A critical role for miR-135a-5p-mediated regulation of *SLC24A2* in neuropathic pain

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Abstract. Neuropathic pain (NP) is a refractory and long-lasting disease caused mostly by peripheral nerve injury. Currently, the mechanism of NP is yet to be elucidated. Intracellular calcium homeostasis is critical for some physiological functions, including the occurrence of NP. NCKX2, encoded by the solute carrier family 4 member 2 (*SLC24A2*) gene, is an important K^+-dependent Na^+-Ca^{2+} exchanger that mediates Ca^{2+} extrusion. The role of NCKX2 in the development of NP is unknown. For this purpose, a sciatic nerve chronic constriction injury (CCI) model was established and it was revealed that the expression levels of *SLC24A2* and its encoded protein NCKX2 were both downregulated in the posterior horn of the spinal cord. Overexpression of *SLC24A2* reduced both mechanical and thermal hyperalgesia and decreased the expression of inflammatory cytokines [interleukin (IL)-1β, IL-6 and tumor necrosis factor-α] in CCI rats. Using bioinformatics analyses, luciferase reporter assays, and a series of behavioral tests, it was demonstrated that the decrease in *SLC24A2* after CCI treatment was directly regulated by increased microRNA (miR)-135a-5p in the spinal cord. Moreover, the effects of miR-135a-5p on NP were *SLC24A2*-dependent. In conclusion, the present results highlighted the suppressive role of NCKX2 in NP, which is mainly regulated by miR-135a-5p and mediates the release of inflammatory cytokines in the dorsal horn of the spinal cord. These findings deepen our understanding of the development of NP and provide novel candidates for NP treatment.

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Introduction

Neuropathic pain (NP) is a refractory and long-lasting disease caused mostly by peripheral nerve injury, which frequently occurs in patients with cancer, those treated with radiochemotherapy or surgery, and patients with AIDS, lumbar disc syndrome, and trauma (1). Although the incidence of NP is high at ~6.9 to 10% in the general population (2), its mechanism is yet to be elucidated, and few effective methods can be used in a complete and definitive way. Accumulating evidence suggests that the dorsal root ganglion and spinal dorsal horn, the key nodes in the pathway of peripheral pain information transmission, are involved in peripheral nerve injury-induced NP (1-3). Exploring the mechanism of molecular cytology changes in these regions is of great significance for understanding neuralgia.

Most cellular processes and physiological functions of neural cells, such as intracellular signal transduction, gene regulation, protein modification, neurotransmitter release and synaptic plasticity, rely on the ubiquitous messenger ionized calcium (Ca^{2+}) (4-6). Previous studies have also demonstrated that the ontogeny of NP is related to the alteration of Ca^{2+} in afferent neurons through the regulation of a number of voltage-gated calcium channels and an increase in Ca^{2+} influx (7-9). The dynamics of intracellular Ca^{2+} are regulated by the activity of a variety of calcium membrane channels and transporters. Among these, Na^+/Ca^{2+} exchangers, which mediate Ca^{2+} extrusion, play a predominant role after neurons experience large Ca^{2+} fluxes (10,11). Na^+/Ca^{2+} exchangers include two families: K^+-independent Na^+/Ca^{2+} (NCX) and K^+-dependent Na^+/Ca^{2+} (NCKX), and NCKX2, which is encoded by solute carrier family 24 member 2 (*SLC24A2*) and is widely expressed in the brain and spinal cord, is a member of the NCKX family (12). Gene-targeted knockout of NCKX2 resulted in motor learning and spatial working memory deficits in mice, which were caused by changes in plasticity at hippocampal Schaffer collateral-CA1 synapses (13). However, the roles of NCKX2 in the process of NP are still unknown.

To this end, the chronic constriction injury (CCI) model was used, and it was revealed that the expression of *SLC24A2* and its encoding protein NCKX2 were both downregulated in the posterior horn of the spinal cord. Overexpression of *SLC24A2* could reduce pain sensitivity and the expression

of inflammatory cytokines in CCI rats. Using bioinformatics analyses, luciferase reporter assays, and a series of behavioral tests, it was demonstrated that the decrease in *SLC24A2* after CCI treatment was directly regulated by increased microRNA (miRNA/miR)-135a-5p in the spinal cord.

Materials and methods

Animals and NP model. All experiments were approved by Ethics Committee of the Second Affiliated Hospital of Soochow University (no. 2018012), and strictly followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th edition). A total of 98 Sprague-Dawley male rats (8-10 weeks old), supplied by the Experimental Animal Center of Soochow University, were housed in a 22-24˚C, 40-60% humidity, specific pathogen-free, 12-h light/dark cycle room, with free access to a standard rat diet and water. The health and behavior of each group of rats were observed daily. The CCI NP model was prepared as previously described (14). Briefly, after being anesthetized with isoflurane (3.5% induction, 2-2.5% maintenance), both sides of the sciatic nerves of rats were exposed and ligated with non-absorbable sutures at 4 sites, with an interval of 1 mm. Sciatic nerves in sham rats were only isolated without ligation, serving as a control. Zero, three and seven days after surgery, the dorsal horn of spinal cords (L4-L6) were harvested under narcosis (isoflurane, 2-2.5% maintenance) for further analysis. Severely inert rats and those with the experiments completed were euthanized immediately by 99.9% carbon dioxide (the air replacement rate was 25% of the container volume per minute) to avoid or alleviate unnecessary suffering or distress. The absence of a heartbeat was used to verify the death of animals.

Intrathecal injection. Rats were anesthetized with isoflurane (3.5% induction, 1.5-2% maintenance), and fixed in the prone position. Following conventional disinfection, atlantooccipital membranes were exposed and a 1-cm length incision was made, then PE-10 polyethylene catheters were slowly implanted into the lumbar interspace through it. Rats with motor function impairment were excluded. Whether the operation was a success was confirmed by the occurrence of bilateral hind limb paralysis after 2% lidocaine injection. Recombinant Lenti-*SLC24A2* (10 μl; Shanghai GeneChem Co., Ltd.), miR-135a-5p agomir or antagomir (10 μM; Guanzhou RiboBio Co., Ltd.) were injected through a microinjection syringe, which linked with the intrathecal catheter, 72 h or 24 h before surgery. NCKX2 antisense oligo-deoxyribonucleotide (AS-ODN) (5'-CCAATGACTCGAATTAGCTT-3', 140 μg/kg) was infused continuously with an osmotic minipump (1 μl/h; RWD Life Science Co., Ltd.) connected to an intrathecal catheter 24 h before surgery (15,16).

Behavioral tests. Mechanical pain was determined by paw withdrawal threshold (PWT) in response to increasing mechanical pressure created by the calibrated Electronic von Frey filament as previously described (17). The pressure for bilateral hind paw withdrawal was recorded and defined as PWT. The cut-off was set at 50 g. The withdrawal threshold was calculated as the average of three continuous tests, with at least 10 min between them. Thermal pain was evaluated by paw withdrawal latency (PWL) in response to light radiant heat. Light was focused on the plantar surface of the bilateral hind paws. The interval between light onset and paw withdrawal was recorded and defined as PWL. The cut-off time was set at 20 sec. The test was repeated at least three times, with a 5-min interval between them, and the average PWLs was considered as the threshold of thermal-evoked pain.

Prediction of the direct binding miRNAs to SLC24A2. Bioinformatics analyses were performed to determine the miRNAs that directly bind to *SLC24A2* using programs in TargetScan (http://www.targetscan.org/vert_72/), miRanda (http://www.microrna.org/microrna/getGeneForm.do), miRDB (http://mirdb.org), PITA (https://genie.weizmann.ac.il/pubs/mir07/index.html) and miRWalk (http://mirwalk.umm.uni-heidelberg.de).

Luciferase reporter assay. The 3'UTR of *SLC24A2* carrying the wild-type binding sites or mutant binding sites of the miR-135a-5p were cloned into the pmirGLO dual-luciferase vector (Promega Corporation). The vectors were transfected into 293T cells by Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) with miR-135a-5p as well as agomir administration (Guanzhou RiboBio Co., Ltd.). After 48 h, cells were harvested and luciferase activity was detected using the Dual-Luciferase assay system (Promega Corporation) according to the manufacturer's instructions. The results were normalized to *Renilla* luciferase activity and expressed as relative luciferase activity.

Reverse transcription-quantitative PCR (RT-qPCR). RNA in lumbar dorsal spinal cord tissues were extracted by TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). Reverse transcription was performed using the PrimeScript RT reagent kit for mRNA or MiScript Reverse Transcription kit for miRNA (Qiagen, Inc.). An ABI PRISM 7500 real-time PCR system and SYBR Premix Ex Taq II (Takara Biotechnology Co., Ltd.) for mRNA or a MiScript SYBR-Green PCR kit (Qiagen, Inc.) for miRNA were used in the PCR reactions. The following thermocycling conditions were used for RT-qPCR: Initial denaturation at 95˚C for 5 min; 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec. GAPDH or U6 served as internal controls, respectively. Primers used were as follows: *SLC24A2* forward (F), 5'-CTGGAGGAGCGAAGGAAAGG-3' and reverse (R), 5'-TGTGAAAGTTCTGGGGCTGAC-3'; GAPDH F, 5'-CAACTTTGGCATCGTGGAAGGG-3' and R, 5'-CAACGGATACATTGGGGGTAGG-3'; miR-135a-5p F, 5'-CTCCTAGGTATGGCTTTTTATTC-3' and R, 5'-TCAACTGGTGTCGTGGAGTC-3'; U6 F, 5'-CTCGCTTCGGCAGCACA-3' and R, 5'-AACGCTTCAC GAATTTGCGT-3'. The relative expression of target genes was calculated by the $2^{-\Delta\Delta Cq}$ method (18).

Western blot analysis. Total protein of lumbar dorsal spinal cord tissues was isolated by N-PER™ Neuronal Protein Extraction reagent (Thermo Fisher Scientific, Inc.) with a protease inhibitor. The protein concentration was determined by the Pierce™ BCA Protein assay kit (Thermo Fisher Scientific, Inc.). Then, 20 μg protein was loaded into each well and separated by 10% SDS-PAGE. Subsequently, the protein blots were transferred onto PVDF membranes. After

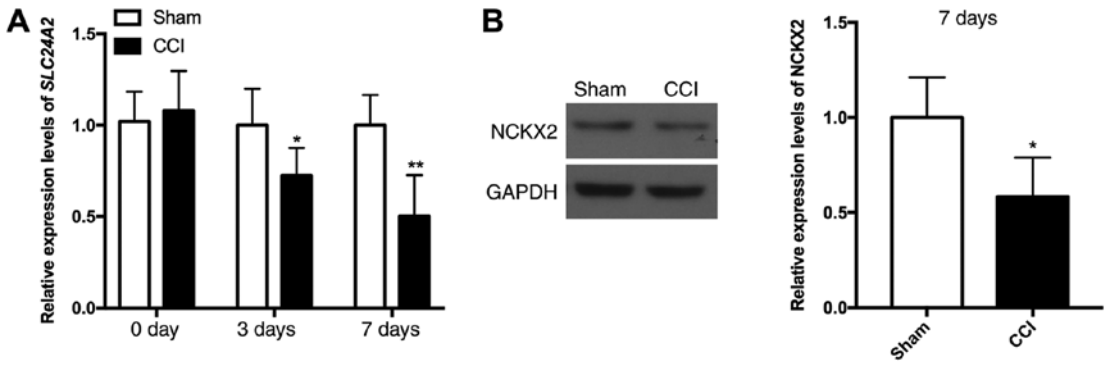

Figure 1. Expression of *SLC24A2* in the dorsal horn of the spinal cord is decreased after CCI. (A) The relative mRNA expression levels of *SLC24A2* in the L4-6 dorsal spinal cord of rats were determined by reverse transcription-quantitative PCR 0, 3 and 7 days after CCI or sham surgery. n=5. (B) The relative protein expression levels of NCKX2 in the L4-6 dorsal spinal cord of rats were determined by western blotting 7 days after CCI or sham surgery. n=5. *P<0.05, **P<0.01 vs. sham group. *SLC24A2*, solute carrier family 4 member 2; CCI, chronic constriction injury; NCKX2, K^+-dependent Na^+/Ca^{2+} exchanger 2.

being blocked by 5% bovine serum albumin (Beijing Solarbio Science & Technology Co., Ltd.) for 2 h at room temperature, the membranes were incubated overnight at 4˚C with the primary antibodies: Rabbit anti-NCKX2 antibody (1:200; ab192419; Abcam) and GAPDH (1:4,000; sc-25778; Santa Cruz Biotechnology, Inc.). The membranes were washed and a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:2,000; ab205718; Abcam) was added followed by incubation at room temperature for 1 h. Finally, the membranes were color developed with ECL (EMD Millipore). The gray value was calculated by Quantity One software (version 4.5.0; Bio-Rad Laboratories, Inc.) normalized to GAPDH.

Enzyme linked immunosorbent assay (ELISA). The protein expression of interleukin (IL)-1β, tumor necrosis factor (TNF)-α and IL-6 in lumbar dorsal spinal cord tissue homogenates were measured using commercially available ELISA kits (SEKM-0002, SEKM-0034 and SEKM-0007; Solarbio Life Science), according to the manufacturer's protocols. The experiment was repeated three times, and the mean value was used.

Statistical analysis. SPSS (version 18.0; SPSS, Inc.) was used for data analysis. Data were presented as the mean ± standard deviation. Comparisons between two groups were estimated by a Student's t-test, whereas multiple group comparisons were carried out by one-way ANOVA analysis with a Bonferroni's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of SLC24A2 in the dorsal spinal cord is decreased after CCI. To investigate the association between *SLC24A2* and NP, RT-qPCR was used to examine the expression levels of *SLC24A2* in the dorsal spinal cord of rats after CCI surgery. The results revealed that *SLC24A2* expression was significantly decreased in CCI rats compared with sham rats 3 and 7 days after surgery (P<0.05 and P<0.01, respectively, Fig. 1A). Moreover, the expression levels of the encoded protein (NCKX2) of the *SLC24A2* gene were determined 7 days after surgery by western blotting. It was revealed that NCKX2 expression was also significantly decreased in the lumbar dorsal spinal cord of CCI rats (P<0.05, Fig. 1B). These results indicated a negative association between *SLC24A2* expression and the progression of NP.

Overexpression of SLC24A2 alleviates NP. To determine the biological effect of *SLC24A2* on NP, *SLC24A2* was overexpressed in the dorsal spinal cord of rats by intrathecal injection of lentivirus carrying *SLC24A2* (Lenti-*SLC24A2*). First, it was demonstrated that the expression levels of *SLC24A2* and its encoded protein NCKX2 were both significantly increased in CCI rats treated with Lenti-*SLC24A2* (both P<0.01, Fig. 2A and B). Then, mechanical and thermal pain responses were evaluated using behavioral experiments. It was determined that mechanical allodynia and thermal hyperalgesia were significantly enhanced in rats subjected to CCI but alleviated by overexpressing *SLC24A2* (P<0.05, Fig. 2C and D). These results indicated a suppressive role of *SLC24A2* in NP.

Overexpression of SLC24A2 decreases the expression of inflammatory cytokines. A previous study revealed that changes in calcium concentration in cells could effectively regulate the inflammatory response (19); therefore, the biological effect of *SLC24A2* on the expression of a number of key inflammatory cytokines, which increase during the development of NP, was subsequently investigated. It was revealed that IL-1β, IL-6 and TNF-α were significantly increased in the dorsal spinal cord of CCI rats, however their expression was reversed in the Lenti-*SLC24A2*-treated group (P<0.05, Fig. 3). The results indicated a suppressive effect of *SLC24A2* on neural inflammation.

SLC24A2 is a direct target gene of miR-135a-5p. To determine whether the decrease in *SLC24A2* after CCI was caused by miRNAs and identify the key regulator, bioinformatics analyses were performed to predict the miRNAs that directly bind to *SLC24A2*. All programs predicted that the seed regions of miR-135a-5p could bind conserved sites in the 3'UTR of *SLC24A2* (Fig. 4B). Moreover, it was revealed that the expression of miR-135a-5p was increased in the CCI rats (P<0.01, Fig. 4A). Therefore, it was next investigated whether miR-135a-5p directly interacts with the 3'UTR of *SLC24A2*.

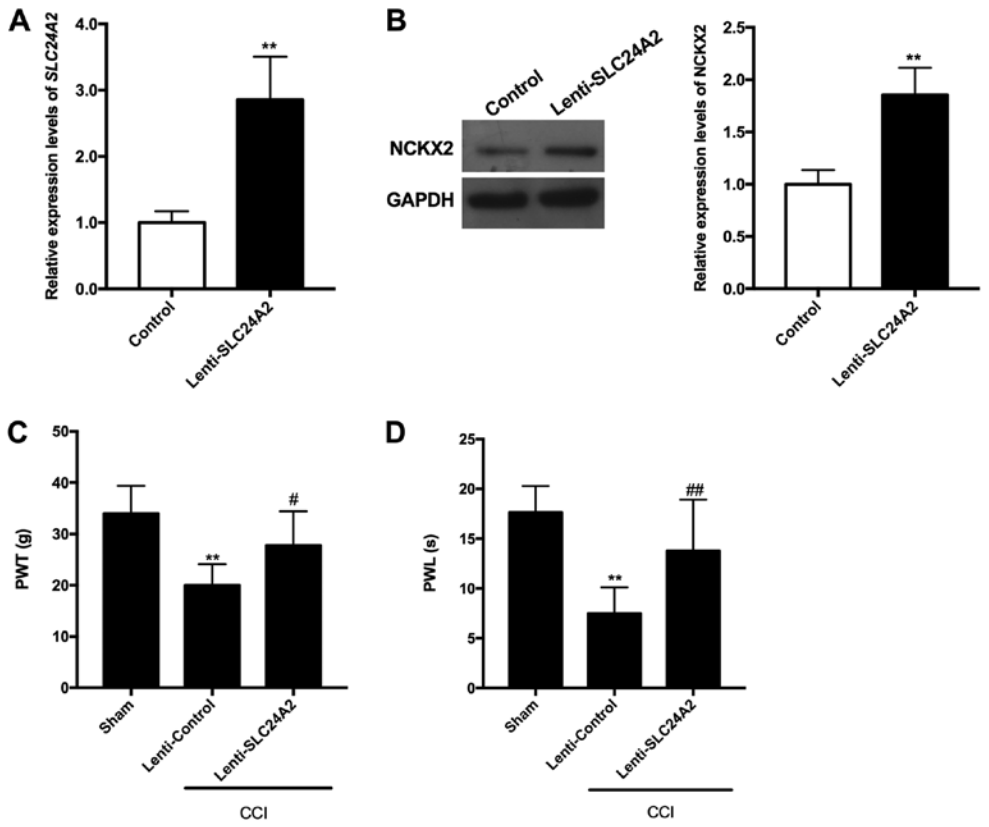

Figure 2. Overexpression of *SLC24A2* alleviates NP. (A) The relative mRNA expression levels of *SLC24A2* in the L4-6 dorsal spinal cord of rats treated with control or Lenti-*SLC24A2* were determined by reverse transcription-quantitative PCR 7 days after CCI surgery. n=5. (B) The relative protein expression levels of NCKX2 in the L4-6 dorsal spinal cord of rats treated with control or Lenti-*SLC24A2* were determined by western blotting 7 days after CCI surgery. n=5. (C) The effect of *SLC24A2* overexpression on mechanical allodynia was evaluated by PWT 7 days after CCI. n=8. (D) The effect of *SLC24A2* overexpression on thermal hyperalgesia was determined by PWL 7 days after CCI. n=8. **P<0.01 vs. control (sham) group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. CCI + Lenti-Control group. *SLC24A2*, solute carrier family 4 member 2; NP, neuropathic pain; CCI, chronic constriction injury; PWT, paw withdrawal threshold; PWL, paw withdrawal latency; NCKX2, K^+-dependent Na^+/Ca^{2+} exchanger 2.

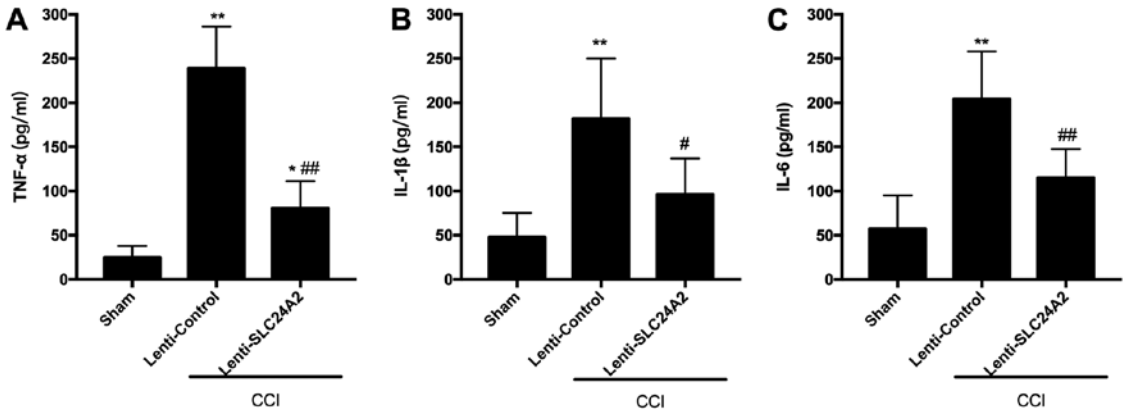

Figure 3. Overexpression of *SLC24A2* decreases the expression of inflammatory cytokines. The protein expression of (A) TNF-α, (B) IL-1β and (C) IL-6 in the L4-6 dorsal spinal cord of rats was determined by ELISA 7 days after CCI. n=6. *P<0.05, **P<0.01 vs. sham group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. CCI + Lenti-Control group. *SLC24A2*, solute carrier family 4 member 2; CCI, chronic constriction injury; IL, interleukin; TNF-α, tumor necrosis factor α.

The results revealed that co-transfection of miR-135a-5p agomir with the luciferase vector containing the *SLC24A2* 3'UTR with the wild-type miRNA binding site led to a significant decrease in relative luciferase activity in 293T cells (P<0.01, Fig. 4B and C). However, when the binding site was mutated, the inhibitory effect of overexpressed miR-135a-5p on relative luciferase activity was blocked (P>0.05, Fig. 4B and C). This result indicated that miR-135a-5p directly targeted the 3'UTR of *SLC24A2*. Furthermore, the effect of up- or downregulation of miR-135a-5p on NCKX2 expression was determined by western blotting. The results revealed that miR-135a-5p agomir treatment significantly decreased the protein levels of NCKX2 in the dorsal spinal cord (P<0.01, Fig. 4D), and the miR-135a-5p antagomir had the opposite effect on NCKX2

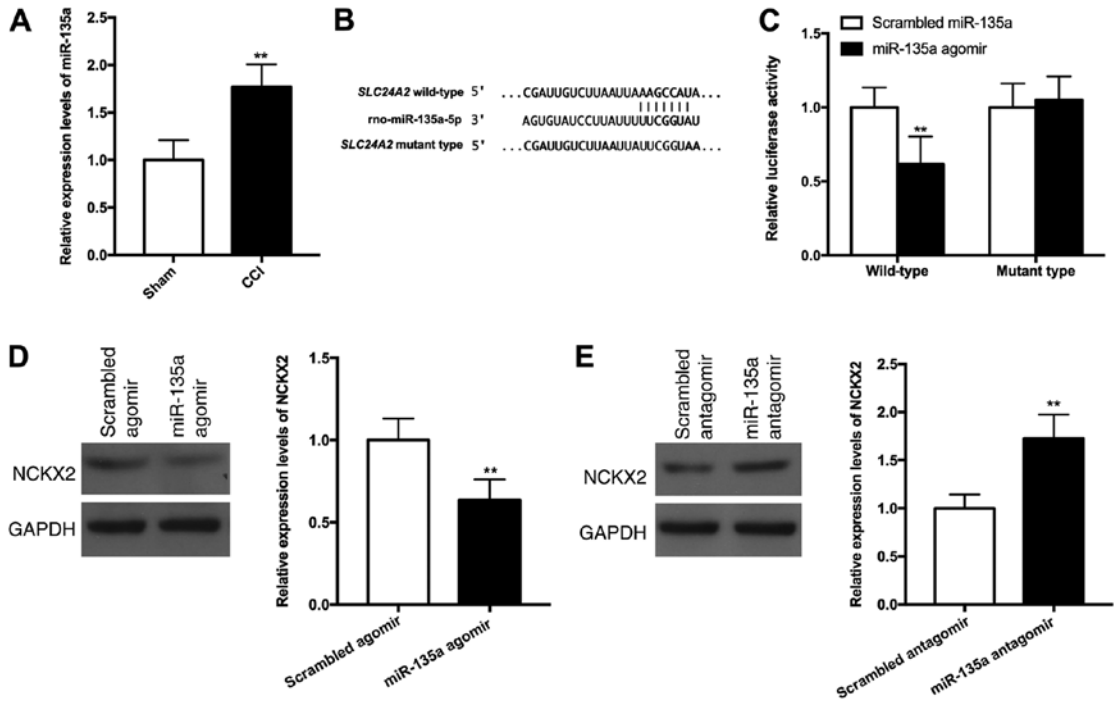

Figure 4. *SLC24A2* is a direct target gene of miR-135a-5p. (A) The relative mRNA expression levels of miR-135a-5p in the L4-6 dorsal spinal cord of rats were determined by reverse transcription-quantitative PCR 7 days after CCI or sham surgery. n=5. **P<0.01 vs. sham group. (B) Schematic diagram of the wild-type or mutant binding sites of miR-135a-5p in the 3'UTR of *SLC24A2*. (C) The relative luciferase activity in 293T cells with different treatments by dual-luciferase reporter assay. n=5. **P<0.01 vs. scrambled miR-135a-5p agomir group. The relative protein expression levels of NCKX2 in the L4-6 dorsal spinal cord of rats treated with (D) miR-135a-5p agomir or (E) miR-135a-5p antagomir were determined by western blotting 7 days after CCI surgery. n=5. **P<0.01 vs. scrambled agomir group. *SLC24A2*, solute carrier family 4 member 2; CCI, chronic constriction injury; miR, microRNA; NCKX2, K^+-dependent Na^+/Ca^{2+} exchanger 2

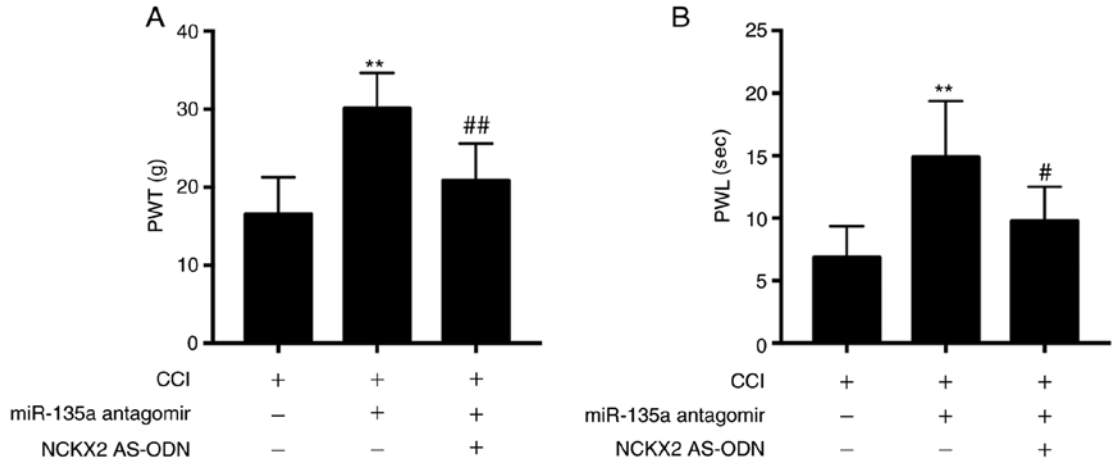

Figure 5. Downregulation of NCKX2 reverses the suppressive effect of the miR-135a-5p antagomir on NP. (A) Downregulation of NCKX2 reverses the suppressive effect of miR-135a-5p antagomir on mechanical allodynia 7 days after CCI. n=8. (B) Downregulation of NCKX2 reverses the suppressive effect of miR-135a-5p antagomir on thermal hyperalgesia 7 days after CCI. n=8. **P<0.01 vs. CCI group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. CCI + miR-135a-5p antagomir group. NP, neuropathic pain; CCI, chronic constriction injury; PWT, paw withdrawal threshold; PWL, paw withdrawal latency; NCKX2, K^+-dependent Na^+/Ca^{2+} exchanger 2; miR, microRNA; AS-ODN, antisense oligo-deoxyribonucleotide.

expression (P<0.01, Fig. 4E). In short, these results indicated that *SLC24A2* is a target gene of miR-135a-5p.

Downregulation of NCKX2 reverses the suppressive effect of miR-135a-5p antagomir on NP. To determine whether miR-135a-5p regulates NP by targeting *SLC24A2*, rescue experiments were performed by co-infecting rats with miR-135a-5p antagomir and NCKX2 AS-ODN. The results revealed that the suppressive effects of the miR-135a-5p antagomir on mechanical allodynia (P<0.01, Fig. 5A) and thermal hyperalgesia (P<0.01, Fig. 5B) were significantly reversed by NCKX2 AS-ODN treatment. Moreover, the decreased expression of the inflammatory cytokines IL-1β (P<0.01, Fig. 6A), IL-6 (P<0.01, Fig. 6B) and TNF-α (P<0.01, Fig. 6C) caused by the miR-135a-5p antagomir was also reversed by NCKX2 AS-ODN. Overall, these results indicated that miR-135a-5p exerted its effect on NP by targeting *SLC24A2* and its encoded protein.

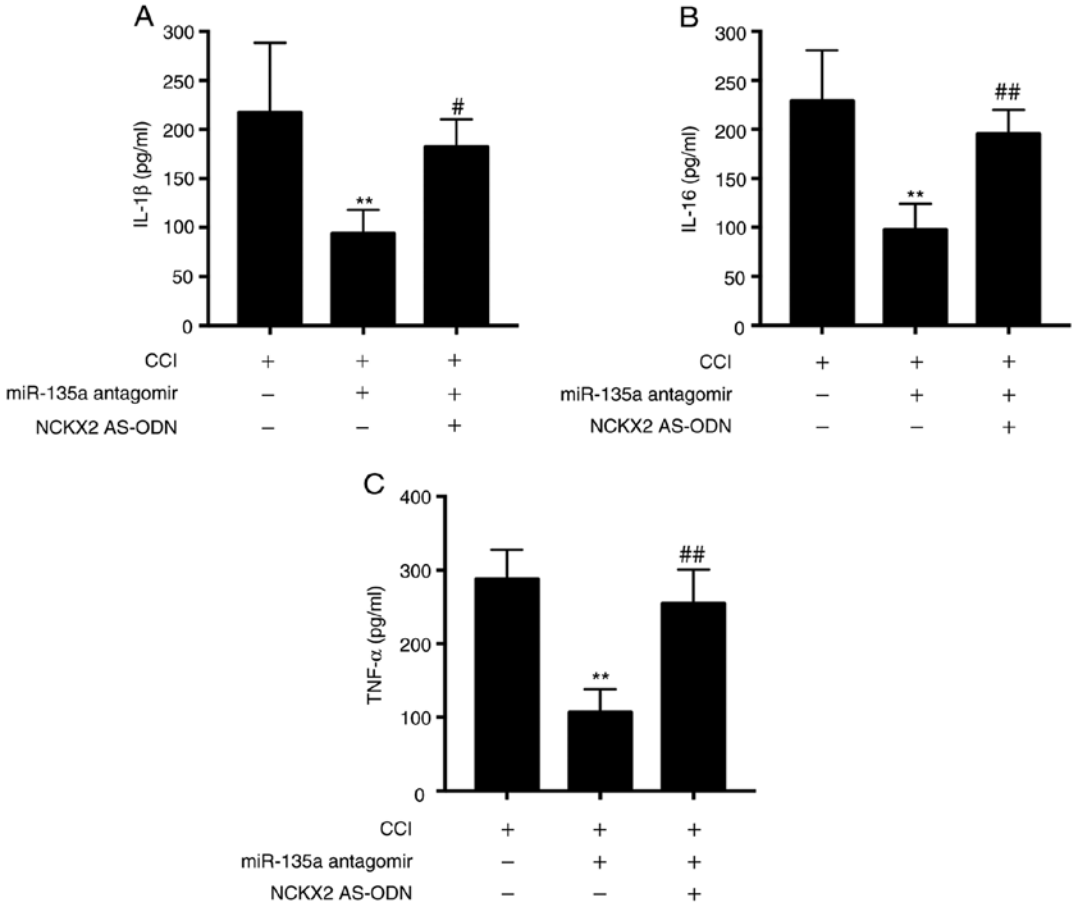

Figure 6. Downregulation of NCKX2 reverses the suppressive effect of miR-135a-5p antagomir on the expression of inflammatory cytokines. Protein expression of (A) IL-1β, (B) IL-6 and (C) TNF-α in the L4-6 dorsal spinal cord of differently treated rats was determined by ELISA 7 days after CCI. n=6. **P<0.01 vs. CCI group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. CCI + miR-135a-5p antagomir group. CCI, chronic constriction injury; NCKX2, K^+-dependent Na^+/Ca^{2+} exchanger 2; miR, microRNA; IL, interleukin; TNF-α, tumor necrosis factor α; AS-ODN, antisense oligo-deoxyribonucleotide.

Discussion

As an important Na^+/Ca^{2+} exchanger, NCKX2 plays a significant role in intracellular calcium clearance and participates in the process of key neurophysiological functions (12,13). To the best of the authors' knowledge, the present study is the first direct evidence that NCKX2 plays a role in the pathologic process of neuralgia, and its expression changes are directly regulated by miR-135a-5p.

NCXs and NCKXs are two major families of Ca^{2+} plasma-membrane exchangers. The NCX family includes three different gene products, NCX1-3, and exchanges Ca^{2+} with Na^+ in either direction to maintain intracellular Ca^{2+} homeostasis (11). The NCKX family is composed of five members, NCKX1-5, which differ from NCXs in their absolute requirement for K^+, and maintain mostly calcium extrusion when the intracellular calcium concentration is >500 nM (20). NCKX2 is the major isoform expressed in neurons (12,21). A previous study demonstrated its physiological effects on hippocampal synaptic plasticity and learning and memory functions (13). In addition, a key role in the pathogenesis of ischemic damage was also revealed in NCKX2-knockout mice (16). However, the roles of NCKX2 during NP have not been indicated. A previous study suggested that NCX members in L4-6 dorsal root ganglion neurons or other sensory neurons are involved in the processes of spinal nerve ligation surgery or chemotherapy-induced peripheral neuropathy pain (22). These results indicated that NCKX2, another type of Na^+-Ca^{2+} exchanger, may also mediate the pathological process of NP. As anticipated, in the present study, using CCI pain models, a significant decrease in NCKX2 mRNA and protein expression in the dorsal horn of the spinal cord was revealed. Overexpression of NCKX2 could relieve the hyperalgesia of rats in thermal and mechanical allodynia. To the best of our knowledge, this is the first study investigating the effect of NCKX2 on NP. Moreover, it was also demonstrated that the roles of NCKX2 in the development of NP may be exerted through regulation of the release of key inflammatory cytokines, including IL-1β, IL-6, and TNF-α. The changes in calcium concentration in cells can effectively regulate the inflammatory response (19), which is important for the occurrence of NP. Thus, blocking the release of inflammatory cytokines by enhancing Ca^{2+} extrusion by NCKX2 may be effective for NP treatment in the future. NF-κB is also an important factor in inflammatory processes and involved in the neuropathic pain (23). Whether NF-κB can be regulated by NCKX2 was not determined in the present study, however in a following study, experiments will be performed to elucidate their association.

In the present study, using a series of bioinformatics analyses, luciferase assays, and behavioral studies, it was

further elucidated that the downregulation of NCKX2 during the progression of NP was related to the upregulation of miR-135a-5p. miR-135a-5p is a highly expressed miRNA in the nervous system (24-29). Mannironi *et al* (24) reported a physiological role for miR-135a in the modulation of presynaptic mechanisms of glutamatergic neurotransmission and a previously unknown mechanism in the amygdala for regulating anxiety-like behavior. In addition, the roles of miR-135a in some nervous system diseases, such as malignant glioma, Alzheimer's disease, Parkinson's disease, cerebral ischemia and temporal lobe epilepsy, have also been demonstrated (25-29). There are few studies revealing the relationship between miR-135a-5p and the pathogenesis of pain. Using miRNA-sequencing analysis of blood samples, Linnstaedt *et al* (30) indicated that the upregulation of miR-135a-5p may be related to persistent pain after motor vehicle collision. In the present study, it was further demonstrated that miR-135a-5p upregulation was involved in NP and that downregulating miR-135a-5p could effectively alleviate mechanical and thermal hyperalgesia. In addition, the increased expression of IL-1β, IL-6 and TNF-α in CCI rats was reversed by regulating the expression of miR-135a-5p, and all these roles were demonstrated to be NCKX2-dependent. These results indicated that miR-135a-5p and its target gene, *SLC24A2*, could be developed into novel effective drug targets for NP treatment.

In conclusion, using the CCI model, it was revealed that miR-135a-5p inhibited the expression of NCKX2 in the dorsal root of the spinal cord and was involved in the progression of NP. Selective regulation of miR-135a-5p or NCKX2 expression could effectively relieve neuralgia. Additional studies should be performed in other pain models to further demonstrate the universal effects of miR-135a-5p or NCKX2 on other types of pain. Moreover, the upstream regulatory factors for miR-135a-5p changes and the downstream effective targets of NCKX2 in the pathogenesis of pain should also be investigated.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XGZ and PJW designed the study and wrote the manuscript. XGZ and HH performed the experiments and statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments were approved by Ethics Committee of the Second Affiliated Hospital of Soochow University (approval no. 2018012), and strictly followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th edition).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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