTTTAGACCTTCTGCA AAAACCTCCTCCTATGGACTGCCC-3, antisense 5-CTAGTTTTTGCAGAAGGTCTAAACAAAAGAAAAACA AGGAGCT-3.

Dual-luciferase Reporter Gene Assays

HEK293T cells were seeded at 1×10^5 cells per well in 24-well plates. The cells were cotransfected with 50 ng/ml firefly luciferase vector and 50-ng control Renilla luciferase vector along with miRNA mimics (30 nM) or scrambled negative controls using the Xtreme GENE siRNA Transfection Reagent (Roche, Switzerland). Luciferase assays were performed 24 h after transfection using the Dual-Glo Luciferase Assay System (Promega, USA).

Fluorescence In Situ *Hybridization, Immunofluorescence, and Immunohistochemistry*

Mice were deeply anesthetized and intracardially perfused with 4% paraformaldehyde. The spinal cord was quickly removed, postfixed with 4% paraformaldehyde, cryoprotected in 30% sucrose, and cut into 30-μm sections. A 5′-DIG (Digoxin)– and 3′-DIG–labeled mature miR-182-5p miRCURY LNA detection probe (5′-DIG-CGGTGTGAGTTCTACCATTGCCAAA-DIG-3′) and a scrambled control probe (5′-DIG-CGATGTGTAACACGTCTATACGCCC-DIG-3′) were synthesized by Exiqon (Denmark). Fluorescence *in situ* hybridization (FISH) was carried out using a FISH kit (Exon, China). To identify the coexpression of ephb1 and miR-182-5p, the sections under FISH were further incubated overnight with primary antibodies against ephb1 (rabbit, 1:500; Abcam; ab129103) at 4°C. The following day, an Alexa Fluor 594–conjugated secondary antibody (Thermo Fisher, USA) was added, and the sections were incubated for 2 h at room temperature. The sections were subsequently washed and mounted for confocal imaging. Fos and phosphorylated extracellular protein kinases (p-ERK) are markers of spinal neuronal sensitization. Fos immunohistochemistry or p-ERK immunofluorescence was carried out as previously reported.[8] The intensity

of p-ERK was calculated using ImageJ. The intensity of the background was subtracted in each section.

Statistical Analysis

Data were expressed as the mean ± SD and analyzed using GraphPad Prism (version 5.0; GraphPad Software Inc., USA). $P < 0.05$ was considered statistically significant. Differences between two groups were analyzed using Student's *t* test. Differences between more than two groups were analyzed using one-way ANOVA followed by the Tukey *post hoc* test. The behavioral data were compared using two-way repeated-measures ANOVA followed by the Tukey *post hoc* test. Correlation analysis was assessed by Pearson correlation test. In response to criticism during peer review, an additional n = 4 samples were conducted to examine the correlation.

Results

miR-182-5p Targets ephb1 in the Spinal Cord

Consistent with previous studies,[4,5] our qPCR results showed that the expression of ephb1 gradually increased from 1 to 14 days after CCI (fig. 1A). Western blotting also confirmed that CCI produced an increase in the expression of spinal ephb1 (fig. 1B). To identify potential miRNAs that may target ephb1, three commonly used online prediction programs (TargetScan, [available at: http://www.targetscan.org/mmu_71/], miRDB, [available at: http://www.mirdb.org/miRDB/], and Pic-Tar, [available at: http://pictar.mdc-berlin.de/]) were employed. The results showed that miR-182-5p, miR-129-5p, and miR-450b-5p potentially target the 3′-UTR of ephb1 mRNA (fig. 1C). To experimentally validate the *in silico* predictions, luciferase reporter assays were performed. The partial 3′-UTR sequence of ephb1 containing the miRNA target regions was cloned downstream of the luciferase gene and then cotransfected with various miRNA mimics. Luciferase activity was significantly inhibited only by the miR-182-5p mimic, indicating the presence of an miR-182-5p target site in the ephb1 3′-UTR (fig. 1D). To further confirm that the decrease in luciferase activity was due to a direct interaction between the miRNA and its putative binding site, we mutated the miR-182-5p-binding site. The results showed that the suppression of luciferase activity induced by miR-182-5p cotransfection was completely abolished in this mutant construct (fig. 1E).

To determine whether these miRNAs were associated with the production of nerve injury–induced nociceptive hypersensitivity, we examined their expression after CCI. The results showed that miR-182-5p gradually declined from 1 to 14 days after CCI (fig. 1F), whereas miR-129-5p and miR-450b-5p were not significantly altered (fig. 1, G and H). The correlation analysis revealed that only miR-182-5p showed a significant inverse correlation with ephb1 expression ($R^2 = 0.90$; $P < 0.05$; fig. 1I). Finally, FISH–immunofluorescence revealed that miR-182-5p and ephb1 were coexpressed in the spine and that the decrease in miR-182-5p expression was accompanied by an increase in ephb1 expression in the CCI group compared with that of the sham group (fig. 1J). Collectively, these *in vitro* and *in vivo* findings indicated that ephb1 is a direct target of miR-182-5p.

The Up-regulation of miR-182-5p Prevented and Reversed CCI-induced Nociceptive Behaviors by Suppressing Spinal Neuronal Sensitization and ephb1 Expression

To investigate the role of miR-182-5p in the induction of nerve injury–induced nociceptive hypersensitivity, two tools, lentivirus (LV-pre-miR-182-5p) and miRNA mimics (miR-182-5p mimic), were used to up-regulate the expression of spinal miR-182-5p. The efficiency of LV-pre-miR-182-5p and the miR-182-5p mimic were validated by qPCR (fig. 2, A and B). Behavioral results showed that from 1 to 3 days after CCI, consecutive intrathecal injections of either LV-pre-miR-182-5p or the miR-182-5p mimic but not the negative control significantly prevented the induction of thermal hyperalgesia and mechanical allodynia (fig. 2, C and D). To explore the effects of miR-182-5p up-regulation on CCI-induced spinal neuronal sensitization and ephb1 expression, we collected the spinal cord and examined changes in the expression of Fos, p-ERK, and ephb1 7 days after CCI. Our data revealed that intrathecal injection of either LV-pre-miR-182-5p or the miR-182-5p mimic but not the scrambled control significantly inhibited the increased expression of spinal Fos (fig. 2E), p-ERK (fig. 2F), and ephb1 (fig. 2G) induced by CCI.

To further investigate the contribution of miR-182-5p up-regulation to the maintenance of nerve injury–induced nociceptive hypersensitivity, we posttreated mice with the miR-182-5p mimic from 7 to 9 days after CCI. The results showed that posttreatment with the miR-182-5p mimic markedly reversed the already established thermal hyperalgesia and mechanical allodynia (fig. 3A). In addition, the expression of spinal Fos (fig. 3B), p-ERK (fig. 3C), and ephb1 (fig. 3D) assessed on day 11 after CCI was strongly inhibited.

The Inhibition of miR-182-5p Facilitated CCI-induced Nociceptive Behaviors by Increasing Spinal Neuronal Sensitization and ephb1 Expression

Since miR-182-5p up-regulation inhibited CCI-induced nociceptive behaviors, does inhibiting miR-182-5p in the spinal cord facilitate nerve injury–induced nociceptive hypersensitivity? To answer this question, we intrathecally injected an miR-182-5p inhibitor into mice daily for 3 days before surgery and then assessed the effects of this injection on CCI-induced nociceptive behaviors. The results showed that the spinal administration of the miR-182-5p inhibitor but not the scrambled control significantly reduced the expression of miR-182-5p (fig. 4A) and promoted the induction of thermal hyperalgesia and mechanical allodynia by CCI (fig. 4B). We then harvested the spinal cords and assessed Fos, p-ERK, and ephb1 expression 1 day after CCI. We found that the inhibition of miR-182-5p significantly

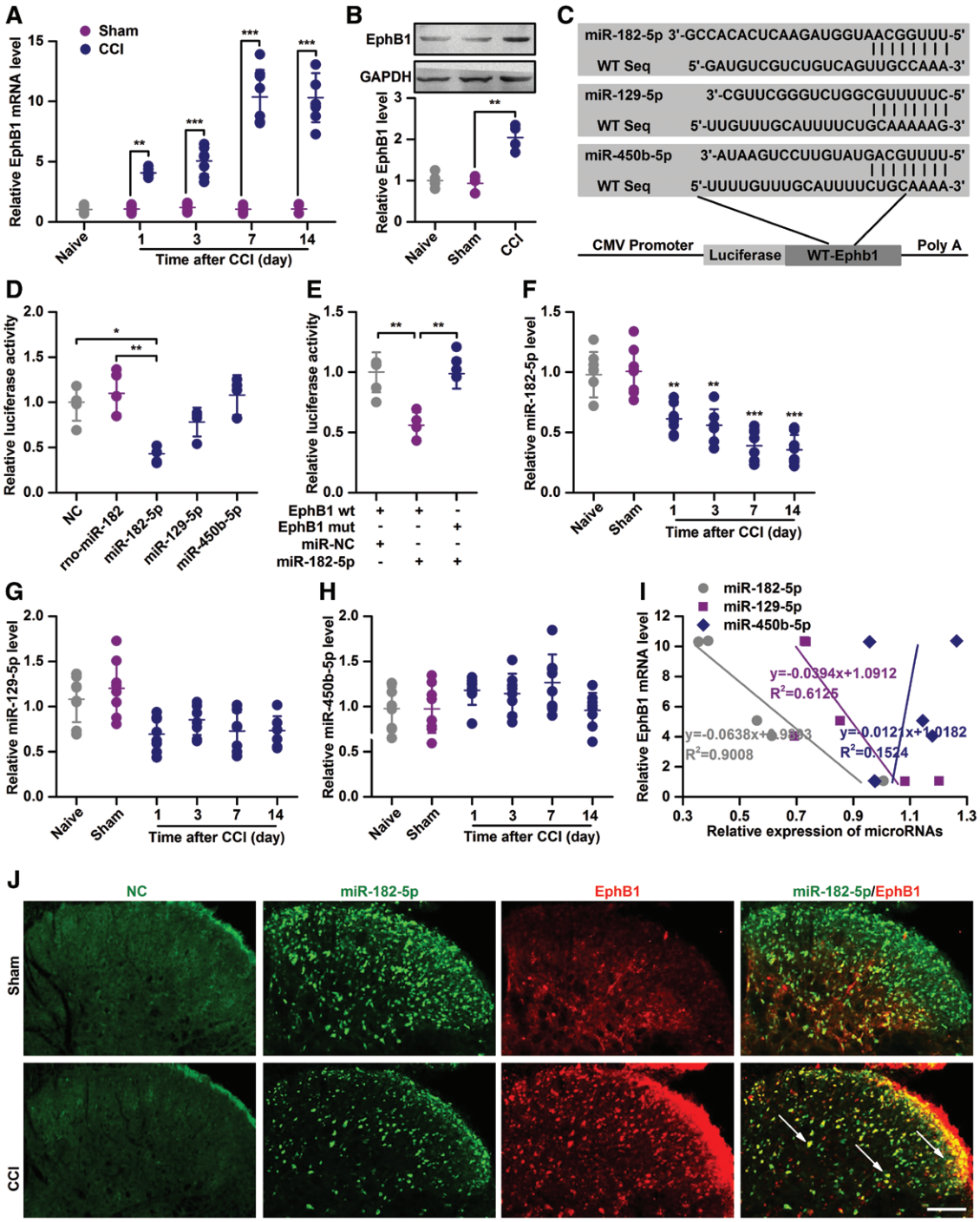

Fig. 1. Micro(mi)R-182-5p targets ephrin type-b receptor 1 (ephb1) in the spinal cord. (*A*) Real-time quantitative polymerase chain reaction showing that ephb1 messenger RNA (mRNA) increased 1, 3, 7, and 10 days after chronic constriction injury (CCI). $^{**}P < 0.01$. $^{***}P < 0.001$. n = 8 mice per group. (*B*) Western blot analysis further validated the increased expression of ephb1 by CCI on day 7. $^{**}P < 0.01$. n = 4 mice per group. (*C*) The 3′-UTR sequences of ephb1 containing the miRNA target regions were cloned downstream of the luciferase gene to generate the luciferase reporter vector. (*D*) miR-182-5p mimic but not miR-129-5p or miR-450b-5p decreased the luciferase activity in HEK293 cells transfected with the ephb1 3′-UTR. $^{*}P < 0.05$. $^{**}P < 0.01$. n = 4 in each group. (*E*) The decreased luciferase activity induced by transfection with miR-182-5p was completely abolished with the mutant ephb1 3′-UTR vector. $^{**}P < 0.01$. n = 4 in each group. (*F–H*) The expression of miR-182-5p, not miR-129-5p and miR-450b-5p, was decreased 1, 3, 7, and 14 days after CCI. $^{**}P < 0.01$. $^{***}P < 0.001$. n = 8 mice per group. (*I*) Correlation analysis showed that miR-182-5p expression was negatively correlated with ephb1 mRNA expression. $R^2 = 0.90$; $^{*}P < 0.05$. *Diamonds*, *squares*, and *circles* represent groups of individuals (mean value) across time. (*J*) Combined fluorescence *in situ* hybridization and immunofluorescence showed that miR-182-5p colocalized with ephb1 in the spinal cord. *Scale bar* = 25 μm.

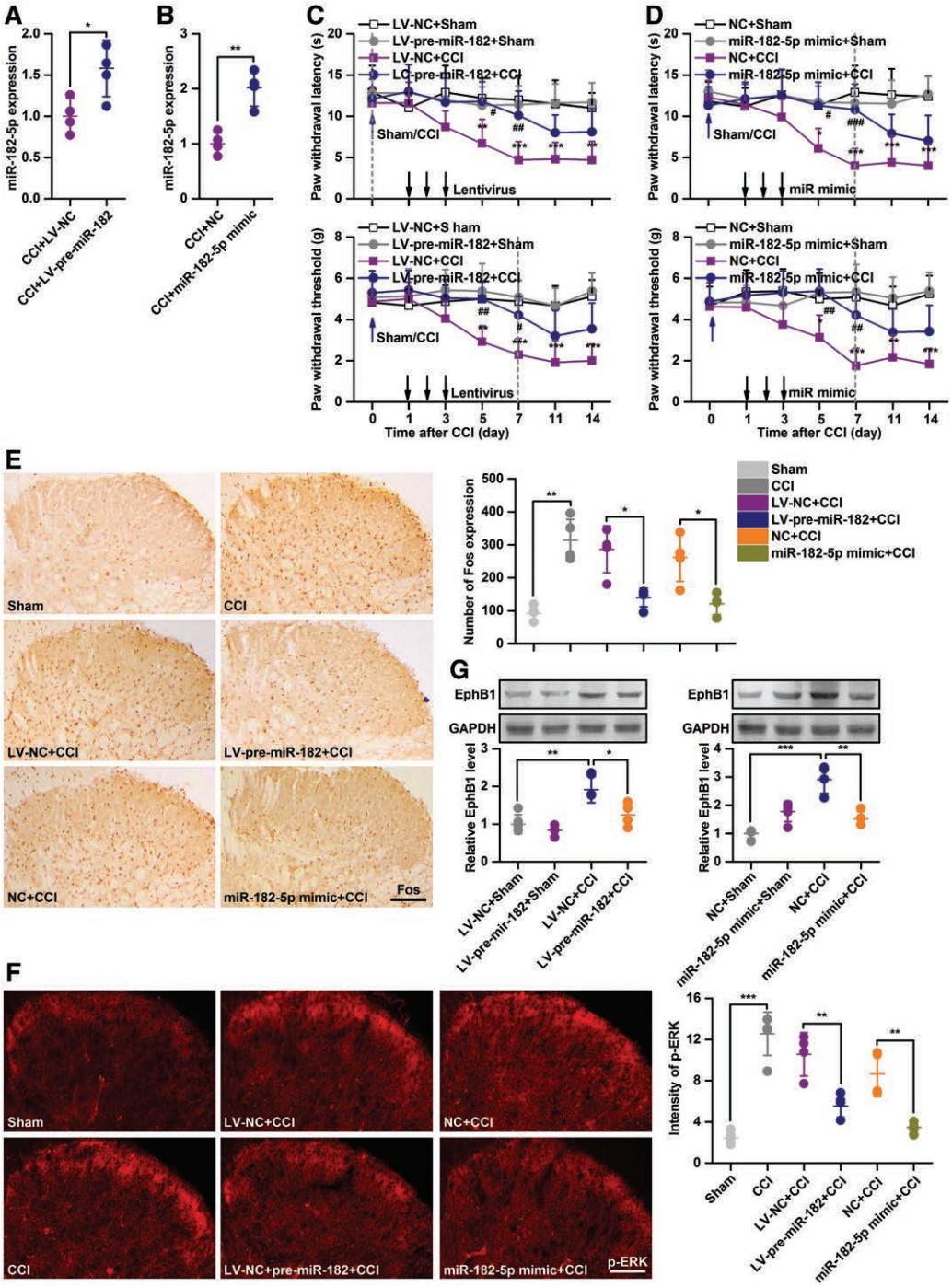

Fig. 2. The up-regulation of micro(mi)R-182-5p prevents nerve injury–induced nociceptive hypersensitivity by inhibiting spinal neuronal sensitization and ephrin type-b receptor 1 (ephb1) expression. (*A*, *B*) The validation of lenti-pre-miR-182 or miR-182-5p mimic transfection efficiency *in vivo*. **P* < 0.05. ***P* < 0.01. n = 4 mice per group. (*C*, *D*) Pretreatment with lenti-pre-miR-182 (intrathecal, 1×10^5 transducing unit/1 μl) or miR-182-5p mimic (intrathecal, 5 μg) for 3 consecutive days (from day 1 to day 3 after chronic constriction injury [CCI]) prevented CCI-induced thermal hyperalgesia and mechanical allodynia. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *versus* the lentiviruses-negative control (LV-NC) + sham or NC + sham group; #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001, *versus* the LV-NC + CCI or NC + CCI group; n = 6 mice per group. (*E*) Immunohistochemical staining showing that treatment with lenti-pre-miR-182 or miR-182-5p mimic significantly inhibited the increased spinal Fos expression induced by CCI. **P* < 0.05, ***P* < 0.01. n = 4 mice per group. (*F*) Immunofluorescence staining showing that treatment with lenti-pre-miR-182 or miR-182-5p mimic significantly inhibited the increased spinal phosphorylated ERK (p-ERK) expression induced by CCI. n = 4 mice per group. (*G*) Western blot showing that lenti-pre-miR-182 or miR-182-5p mimic treatment depressed the increased spinal ephb1 expression induced by CCI. **P* < 0.05. ***P* < 0.01. ****P* < 0.001. n = 4 mice per group. Fos, p-ERK, and ephb1 were measured on day 7 after CCI. LV-pre-miR-182-5p, recombinant lentiviruses containing pre-miRNA-182-5p.

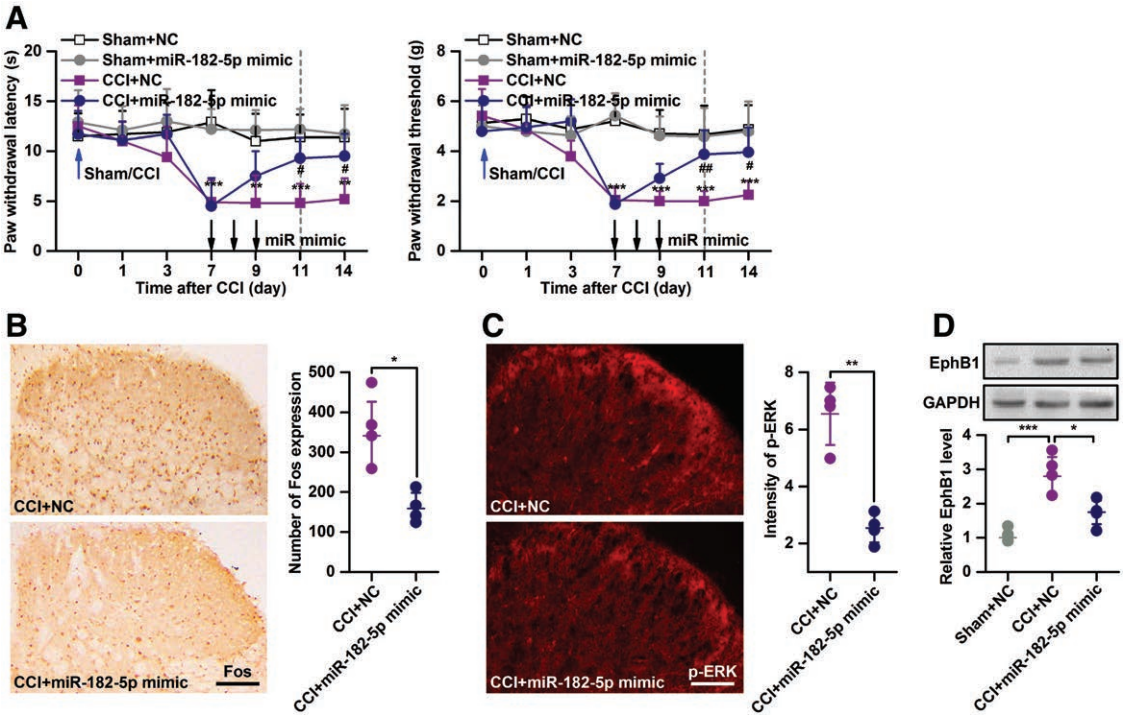

Fig. 3. Micro(mi)R-182-5p up-regulation reverses chronic constriction injury (CCI)–induced nociceptive behaviors by suppressing spinal neuronal sensitization and ephrin type-b receptor 1 (ephb1) expression. (*A*) Posttreatment with miR-182-5p mimic (intrathecal, 5 μg) for 3 consecutive days (from day 7 to day 9 after CCI) significantly reduced CCI-induced thermal hyperalgesia and mechanical allodynia. $^{**}P < 0.01$, $^{***}P < 0.001$, *versus* the sham + negative control (NC) group; $\#P < 0.05$, $\#\#P < 0.01$, *versus* the CCI + NC group; n = 6 mice per group. (*B*) Immunohistochemical staining showing that posttreatment with lenti-pre-miR-182 or miR-182-5p mimic significantly inhibited the increased spinal Fos expression induced by CCI. $^{*}P < 0.05$. n = 4 mice per group. (*C*) Immunofluorescence staining showing that posttreatment with lenti-pre-miR-182 or miR-182-5p mimic significantly inhibited the increased spinal phosphorylated ERK (p-ERK) expression induced by CCI. n = 4 mice per group. (*D*) Western blot showing that lenti-pre-miR-182 or miR-182-5p mimic posttreatment depressed the increased spinal ephb1 expression induced by CCI. $^{*}P < 0.05$. $^{***}P < 0.001$. n = 4 mice per group. Fos, p-ERK, and ephb1 were measured on day 11 after CCI. LV-pre-miR-182-5p, recombinant lentiviruses containing pre-miRNA-182-5p.

increased the expression of spinal Fos (fig. 4C), p-ERK (fig. 4D), and ephb1 (fig. 4E) induced by CCI.

To further determine the role of ephb1 in mediating nociceptive hypersensitivity after inhibition of miR-182-5p, we pretreated or posttreated animals with siRNA to knock down ephb1 before or after intrathecal injection with an miR-182-5p inhibitor and then measured their behavioral responses. We found that knocking down ephb1 significantly inhibited or reversed the thermal hyperalgesia and mechanical allodynia induced by inhibiting spinal miR-182-5p in CCI mice (fig. 4, F and G), suggesting that the increased expression of ephb1 mediated the nociceptive behaviors induced by the inhibition of miR-182-5p. Moreover, treatment with ephb1-Fc to block the spinal ephb1 receptor also inhibited or reduced the thermal hyperalgesia and mechanical allodynia induced by the inhibition of spinal miR-182-5p in CCI mice (fig. 4, H and I). These findings, combined with previous results, suggest that miR-182-5p contributes to the modulation of nerve injury–induced nociceptive hypersensitivity by targeting the ephb1 receptor.

miR-182-5p Down-regulation and ephb1 Up-regulation after CCI Were Mediated by the NMDA Receptor

NMDA receptor activation in the spinal cord is usually considered to be the trigger for nerve injury–induced central sensitization and nociceptive behaviors.[16–18] Therefore, we investigated whether the NMDA receptor was responsible for the down-regulation of miR-182-5p and up-regulation of ephb1 in the spinal cord after CCI. Our results showed that pretreatment with MK801, which is a noncompetitive NMDA receptor antagonist, significantly prevented both the down-regulation of miR-182-5p and the up-regulation of ephb1 after CCI (fig. 5, A and B). In contrast, spinal administration of NMDA decreased spinal miR-182-5p and ephb1 expression in naive mice (fig. 5, C and D). These findings indicate that CCI-induced miR-182-5p down-regulation and ephb1 up-regulation are mediated by the NMDA receptor.

Discussion

Previous studies have indicated that up-regulation of the ephb1 receptor in the spinal cord is required for the

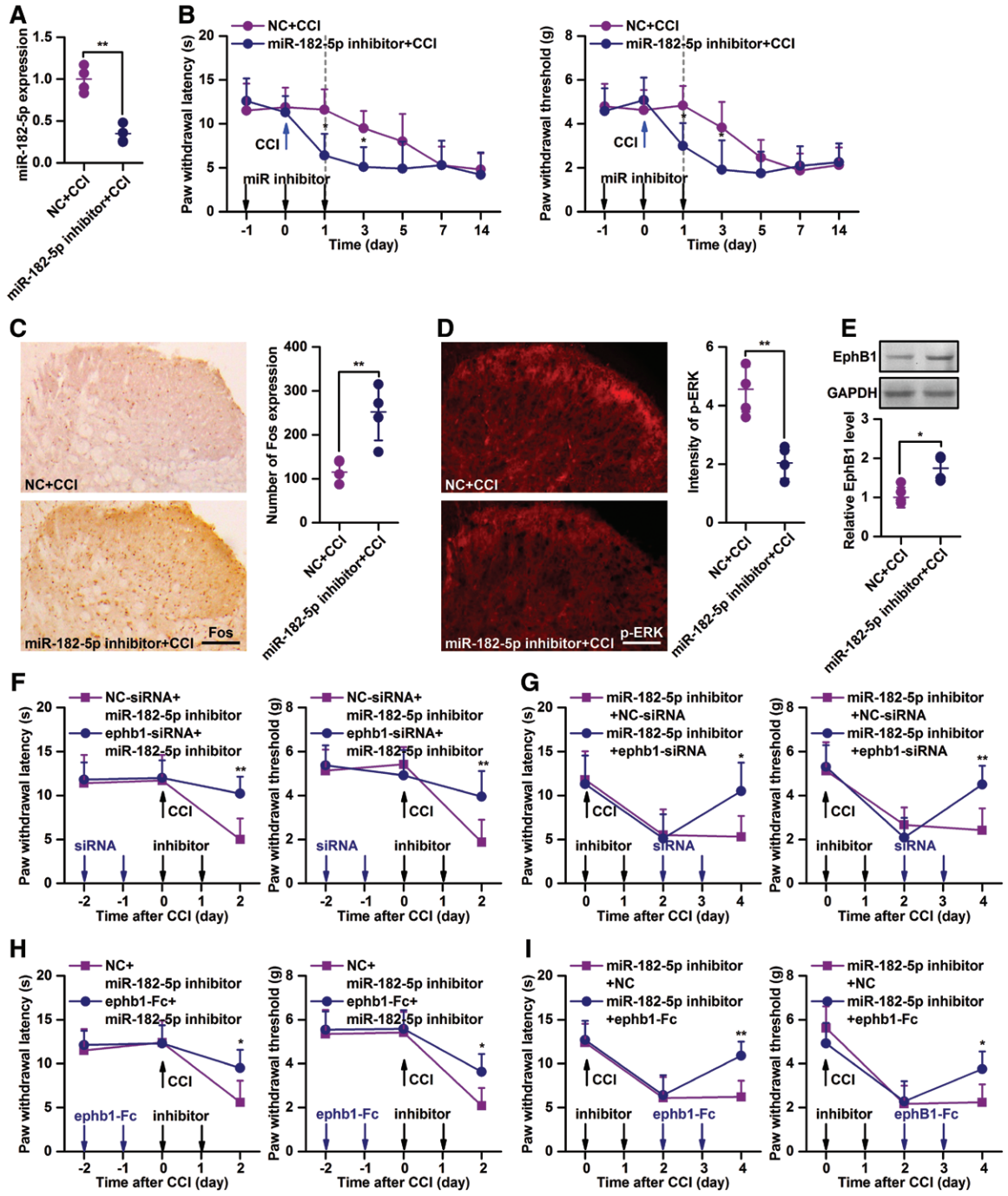

Fig. 4. miR-182-5p inhibition facilitates chronic constriction injury (CCI)–induced nociceptive behaviors by increasing spinal neuronal sensitization and ephrin type-b receptor 1 (ephb1) expression. (*A*) The validation of miR-182-5p inhibitor transfection efficiency *in vivo.* $^{**}P < 0.01$. n = 4 mice per group. (*B*) Intrathecal injection of miR-182-5p inhibitor (intrathecal, 5 μg) for 3 consecutive days before CCI promoted the induction of CCI-induced thermal hyperalgesia and mechanical allodynia. $^{*}P < 0.05$, *versus* the negative control (NC) group, n = 6 mice per group. (*C*) Immunohistochemical staining showing that miR-182-5p inhibitor administration increased spinal Fos expression. $^{**}P < 0.01$. n = 4 mice per group. (*D*) Immunofluorescence staining showing that miR-182-5p inhibitor administration increased spinal phosphorylated ERK (p-ERK) expression. n = 4 mice per group. (*E*) Western blot showing that miR-182-5p inhibitor administration increased spinal ephb1 expression. $^{*}P < 0.05$. n = 4 mice per group. Fos and ephb1 were measured on day 1 after CCI. (*F*, *G*) Knockdown of ephb1 by siRNA (5 μg) significantly inhibited or reduced thermal hyperalgesia and mechanical allodynia induced by the miR-182-5p inhibitor in CCI mice. $^{*}P < 0.05$. $^{**}P < 0.01$. n = 6 mice per group. (*H*, *I*) Blockade of ephb1 by ephb1-Fc (0.5 μg) significantly inhibited or reduced thermal hyperalgesia and mechanical allodynia induced by the miR-182-5p inhibitor in CCI mice. $^{*}P < 0.05$. $^{**}P < 0.01$. n = 6 mice per group.

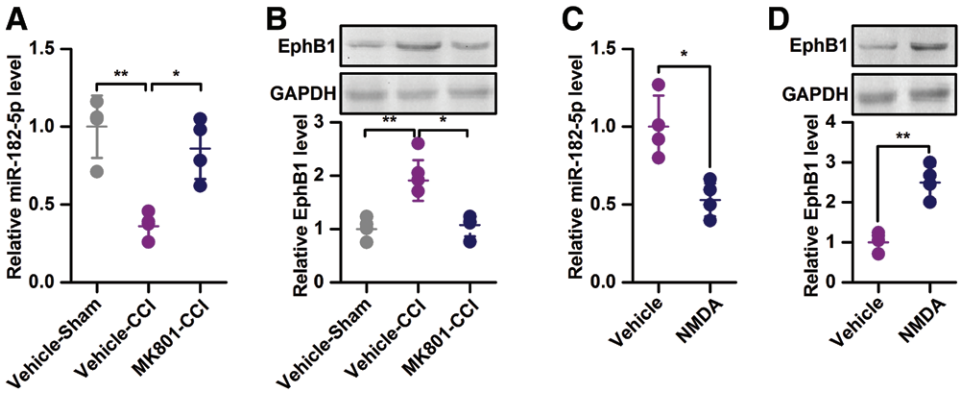

Fig. 5. Micro(mi)R-182-5p down-regulation and ephrin type-b receptor 1 (ephb1) up-regulation induced by chronic constriction injury (CCI) are mediated by the *N*-methyl-D-aspartate (NMDA) receptor. (*A* and *B*) Intrathecal injection of MK801 (2.5 μg, from day 1 to day 3 after CCI) prevented CCI-induced miR-182-5p down-regulation (*A*) and ephb1 up-regulation (*B*) in the spinal cord. **P* < 0.05. ***P* < 0.01. n = 4 mice per group. miR-182-5p and ephb1 were measured on day 7 after CCI. (*C* and *D*) Intrathecal injection of NMDA (0.5 μg) for 3 days in naive mice decreased miR-182-5p expression (*C*) and increased ephb1 expression (*D*). **P* < 0.05. ***P* < 0.01. n = 4 mice per group. miR-182-5p and ephb1 were measured on day 4 after NMDA injection.

development and maintenance of nerve injury–induced nociceptive hypersensitivity. The current study further revealed that this aberrant expression of spinal ephb1 induced by peripheral nerve injury is modulated by miR-182-5p.

miRNA in the Spinal Cord Contributed to Nerve Injury–induced Nociceptive Hypersensitivity

Central sensitization, which is characterized by an increase in neuronal excitability, is an important mechanism in nerve injury–induced nociceptive hypersensitivity.[19] The induction and maintenance of central sensitization largely depend on the aberrant expression of ion channels, receptors, and intracellular signaling pathways.[19] However, the regulatory mechanisms controlling this abnormal gene expression remain unclear. Recently, increasing studies have indicated that miRNAs, as regulators of gene expression, are associated with a variety of neurophysiologic and neuropathologic processes such as synaptic development, neuronal plasticity, and CNS disease.[20–22] In addition, there is also a strong connection between miRNAs and nociceptive sensitization.[10] In the dorsal root ganglion, conditional deletion of the Dicer enzyme, which is important for miRNA processing, results in a significant decrease in the inflammatory nociceptive response.[23] Inhibition of miR-195 in the spinal cord has been reported to attenuate nerve injury–induced allodynia and hyperalgesia,[24] whereas increasing the expression of miR-23b led to exaggerated nerve injury–induced hyperalgesia.[25] These findings demonstrate that miRNAs are a key regulator of nociceptive hypersensitivity. In the current study, by using a CCI-induced nerve injury model, we found that spinal miR-182-5p expression gradually decreased after the development of nerve injury–induced nociceptive hypersensitivity and that up-regulating miR-182-5p prevented and reversed nerve injury–induced allodynia and hyperalgesia, suggesting a correlation between miR-182-5p and nerve injury–induced nociceptive hypersensitivity.

Spinal miR-182-5p Regulates Nerve Injury–induced Nociceptive Hypersensitivity by Targeting the ephb1 Receptor

miRNAs do not encode any proteins; instead, they typically bind to the 3′-UTRs of mRNAs to regulate gene expression posttranscriptionally.[26] In the current study, although bioinformatics analysis identified several miRNAs that target the 3′-UTR of ephb1 mRNA, miR-182-5p was shown to be the most likely candidate. The sequence alignments of miR-182-5p and its binding site in the 3′-UTR of ephb1 is highly conserved in mammals, especially in the seed region, which showed 100% conservation. miR-182-5p but not miR-129-5p or miR-450b-5p reduced the luciferase activity of the ephb1 reporter gene. Only miR-182-5p was inversely correlated with the expression of ephb1 after CCI. FISH–immunofluorescence also revealed that miR-182-5p colocalized with ephb1 in the spine. Furthermore, up-regulating miR-182-5p in the spinal cord decreased ephb1 expression and reduced CCI-induced nociceptive behaviors as well as spinal neuronal sensitization. In contrast, inhibiting miR-182-5p increased the expression of spinal ephb1 and facilitated CCI-induced nociceptive behaviors. These results indicated that spinal miR-182-5p is involved in the modulation of nerve injury–induced nociceptive hypersensitivity by targeting ephb1.

NMDA Receptor–mediated miR-182-5p Down-regulation and ephb1 Up-regulation in Nerve Injury–induced Nociceptive Hypersensitivity

Although increasing evidence supports the alteration of miRNA expression in nociceptive hypersensitivity, little is known about how miRNA gene expression is regulated in this process. Under pain conditions, the NMDA receptor is known to be important for triggering nociceptive behavior–related plasticity.[27,28] The induction of nociceptive sensitization is usually accompanied by the increased activation and expression of the NMDA receptor in the spinal cord.[17,18]

Therefore, NMDA receptor activation may be the upstream mechanism causing miR-182-5p alterations after CCI. In this study, we found that spinal administration of MK801, which is a noncompetitive NMDA receptor antagonist, markedly decreased spinal miR-182-5p and ephb1 expression. In contrast, intrathecal injection of NMDA decreased spinal miR-182-5p and ephb1 expression in naive mice. These data imply that miR-182-5p down-regulation and ephb1 up-regulation are mediated by NMDA receptors during peripheral nerve injury. A recent study by Jiang *et al.*[14] found that the NMDA receptor is also critical for the down-regulation of miR-186-5p after spinal nerve ligation. Blockade of the NMDA receptor prevented the down-regulation of miR-186-5p and the subsequent up-regulation of C-X-C motif chemokine 13. Therefore, NMDA receptor–mediated miRNA regulation may be involved in mediating the pathologies associated with nerve injury–induced nociceptive hypersensitivity. Interestingly, previous studies have indicated that ephb1 receptor activation enhances the activity of the NMDA receptor during peripheral nerve injury. Therefore, we propose a vicious cycle involved in nerve injury–induced nociceptive hypersensitivity that includes the NMDA receptor, miR-182-5p, and the ephb1 receptor.

miR-182 and Other Diseases

Emerging evidence has shown that miR-182, as a posttranscriptional regulator, plays an important role in the regulation of gene expression. For example, in breast cancer cells, the miR-182-mediated down-regulation of BRCA1 impedes DNA repair and may impact breast cancer therapy.[29] miR-182-5p protects inner ear hair cells from cisplatin-induced apoptosis by inhibiting FOXO3a gene expression.[30] Aberrant miR-182 expression promotes melanoma metastasis by repressing FOXO3 and microphthalmia-associated transcription factor.[31] Moreover, recent studies have also reported that miR-182 may be involved in some nervous system diseases, including glioma tumorigenesis,[32] diabetic corneal nerve regeneration,[33] and Schwann cell proliferation and migration after sciatic nerve injury.[34] The current study broadens our knowledge of the functional role of miR-182 in the CNS. In the current study, for the first time, we found that spinal miR-182-5p was involved in the modulation of nerve injury–induced nociceptive hypersensitivity by using a CCI-induced nerve injury model.

There are several limitations in this study. First, miR-182 expression patterns were not colocalized by cell type, which certainly warrants further investigation. Second, luciferase reporter assays were performed in human embryonic kidney cells, which may not hold the same relevance in CNS-derived cell lines. Third, the effect of miR-182-5p on nerve injury–induced nociceptive hypersensitivity was our primary focus; thus, effects on other models, such as inflammation or cancer-induced nociceptive hypersensitivity, were not assessed.

In conclusion, the current study demonstrated that the decrease in spinal miR-182-5p was involved in the modulation of nerve injury–induced nociceptive hypersensitivity through the ephb1 receptor. This finding may lead to the discovery of novel drug targets for the prevention and treatment of nerve injury–induced nociceptive hypersensitivity.

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Competing Interests

The authors declare no competing interests.

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References

1. Lin L, Lesnick TG, Maraganore DM, Isacson O: Axon guidance and synaptic maintenance: Preclinical markers for neurodegenerative disease and therapeutics. Trends Neurosci 2009; 32:142–9
2. Klein R: Eph/ephrin signaling in morphogenesis, neural development and plasticity. Curr Opin Cell Biol 2004; 16:580–9
3. Vasileiou I, Adamakis I, Patsouris E, Theocharis S: Ephrins and pain. Expert Opin Ther Targets 2013; 17:879–87
4. Kobayashi H, Kitamura T, Sekiguchi M, Homma MK, Kabuyama Y, Konno S, Kikuchi S, Homma Y: Involvement of EphB1 receptor/EphrinB2 ligand in neuropathic pain. Spine (Phila Pa 1976) 2007; 32:1592–8
5. Song XJ, Cao JL, Li HC, Zheng JH, Song XS, Xiong LZ: Upregulation and redistribution of ephrinB and EphB receptor in dorsal root ganglion and spinal dorsal horn neurons after peripheral nerve injury and dorsal rhizotomy. Eur J Pain 2008; 12:1031–9
6. Song XJ, Zheng JH, Cao JL, Liu WT, Song XS, Huang ZJ: EphrinB-EphB receptor signaling contributes to neuropathic pain by regulating neural excitability and spinal synaptic plasticity in rats. Pain 2008; 139:168–80
7. Ruan JP, Zhang HX, Lu XF, Liu YP, Cao JL: EphrinBs/EphBs signaling is involved in modulation of spinal nociceptive processing through a mitogen-activated protein kinases-dependent mechanism. Anesthesiology 2010; 112:1234–49
8. Yu LN, Zhou XL, Yu J, Huang H, Jiang LS, Zhang FJ, Cao JL, Yan M: PI3K contributed to modulation of spinal nociceptive information related to ephrinBs/EphBs. PLoS One 2012; 7:e40930
9. Ambros V: microRNAs: Tiny regulators with great potential. Cell 2001; 107:823–6
10. Andersen HH, Duroux M, Gazerani P: MicroRNAs as modulators and biomarkers of inflammatory and neuropathic pain conditions. Neurobiol Dis 2014; 71:159–68
11. Lutz BM, Bekker A, Tao YX: Noncoding RNAs: New players in chronic pain. Anesthesiology 2014; 121:409–17

12. Favereaux A, Thoumine O, Bouali-Benazzouz R, Roques V, Papon MA, Salam SA, Drutel G, Léger C, Calas A, Nagy F, Landry M: Bidirectional integrative regulation of Cav1.2 calcium channel by microRNA miR-103: Role in pain. EMBO J 2011; 30:3830–41
13. Lu Y, Cao DL, Jiang BC, Yang T, Gao YJ: MicroRNA-146a-5p attenuates neuropathic pain via suppressing TRAF6 signaling in the spinal cord. Brain Behav Immun 2015; 49:119–29
14. Jiang BC, Cao DL, Zhang X, Zhang ZJ, He LN, Li CH, Zhang WW, Wu XB, Berta T, Ji RR, Gao YJ: CXCL13 drives spinal astrocyte activation and neuropathic pain via CXCR5. J Clin Invest 2016; 126:745–61
15. Zhou XL, Wang Y, Zhang CJ, Yu LN, Cao JL, Yan M: PKA is required for the modulation of spinal nociceptive information related to ephrinB-EphB signaling in mice. Neuroscience 2015; 284:546–54
16. Zhuo M: Long-term potentiation in the anterior cingulate cortex and chronic pain. Philos Trans R Soc Lond B Biol Sci 2014; 369:20130146
17. Woolf CJ, Thompson SW: The induction and maintenance of central sensitization is dependent on N-methyl-D-aspartic acid receptor activation; implications for the treatment of post-injury pain hypersensitivity states. Pain 1991; 44:293–9
18. Petrenko AB, Yamakura T, Baba H, Shimoji K: The role of N-methyl-D-aspartate (NMDA) receptors in pain: A review. Anesth Analg 2003; 97:1108–16
19. Latremoliere A, Woolf CJ: Central sensitization: A generator of pain hypersensitivity by central neural plasticity. J Pain 2009; 10:895–926
20. Kosik KS: The neuronal microRNA system. Nat Rev Neurosci 2006; 7:911–20
21. Bicchi I, Morena F, Montesano S, Polidoro M, Martino S: MicroRNAs and molecular mechanisms of neurodegeneration. Genes (Basel) 2013; 4:244–63
22. Ksiazek-Winiarek DJ, Kacperska MJ, Glabinski A: MicroRNAs as novel regulators of neuroinflammation. Mediators Inflamm 2013; 2013:172351
23. Zhao J, Lee MC, Momin A, Cendan CM, Shepherd ST, Baker MD, Asante C, Bee L, Bethry A, Perkins JR, Nassar MA, Abrahamsen B, Dickenson A, Cobb BS, Merkenschlager M, Wood JN: Small RNAs control sodium channel expression, nociceptor excitability, and pain thresholds. J Neurosci 2010; 30:10860–71
24. Shi G, Shi J, Liu K, Liu N, Wang Y, Fu Z, Ding J, Jia L, Yuan W: Increased miR-195 aggravates neuropathic pain by inhibiting autophagy following peripheral nerve injury. Glia 2013; 61:504–12
25. Im YB, Jee MK, Choi JI, Cho HT, Kwon OH, Kang SK: Molecular targeting of NOX4 for neuropathic pain after traumatic injury of the spinal cord. Cell Death Dis 2012; 3:e426
26. Dong H, Lei J, Ding L, Wen Y, Ju H, Zhang X: MicroRNA: Function, detection, and bioanalysis. Chem Rev 2013; 113:6207–33
27. Wu LJ, Zhuo M: Targeting the NMDA receptor subunit NR2B for the treatment of neuropathic pain. Neurotherapeutics 2009; 6:693–702
28. Zhuo M: Plasticity of NMDA receptor NR2B subunit in memory and chronic pain. Mol Brain 2009; 2:4
29. Moskwa P, Buffa FM, Pan Y, Panchakshari R, Gottipati P, Muschel RJ, Beech J, Kulshrestha R, Abdelmohsen K, Weinstock DM, Gorospe M, Harris AL, Helleday T, Chowdhury D: miR-182-mediated downregulation of BRCA1 impacts DNA repair and sensitivity to PARP inhibitors. Mol Cell 2011; 41:210–20
30. Li Y, Li A, Wu J, He Y, Yu H, Chai R, Li H: MiR-182-5p protects inner ear hair cells from cisplatin-induced apoptosis by inhibiting FOXO3a. Cell Death Dis 2016; 7:e2362
31. Segura MF, Hanniford D, Menendez S, Reavie L, Zou X, Alvarez-Diaz S, Zakrzewski J, Blochin E, Rose A, Bogunovic D, Polsky D, Wei J, Lee P, Belitskaya-Levy I, Bhardwaj N, Osman I, Hernando E: Aberrant miR-182 expression promotes melanoma metastasis by repressing FOXO3 and microphthalmia-associated transcription factor. Proc Natl Acad Sci USA 2009; 106:1814–9
32. Xue J, Zhou A, Wu Y, Morris SA, Lin K, Amin S, Verhaak R, Fuller G, Xie K, Heimberger AB, Huang S: miR-182-5p induced by STAT3 activation promotes glioma tumorigenesis. Cancer Res 2016; 76:4293–304
33. Wang Y, Zhao X, Wu X, Dai Y, Chen P, Xie L: microRNA-182 mediates Sirt1-induced diabetic corneal nerve regeneration. Diabetes 2016; 65:2020–31
34. Yu B, Qian T, Wang Y, Zhou S, Ding G, Ding F, Gu X: miR-182 inhibits Schwann cell proliferation and migration by targeting FGF9 and NTM, respectively at an early stage following sciatic nerve injury. Nucleic Acids Res 2012; 40:10356–65