



Inhibition of MicroRNA-15a/16 Expression Alleviates Neuropathic Pain Development through Upregulation of G Protein-Coupled Receptor Kinase 2

Tao Li¹, Yingchun Wan², Lijuan Sun², Shoujun Tao³, Peng Chen¹, Caihua Liu⁴, Ke Wang⁵, Changyu Zhou⁶ and Guoqing Zhao^{1,*}

¹Department of Anesthesiology, China-Japan Union Hospital, Jilin University, Jilin 130033,

²Department of Endocrinology, China-Japan Union Hospital, Jilin University, Jilin 130033,

³Department of Anesthesiology, Affiliated Hangzhou First People's Hospital, Zhejiang University School of Medicine, Zhejiang 310006,

⁴Department of Anaesthesiology, The Central Hospital of Wuhan Affiliated with Tongji Medical College of Huazhong University of Science and Technology, Hubei 430014,

⁵Department of Gynaecology and Obstetrics, China-Japan Union Hospital, Jilin University, Jilin 130033,

⁶Department of Gastroenterology, China-Japan Union Hospital, Jilin University, Jilin 130033, China

Abstract

There is accumulating evidence that microRNAs are emerging as pivotal regulators in the development and progression of neuropathic pain. MicroRNA-15a/16 (miR-15a/16) have been reported to play an important role in various diseases and inflammation response processes. However, whether miR-15a/16 participates in the regulation of neuroinflammation and neuropathic pain development remains unknown. In this study, we established a mouse model of neuropathic pain by chronic constriction injury (CCI) of the sciatic nerves. Our results showed that both miR-15a and miR-16 expression was significantly upregulated in the spinal cord of CCI rats. Downregulation of the expression of miR-15a and miR-16 by intrathecal injection of a specific inhibitor significantly attenuated the mechanical allodynia and thermal hyperalgesia of CCI rats. Furthermore, inhibition of miR-15a and miR-16 downregulated the expression of interleukin-1 β and tumor-necrosis factor- α in the spinal cord of CCI rats. Bioinformatic analysis predicted that G protein-coupled receptor kinase 2 (GRK2), an important regulator in neuropathic pain and inflammation, was a potential target gene of miR-15a and miR-16. Inhibition of miR-15a and miR-16 markedly increased the expression of GRK2 while downregulating the activation of p38 mitogen-activated protein kinase and NF- κ B in CCI rats. Notably, the silencing of GRK2 significantly reversed the inhibitory effects of miR-15a/16 inhibition in neuropathic pain. In conclusion, our results suggest that inhibition of miR-15a/16 expression alleviates neuropathic pain development by targeting GRK2. These findings provide novel insights into the molecular pathogenesis of neuropathic pain and suggest potential therapeutic targets for preventing neuropathic pain development.

Key Words: GRK2, miR-15a/16, Neuropathic pain, p38 MAPK

INTRODUCTION

Neuropathic pain is a neurological disease characterized by hyperalgesia and allodynia of the somatosensory system (Haanpaa *et al.*, 2011). Neuropathic pain has emerged as a global problem affecting approximately 6.9-10% of the population worldwide, with enormous socioeconomic costs (Denk and McMahon, 2012; van Hecke *et al.*, 2014). Despite advances in

drug development for neuropathic pain, treatment efficacy is still limited (O'Connor and Dworkin, 2009). Neuropathic pain is a complicated disease and the molecular mechanisms underlying its pathogenesis remain poorly understood. Therefore, a better understanding of the molecular pathogenesis of neuropathic pain, and the search for novel and effective targets for treatment, are essential.

MicroRNAs (miRNAs), non-coding RNAs, are emerging as

Open Access <https://doi.org/10.4062/biomolther.2018.073>

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Received Apr 30, 2018 Revised Jul 24, 2018 Accepted Aug 14, 2018

Published Online Jun 13, 2019

***Corresponding Author**

E-mail: zhaoguoqing_cjuh@163.com

Tel: +86-0431-84995999, Fax: +86-0431-84995999

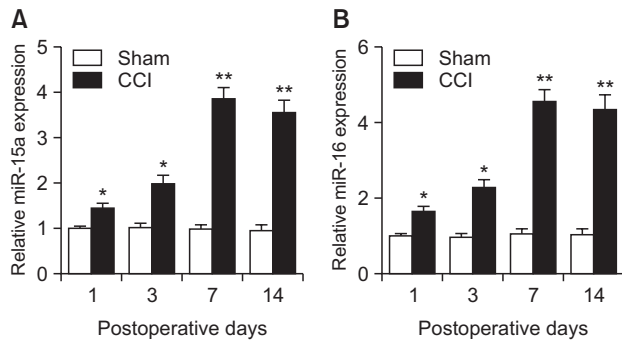


Fig. 1. Expression profile of miR-15a/16 in CCI rats. Relative expression of miR-15a (A) and miR-16 (B) in spinal cords from sham or CCI rats were detected by RT-qPCR. At postoperative days 1, 3, 7, and 14, the L4-L5 lumbar spinal cords were removed for investigation. * $p < 0.05$ and ** $p < 0.01$ versus sham.

novel and important mediators for gene expression (Ambros, 2004). Mature miRNAs consist of 19-25 nucleotides which can bind to the 3'-untranslated region (UTR) of target mRNA, with complementary base-pair sequences, leading to mRNA degradation and translational repression (Bartel, 2004). miRNAs are highly involved in the development and progression of various diseases via regulation of various biological activities and may be potential and critical targets for treatment (Krol *et al.*, 2010). There is accumulating evidence that miRNAs play a pivotal role in the pathogenesis of neuropathic pain and are novel targets for prevention and treatment (Andersen *et al.*, 2014; Sakai and Suzuki, 2014; Jiangpan *et al.*, 2016). Therefore, a better understanding of the regulatory network of miRNAs involved in the pathogenesis of neuropathic pain will aid development of novel therapeutic strategies.

G protein-coupled receptor kinase 2 (GRK2), also known as beta-adrenergic receptor kinase 1, is an important in regulating the responsiveness of G protein-coupled receptors (GPCRs) (Lombardi *et al.*, 2002). GRK2 can prevent agonist-induced overstimulation via desensitization of a series of GPCRs, while a deficiency of GRK2 prolongs the signaling in response to activation of GPCRs (Vroon *et al.*, 2006). Moreover, GRK2 is involved in regulating various intracellular signaling pathways, such as mitogen activated protein kinases (MAPK) and PI3K/Akt (Peregrin *et al.*, 2006; Zhang *et al.*, 2017). Accumulating evidence has suggested that GRK2 plays an important role in various diseases associated with inflammation (Penela *et al.*, 2008; Lucas *et al.*, 2015; Woodall *et al.*, 2016). Notably, GRK2 is also reported to regulate the development and progression of neuropathic pain. In a rodent model of neuropathic pain, GRK2 expression is decreased in the lumbar spinal cord (Kleibeuker *et al.*, 2007). Additionally, neuropathic pain is prolonged in GRK2-deficient mice (Willemen *et al.*, 2010; Wang *et al.*, 2011). Therefore, GRK2 may serve as a promising target for treatment of neuropathic pain (Kavelaars *et al.*, 2011).

The miR-15a/16 cluster located on chromosome 13q14 is a highly conserved miRNA group that plays an important role in various diseases, such as cancer, cardiovascular disease, and neurological disease (Calin *et al.*, 2008; Spinetti *et al.*, 2013; Yang *et al.*, 2017b). However, whether miR-15a/16 is involved in regulating neuropathic pain remains unclear. In this study, we aimed to investigate the potential role of miR-15a/16

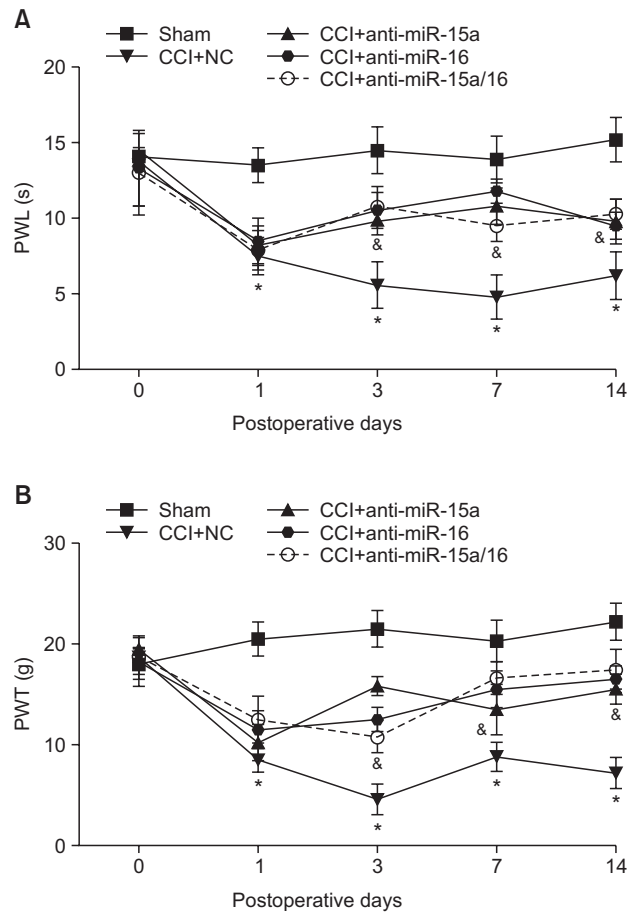


Fig. 2. Inhibition of miR-15a/16 alleviates neuropathic pain development in CCI rats. (A) Thermal hyperalgesia was determined by the PWL in response to radiant heat stimulation. (B) Mechanical allodynia was determined by the PWT in response to Von Frey hair stimulation. Anti-miR-15a, anti-miR-16, or the negative control (NC) were intrathecally injected into CCI rats. * $p < 0.05$ versus sham, $^{\circ}p < 0.05$ versus CCI+NC.

in the development and progression of neuropathic pain.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley (SD) rats (weighing 200-250 g, aged 7-8 weeks) were purchased from Experimental Animal Center of Jilin University. The rats were housed in separate cages ($20 \pm 1^{\circ}\text{C}$; 12-h light/dark cycle) with *ad libitum* access to water and food. The animal procedures were performed in accordance with the guidelines of the International Association for the Study of Pain and the National Institute of Health Guide for the Care and Use of Laboratory Animals. This study was authorized and approved by the Institutional Animal Care and Use Committee of China-Japan Union Hospital.

Neuropathic pain model

Neuropathic pain in rats was induced by CCI based on procedures described previously (Bennett and Xie, 1988). The rats were allowed to acclimate to their environment for

2 days prior to the experiments. The rats were anesthetized by an intraperitoneal injection of phenobarbital sodium (40 mg/kg). The sciatic nerves on both sides were revealed by blunt dissection and isolated from surrounding tissues. The sciatic nerves were loosely ligated using a 4-0 catgut thread with about 1 mm between ligatures. A sham surgery was performed with the sciatic nerve revealed but not ligated. Rats with sham surgery were used as controls. After the surgery, the muscle and skin layers were sutured with thread and the area of surgery was sterilized with iodine.

Intrathecal injection procedure

The rats were anesthetized and a Hamilton syringe with a 30-gauge needle was inserted into the subarachnoid space of the spinal cord between the L4 and L5 lumbar. Proper location of the intrathecal implantation was systemically confirmed by injection of 2% lidocaine to induce bilateral hind limb paralysis. Intrathecal delivery of miR-15a/16 antagomir (anti-miR-15a, anti-miR-16, or a mixture of miR-15a and miR-16) was performed using a microinjection syringe linked to the intrathecal catheter. After the experiments, the L4-L5 lumbar spinal cords were dissected for biological detection.

Real-time quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer’s instructions. The reverse transcription of the total RNA was conducted using M-MLV reverse transcriptase (Takara, Dalian, China) for mRNA detection and the TaqMan miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) for miRNA detection. The amplification of cDNA was performed using SYBR Green PCR Master Mix (Applied Biosystems) following the thermal cycling conditions: initial denaturation (94°C, 5 min), 40 cycles at 94°C (20 s), 55°C (30 s) and 72°C (30 s), and a final cycle at 72°C (5 min). GAPDH and U6 were used as reference genes for normalization of mRNA and miRNA expression, respectively. The relative expression was calculated using the 2^{-ΔΔCt} method.

Measurement of pain threshold

Thermal hyperalgesia, indicated by paw withdrawal latencies (PWL) in response to radiant heat stimulation, was determined using a pain threshold detector. Mechanical allodynia, indicated by paw withdrawal threshold (PWT) in response to the mechanical stimulus, was detected using Von Frey hair (IITC, Woodland Hills, CA, USA). The experimental detections were performed according to standard procedures described previously (Hargreaves *et al.*, 1988; Chaplan *et al.*, 1994).

Enzyme-linked immunosorbent assay (ELISA)

The protein concentrations of IL-1β and TNF-α in lumbar spinal cords were determined using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) as per the protocol recommended by the manufacturer.

Luciferase reporter assay

293T cells purchased from Stem Cell Bank of Chinese Academy of Sciences (Shanghai, China) were used to perform the luciferase reporter assay. The 293T cells were cultured in DMEM (Gibco, Rockville, MD, USA), containing 10% fetal bovine serum and a 1% penicillin/streptomycin mix, and

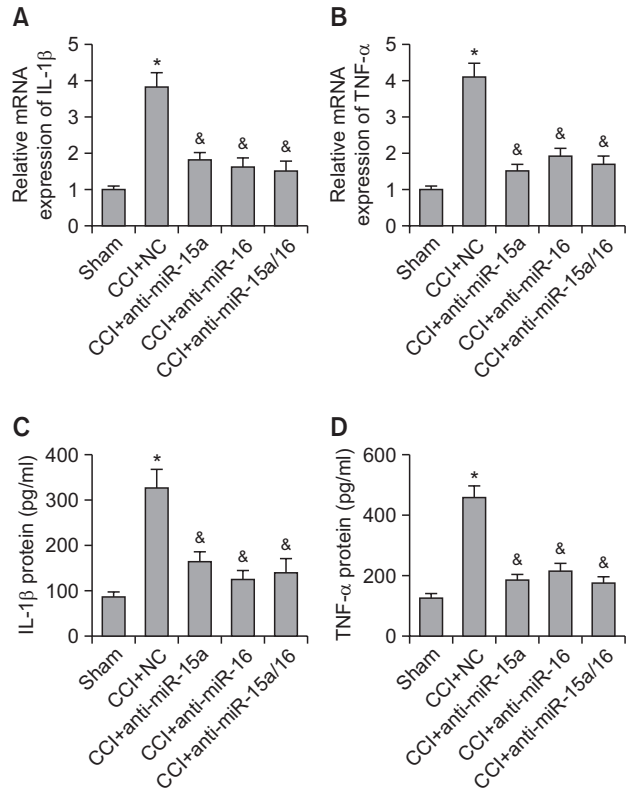


Fig. 3. Inhibition of miR-15a/16 suppresses neuroinflammation in CCI rats. Relative mRNA expression of IL-1β (A) and TNF-α (B) in the spinal cord was assessed by RT-qPCR analysis on postoperative day 7. Protein concentration of IL-1β (C) and TNF-α (D) in the spinal cord was determined by the ELISA method on postoperative day 7. **p*<0.05 versus sham, &*p*<0.05 versus CCI+NC.

grown in a humidified atmosphere with 95% air and 5% CO₂ at 37°C. The fragments of GRK2 3'-UTRs harboring the predicted wild-type seed-matched (WT) or mutant (MT) binding sites were inserted into pmirGLO dual-luciferase reporter plasmids (Promega, Madison, WI, USA). The WT or MT constructs were cotransfected with miR-15a/16 mimics (RiboBio, Guangzhou, China) into 293T cells using Lipofectamine 2000 (Invitrogen) as per the protocols of the manufacturer. After incubation for 48 h, the luciferase reporter activity was determined using the dual-luciferases reporter system (Promega).

Western blot analysis

Tissues were homogenized in cold phosphate-buffered saline for protein extraction. Protein concentrations were measured using a BCA protein assay kit (Beyotime Biotechnology, Haimen, China). Equal amounts of protein (40 μg) from each sample were separated by electrophoresis with 10% sodium dodecyl sulfate polyacrylamide gel. The separated proteins were transferred into a polyvinylidene fluoride (PVDF) membrane by the electro-blotting method. Thereafter, the membrane was blocked using 5% skimmed milk for 1 h at 37°C, followed by incubation with primary antibodies (4°C, overnight). The primary antibodies, including anti-GRK2, anti-p38, anti-p-p38, and anti-GAPDH, were purchased from Cell Signaling Technology (Danvers, MA, USA). After washing with

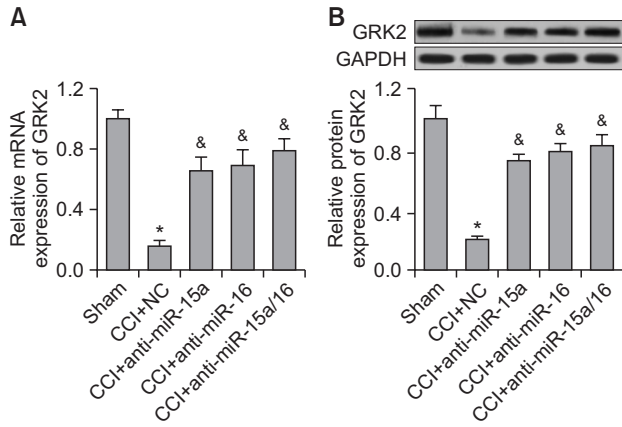


Fig. 5. Inhibition of miR-15a/16 upregulates the expression of GRK2 in CCI rats. (A) Relative mRNA expression of GRK2 in the spinal cords was measured by RT-qPCR at postoperative day 7. (B) Relative protein expression of GRK2 in the spinal cords was determined by Western blot at postoperative day 7. * $p < 0.05$ versus sham, & $p < 0.05$ versus CCI+NC.

decreased the luciferase activity of the reporter plasmid containing the predicted wild-type seed region for miR-15a and miR-16 (Fig. 4C). However, these effects were not observed in reporter plasmids containing mutant binding sites in the seed region of the GRK2 3'-UTR (Fig. 4C). Collectively, these data suggest that miR-15a/16 directly binds to the 3'-UTR of GRK2.

Inhibition of miR-15a/16 upregulates the expression of GRK2 in CCI rats

Considering the interaction between miR-15a/16 and the GRK2 3'-UTR, we investigated the effect of miR-15a/16 inhibition on GRK2 expression in CCI rats. The results showed that CCI surgery induced a significant decrease in GRK2 mRNA and protein expression in rat spinal cords (Fig. 5A, 5B). Interestingly, inhibition of miR-15a/16 significantly promoted the expression of GRK2 in CCI rats (Fig. 5A, 5B). Overall, these results suggest that miR-15a/16 inhibition can promote the expression of GRK2 in CCI rats.

Inhibition of miR-15a/16 suppresses the activation of p38 MAPK

GRK2 has been reported to regulate neuropathic pain and the inflammatory response associated with regulation of p38 MAPK (Peregrin *et al.*, 2006). We investigated whether inhibition of miR-15a/16 has a regulatory effect on p38 MAPK. We found that the phosphorylation of p38 MAPK was significantly increased in CCI rats (Fig. 6A-6D). Inhibition of miR-15a/16 showed no obvious effect on total protein expression of p38 MAPK (Fig. 6A, 6B). However, inhibition of miR-15a/16 significantly inhibited phosphorylation of p38 MAPK in CCI rats (Fig. 6A, 6C, 6D). In addition, we further found that inhibition of miR-15a/16 significantly decreased the phosphorylation of NF- κ B p65 in CCI rats (Fig. 6E-6H). These data suggest that miR-15a/16 inhibition alleviates neuropathic pain that may be associated with regulation of GRK2/p38 MAPK/NF- κ B signaling.

Knockdown of GRK2 eliminates the protective effects of miR-15a/16 inhibition in neuropathic pain rats

To validate whether inhibition of miR-15a/16 alleviates neu-

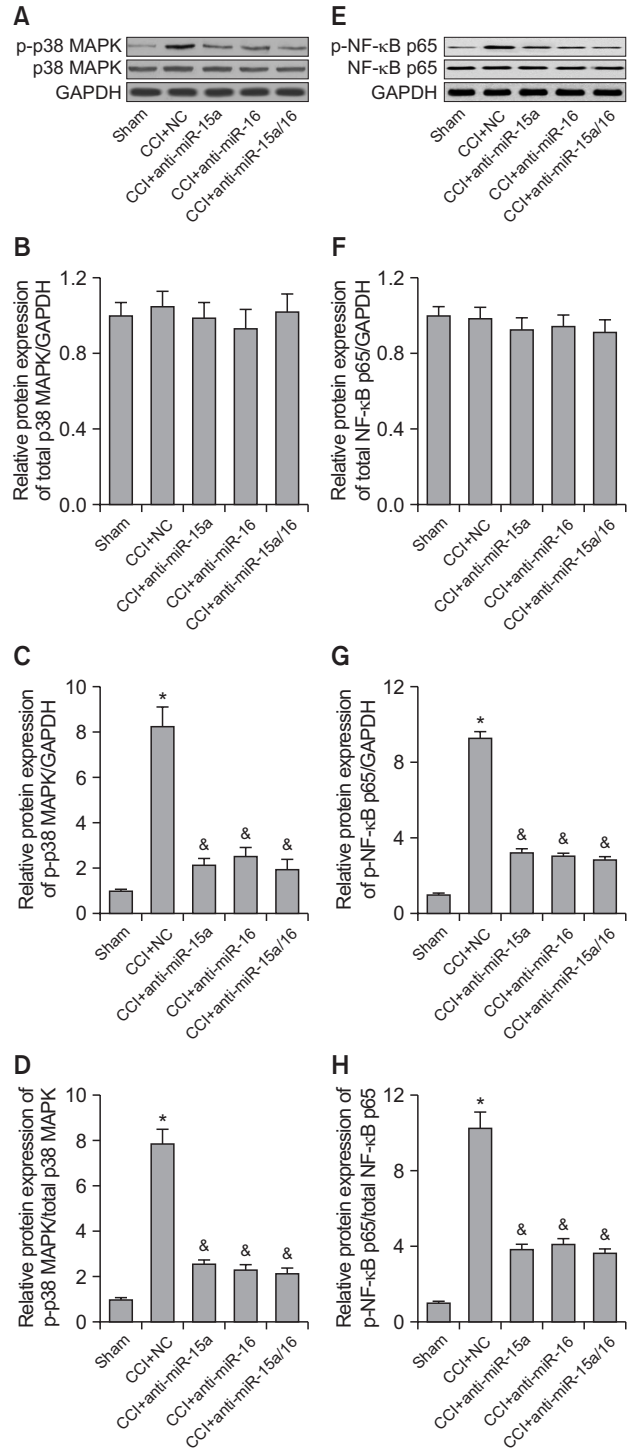


Fig. 6. Inhibition of miR-15a/16 suppresses the phosphorylation of p38 MAPK. (A) Western blot analysis of total p38 and phosphorylated p38 (p-p38) MAPK expression in the spinal cords from sham and CCI rats at postoperative day 7. Quantitative analysis of protein expression of total p38/GAPDH (B), p-p38 MAPK/GAPDH (C) and p-p38 MAPK/total p38 MAPK (D). (E) Western blot analysis of total NF- κ B p65 and phosphorylated NF- κ B p65 (p-NF- κ B p65) expression in the spinal cords from sham and CCI rats at postoperative day 7. Quantitative analysis of protein expression of total NF- κ B p65/GAPDH (F), p-NF- κ B p65/GAPDH (G) and p-NF- κ B p65/total NF- κ B p65 (H). * $p < 0.05$ versus sham, & $p < 0.05$ versus CCI+NC.

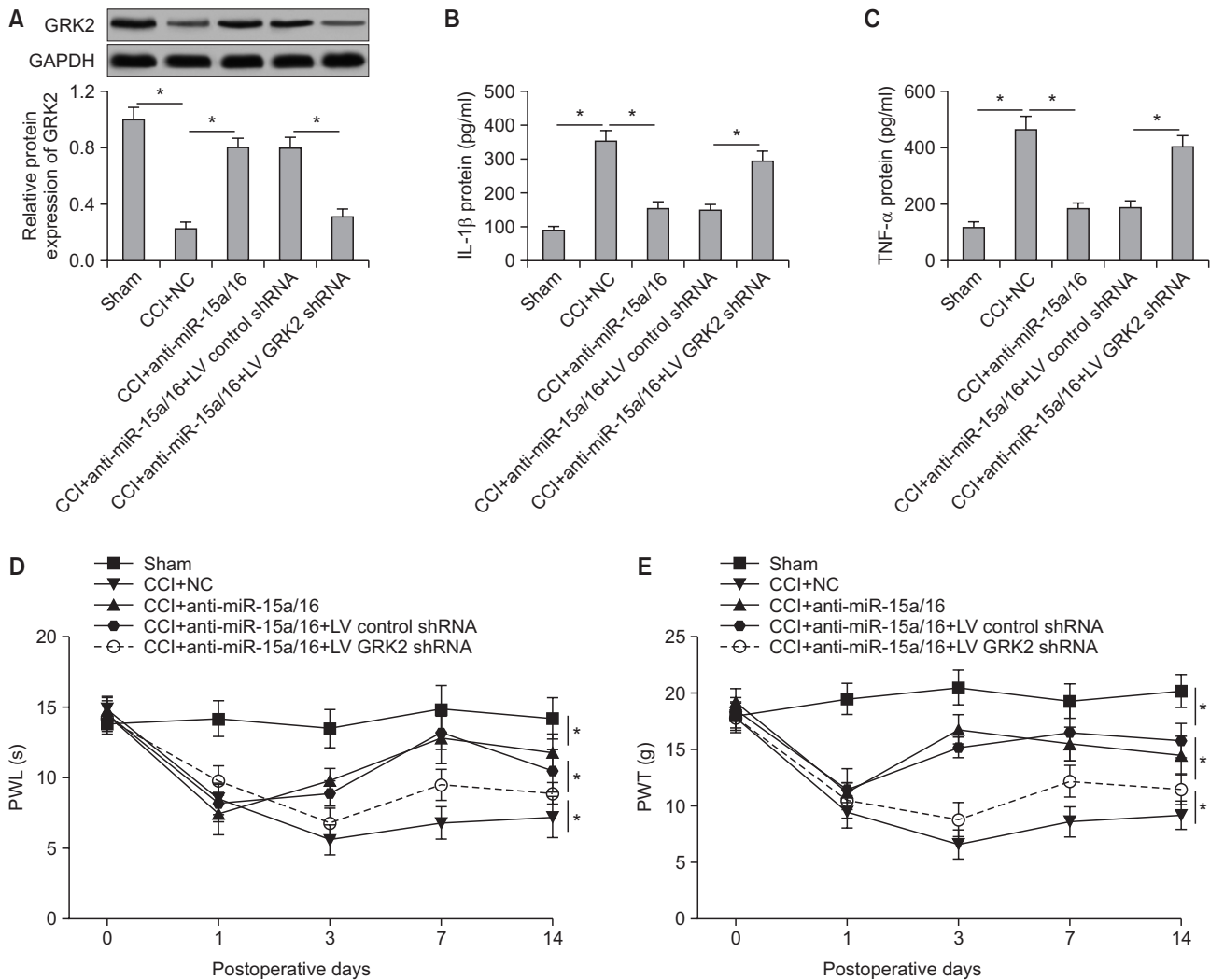


Fig. 7. Inhibition of miR-15a/16 alleviates neuropathic pain through upregulation of GRK2. CCI rats were treated with anti-miR-15a/16 combined with LV-GRK2 shRNA by intrathecal injection. (A) Relative protein expression of GRK2 was detected by Western blot at postoperative day 7. Protein expression of IL-1 β (B) and TNF- α (C) was measured by the ELISA method. (D) Thermal hyperalgesia in rats was determined by the Pawl in response to radiant heat stimulation. (E) Mechanical allodynia in rats was determined by the PWT in response to Von Frey hair stimulation. * $p < 0.05$.

ropathic pain through promoting GRK2 expression, we investigated the effect of GRK2 silencing on miR-15a/16 inhibition-mediated effects. LV-GRK2 shRNA was used to silence the expression of GRK2. We found that knockdown of GRK2 significantly blocked the promotive effect of miR-15a/16 inhibition on GRK2 expression in CCI rats (Fig. 7A). Furthermore, our results showed that knockdown of GRK2 eliminated the inhibitory effect of miR-15a/16 inhibition on neuroinflammation (Fig. 7B, 7C) and neuropathic pain (Fig. 7D, 7E) in CCI rats. Collectively, these results suggest that inhibition of miR-15a/16 alleviates neuropathic pain through upregulation of GRK2.

Knockdown of GRK2 reverses the inhibitory effect of miR-15a/16 inhibition on p38 MAPK activation

To investigate whether inhibition of miR-15a/16 suppresses the activation of p38 MAPK through regulation of GRK2, we evaluated the effect of GRK2 knockdown on the miR-15a/16

inhibition-mediated effect on the phosphorylation of p38 MAPK. As expected, we found that knockdown of GRK2 markedly reversed the inhibitory effect of miR-15a/16 inhibition on the phosphorylation of p38 MAPK (Fig. 8A-8D), indicating that GRK2 contributes to miR-15a/16 inhibition-mediated p38 MAPK phosphorylation.

DISCUSSION

There is a growing body of evidence that miRNAs are critical regulators in neuropathic pain development, and are potential therapeutic targets for neuropathic pain prevention in rodent models (Su *et al.*, 2017; Yang *et al.*, 2017a; Ji *et al.*, 2018). However, the precise role of miRNAs in neuropathic pain remains largely unknown. In this study, we newly identified miR-15a/16 as a regulator in neuropathic pain. We found

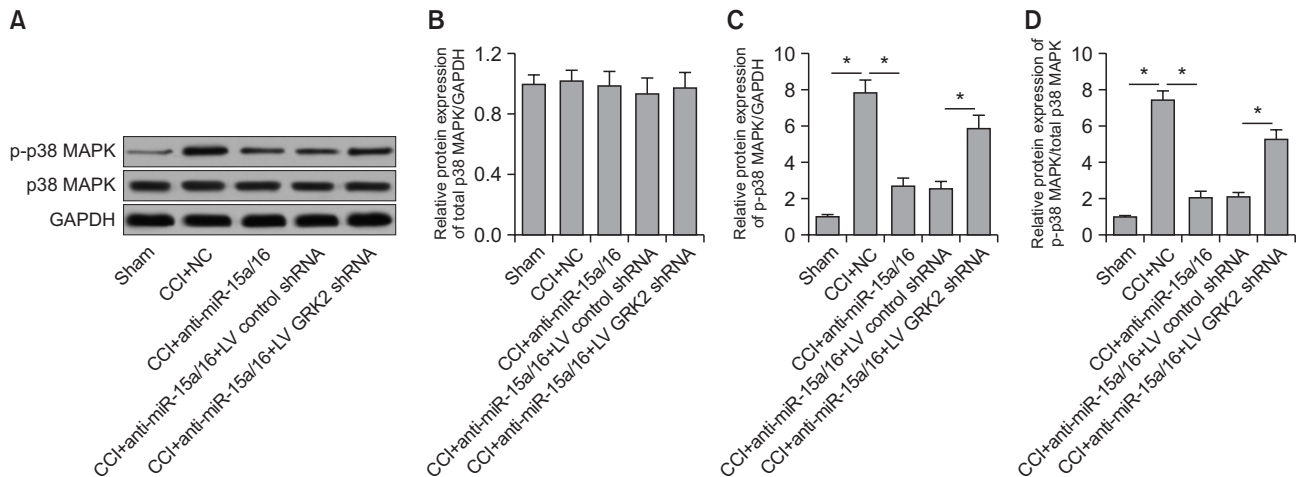


Fig. 8. Knockdown of GRK2 reverses the inhibitory effect of miR-15a/16 inhibition on p38 MAPK activation. CCI rats were treated with anti-miR-15a/16 in combination with LV-GRK2 shRNA by intrathecal injection. (A) Relative protein expression of total p38 and p-p38 MAPK were observed by Western blot at postoperative day 7. Quantitative analysis of protein expression of total p38 MAPK/GAPDH (B), p-p38 MAPK/GAPDH (C) and p-p38 MAPK/total p38 MAPK (D). * $p < 0.05$.

that both miR-15a and miR-16 expression was upregulated in CCI rats, and inhibition of miR-15a/16 had a protective effect on CCI-induced neuropathic pain. The underlying molecular mechanism is associated with the regulatory effect of miR-15a/16 on GRK2 expression. Our study will allow further development of therapeutic strategies for neuropathic pain.

Increasing evidence suggests that miR-15a/16 plays an important role in various diseases (Yue and Tigyi, 2010), especially those involving inflammatory responses. One study showed that miR-15a/16 regulates phagocytosis and Toll-like receptor 4-mediated pro-inflammatory cytokine/chemokine release in macrophages, suggesting an important role of miR-15a/16 in sepsis (Moon *et al.*, 2014). Moreover, miR-15a/16 regulates high glucose-induced pro-inflammatory signaling in human retinal endothelial cells (Ye *et al.*, 2016). Interestingly, inhibition of miR-15a/16 has been shown to ameliorate ischemic brain injury in experimental stroke associated with inhibition of pro-inflammatory cytokines (Yang *et al.*, 2017b). However, little is known about the role of miR-15a/16 in neuropathic pain. In this study, we showed that miR-15a/16 expression was significantly increased in CCI rats and the inhibition of miR-15a/16 alleviated the progression and development of neuropathic pain. Moreover, we showed that inhibition of miR-15a/16 also inhibited the expression of IL-1 β and TNF- α , suggesting that miR-15a/16 is involved in regulating inflammation in neuropathic pain. Conversely, a recent study reported that miR-16 inhibits inflammatory pain by targeting Ras-related protein 23 (Chen *et al.*, 2016). Therefore, the exact role of miR-15a/16 in neuropathic pain and neuroinflammation requires further investigation. Nevertheless, our study suggests that inhibition of miR-15a/16 attenuates neuropathic pain in a rat model.

Studies have shown that GRK2 plays an important role in the neuroinflammatory processes of the nervous system. GRK2 expression is decreased in hypoxic-ischemic brain damage, which precedes the loss of neurons (Lombardi *et al.*, 2004). Beta-amyloid peptide induces a decrease in GRK2 expression in the temporal cortex, which is associated with activation of microglia-mediated neuroinflammation, indicating

an important role of GRK2 in the pathogenesis of Alzheimer's disease (Suo *et al.*, 2004). Moreover, reduced expression of GRK2 is associated with the pathogenesis of Parkinson disease (Ahmed *et al.*, 2008). These findings suggest that GRK2 exerts a neuroprotective effect in neurological diseases. The function of GRK2 in neuropathic pain has also been widely studied. Kleibecker *et al.* (2007) first reported that GRK2 expression was decreased in the lumbar spinal cord of neuropathic pain rats induced by CCI, and decreased expression of GRK2 could potentiate inflammation-induced mechanical allodynia. In GRK2-deficient mice, microglia/macrophage GRK2 expression was reduced in the lumbar spinal cord during neuropathic pain, alongside increased microglial activation and pro-inflammation signaling in the spinal cord (Eijkelkamp *et al.*, 2010). Willemsen *et al.* (2012) reported that GRK2-deficient mice were associated with increased levels of pro-inflammatory M1 activation markers in spinal cord microglia/macrophages, which contribute to the persistent microglia activation during neuropathic pain. These findings suggest that GRK2 is a novel and potential target for treatment of neuropathic pain. The decreased expression of GRK2 is related to pathogenesis of neuropathic pain. However, the upstream regulation of GRK2 remains unknown. In this study, we found that GRK2 was a target gene of miR-15a/16. We found that miR-15a/16 expression was significantly upregulated in CCI rats, which may contribute to the downregulation of GRK2 during neuropathic pain. As expected, we showed that inhibition of miR-15a/16 significantly upregulated the expression of GRK2 in the spinal cord of CCI rats. Moreover, knockdown of GRK2 eliminated the miR-15a/16 inhibition-mediated protective effect. Our study may provide novel insights into understanding the dysregulation of GRK2 in the pathogenesis of neuropathic pain.

GRK2 has been reported to inhibit pro-inflammation by inhibiting the activation of p38 MAPK (Peregrin *et al.*, 2006). In GRK2-deficient microglia, LPS promotes TNF- α expression through a p38 MAPK-dependent pathway (Nijboer *et al.*, 2010). The increased phosphorylation and activation of p38 MAPK in the spinal cord contributes to the development and

progression of neuropathic pain (Tsuda *et al.*, 2004; Svensson *et al.*, 2005). In GRK2-deficient mice, chronic hyperalgesia is associated with increased phosphorylation of p38 MAPK and TNF- α in the spinal cord (Eijkelkamp *et al.*, 2010). The p38 MAPK inhibitor can inhibit IL-1 β -induced hyperalgesia in GRK2-deficient mice (Willemen *et al.*, 2010). In line with these findings, our results showed that miR-15a/16 inhibition attenuated neuropathic pain and neuroinflammation through promotion of GRK2 expression, which impeded the activation of p38 MAPK in CCI rats.

As well-known, one miRNA can target numerous target genes and one target gene can be targeted by various miRNAs. In this study, we showed that inhibition of GRK2 partially reversed the miR-15a/16 inhibition-mediated suppressive effect on neuropathic pain and neuroinflammation, indicating that other target gene of miR-15a/16 may be involved in this process. Therefore, the precise regulatory mechanism of miR-15a/16 in neuropathic pain remains to be determined.

Taken together, our results show that epigenetic regulation of GRK2 by miR-15a/16 contributes to the dysregulation of GRK2 during the development and progression of neuropathic pain. Inhibition of miR-15a/16 attenuates neuropathic pain and neuroinflammation through promotion of GRK2 expression and inhibition of the activation of p38 MAPK. These findings provide novel insights into the molecular pathogenesis of neuropathic pain and will allow further development of promising and effective therapeutic strategies for the treatment and prevention of neuropathic pain.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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