MiR-106b-5p represses neuropathic pain by regulating P2X4 receptor in the spinal cord in mice

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Research

Keywords: P2X4R, miR-106b-5p, neuropathic pain, spinal cord, spared nerve injury.

DOI: https://doi.org/10.21203/rs.3.rs-41480/v1

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Abstract

Background: P2X4 receptor (P2X4R)-mediated spinal microglial activation makes a critical contribution to pathologically enhanced pain processing in the dorsal horn. It can be upregulated under conditions of neuropathic pain. However, the specific mechanism of pathogenesis and potential molecular targets has not yet been made explicit. MicroRNAs (miRNAs) are commonly recognized as indicators in neuropathic pain pathophysiology.

Methods: We established the pain model of spared nerve injury (SNI), and the 50% paw withdrawal thresholds (PWMTs) were used to assess behavior of mouse. MiRNA expression profiling was performed to detect differential expressed miRNA. The western-bolt and quantitative real time PCR to examine P2X4R and miRNA expression in the mouse. Dual-luciferase reporter assays confirmed the correlation between P2X4R and miRNA. Fluorescence in situ hybridization was used to show location between P2X4R and miRNA.

Results: In the present study, we found that P2X4R was up-regulated in the spinal dorsal horn of mice following spared nerve injury (SNI), and we identified 69 miRNAs (46 up-regulated and 23 down-regulated miRNAs) were differently expressed (fold change > 2, \( P < 0.05 \)). P2X4R was a major target of miR-106b-5p (one of down-regulated miRNAs in SNI) with bioinformatics technology and quantitative real time PCR analysis validated the expressed change of miR-106b-5p, and dual-luciferase reporter assays confirmed the correlation between them. Fluorescence in situ hybridization showed that miR-106b-5p was co-localized with P2X4R in the spinal cord. Transfection with miR-106b-5p mimic on BV2 cells reversed the up-regulation of P2X4R induced by LPS. Moreover, miR-106b-5p overexpression significantly attenuated neuropathic pain induced by SNI, with decreased expression of P2X4R mRNA and protein in the spinal cord.

Conclusion: Taken together, our results suggest that miR-106b-5p can serve as an important regulator of neuropathic pain development by targeting P2X4R.

Introduction

Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage by the International Association for the Study of pain [1], and neuropathic pain is defined as “pain caused by a lesion or disease of the somatosensory nervous system” [2]. Neuropathic pain treatment is a great challenge and the outcomes remain unsatisfactory, mainly due to barely understood pathogenesis and a lack of well-defined molecular targets [3, 4].

P2X receptors are non-selective cation channels that open in response to adenosine triphosphate (ATP) binding, allowing the rapid flow of ions (K\(^+\), Na\(^+\), Ca\(^{2+}\)) across the membrane [5]. Among the seven P2X receptors, P2X4 receptor (P2X4R) display the most widespread tissue distribution and expression in neurons and the immune system [6]. Studies have found that P2X4R is closely related to the immune system and plays vital functions through monocytes, macrophages, dendritic cells, neutrophils, and T lymphocytes [7]. Purinergic receptor-mediated spinal microglial functions make a critical role in pain processing [8, 9]. A great deal of evidence has shown that P2X4R plays a vital role in the process of neuropathic pain [10, 11]. Our previous study has found that ATP activates p38 mitogen-activated protein kinase (p38 MAPK) by binding to endogenous P2X4R in...
microglia and subsequently increases the expression of P2X₄R, thus promoting long-term potentiation [12], which is the basis of the hypersensitization of nociceptive neurons in the spinal cord. A previous study demonstrated that the blockade of P2X₄R inhibits neuropathic pain-related behavior by preventing MMP-9 activation, then pronociceptive interleukins (IL-1β, IL-18, IL-6) release in CCI-induced rat model, suggesting that P2X₄R may indeed play a significant role in neuropathic pain development by modulating neuroimmune interactions in the spinal cord and DRG [11]. Although these studies have partly elucidated the mechanism of altered P2X₄R expression, the specific mechanism are not complete clear.

MiRNAs are highly deregulated in neuropathic pain, and might be a critical molecule for treatment [13, 14]. MiRNAs can negatively regulate gene expression by translationally targeting the targeted mRNAs [15–17], and can serve as indicators in neuropathic pain pathophysiology [18–20]. It has been found that miR-98 might depress neuropathic pain development through modulating high mobility group AT-hook 2 (HMGA2) [21] and that miR-30b is involved in the development of neuropathic pain, probably by regulating the expression of Nav1.3 [3]. However, whether miRNA is involved in the pathogenesis of neuropathic pain by regulating the P2X₄R has not been discovered. In the present study, P2X₄R was up-regulated and a microarray analysis was constructed to identify differential expressed (DE) miRNAs in the spinal dorsal horn following spared nerve injury (SNI). Moreover, the interaction of potential miRNA and P2X₄R was evaluated to clarify the underlying mechanisms involved in the development of neuropathic pain.

**Materials And Methods**

**Animals**

Male Balb/c mice aged 8 weeks were purchased from Animal Experimental Center, Sun Yat-sen University, China (Guangzhou, China). Mice were housed in separated cages and the rooms were kept at 24 ± 1°C (297 ± 1 K) temperature and 50–60% humidity, under a 12/12 light-dark cycle and with free access to food and water ad libitum. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Guangzhou Medical University and were carried out in accordance with the guidelines of the National Institutes of Health on animal care and the ethical guidelines for investigation of experimental pain in conscious animal.

**Cell Culture And Transfection**

Culture and transfection of BV2 cells were carried out as described elsewhere [22]. All cells were collected in high glucose Medium (Gibco) with 10% FBS (JRScientific, Woodland, CA, USA), 100 mg/mL streptomycin, and 100 units/mL penicillin (Quality Biological, Gaithersburg, MD, USA). The cells were incubated at 37 C, 95% O2, and 5% CO2 with a seeding density of 1×10⁵ BV2 cells/mL. One day later, miRNAs mimic or inhibitor (RiboBio, Guangzhou, China) were transfected in the cells. 120 ul Opti-Mem was used to dilute miR-106b-5p mimic (50 nM)/inhibitor (100 nM) or negative control. The sequences for miR-106b-5p mimic are as follow, forward: 5'-UAAAGUGCUGACAGUGCAGAU-3', Reverse: 5'-AUUUCACGACUGUCAGUCUA-3'; the sequences for miR-106b-5p inhibitor is 5'-AUUUCACGACUGUCAGUCUA-3'. The final concentrations of mimic and inhibitor were 50 nM and 100 nM, respectively. 120 ul Opti-Mem was simultaneously used to dilute 7.5 uL RNAiMAX (Invitrogen, Carlsbad, CA), then the two solutions were mixed. After 5 min, the mixture was placed into each 2 mL well and
high glucose medium was added. 2 ml LPS (100 ng/mL, Sigma) was added to each 2 mL well 6 h before the cells were collected 48 h later for qPCR and western-blot examinations.

Cell Proliferation Assay

Cell proliferation was evaluated using a cell counting kit-8 (CCK-8) assay (Beyotime, China). BV2 cells (5 × 10^3) were plated onto 96-well plates, then the LPS or miRNA mimic or miRNA inhibitor were added in the BV2 cells. Each well contained 100 µL of medium supplemented with 10 µL of CCK8 solution. The absorbance at 450 nm was measured to assess the degree of cell proliferation after incubating the cells at 37 °C for 1 hr.

Luciferase Assay

A dual luciferase reporter assay was performed as outlined for a previous procedure [23]. The pmirGLO dual-luciferase vector (pmirGLO vector), which contained both the firefly luciferase gene and the renilla luciferase gene, was purchased from Promega (Madison, WI, USA). P2X₄R 3’UTR, including the predicted binding sites of miR-106b-5p, was inserted into the 3’UTR region downstream of the firefly luciferase gene of the pmirGLO vector (pmir-GLO-UTR). HEK293 cells were cultivated in high glucose medium (Gibco) with 10% FBS (JRScientific, Woodland, CA, USA), 100 mg/mL streptomycin, and 100 units/mL penicillin (Quality Biological, Gaithersburg, MD, USA). The cells were incubated in a humidified incubator with 5% CO2 at 37°C. When the HEK293T cells had a confluence of 70–80%, A P2X₄R-luciferase-reporter construct (pmiRGLO-P2X₄R 3’-UTR vector), containing the miR-106b-5p binding motif, was co-transfected with miR-106b-5p mimic at different doses of 10, 20, and 50 nM (20 umol/L) or NC mimic into HEK293 cells by using RNAiMAX (Invitrogen). After another 48 h of culture, we used 1 * passive lysis buffer to lyse the transfected cells, and 20 ul supernatant was achieved for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). The ratio of firefly activity to renilla activity was recognized as relative reporter activity. Experiments were performed in triplicate and repeated three times.

Surgical Procedures And Drug Infusion

SNI surgeries were performed to mice according to our previous work [24]. In brief, mice were deeply anesthetized after the behavioral tests. The skin of the lateral thigh was incised and the biceps femoris muscle was dissected bluntly to expose the left sciatic nerve and its three terminal branches (the sural, common peroneal and tibial nerves). The common peroneal and the tibial nerves were tightly ligated with 5−0 silk. Then, the nerve was transected distal to the ligation, and 2−4 mm length of nerve fiber was removed. Great care was taken to avoid any contact with or stretching of the intact sural nerve. The wound was closed in two layers. The sham group was given all procedures except ligation and transection.

The method of intrathecal injections was described previously [25]. In brief, mice were restrained with the left hand and the injection was performed with the right hand. The vertebral landmarks for L5 and L6 vertebrae were identified by palpation. An injection into the subarachnoid space between the L5 and the L6 vertebrae was done via a 27-gage needle. Entry of the needle was confirmed with the presence of a tail flick. The injection
volume of all other compounds was 5 µl. In one part, the mice were divided into three groups: sham + scramble (saline), SNI + scramble (saline) and SNI + miR-106b-5p agomir (a selective mimic of miR-106b-5p). In another part, the mice were divided into two groups, naïve + scramble and naïve + miR-106b-5p antagomir. Beginning 1 day after naïve or 7 days after SNI surgery, continuous intrathecal infusion was delivered once a day for 3 days, from day 1 to 3 for naïve mice and from day 7 to 9 for SNI mice. Mice with neurological deficits were excluded from the experiment.

Behavior Tests

The 50% paw withdrawal thresholds (PWMTs) were measured on days 0, 3, 5, and 7 for the evaluation of SNI model or on days 0, 5, 7, 8, 9, 10, and 14 during continuous miR-106b-5p agomir injection following SNI surgery. They were measured on days 0, 1, 2, and 3 during continuous miR-106b-5p antagomir injection in naïve mice, always between 8 and 10 AM in the morning. PWMT was assessed with method described previously [26]. The filament force evoking paw withdrawals for more than 3 times in a round of testing was defined as the mechanical threshold. The cutoff force was 4 g. The observation of a positive response (paw lifting, shaking, or licking) within 5 s.

MiRNA Microarray Analysis

The method was described as our previous study [13]. MiRNA expression profiling was performed using the RibonArray platform (RiboBio, Guangzhou, China). In brief, 1 µg of total RNA was labeled with a Cy3 using a ULS™ microRNA Labeling Kit (Kreatech, Amsterdam, Netherlands) and hybridized on the microarray. Based on Sanger miRBase version 19.0 database, RiboBio designed 1263 specific oligos for 1281 mouse miRNA, where 1263 are non-redundant sequence. In addition, 54 RibonArray™ internal controls were used. We also put some probes for location identify function. T-test P-value of < 0.05 and fold-change (> 2) were applied to determine two differentially expressed (DE) sets of genes of six experimental samples.

Rna Extraction And Quantitative Real Time Pcr (qpcr)

Total RNA was extracted using the Trizol method [24, 27]. The quality and quantity of RNA were measured using a Nanodrop Spectrophotometer (Thermo Scientific) and samples with an absorbance ratio at 260/280 between 1.8–2.2 were considered acceptable. RNA degradation was not assessed. RNA dilutions were made up in nuclease-free water. For analysis of mRNA, the method was described previously [24, 27]. Reverse transcription was generated using the SuperScriptTM III Reverse Transcriptase (Invitrogen) with a Gene Amp PCR System 9700 (Applied Biosystems). For miRNA analysis, 1 ug RNA was used for reverse transcription by miRNA 1st Strand cDNA Synthesis Kit (by stem-loop) (Vazyme, #MR101) according to the instructions. Quantitative Real-time PCR (qPCR) was carried out on a real-time detection instrument ViiA 7 Real-time PCR System (Applied Biosystems) using 2X PCR master mix (Arraystar). All experiments were replicated three times. The relative expression of genes was calculated based on the $2^{-\Delta\Delta Ct}$ method using the mouse housekeeping GAPDH/U6 gene as an endogenous control. Expression ratios were subjected to a log2 transform to produce fold change data.
Western Blot

To ensure a sufficient amount of protein, BV2 cells and a section of ipsilateral Lumbar enlargement was prepared. Based on established protocol [28], tissues were homogenized in a RIPA buffer (Sangon, Shanghai, China). After centrifugation at 12,000 × g for 15 min at 4 °C, the supernatant was collected to analyze cytosolic proteins, and protein concentrations were determined with a BCA Kit (Thermo Fisher Scientific, Rockford, IL, USA). The contents of the proteins in the samples were measured using the Bio-Rad protein assay (Bio-Rad) and were then heated at 99 °C for 5 min. Samples of 20 mg total protein were separated by 10% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred onto a polyvinylidene difluoride membrane. After the membranes were blocked with 5% Milk in Tris-buffered PBS containing 0.1% Tween-20 for 1 h, then rabbit anti-P2X4R (1:1000, Alomone Labs), rabbit anti-tubulin (1:10000, Sigma), and rabbit anti-GAPDH (1:5000, Cell Signaling Technology) primary antibodies would be used. The proteins were detected using horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:1000, Jackson) and visualized using Western peroxide reagent and luminol/enhancer reagent (Clarity Western ECL Substrate, Bio-Rad). The intensity of the blots was quantified via densitometry using Image J software. All cytosol protein bands were normalized to tubulin or GAPDH.

Fluorescence In Situ Hybridization

The fluorescence in situ hybridization technology was performed by Servicebio (wuhan, China). The spinal cord of animals was sectioned at 3 mm, and sections were dehydrated in series of ethanol washes (70%, 85%, and 100%) and air-dried. After incubated in hybridization solution at 37 °C for 1 h, the sections were incubated overnight in hybridization solution with 6 ng/µL of DIG (488) labeled probes for miR-106b-5p (5’-DIG-ATCTGCACTGTACAGACTTTA-DIG-3’) at 37 °C. Following hybridization, the sections were washed twice at 37 °C with 2 × SSC for 10 min, then twice at 37 °C with 1 × SSC for 5 min and in 0.5 × SSC at room temperature for 10 min. After they were blocked for 30 min at room temperature, we incubated them with anti-DIG-HRP at 37 °C for 50 min. Finally, FITC-TSA was added at dark for 5 min. To identify the cell types expressing miR-106b-5p, the above sections were incubated overnight at 4 °C with primary antibodies against ATP-gated cation channel protein (P2X4R, rabbit, 1:100, ), the ionized calcium-binding adapter molecule (IBA-1, rabbit, 1:300;), glial fibrillary acidic protein (GFAP, mouse, 1:1000;) or the neuronal-specific nuclear protein (NeuN, mouse, 1:50;) for double staining as described previously.

Statistical analysis

The data are presented as means ± SEM. For comparisons between two groups, the P value was evaluated and calculated using a two-tailed unpaired t-test. When there were multiple groups involved, a one-way analysis of variance (ANOVA) was used; multiple factors were compared using two-way analysis of variance (ANOVA). Values of P < 0.05 were considered statistically significant.

Results

Up-regulation of P2X4R in the spinal cord after SNI
Compared to baseline PWMTs observed on day −1d, SNI induced a conspicuous reduction in PWMTs ($P < 0.001$, Fig. 1A). In contrast, no changes in the PWMTs were observed in the sham group during the observation period. To determine the expression of P2X$_4$R in the spinal cord of SNI mice, qPCR and western-blot analysis were performed. Compared to the sham group, the SNI group showed an obvious up-regulation of P2X$_4$R mRNA expression in the spinal cord ($P < 0.05$, Fig. 1B). The western-blot results showed that the P2X$_4$R protein strongly increased after nerve injury ($P < 0.01$, Fig. 1C), which was consistent with the data from the behavioral test. As a consequence, the increased expression of P2X$_4$R mRNA and protein in the spinal cord of SNI mice confirmed the potential ability of P2X$_4$R to aggravate SNI-induced neuropathic pain.

The Differentially Expressed Mirnas In Neuropathic Pain Mice

To investigate the expression of miRNAs in neuropathic pain, the expression levels of miRNAs in the spinal cord of neuropathic pain mice with microarray were analyzed. Results showed 69 miRNAs were differently expressed (fold change > 2.0, $P < 0.05$), including 46 up-regulated miRNAs (Fig. 2A) and 23 down-regulated miRNAs (Fig. 2B).

Focus on the downregulated miRNAs, we discovered that P2X$_4$R was the target of miR-106b-5p using TargetScan software. The matched seed sequences between miR-106b-5p and P2X$_4$R 3’UTR were highly matched between human and mice (Fig. 2C). The TargetScan software demonstrated that the seed sequence for the miR-106b-5p position (1–7) was paired with P2X$_4$R 3’UTR from 363 bps to 388 bps in humans and from 1165 bps to 1190 bps in the mice. Then, the expression of miR-106b-5p in SNI mice was verified by the qPCR method, and the results were consistent with those of the microarray. MiR-106b-5p showed a down-regulation trend in the spinal dorsal horn of the SNI mice ($P < 0.01$, Fig. 2D). To verify whether miR-106b-5p targets P2X$_4$R 3’UTR, a dual-luciferase reporter vector containing the sequence of P2X$_4$R 3’UTR was designed (pmirGLO P2X$_4$R 3’UTR). When transfecting the P2X$_4$R 3’UTR vector with three different doses of miR-106b-5p mimic (10, 20 and 50 nM) into HEK293 cells with RNAiMax, the miR-106b-5p mimic reduced relative luciferase activity in a dose-dependent manner ($P < 0.05$, Fig. 2E).

MiR-106b-5p mimic transfection inhibits the expression of P2X$_4$R mRNA in BV2 cells

The result was consistent with previous study [22], BV2 cells were activated after stimulating by LPS (Fig. 3A-B). Further, the cell proliferation was verified by using CCK-8 assay. The results showed that BV2 cells viability increased significantly in a dose-dependent relationship with LPS ($P < 0.05$, Fig. 3C). Besides, miR-106b-5p mimic or inhibitor did not affect BV2 cells viability ($P > 0.05$, Fig. 3D), which indicated miR-106b-5p mimic or inhibitor has no toxic effect on BV2 cells viability.

Moreover, to determine whether miR-106b-5p regulate the expression of P2X$_4$R, miR-106b-5p mimic was transfected in BV2 cells. LPS (2 mL, 100 ng/mL) was used to stimulate the BV2 cells 42 h later. The levels of miR-106b-5p and P2X$_4$R mRNA were measured by qPCR, and the changes in P2X$_4$R protein expression were determined by western-blot after transfection at 48 h. Compared to the naïve non-transfected group, LPS
stimulation induced a significant increase in P2X₄R at the mRNA (P< 0.01, Fig. 4B) and protein level (P< 0.05, Fig. 4C), while a reduction of miR-106b-5p was observed (P< 0.05, Fig. 4A). However, the overexpression of miR-106b-5p reversed the up-regulation of P2X₄R mRNA (P< 0.05, Fig. 4B) and P2X₄R protein (P< 0.01, Fig. 4C) by transfecting the miR-106b-5p mimic. Moreover, the transfection of miR-106b-5p mimic increased the expression of miR-106b-5p (P< 0.0001, Fig. 4A), but did not influence the changes of P2X₄R mRNA and protein (P> 0.05, Fig. 4B and 4C) in untreated BV2 cells.

In addition, we found that miR-106b-5p inhibitor transfection up-regulated P2X₄R mRNA (P< 0.001, Fig. 4E) and P2X₄R protein (P< 0.01, Fig. 4F), while it down-regulated miR-106b-5p (P< 0.0001, Fig. 4D). Taken together, it has been demonstrated that miR-106b-5p may suppress the expression of P2X₄R mRNA through its binding with P2X₄R 3’UTR.

**P2X₄R are co-localized with miR-106b-5p in spinal cord of SNI mice**

In order to define the localization of miR-106b-5p, the immunofluorescence in situ hybridization was performed in the spinal cord. As shown in Fig. 5, miR-106b-5p with P2X₄R, IBA1 (a marker for microglia cells), GFAP (a marker for glial cells), and NeuN (a marker for neurons) were stained. Results showed that miR-106b-5p was mainly co-localized with P2X₄R, IBA1 and NeuN, but not with GFAP.

**Intrathecal miR-106b-5p agomir attenuates neuropathic pain and inhibits the expression of P2X₄R in the spinal cord in SNI mice**

To assess the exact impact of miR-106b-5p on neuropathic pain, miR-106b-5p agomir was intrathecally delivered to SNI mice on day 7, consecutively for 3 days. As shown in Fig. 6B, qPCR results confirmed that miR-106b-5p agomir up-regulated the expression of miR-106b-5p (P< 0.01, Fig. 6B) in SNI mice. At day 7 after SNI, 50% paw withdrawal threshold was significantly decreased, indicating neuropathic pain was established (P< 0.0001, Fig. 6A). From day 3 after miR-106b-5p agomir administration, the decreased 50% paw withdrawal threshold induced by SNI were increased (P< 0.05, Fig. 6A), whereas the scrambled miRNA had no effect (P> 0.05, Fig. 6A).

Furthermore, we tested whether miR-106b-5p agomir could repress the expression of P2X₄R. QPCR and western-blot analysis showed that miR-106b-5p agomir reversed the up-regulation of P2X₄R both RNA (P< 0.05, Fig. 6C) and protein levels (P< 0.001, Fig. 6D) in SNI mice.

**Intrathecal miR-106b-5p antagonir induces pain behaviors and increases the expression of P2X₄R in the spinal cord of naïve mice**
To further explore the regulation of P2X₄R by miR-106b-5p, miR-106b-5p were down-regulated in naïve mice by intrathecal injection with miR-106b-5p antagonim (P < 0.05, Fig. 7B). MiR-106b-5p antagonim was applied to naïve mice for 3 days and the sensitivity to the mechanical thresholds were observed. We found that the 50% paw withdrawal threshold was significantly lower in miR-106b-5p antagonim treated mice than that of scrambled miRNAs treated mice (P < 0.01, Fig. 7A), demonstrating that miR-106b-5p antagonim produced pain behaviors in naïve mice. Furthermore, the spinal cord tissue was harvested at day 3 to assess the expression of P2X₄R at the mRNA and protein levels. As shown in Fig. 7, intrathecal miR-106b-5p antagonim increases the expression of P2X₄R mRNA (P < 0.01, Fig. 7C), as well as P2X₄R protein (P < 0.05, Fig. 7D) in naïve mice.

Discussion

Nervous system damage results in neuropathic pain, and miRNAs are reported to regulate neuropathic pain. In our study, P2X₄R mRNA and protein were increased in the SNI mouse model. Then, 69 DE miRNAs were identified in SNI mice; among them, we also found that miR-106b-5p was decreased and that it could repress neuropathic pain development by targeting P2X₄R. Our findings indicated that miR-106b-5p could be used as a novel therapeutic target of neuropathic pain through regulating P2X₄R.

As is already known, purinergic receptor-mediated spinal microglial functions contribute significantly to pathologically enhanced pain management. [8, 9]. Consistent with previous study, the expression of spinal P2X₄R was up-regulated in SNI rats and DNP rats [29–31]. Additionally, P2X₄R was mostly expressed in microglial cell [32, 33]. Some researchers found that duloxetine, a drug that inhibits neurotransmitters and microglial P2X₄R function and alleviates neuropathic pain after peripheral nerve injury [10]. Our results together with these findings, show that P2X₄R is upregulated in neuropathic pain model, which strongly suggests that P2X₄R plays a crucial role in neuropathic pain. Despite recent advances, understanding the transcriptional or translational regulatory mechanisms underlying changes in the expression and function of P2X₄R under neuropathic pain conditions remains a major challenge.

MiRNAs are commonly recognized as mediators of gene expression and can serve as indicators in neuropathic pain pathophysiology [18–20]. There has been a focus on studies associated miRNAs with chronic neuropathic pain states. For example, miR-98 might depress neuropathic pain development through modulating HMGA2 [21]. MiRNAs are highly deregulated in neuropathic pain. [13, 14] and might be critical molecules for treatment. These findings provide us with information that the mechanism of P2X₄R in neuropathic pain can be investigated from the perspective of miRNA.

We identified 69 miRNAs showing differential expression in the spinal cord after SNI by microarray analysis (fold change > 2, P < 0.05, Fig. 2A and B). Based on common mechanisms, miRNA plays a regulatory role mainly by silencing its target genes [34, 35], thus we focused on the down-regulated miRNAs in our present study. Among the down-regulated miRNAs, miR-106b-5p was found to target P2X₄R through TargetScan software (Fig. 2C). Consistent with the results of the microarray, miR-106b-5p was decreased in SNI mice by the qPCR method (Fig. 2D). Interestingly, miR-106b-5p was also dysregulated in some psychiatric disorder diseases, such as Alzheimer's disease and attention-deficit hyperactivity disorder [36, 37]. Further, the dual-luciferase assay verified that miR-106b-5p negatively regulated P2X₄R through combing with P2X₄R 3'UTR.
Moreover, fluorescence in situ hybridization showed that miR-106b-5p was co-localized with P2X₄R, microglia and neurons in mice spinal cords (Fig. 4). A lot of evidence suggests that P2X₄R are almost expressed in microglia to play a vital role in pain development [8, 38], but also are also expressed in DRG neurons during chronic inflammatory pain [39]. Our results are consistent with these studies and provide evidence for the interaction between miR-106b-5p and P2X₄R.

In our study, BV2 cells were activated after stimulating by LPS (Fig. 3A-B), which are consistent with classical theory [22]. And the viability of BV2 cells increased in a dose-dependent manner following LPS treatment (P<0.05, Fig. 3C). BV2 cells are typically microglia cell lines, and P2X₄R is almost exclusively expressed in microglia [10]. Further, we found that P2X₄R is increased at both the mRNA and protein level under LPS-induced inflammatory conditions in BV2 cells (Fig. 4B and C). What's more, the BV2 cells transfected with miR-106b-5p mimic reversed the up-regulation of P2X₄R induced by LPS (Fig. 4B and C). These data indicated that miR-106b-5p regulates P2X₄R at the transcription level in BV2 cells. However, transfecting miR-106b-5p mimic did not alter the level of P2X₄R in naïve BV2 cells (Fig. 4B and C, P>0.05).

Further, we demonstrated that miR-106b-5p alleviated pain by inhibiting P2X₄R through the changes in molecular levels in SNI mice. Meanwhile, the increased expression of P2X₄R was reversed (Fig. 6C and D) with intrathecal administration of miR-106b-5p agomir in SNI mice. To further determine the role of miR-106b-5p in the SNI-induced neuropathic pain, we found that intrathecal administration of miR-106b-5p antagonir contributed to pain behaviors (Fig. 7A) and the up-regulation of P2X₄R (Fig. 7C and D) in naïve mice. These results revealed that miR-106b-5p may function as an effective therapeutic target for treating neuropathic pain through regulating P2X₄R.

The activated P2X₄R causes the phosphorylation of p38-MAPK, and the release of BDNF, all of which are essential to the persistence of pain hypersensitivity after nerve injury. Mounting evidence suggests that P2X₄R may regulate the progress of neuropathic pain through the p38MAPK, ERK1/2, and PI3K/Akt pathways, which were core pathways in the development of neuropathic pain [11, 40–42]. Meanwhile, miR-106b could promote cervical cancer progression by modulating the expression of the GSK3β, VEGFA, and PTK2 genes. Importantly, these three genes play crucial roles in PI3K-Akt signaling, focal adhesion and cancer [43]. Further, the expression of miR-106b was inversely correlated with TGF-β type II receptor protein level; and miR-106b can directly inhibit the TGF-β type II receptor translation in vitro [44], while TGF-β receptor binding is an important molecular function in diabetic neuropathic pain from our previous study [27]. This accumulated evidence reveals that miR-106b-5p might regulate the expression of P2X₄R in neuropathic pain through some important pathways. Therefore, further investigations illustrating the potential mechanism would provide a new direction and serviceable theoretical foundation for the clinical intervention of neuropathic pain.

**Conclusion**

The biological role of miR-106b-5p in neuropathic pain progression was the focus of our present research. We observed that miR-106b-5p alleviated neuropathic pain development by down-regulating P2X₄R. P2X₄R can enable neuropathic pain development, which can be reversed by miR-106b-5p in vitro. Our data revealed that
miR-106b-5p may function as an effective therapeutic target for treating neuropathic pain through regulating P2X4R.

**Declarations**

**Authors’ Contributions**

H.D. (Huiying Du) and X.T. (Xinran Tan) performed the Western blot, FISH, cell culture and transfection, cell proliferation assay, luciferase assay, animal experiments and behavioral tests. H.D. and X.W. (Xuhong Wei) analyzed the data. Q.G. (Qingjuan Gong) and Z.Y (Zhongmin Yuan) designed and supervised the study. H.D. and Q.G. wrote the manuscript. All authors reviewed the manuscript.

**Funding**

This work was supported by grants from the Natural Science Foundation of Guangdong (NO. 2018A030313532), the National Natural Science Foundation of China (NO. 31100805).

**Availability of data and materials**

The datasets generated and/or analyzed in present study will be available from the corresponding author upon reasonable request from any qualified investigator.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Conflicts of interest**

The authors report no conflicts of interest in this work.

**Acknowledgements**

Not applicable

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Figures
Figure 1

The pain behavior of neuropathic pain induced by SNI and the change in expression of P2X4R mRNA and protein. (A) Responses of the paw to mechanicals. *** $P < 0.001$, **** $P < 0.0001$ vs. sham. Two-way ANOVA, $n = 6$ mice. (B) The increased expression of P2X4R mRNA in the spinal cord of SNI mice. $t_4 = 3.539$, * $P < 0.05$, $n = 3$ mice. (C) The increased expression of P2X4R protein in the spinal cord of SNI mice. $t_4 = 4.670$, ** $P < 0.01$. Two-tailed unpaired t-test, $n = 3$ mice.
**A**

![Heatmap](image)

**B**

![Heatmap](image)

**C**

- has 363-388 of P2X4R 3'UTR
  - 5' ACGGCCGGCUGACUGUUCAAGCAGUUUA 3'
- has-miR-106b
- mmu-miR-106b

**D**

![Bar graph](image)

**E**

![Bar graph](image)
Figure 2

MiR-106b-5p was downregulated in neuropathic pain induced by SNI, and miR-106b-5p directly targets P2X4R 3’UTR. (A-B) The heat map showed the differential expression of miRNA in SNI mice (fold change > 2.0, P < 0.05). (C) The matched seed region between miR-106b-5p and P2X4R 3’UTR predicted by software in red. (D) The decreased expression of miR-106b-5p in the spinal cord of SNI mice. Two-tailed unpaired t-test, t4 = 4.862, ** P < 0.01, n = 3 mice. (E) Transfection of miR-106b-5p mimic with wild-type P2X4R 3’UTR plasmid vector reduced relative luciferase activity, but no change in luciferase activity was detected in the scramble group, * P < 0.05, ** P < 0.01, *** P < 0.01 vs. vehicle, ### P < 0.001 vs. scramble. One-way ANOVA, n = 3.
Figure 3

Morphology of BV2 cells observed under microscope and effect of LPS or miR-106b-5p mimic or inhibitor on BV2 microglia cell viability. (A) The picture shows normal morphology of BV2 cells. (B) The picture presents the morphology of LPS-stimulated BV2 cells. (C) Cell viability was evaluated by CCK-8 assay. The affection of LPS on cell growth was denoted as a percentage of normal control, ** P < 0.05, **** P < 0.0001 vs. naïve. One-way ANOVA, n = 8. (D) Compared with naïve group, the affection of those compounds on cell growth showed no different. P > 0.05, One-way ANOVA, n = 5.
Figure 4

MiR-106b-5p regulated the expression of P2X4R in BV2 cells. (A-C) The relative expression of miR-106b-5p (A) and P2X4R mRNA (B) and protein (C) treated with miR-106b-5p mimic/scramble in BV2 cells. * P < 0.05, ** P < 0.01, **** P < 0.0001 vs. naïve; ## P < 0.01, #### P < 0.0001 vs. LPS. One-way ANOVA, n = 3 mice. (D-F) The relative expression of miR-106b-5p (D) and P2X4R mRNA (E) and protein (F) treated with miR-106b-5p inhibitor/scramble in BV2 cells. ** P < 0.01, *** P < 0.001, **** P < 0.0001 vs. naïve. One-way ANOVA, n = 3 mice.
Figure 5

MiR-106b-5p regulated the expression of P2X4R in BV2 cells. (A-C) The relative expression of miR-106b-5p (A) and P2X4R mRNA (B) and protein (C) treated with miR-106b-5p mimic/scramble in BV2 cells. * P < 0.05, ** P < 0.01, **** P < 0.0001 vs. naïve; ## P < 0.01, #### P < 0.0001 vs. LPS. One-way ANOVA, n = 3 mice. (D-F) The relative expression of miR-106b-5p (D) and P2X4R mRNA (E) and protein (F) treated with miR-106b-5p inhibitor/scramble in BV2 cells. ** P < 0.01, *** P < 0.001, **** P < 0.0001 vs. naïve. One-way ANOVA, n = 3 mice.
Figure 6

MiR-106b-5p agomir down regulated P2X4R and alleviated neuropathic pain. (A) The paw mechanical withdraw thresholds were tested in mice. **** P < 0.0001 vs. sham + scramble; # P < 0.05, ## P < 0.01 vs. SNI + scramble. Two-way ANOVA, n = 6 mice. (B-C) The relative expression of miR-106b-5p (B) and P2X4R mRNA (C) with intrathecal miR-106b-5p agomir/scramble in the spinal cord of SNI mice was determined by qPCR. * P < 0.05, ** P < 0.01 vs. sham + scramble; # P < 0.05, ## P < 0.01 vs. SNI + scramble. One-way ANOVA, n = 3 mice. (D) P2X4R protein expression in the spinal cord of SNI mice at 14 days injected with intrathecal miR-106b-5p agomir, t4 = 9.357, *** P < 0.001. Two-tailed unpaired t-test, n = 3 mice.
Figure 7

P2X4R was upregulated by miR-106b-5p antagonir with pain behavior. (A) The paw mechanicals withdraw thresholds were tested in mice. ** P <0.01, *** P <0.001, **** P < 0.0001 vs naïve + scramble. Two-way ANOVA, n = 3 mice. (B) The relative expression of miR-106b-5p at 3 days in the spinal cord of mice, t4 = 3.213, * P < 0.05. (C) The relative expression of P2X4R mRNA at 3 days in the spinal cord of mice, t4 = 6.151, ** P < 0.01. (D) The relative expression of P2X4R protein at 3 days in the spinal cord of mice, t4 = 4.330, * P < 0.05. Two-tailed unpaired t-test, n = 3 mice.