RESEARCH PAPER

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miR-488-3p alleviates neuropathic pain by regulating target gene ROCK1

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The function of microRNA (miRNA) in neuropathic pain (NP) has received widespread attention. The current research sought to address the contribution of miR-488-3p in NP and its downstream mechanisms. The NP rat model was constructed by chronic constriction injury (CCI) surgery in rats. Regulation of miR-488-3p or Rho-associated coiled-coil-containing protein kinase 1 (ROCK1) in rats by intrathecal injection of lentivirus or plasmid. Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) to examine the levels of miR-488-3p and ROCK1 in the dorsal root ganglion (DRG). Enzyme-linked immunosorbent assay (ELISA) to monitor the secretion of pro-inflammatory and anti-inflammatory factors. Paw withdrawal threshold (PWT) and paw withdrawal latency (PWL) for the evaluation of mechanosensitive and thermal nociceptive hypersensitivity of NP behaviors. Validation of molecular mechanism between miR-488-3p and ROCK1 using RNA immunoprecipitation assay and dual-luciferase reporter (DLR) assay. miR-488-3p was vigorously less expressed in the DRGs of CCI rats, while ROCK1 was upregulated (P<0.05). Elevated miR-488-3p alleviated the decrease of PWL and PWT in CCI rats, inhibited the secretion of pro-inflammatory factors, and enhanced anti-inflammatory factors levels. Mechanistically, ROCK1 was the target of miR-488-3p. Raised ROCK1 partially attenuated the mitigating effect of miR-488-3p on NP behavior and the suppression of inflammatory responses in rats (P<0.05). Current research demonstrated that miR-488-3p may be a novel therapeutic target for NP.

Key words: neuropathic pain, miR-488-3p, ROCK1, inflammation

INTRODUCTION

Neuropathic pain (NP) is a chronic disease caused by damage and dysfunction of the peripheral and central nervous system, including spontaneous pain (persistent pain and paroxysmal pain) and evoked pain (nociceptive hypersensitivity) (Dai et al., 2020). The prevalence of NP is as high as 7-10% (Gada et al., 2021) and causes losses of $600 billion annually (Hu et al., 2021). NP is characterized by complex pathogenesis, inadequate existing treatment options, and low efficiency of clinical intervention. Therefore, a better insight into the underlying molecular mechanisms of NP is necessary to develop newer and more effective therapeutic approaches.

MicroRNAs (miRNAs) are endogenous non-coding single-stranded RNAs that regulate gene expression by suppressing translation or promoting the degradation of target mRNAs. miRNAs are aberrantly expressed in tumors (Luo et al., 2023), NP (Hori et al., 2016), atherosclerosis (Li et al., 2021a), and diabetes (Ban et al., 2020). miR-488 is located on human chromosome 1q25.2 and previous studies have proven its potential neuroprotective effects. miR-488-3p is engaged in regulating the progression of glioma (Xue et al., 2018) and neuroblastoma (Muinos-Gimeno et al., 2011). Diminished miR-488-3p promotes neuronal damage and neuro-inflammation occurrence in epilepsy (Wen et al., 2023). Meanwhile, upregulated miR-488-3p exerts neuroprotective effects in ischemic stroke by inhibit-

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ing neuronal cell death (Zhou et al., 2021). Additionally, miR-488-3p was typically downregulated in spinal cord injury, a common complication of NP (Li et al., 2021b). Most importantly, small RNA sequencing performed by Dai et al. in 2019 revealed differentially expressed miRNAs in the L3-L6 dorsal root ganglion (DRG) of NP rats and found miR-488-3p to be significantly downregulated. Nevertheless, the specific actions and potential mechanisms of miR-488-3p in NP are not clear.

Here we explore the possible role of miR-488-3p in NP and its potential mechanism of action, to provide a reference for the effective treatment of NP.

METHODS

Animals

Sprague-Dawley (SD) rats (male, weight 200-250 g) were obtained from the Shanghai Animal Experimental Center. All rats were kept in a sterile environment with 24 ±3°C, natural light/dark cycle, and cage changes twice a week. Animal experiments were approved by the Institutional Animal Care and Use Committee of Xiangtan Central Hospital (approval No. 2021-05--011) and executed in compliance with NIH guidelines for the use and care of animals and to minimize discomfort in rats.

Experimental grouping

To examine the potential effect of miR-488-3p in NP, 40 SD rats were divided into 4 groups (10 rats/per group) according to the random number table method: Sham group, Chronic Constriction Injury (CCI) group, CCI+LV-miR-448-3p, and CCI+LV-NC group. A second batch of animal studies was conducted to explore the levels of Rho-associated coiled-coil-containing protein kinase 1 (ROCK1) in NP. 40 SD rats were divided into the Sham group, CCI+LV-NC group, CCI+LV-miR-488-3p+oe-NC, and CCI+LV-miR-488-3p+oe-ROCK1 group. Among them, lentiviral vector overexpressing miR-488-3p (LV-miR-488-3p), negative control viral vector (LV-NC), ROCK1 overexpressing plasmid pcDNA3.1-ROCK1 (oe-ROCK1) and its negative control pcDNA3.1-NC (oe-NC) were constructed by GenePharm. Lentiviral (5 μL at a titer of 3.0×10^8 TU/mL) and plasmid (3 μL at 0.005 mg/μL) intrathecal injections were performed every 24 h before 72 h of model construction according to previous studies (Qiu et al., 2020; Li et al., 2021c).

Intrathecal injection

Rats were anesthetized by intraperitoneal injection of pentobarbital (40 mg/kg) and placed on the operating table. A PE-10 polyethylene catheter was inserted into the spinal subarachnoid space to L4-L6. Bilateral hind limbs were paralyzed by intrathecal injection of 2% lidocaine (Xylocaine, Astra Zeneca), and successful catheter insertion was confirmed when the hind limbs were paralyzed within 30 s. The lentivirus and plasmid are injected into the rat through a microinjector linked to an intrathecal catheter. Sham group rats were injected intrathecally with an equal volume of saline as the control group.

CCI rat model

The CCI model construction is based on previous studies. Rats were anesthetized by intraperitoneal injection of pentobarbital (40 mg/kg). The right hind leg hair was shaved off with an electric razor and disinfected with 75% alcohol. A 2 cm skin incision was made at the outer edge of the femur in the middle of the right thigh. The fascia and muscles were bluntly separated to disclose the sciatic nerve trunk. Four ligatures were performed with 4-0 non-absorbable silk threads (ETHICON, China), each spaced about 1 mm apart, leaving only constriction marks without blocking the blood supply, and a slight tremor of the leg muscles was sufficient. Finally, the surgical incision is sutured and placed on a heating pad to help the recovery of body temperature. The successful construction of the CCI-induced NP rat model was confirmed when the rats showed lameness, spontaneous foot lifting, and spontaneous, and when there was a significant decrease in the pain behavior indexes of the rats (Zhong et al., 2018).

Behavioral testing

Assessment of rat mechanosensitive by paw withdrawal threshold (PWT) at days 0, 3, 7, 14, and 24 after CCI surgery. Rats were positioned in a plexiglass viewing chamber with a metal mesh bottom, and calibrated Von Frey wires were attached to the plantar surface of the hind paws. The diameter of the filaments was recorded when the rat retracted the claw. Assessment of thermal nociceptive hypersensitivity in rats by radiometric pyrometry detection of paw withdrawal latency (PWL). Rats were placed in plexiglass boxes and subjected to thermal radiation stimulation at intervals of 20 s to prevent tissue damage. PWL was

defined as the time interval between stimulus onset and hid paw retraction, with sudden paw retraction, licking, and shaking all considered positive responses. At the end of the behavioral test, rats were euthanized by intraperitoneal injection of an overdose of sodium pentobarbital (200 mg/kg), and the right L4-L5 dorsal root ganglion (DRG) was dissected for subsequent testing.

Real-time quantitative reverse transcription polymerase-chain-reaction (RT-qPCR)

RNAiso plus (Takara, Japan) was added to DRG tissues to extract and isolate total RNA, and the NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, USA) monitored RNA quality. 1 μg RNA was reversed to cDNA through the FastKing gDNA Dispelling RT SuperMix kit (TIANGEN) or micute Plus miRNA First-Strand cDNA kit (TIANGEN). The cDNA was then mixed with primer and SuperReal PreMix Plus (SYBR Green) kit (TIANGEN) or miRcute Plus miRNA qPCR Kit (SYBR Green) kit (TIANGEN, China) and amplified on Step one plus (Applied Biosystem, USA). U6 and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were conducted as a reference for normalizing data at the miRNA and target gene levels respectively. Each sample was tested in triplicate and calculated using the $2^{-\Delta\Delta Ct}$ method. The RT-qPCR cycling conditions consisted of 95°C for 2 min; then 35 cycle amplification for 20 s at 95°C, 30 s at 55°C, and 15 s at 72°C; followed by 1 min at 72°C. The primer sequences are presented as below:
miR-488-3p: 5'-CGGGGCAGCUCAGUACAG-3' (forward) and 5'-CAGTGCGTGTCGTGGAGT-3' (reverse);
U6: 5'-CTCGCTTCGGCAGCACA-3' (forward) and 5'-AACGCTTCACGAATTTCGCT-3' (reverse);
ROCK1: 5'-CTGCTGACTGAGCGAACACT-3' (forward) and 5'-ACCACGCTTGACAGGTTCTT-3' (reverse);
GAPDH: 5'-ACAACTTTGGTATCGTGGAAGG-3' (forward) and 5'-GCCATCACGCCACAGTTTC-3' (reverse).

Enzyme-linked immunosorbent assay (ELISA)

DRGs from L4-L5 of rats were collected and lysed by adding RIPA buffer (10 μl/mg; Beyotime, China) containing 1% protein inhibitor (Beyotime, China). The supernatant was collected after centrifugation. The concentration of the pro-inflammatory factors interleukin (IL)-1β (#ab255730), IL-6 (#ab100772), tumor necrosis factor (TNF)-α (#ab236712), and the anti-inflammatory factors IL-10 (#ab214566) and IL-4 (#ab100770, Abcam, USA) were subsequently measured using commercial assay kits, and the changes in absorbance values were measured on an enzyme marker (SpectraMax M5, Molecular Devices, USA) with an optical density (OD) 490 nm.

Dual-luciferase reporter (DLR) assay

The bioinformatics website TargetScan 8.0 predicted potential target genes for miR-488-3p and found a potential binding site for ROCK1. The 3' UTR fragment of ROCK1 containing the predicted potential binding site was subcloned into the pmiR-RB-REPORT luciferase reporter vector to form a recombinant wild-type luciferase reporter plasmid WT-ROCK1. Site-mutated ROCK1 constitutes the recombinant mutant luciferase reporter plasmid Mut-ROCK1. 293T cells were inoculated into 48-well plates and WT-ROCK1 or Mut-ROCK1 was co-transfected with miR-488-3p mimic (#miR10004763-1-5) or mimic NC (#miR1N0000001-1-5, Ribobio, China) using Lipofectamine 3000 (ThermoFisher, USA). After 48 h, luciferase assays were examined by luciferase reporter analysis system.

RNA immunoprecipitation (RIP) assay

Magna RIPTM RNA-Binding protein immunoprecipitation kit (Millipore Sigma, USA) was conducted to RIP assay. In brief, DRG tissues were lysed in RIP buffer and the upper lysate was collected by centrifugation. The extracts were incubated with magnetic beads binding to human Ago antibodies or negative control IgG. Then, proteinase K was an addition to extract the proteins to obtain co-precipitated RNA, which was purified and subjected to RT-qPCR.

Statistical analysis

Statistical analyses were conducted with GraphPad Prism version 9.0 (GraphPad Software, La Jolla, USA). The data are provided as mean ± SD after three biological replicates. Two sets of data were analyzed using. Repeated measurement ANOVA was performed on the data at different time points between the groups. ANOVA analysis was carried out to evaluate the statistical differences between multiple groups. $P<0.05$ considers the results to be statistically significant.

RESULTS

The miR-448-3p level was restrained in the CCI rats

NP model was successfully conducted by CCI. A substantial decline in PWL and PWL was observed in CCI rats at 0, 3, 7, 14, and 21 days postoperatively (P<0.05, Fig. 1A-B). ELISA assays were carried out to qualify the levels of neuroinflammatory factors in rat DRGs after 14 days of surgery. The neuro-pro-inflammatory factors TNF-α, IL-1β, and IL-6 were distinctly upregulated in the CCI group, while the anti-inflammatory factors IL-4 and IL-10 were typically downregulated (P<0.05, Fig. 1C-D). Additionally, we explored miR-488-3p expression at different postoperative times. As displayed in Fig. 1E, the expression of miR-488-3p was prominently declined in CCI rats with time dependence. The findings manifested that dysregulated miR-488-3p is implicated in the pathogenesis of NP.

Enhanced levels of miR-488-3p attenuate NP behavior in CCI rats

LV-miR-488-3p was intrathecally injected to explore the actions of miR-488-3p in NP. miR-488-3p was successfully upregulated in the DRGs of CCI+LV-miR-488-3p groups (P<0.05, Fig. 2A). Both reduced PWT and PWL in CCI rats were partially restored by upregulated miR-488-3p compared to the CCI+LV-NC group (P<0.05, Fig. 2B-C).

Elevated miR-488-3p mitigated the overactivated inflammation in CCI rats

The influence of miR-488-3p on neuroinflammatory responses was subsequently analyzed. As indicated in Fig. 3A, secretion of the neuroinflammatory factors TNF-α, IL-1β, and IL-6 was prominently promoted on day 21 of CCI construction but this promotion was typically suppressed by the elevated miR-488-3p (P<0.05).

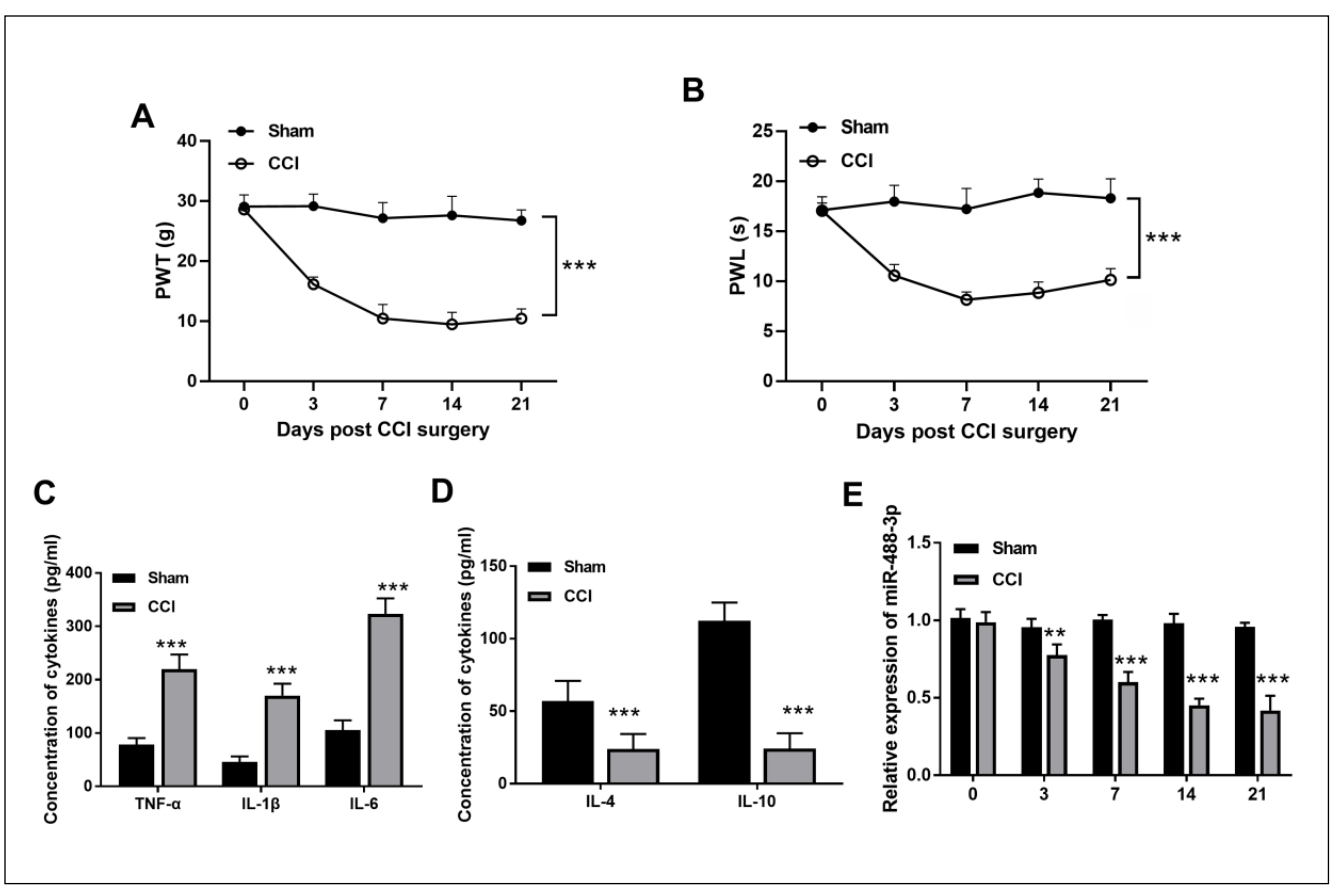

Fig. 1. The miR-488-3p levels were dysregulated in the CCI rats. Mechanical allodynia (A) and thermal hyperalgesia (B) in rats was monitored on day 0, 3, 7, 14, and 21. The level of neuroinflammatory factor (C) and anti-inflammatory factors (D) were monitored by ELISA assay on day 21 of CCI model construction. (E) The levels of miR-488-3p in the CCI rats of DGRs were measured on days 0, 3, 7, 14, and 21. ***P<0.001 *vs.* Sham group.

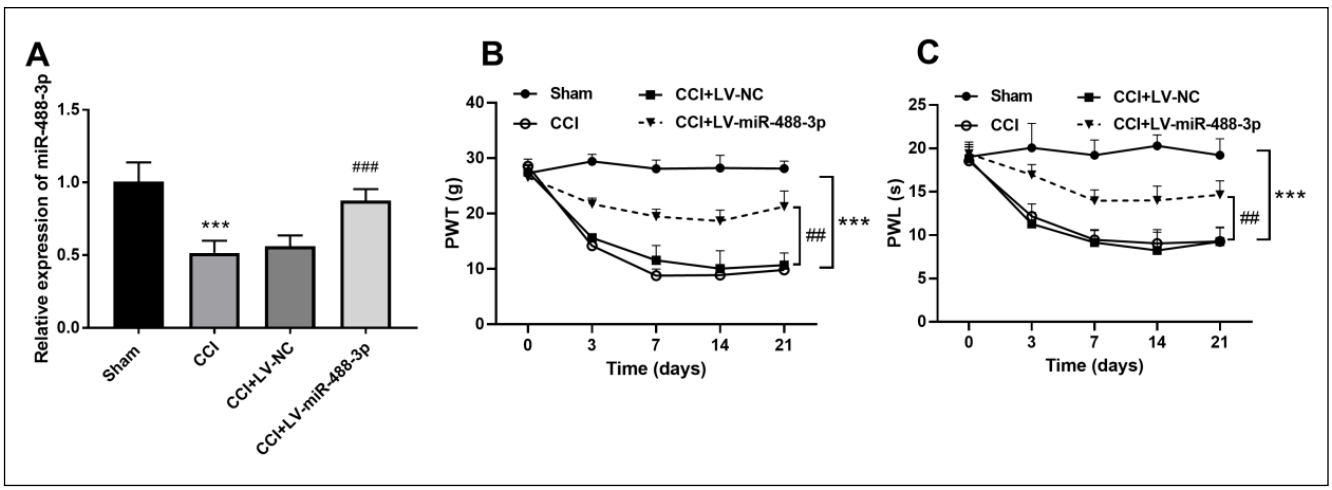

Fig. 2. Increased miR-488-3p levels attenuated NP behavior. A. miR-488-3p levels in rat DRGs after intrathecal injection of LV-miR-488-3p were to be explored on day 21 of CCI model construction. Mechanical allodynia (B) and thermal hyperalgesia (C) in rats were monitored after intrathecal injection of LV-miR-488-3p. ***P<0.001, *vs.* CCI group; ##P<0.01 *vs.* CCI+LV NC group.

The reduced IL-4 and IL-10 levels in the CCI group were partially restored by the elevated miR-488-3p (P<0.05, Fig. 3B).

ROCK1 was a target of miR-488-3p and suppressed by miR-488-3p

Bioinformatics analysis led to the prediction of ROCK1 as a target gene for miR-488-3p. The putative binding sequences are presented in Fig. 4A. DLR assay revealed that elevated miR-488-3p distinctly reduced the luciferase activity of ROCK1-WT (P<0.05, Fig. 4B). miR-488-3p and ROCK1 were considerably enriched in the Ago2 antibody compared to the negative control IgG (P<0.05, Fig. 4C). Moreover, the ROCK1 levels were up-regulated in the DRGs of CCI rats with the time-dependent (P<0.05, Fig. 4D). Spearman correlation coefficient identified that miR-488-3p was negatively correlated with ROCK1 in 14-day CCI rats models (r=-0.728, P=0.017, Fig. 4E). And elevated ROCK1 in DRGs of CCI rats were suppressed by increased miR-488-3p (P<0.05, Fig. 4F).

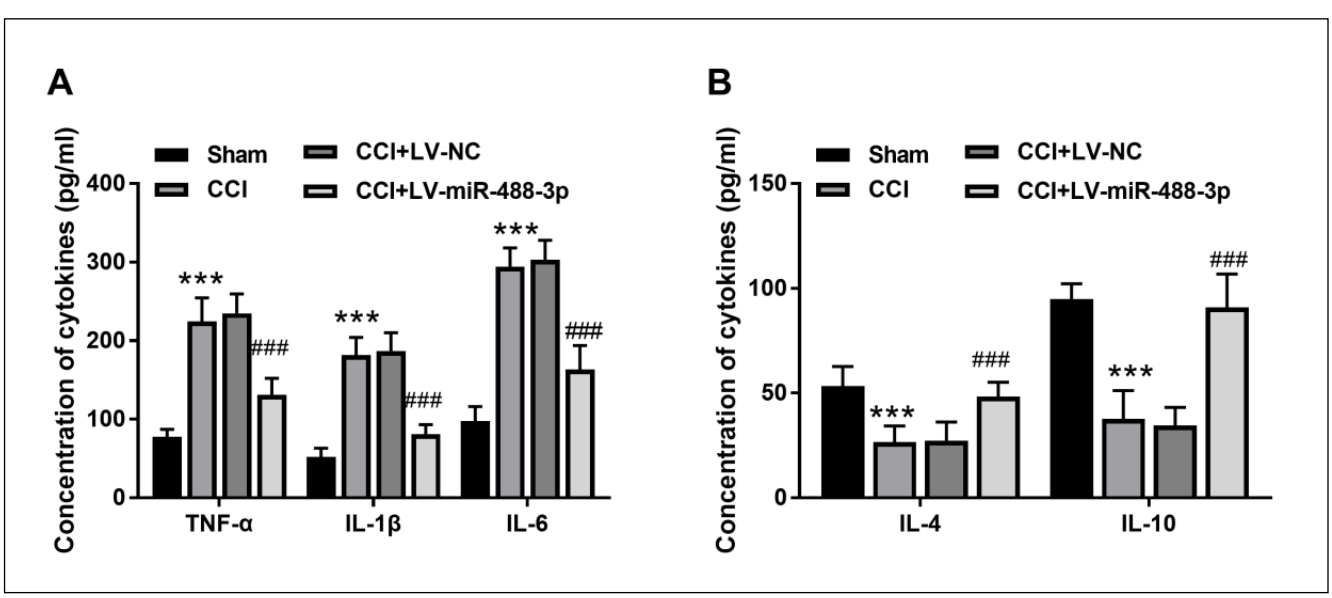

Fig. 3. The effects of overexpression of miR-488-3p on neuro-proinflammatory (A) and neuroinflammatory factor levels (B) were explored by the ELISA on day 21 of CCI model construction. ***P<0.001 *vs.* CCI group; ###P<0.001 *vs.* CCI+LV NC group.

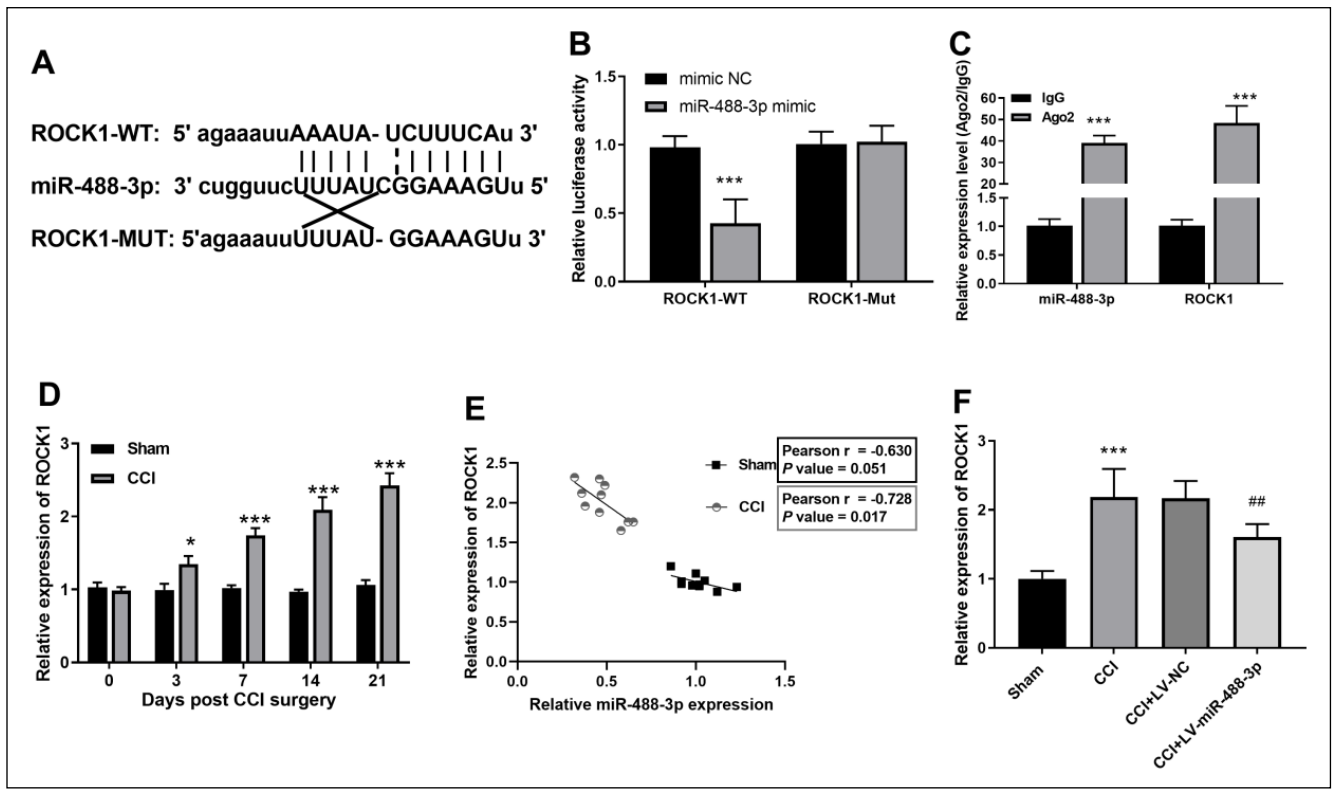

Fig. 4. ROCK1 was the target of miR-488-3p. (A) ROCK1 and miR-488-3p binding sites. (B) DLR assay was conducted to examine the miR-488-3p target to ROCK1. (C) Enrichment of miR-488-3p and ROCK1 in the immunoprecipitation complex was detected by RIP assay. (D) The expression of ROCK1 in DRGs of CCI rats. (E) Spearman correlation coefficient identified that miR-488-3p was negatively correlated with ROCK1 in 14-day CCI rats. (F) The levels of ROCK1 after intrathecal injection of LV-miR-488-3p were analyzed by RT-qPCR on day 21 of CCI model construction. ***P<0.001 *vs.* NC or IgG or sham; ###P<0.001 *vs.* CCI+LV NC group.

Elevated ROCK1 partially reversed the mitigating effect of miR-488-3p on NP

To elucidate the function of ROCK1, overexpression of the ROCK1 plasmid was intrathecally injected into rats. ROCK1 levels were persistently higher in the CCI+LV-miR-488-3p+oe-ROCK1 group compared to the CCI+LV-miR-488-3p+oe-NC group (P<0.05, Fig. 5A). The alleviating effect of miR-488-3p on both PWL and PWT in CCI rats was typically suppressed by elevated ROCK1 (P<0.05, Fig. 5B-C). In terms of neuroinflammation, the over-secretion of proinflammatory factors and inhibition of anti-inflammatory factors in CCI rats were typically reversed by upregulation of miR-488-3p, but this reversal was attenuated by elevated ROCK1 (P<0.05, Fig. 5D-E).

DISCUSSION

Accumulating evidence points to an essential role for miRNA in NP. For instance, lncRNA MIAT promotes NP progression by targeting miR-362-3p (Zhang et al., 2022a). Salvianol alleviates NP by suppressing CCI-induced astrocyte activity *via* miR-15a (Cai et al., 2022). Increasing research highlights the usefulness of miR-488-3p in disease pathological processes, including gastric cancer (Luo et al., 2023), esophageal cancer (Hu et al., 2023), and endometriosis (Xu et al., 2022). However, miR-488-3p has attracted attention as a neuroprotective miRNA. MiR-488-3p diminished neuronal injury in an epileptic cell model (Wen et al., 2023) and elevated miR-488-3p suppresses neuronal cell death and alleviates brain infarction in mice with ischemic stroke (Zhou et al., 2021). Brain-derived extracellular vesicles containing miR-488-3p are involved in traumatic brain injury (Ko et al., 2020), and their levels correlate with the malignant behavior of gliomas (Xue et al., 2018). Furthermore, miR-488 is dysregulated in patients with Parkinson's disease, an associated complication of NP (Tatura et al., 2016). While spinal cord injury is another common complication of NP, dysregulated miR-488-3p in spinal cord injury was identified by Chen et al. in 2022 and Li et al. in 2021, respectively. Of more interest

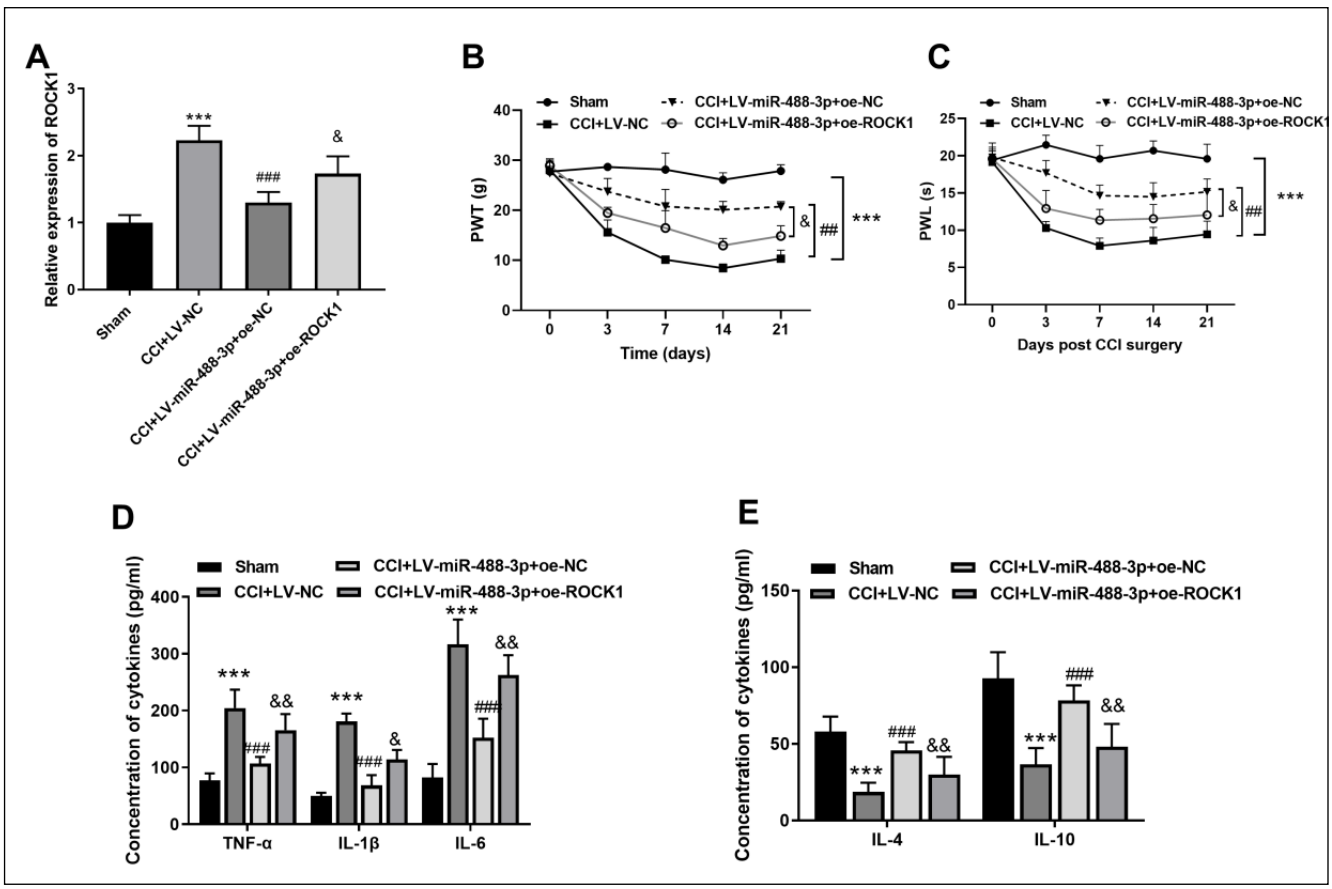

Fig. 5. Elevated ROCK1 partially diminished the mitigation of miR-488-3p on NP. A. The levels of ROCK1 in DRGs of CCI rats on day 21 of model construction after intrathecal injections of oe-ROCK1 plasmids. Mechanical allodynia (B) and thermal hyperalgesia (C) in rats were monitored after intrathecal injection of oe-ROCK1 plasmids. ELISA assay was conducted the examine the levels of proinflammatory factors (D) and anti-inflammatory factors (E) on day 21 of model construction. ***P<0.001 *vs.* mimic NC or IgG or sham; ###P<0.001 *vs.* CCI+LV NC group; &P<0.05, &&P<0.01 *vs.* CCI+LV-miR-488-3p+oe-NC.

to us is the small RNA sequencing of NP rats by Dai et al. in 2019 and the revelation of significant downregulation of miR-488-3p. Hence, we focused on the potential actions and mechanisms of miR-488-3p in NP.

CCI model is the most commonly used to simulate peripheral nerve injury in NP and produces a partially de-innervated sciatic nerve, allowing analysis of NP behaviors including mechanical nociceptive hyperalgesia and thermal nociceptive hyperalgesia, with good reproducibility and stability (Zhang et al., 2019). Additionally, disturbances in any part of the sciatic nerve travel process can cause sciatic nerve pain, most commonly at the L4-L5 of the DRG (Zheng et al., 2015), which is why we chose this site for our follow-up research. Consistent with most previous studies, NP behavioral indicators of PWT and PWL were distinctly reduced in CCI rats, suggesting that mechanical ectopic pain and thermal nociceptive hyperalgesia became more sensitive and vulnerable in rats. And the miR-488-3p levels decreased progressively with time after CCI surgery. Therefore, in vivo studies were performed by intrathecal injection of miR-488-3p lentivirus, and the rats showed good mental status and no change in body weight. However, when miR-488-3p levels were increased, pain behaviors were usually reduced in rats.

Nerve injury results in a wide range of complex changes at the site of injury including the recruitment of immune cells, the induction and release of multiple inflammation mediators, and the expression of neuronal excitatory regulators. Inflammation is an essential mechanism of NP and, blocking the overproduction of inflammation is considered an excellent strategy to alleviate NP (Jin et al., 2021). And the actions of miR-488-3p in inflammation have been discussed. miR-488-3p was found to be enhanced in gouty arthritis (Zhou et al., 2017) and acts as a sponge for lncRNA PVT1 involved in chondrocyte apoptosis in osteoarthritis (Li et al., 2017). miR-488-3p also participated in the neuroinflammato-

ry response in epilepsy (Wen et al., 2023). In our study, we found that the secretion of pro-inflammatory factors was typically activated, and the secretion of anti-inflammatory factors was distinctly inhibited in CCI rats, suggesting an over-activation of the neuroinflammatory response in the NP. However, this activation state was significantly inhibited by high expression of miR-488-3p, suggesting that miR-488-3p noticeably attenuated inflammatory damage in NP.

ROCK1 is a classical serine-threonine kinase located on chromosome 18q11.1 that regulates the cytoskeleton by increasing actin filament stability and myosin contraction through post-phosphorylation of downstream substrates (Ghafouri-Fard et al., 2022) and is associated with cell morphology and polarity (Kumar et al., 2022). Suppression of ROCK1 activity has potential therapeutic effects in a variety of diseases, including cancer (Liu et al., 2023), asthma (Lin et al., 2023), diabetic cardiomyopathy (Zhang et al., 2022b), and pulmonary hypertension (Zhao et al., 2021). ROCK1 is associated with nerve damage. Dexmedetomidine reverses MTX-induced oxidative stress and exerts hippocampal neuronal protection through the modulation of ROCK1-related signaling pathways (Taha et al., 2023). ROCK1 also engaged in the protective effects of circ-Arhgap5 RNA silencing on neuronal injury induced by cerebral ischemia-reperfusion (Wang et al., 2023). ROCK1 is also involved in inflammatory disease. For instance, ROCK1 is typically up-regulated in patients with multiple sclerosis, a chronic inflammatory disease of the central nervous system (Saeidi et al., 2023). Increased levels of ROCK1 and regulation of pro-inflammatory factors in acute lung injury due to sepsis (Li et al., 2023). ROCK1 also regulates UVB-induced dermatitis (Li et al., 2022c). Additionally, ROCK1 is involved in the advance of Alzheimer's disease, a complication associated with NP (Ruan et al., 2021), while also participating in the return of spinal sensory function in spinal cord injury, another complication of NP (Wang et al., 2020). Ferulic acid alleviates sciatic nerve pain by inhibiting several target genes such as ROCK1 and COX2 (Zhang et al., 2022c). Given the potential actions of ROCK1 in neuronal injury, neuroinflammation, and complications of NP, we are interested in the potential role of ROCK1 in NP. We predicted that ROCK1 is a target gene for miR-488-3p, which is consistent with the results in pre-eclampsia (Zhang et al., 2022d) and endometriosis (Xu et al., 2022). We also identified that ROCK1 was noticeably elevated in CCI rats, but the elevation could be noticeably inhibited by miR-488-3p, suggesting that miR-488-3p may alleviate NP by inhibiting ROCK1. Subsequently, overexpression of ROCK1 levels in CCI diminished the mitigating effect of miR-488-3p on NP behavior as well as neuroinflammatory damage. Finally, some limitations exist in this study. Firstly, it failed to validate the clinical significance of miR-488-3p in patients with NP. Furthermore, failure to deeply examine the signaling pathway of miR-488-3p/ROCK1 affecting NP. These contents will be focused on our next study.

CONCLUSION

This article presents evidence that aberrant miR-488-3p takes a pivotal role in NP. miR-488-3p alleviates NP behavior and neuroinflammation by targeting ROCK1 in CCI rats. The preliminary suggestion is that miR-488-3p may be a new therapeutic option for NP treatment.

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