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Nerve injury-induced upregulation of miR-21 in the primary sensory neurons contributes to neuropathic pain in rats

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ABSTRACT

Neuropathic pain is intractable chronic pain caused by damage to the somatosensory system. Peripheral nerve injury of the primary sensory neurons changes expressions of multiple microRNAs that affect many aspects of cellular functions by regulating specific gene expressions. miR-21, a well-characterized oncogenic miRNA, is consistently upregulated after peripheral nerve injury in the dorsal root ganglion (DRG), where cell bodies of primary sensory neurons exist. However, their causal relationship to the pain is fully unknown. In this study, we therefore investigated the miR-21 expression in the DRGs along with the time course of neuropathic pain and its involvement in the neuropathic pain. Neuropathic pain was induced in rats by specific ligation of the left fifth lumbar spinal nerve. After the injury, miR-21 expression in the injured DRG neurons, but not in the neighboring uninjured DRG neurons, was persistently upregulated following the pain development. Intrathecal administration of interleukin-1β also increased the miR-21 expression in the DRG. Both mechanical allodynia and thermal hyperalgesia in the neuropathic pain were attenuated by intrathecal administration of miR-21 inhibitor. miR-21 is specifically upregulated in the injured DRG neurons and causally involved in the late phase of neuropathic pain. Therefore, miR-21 and its modulatory system may be a therapeutic target for intractable chronic neuropathic pain.

1. Introduction

Neuropathic pain is chronic pain caused by lesions or diseases to the somatosensory system. Since neuropathic pain is resistant to currently available analgesics in contrast to pain caused by inflammation of peripheral organs, it remains a major clinical problem and development of effective analgesics is required. One reason, to make the therapy challenging, can be multiple molecular mechanisms underlying the neuropathic pain. Expression and functional changes in a wide variety of molecules are induced in the primary sensory neurons by nerve injury [1,2].

MicroRNAs (miRNAs) are non-coding functional RNAs that negatively regulate multiple gene expressions. They generally recognize the 3′-UTR of mRNAs in a sequence-specific manner to post-transcriptionally inhibit the protein expressions of target mRNAs. One type of miRNA is predicted to typically target hundreds of mRNAs [3]. Thus, the expression changes of miRNAs concurrently modulate many gene expressions and considerably affect cellular functions as well described in development and cancers [4]. However, their physiological and pathological involvement in neuropathic pain remains largely unknown, although miRNAs, such as miR-103 in spinal neurons [5], have been expected as a novel target for analgesics [6]. In the dorsal root ganglion (DRG), where the cell bodies of primary sensory neurons exist, massive changes in miRNA expressions are reported after peripheral nerve injury [7–11]. Among the miRNAs dysregulated in the DRG, miR-21 expression is consistently shown to increase after multiple types of peripheral nerve injury [7–9]. miR-21 has a wide range of physiological and pathological functions including immune system and cardiovascular diseases [12,13]. Especially, miR-21 has been well elucidated as an oncogenic miRNA upregulated in almost all the kinds of carcinoma cells [14]. In contrast, its functional significance in neuronal cells is poorly understood. miR-21 is shown to modulate axon growth of DRG neurons *in vitro*, consistent with the upregulation after axotomy [7]. miR-21 protects apoptotic cell death of cortical neurons after oxygen and glucose deprivation *in vitro* [15]. However, it remains fully unknown whether miR-21 expression is causally related to the pain behavior caused by neuronal damage. Here, we examined the miR-21 expression in the DRGs along with the time course of neuropathic pain and the involvement of miR-21 in the neuropathic pain.

Abbreviations: CCI, chronic constriction injury; DRG, dorsal root ganglion; ERK, extracellular signal-regulated kinase; IL, interleukin; L4, lumbar fourth; L5, lumbar fifth; miRNA, microRNA; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; SNL, spinal nerve ligation.

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2. Materials and methods

2.1. Animal models

All the experimental procedures were approved by our Institutional Committee on Laboratory Animals (Approval number, 23-107) and performed in accordance with the guidelines of the International Association for the Study of Pain [16]. Male Sprague–Dawley rats (5 weeks of age) were used for all experiments. The animals were singly housed and allowed food and water *ad libitum*. All the surgeries were performed on the rats under deep anesthesia following intraperitoneal administration of sodium pentobarbital (50 mg/kg). To create the neuropathic pain model, lumbar fifth (L5) spinal nerve ligation (SNL) or chronic constriction injury (CCI) of sciatic nerve was performed on the left side of rats [17,18]. For SNL, the L5 spinal nerve was exposed and tightly ligated with 4–0 silk thread in two regions separated by about 1 mm. For CCI, the sciatic nerve was loosely ligated in four regions. The right side was left intact as a control.

2.2. Behavioral tests

Paw withdrawal response to mechanical stimuli was measured using a set of von Frey filaments (Muromachi Kikai, Tokyo, Japan). Each rat was placed on a metallic mesh floor covered with a plastic box, and a von Frey monofilament was applied from under the mesh floor to the plantar surface of the hind paw. The weakest force (g) inducing withdrawal of the stimulated paw at least three times in five trials was referred to as the paw withdrawal threshold. The Plantar Test (Ugo Basile, Varese, Italy) was used to examine thermal hyperalgesia. Each rat was placed on a glass plate with a radiant heat generator underneath and the latency of paw withdrawal from the heat stimulus was measured twice separated by a 5-min interval. The average value was used as the latency of response. Both tests were performed in a blind fashion.

2.3. Quantitative PCR

All the procedures were basically performed according to the corresponding manufacturers' protocols. Lumbar fourth (L4) and L5 DRGs were removed from rats under deep anesthesia with pentobarbital. Total RNA was extracted from the DRGs using RNAiso plus (Takara Bio, Shiga, Japan). Total RNA (10 ng) was reverse-transcribed with a mature miR-21-specific stem-loop primer (Life Technologies, Carlsbad, CA, USA) using a TaqMan MicroRNA Reverse Transcription Kit (Life Technologies). PCR mixture was prepared using TaqMan Universal PCR Master Mix and premixed TaqMan probe and primer pair specific for miR-21 included in the TaqMan MicroRNA Assays (Life Technologies). The amplification efficiency per one PCR cycle was obtained by assaying serially-diluted samples (four points at 1:5 dilution) and the relative expression was calculated.

2.4. In situ hybridization

Rats were deeply anesthetized with intraperitoneal pentobarbital and perfused transcardially with phosphate-buffered saline (PBS; pH 7.4) and then fresh 4% paraformaldehyde in PBS. DRGs were post-fixed overnight and cryoprotected in 20% sucrose in PBS at 4 °C overnight. Subsequently, they were rapidly frozen in dry ice/acetone, cut into 10-μm sections using a cryostat (Leica Microsystems, Wetzlar, Germany). miR-21-specific and negative-control locked nucleic acid probes conjugated with digoxigenin at the 3′ terminal were obtained from Exiqon. Tissues were pretreated with 1 μg/ml proteinase K (Merck, Darmstadt, Germany) at 37 °C for 5 min, fixed by 4% paraformaldehyde in PBS and then hybridized with the probe (50 nM) in hybridization buffer (50% formamide, 5× SSC, pH 4.5, 1% SDS, 50 μg/ml heparin, and 50 μg/ml yeast RNA) at 50 °C overnight. Slides were rinsed with a wash buffer (50% formamide, 5× SSC, pH 7.0, and 1% SDS) at 51 °C for 30 min and subsequently washed three times with another wash buffer (50% formamide and 2× SSC, pH 7.0) at 51 °C for 30 min each. Slides were then incubated with 0.5% blocking solution (Roche Applied Science, Penzberg, Germany) at room temperature for 1 h and subsequently with a sheep anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche Applied Science) at 4 °C overnight. Slides were stained with the BM purple alkaline phosphatase substrate solution (Roche Applied Science) containing 2 mM levamisole at room temperature for 5 days. The negative-control probe did not yield any signal in the intact L5 DRG (data not shown), confirming the sequence-specific staining.

2.5. Intrathecal drug administration

Intrathecal catheterization was performed on intact rats several days before SNL operation as previously described [19]. A polyethylene catheter (PE-10) filled with sterile saline was inserted from the atlanto-occipital membrane into the subarachnoid space. The catheter was then passed to reach the lumbar enlargement of the spinal cord. Another tip of catheter was tightly tied and implanted subcutaneously until 7 days after SNL. Then, the outer tip of catheter was attached with an osmotic pump (Alzet, Cupertino, CA, USA). The pump continuously delivered miR-21 inhibitor (Life Technologies) or negative-control miRNA inhibitor dissolved in saline for 3 days (1 nmol/day). For interleukin (IL)-1β administration, the catheter attached with the osmotic pump delivering IL-1β (200 ng/day) or saline as a control was inserted in intact rats. Rats showing no apparent dysfunction were used for experiments.

2.6. Statistical analysis

Values are expressed as means ± s.e.m. The paired *t*-test was used to compare the values of paw withdrawal threshold and miR-21 expression level between the intact and SNL or CCI sides. The unpaired *t*-test was used to compare the values of paw withdrawal threshold and latency and miR-21 expression level between control and IL-1β or anti-miR-21 treatments. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. miR-21 increases in the injured DRG neurons in the late phase of neuropathic pain

The paw withdrawal threshold to mechanical stimulus was decreased on the SNL side 3 days after SNL and remained decreased for at least 14 days, while unchanged on the intact side (Fig. 1A). Then, we followed the time course of miR-21 expression change in the L5 DRG after SNL using quantitative RT-PCR. The miR-21 expression was increased in the L5 DRG of SNL side at days 7 and 14 after SNL (Fig. 1B). In contrast, the miR-21 expression was unchanged at day 3 (Fig. 1B). Furthermore, we examined the miR-21 expression in the SNL-spared neighboring L4 DRG, because the neighboring uninjured L4 DRG neurons are shown to contribute to neuropathic pain as well [20–22]. However, the miR-21 expression was unchanged in the uninjured L4 DRG of SNL side (Fig. 1B). We also examined the miR-21 expression in another neuropathic pain model. After CCI, the paw withdrawal threshold to mechanical stimulus was also decreased on the CCI side (Fig. 1C).

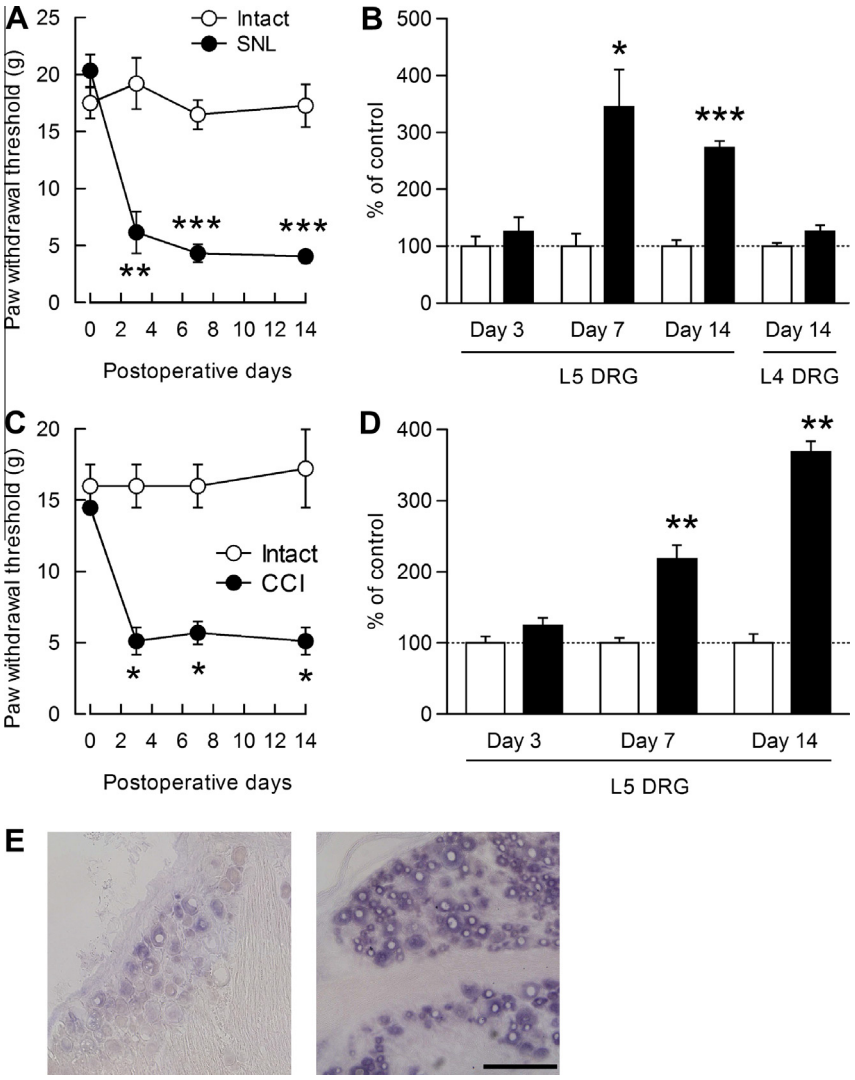

Fig. 1. miR-21 expression is increased in the injured DRG neurons in the neuropathic pain condition. (A and C) The paw withdrawal responses to mechanical stimuli were evaluated on the intact and SNL (A; $n = 6$) or CCI (C; $n = 6$) sides. (B and D) Quantitative RT-PCR for miR-21 expression in the DRGs after SNL (B; $n = 4$–7) and CCI (D; $n = 4$). Data are presented as percentages of the levels on the intact side. (E) Representative images of *in situ* hybridization for miR-21 in the L5 DRG of intact (left) and SNL (right) rats at 14 days after SNL. Scale bar, 100 μm. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to the values on the intact side by the paired *t*-test.

Consistently, the miR-21 expression was increased in the L5 DRG of CCI side at days 7 and 14, but not at day 3, despite the time course of expression change was slightly distinct (Fig. 1D). To reveal miR-21-expressing cells in the injured DRG, we performed *in situ* hybridization for miR-21 in the L5 DRG of intact and SNL rats. Consistent with the quantitative RT-PCR results, L5 DRG neurons showed increased miR-21 signals at 14 days after SNL (Fig. 1E), as observed in axotomy [7].

3.2. IL-1β induces the miR-21 expression in the DRG

IL-1β is shown to induce the expression of miR-21 in MIN6 cells and human pancreatic islets [23]. Since IL-1β is well known to involve in the pathogenesis of neuropathic pain [24], we investigated the involvement of IL-1β in the miR-21 upregulation in the neuropathic pain state. Intrathecal administration of IL-1β for 3 days induced mechanical allodynia in intact rats (Fig. 2A). In rats injected with IL-1β for 3 days, the miR-21 expression was significantly increased in the L5 DRG (Fig. 2B).

3.3. Blockade of miR-21 alleviates the neuropathic pain

To address the contribution of miR-21 to the maintenance of neuropathic pain, we commenced an intrathecal administration of miR-21 inhibitor, a single-stranded and chemically-modified RNA that specifically binds miR-21 and inhibits its function. The miR-21 inhibitor was continuously administered using osmotic pump from day 7 after SNL when the neuropathic pain was established. At 3 days after miR-21 inhibitor administration, both mechanically allodynia and thermal hyperalgesia were partially attenuated on the SNL side (Fig. 3A). The paw withdrawal thresholds and latencies to mechanical and thermal stimuli, respectively, were unaffected on the intact side by the miR-21 inhibitor (Fig. 3B).

4. Discussion

We have shown that miR-21 is upregulated in the injured DRG, but not adjacent injury-spared DRG in the SNL rats, although both DRGs are involved in the neuropathic pain [20–22]. In addition,

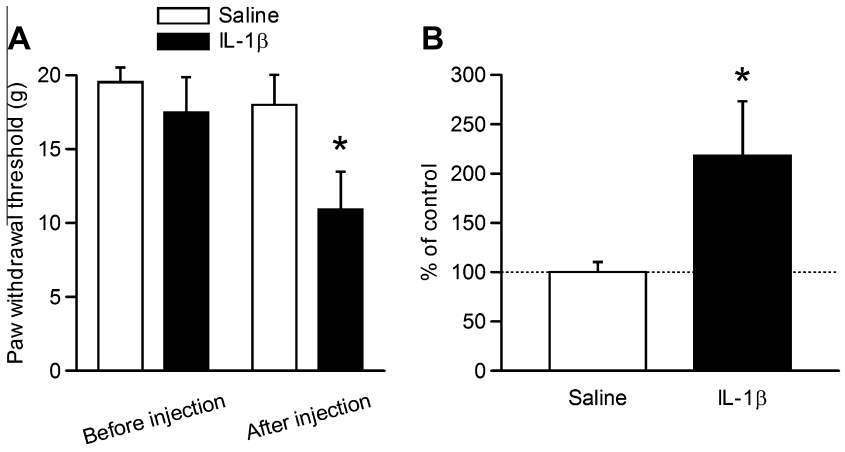

Fig. 2. miR-21 expression is increased in the DRG after intrathecal IL-1β administration. (A) The paw withdrawal responses to mechanical stimuli were evaluated on the left side in rats with saline- or IL-1β-injection for 3 days (n = 5–6). (B) Quantitative RT-PCR for miR-21 expression in the left L5 DRGs after saline- or IL-1β-injection for 3 days. Data are presented as percentages of the levels in the saline-injected rats (n = 5–6). $^{*}P < 0.05$ compared to the values of saline-injected rats by the unpaired t-test.

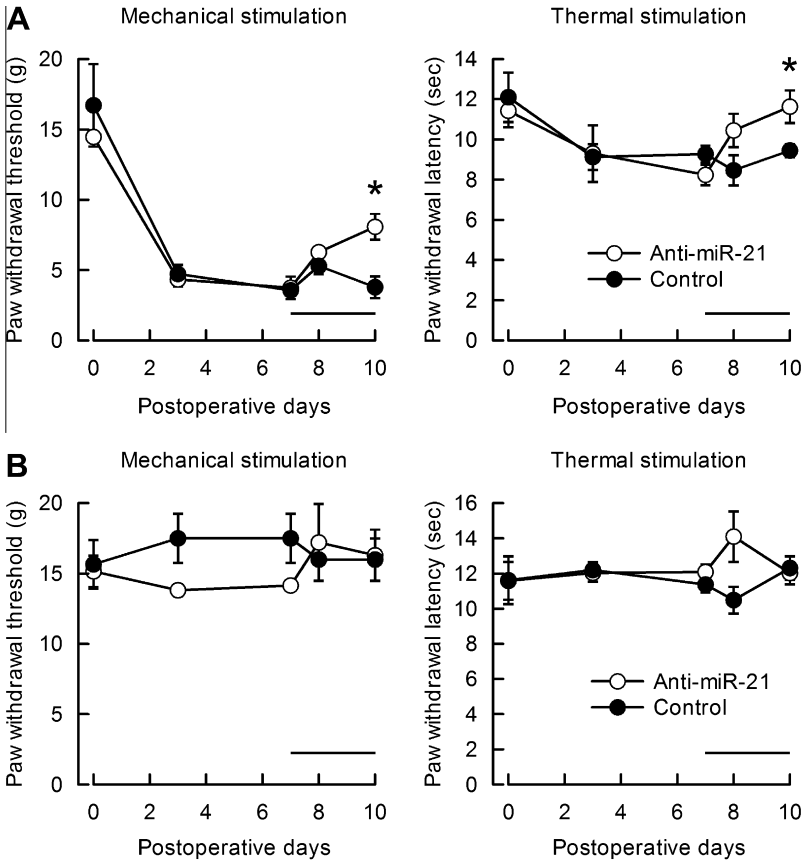

Fig. 3. miR-21 inhibition alleviates the neuropathic pain. The paw withdrawal responses to mechanical and thermal stimuli were evaluated on the SNL (A) and intact (B) sides (n = 4–6). Control or anti-miR-21 inhibitor was continuously administered from day 7 after SNL, as indicated by horizontal bars. $^{*}P < 0.05$ compared to the values of control-treated rats by the unpaired t-test.

CCI, another type of injury that causes the neuropathic pain, increased the miR-21 expression. Axotomy or crush injury of peripheral nerve is also reported to increase the miR-21 expression [7–9]. Therefore, miR-21 expression may be consistently increased in DRG neurons upon damages to neuronal cell itself. In fact, miR-21 expression is reportedly upregulated by several neuronal damages in the central nervous system. miR-21 is increased in cortical primary neurons after ischemic injury by oxygen and glucose deprivation *in vitro* [25] and in neurons after ischemic injury *in vivo* [15]. miR-21 is upregulated in the hippocampus by exposure to ionizing radiation [26] and after traumatic brain injury [27]. Furthermore, miR-21 is reportedly upregulated in possible cerebral neurons in patients with human immunodeficiency virus-associated dementia and miR-21 therefore may be induced in neurons by excitotoxic prolonged N-methyl-D-aspartate receptor stimulation [28]. Thus, these results suggest that miR-21 upregulation is consistently induced in many types of neurons by a variety of damages and is therefore important for functional

changes in the damaged neurons. Consistently, miR-21 upregulation in the DRG neurons was involved in the neuropathic pain in this study. However, detailed molecular mechanisms of miR-21 upregulation after nerve injury are unclear, because miR-21 expression is controlled by diverse molecular mechanisms. Several enhancer elements including binding sites for AP-1, PU.1, C/EBPα, NFI, SRF, p53 and STAT3 are found in the promoter region of miR-21 [29]. miR-21 also increased due to the enhanced processing of pri-miR-21 transcript by Drosha [30]. One plausible candidate responsible for miR-21 upregulation in the injured DRG neurons is AP-1, since IL-1β, which increased the miR-21 expression in the DRG, induces AP-1 activation and is causally involved in the neuropathic pain. IL-1β is also shown to induce the miR-21 expression in MIN6 cells and human pancreatic islets [23]. In addition, NGF, a well-known neurotrophic factor for a subpopulation of DRG neurons, is shown to increase the miR-21 expression in PC12 cells possibly through AP-1 [31]. Considering the importance of miR-21 in a variety of organs [4,12], clarifying the nerve injury-specific molecular mechanisms for miR-21 upregulation will provide specific means for suppressing the miR-21 functions in neurons to alleviate the neuropathic pain.

miR-21 upregulation substantially contributed to the neuropathic pain possibly through translational suppression. Since many genes are predicted and verified as a target for miR-21 [32,33], miR-21 may participate in the neuropathic pain through downregulation of multiple target genes. For example, miR-21 is shown to target negative regulators of matrix metalloproteinases (MMPs), Reck [34] and timp-3 [34–36]. Since the increase in the MMP activity contributes to neuropathic pain [37], miR-21 upregulation may have a role in the neuropathic pain through regulating MMP activity. In addition, miR-21 targets an endogenous inhibitor of phosphatidylinositol 3-kinase, PTEN [38], which is decreased after peripheral nerve injury [39] and involved in the neuropathic pain [40]. Furthermore, miR-21 suppresses sprouty 1, sprouty 2 and Btg2, negative regulators for extracellular signal-regulated kinase (ERK) signaling pathway [13,41–43]. Although ERK is phosphorylated only in a part of injured large DRG neurons and satellite cells after peripheral nerve injury [44], miR-21 may be involved in the neuropathic pain through downregulation of these ERK signaling pathway inhibitors. Thus, targeting the miR-21 function has a potential to suppress multiple pain-promoting pathways at a time.

In conclusion, we have shown the nerve injury-dependent miR-21 upregulation in the DRG neurons. miR-21 upregulation partially contributed to the late phase of neuropathic pain. These findings suggest that miR-21 itself and/or its regulatory mechanisms can be a therapeutic target for intractable chronic neuropathic pain.

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