Downregulation of microRNA-218 relieves neuropathic pain by regulating suppressor of cytokine signaling 3

LONGYUN LI and GUOQING ZHAO

Department of Anesthesiology, China-Japan Union Hospital of Jilin University, Changchun, Jilin 130033, P.R. China

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Abstract. Neuropathic pain is an incapacitating disease that affects a large number of people worldwide, but effective therapies have not yet been established. microRNAs (miRs) are short non-coding RNAs that participate in several biological processes and states, including neuropathic pain. Nevertheless, the precise role of miRs in regulating neuropathic pain remains largely unknown. In the present study, we investigated the role of miR-218 in neuropathic pain using a rat model of chronic constriction injury (CCI). miR-218 expression was induced and studied in the spinal cord and microglial cells of rats with CCI. We noted that downregulation of miR-218 by a specific miR-218 inhibitor significantly attenuated mechanical allodynia, thermal hyperalgesia, and proinflammatory cytokine release in CCI rats. A dual-luciferase reporter assay, RT-qPCR, and western blot analysis results demonstrated that miR-218 directly targeted the 3'-UTR of the suppressor of cytokine signaling 3 (SOCS3) and regulated mRNA and protein expression of SOCS3. Treatment with miR-218 inhibitors inactivated Janus kinase/signal transducer and activator of transcription 3 (STAT3) signaling in rats with CCI in vivo. Moreover, miR-218 inhibitors significantly inhibited the activation of microglial cell STAT3 signaling and downstream proinflammatory genes in microglial cells. These results suggest that miR-218 regulated neuropathic pain and neuroinflammation by regulating SOCS3 expression, which negatively mediated STAT3 signaling. Thus, we propose that silencing of miR-218 may be a promising and novel treatment for neuropathic pain.

Introduction

Chronic neuropathic pain caused by peripheral nerve injury is an incapacitating disease that affects a considerable proportion of the human population worldwide (1,2). Generally, neuropathic pain is characterized by increased allodynia and hyperalgesia in response to external stimuli (3). The clinical treatment of neuropathic pain remains a challenge in medical care due to its complex mechanism.

It has been suggested that neuroinflammation in the spinal cord plays an important role in the pathogenesis of neuropathic pain (4). The induced expression of proinflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 after nerve injury contributes to increase neuroinflammation and the development of neuropathic pain (5). Neuropathic pain involves numerous signaling pathways, such as the Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) pathway, which is activated after peripheral nerve injury (6). STAT3 is widely expressed in the nervous system and activated after nerve injury, and activated STAT3 is involved in the neuroinflammatory responses (7). The rapid activation of STAT3 signaling and downstream genes after nerve damage plays a critical role in the induction of neuropathic pain (8). Therefore, STAT3 is a potential target for the effective treatment of neuropathic pain.

The suppressor of cytokine signaling (SOCS) protein has previously been reported to negatively regulate the JAK/STAT signaling cascade (9). To date, at least eight members of the SOCS family have been identified, of which SOCS1 and SOCS3 are the most effective members in terms of negatively regulating several inflammatory signaling pathways, particularly the JAK/STAT signaling pathway (9). The induction of SOCS3 inhibits STAT1 activation in hepatic cells infected with the hepatitis C virus (10). By contrast, overexpression of SOCS3 attenuates STAT1 and STAT3 activation, as well as inflammation in mice with atherosclerosis (11). Moreover, overexpression of SOCS3 blocks the JAK/STAT signaling pathway, thereby significantly improving the pathophysiological consequences mediated by STAT3 signaling (12-14). Using a rat model of neuropathic pain, it has been noted that overexpression of SOCS3 blocks the JAK/STAT3 signaling pathway and consequently attenuates neuroinflammation and mechanical allodynia (8). Therefore, for treatment of neuropathic pain, the development of novel agents targeting SOCS3 to inhibit the JAK/STAT3 signaling pathway is of great importance.

Correspondence to: Professor Guoqing Zhao, Department of Anesthesiology, China-Japan Union Hospital of Jilin University, 126 Xiantai Road, Changchun, Jilin 130033, P.R. China
E-mail: zhaoguqing1965@sina.com

Abbreviations: miRs, microRNAs; SOCS3, suppressor of cytokine signaling 3; TNF, tumor necrosis factor; IL, interleukin; JAK, Janus kinase; STAT3, signal transducer and activator of transcription 3; CCI, chronic constriction injury; ITGAM, integrin alpha M; COX2, cyclooxygenase 2; CCL2, chemokine (C-C motif) ligand 2

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miRNAs (miRs), which are short and non-coding RNAs with ~21 nucleotides, have gained particular attention due to the regulatory effect which they exert on target genes by inhibiting protein translation (15,16). miRs are expressed in the majority of organisms (17) and participate in numerous biological processes (15,18,19). In recent years, the roles of miRs in neuropathic pain have been discussed, as it has been noted that various miRs were differentially expressed during neuropathic pain development (20). In a mouse model of diabetic neuropathic pain, approximately 21 miRs were significantly downregulated, whereas another 21 miRs were upregulated in spinal cord tissues, and their target genes were potential inflammation regulators (21). The expression of approximately 63 miRs was significantly altered in root ganglia after spinal nerve ligation (22). Thus, we suggest that these abnormal miRs are promising targets for the treatment of neuropathic pain.

In the present study, SOCS3 was predicted as a candidate target gene of miR-218 through bioinformatics analysis. We also speculated that miR-218 is involved in neuropathic pain, and that regulation of SOCS3 expression by targeting miR-218 may be a potential treatment for neuropathic pain. To assess these hypotheses, we detected the expression patterns of miR-218 during the development of neuropathic pain. We found that miR-218 was highly expressed in the spinal cord and microglia in our rat model. As expected, inhibiting miR-218 expression with a specific miR-218 inhibitor effectively improved neuropathic pain behavior and attenuated neuroinflammation. The regulatory effect of miR-218 on SOCS3 expression was confirmed through a dual-luciferase reporter assay. Treatment with an miR-218 inhibitor significantly upregulated the expression of SOCS3 and downregulated the STAT3 signaling pathway in the spinal cord and microglia. These results suggest that inhibiting miR-218 effectively attenuated neuropathic pain, possibly by upregulating SOCS3 expression and inhibiting the JAK/STAT3 inflammatory signaling pathway.

Materials and methods

Animals. A total of thirty adult female Sprague-Dawley rats (180-220 g) purchased from The Experimental Animal Centre of Jilin University (Changchun, China) were used in the present study. The rats were housed in individual cages at 23.0±1˚C with a 12/12-h light/dark cycle and were given free access to water and food. All animal protocols were conducted in accordance with the Institutional Animal Care and Use Committee approved by Jilin University.

Chronic constriction injury (CCI). The rats were randomly divided into three groups: the sham-operated group (n=10), CCI + miR-scrambled (n=10) group, and the CCI + miR-218 inhibitor group (n=10). A rat model of neuropathic pain was established by CCI as previously described (23). The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg). The midline was incised, and the left sciatic nerve was exposed. A 4.0 catgut thread was used to ligate the left sciatic nerve at four sites at intervals of 1 mm. The wound was closed using absorbable sutures. Sham-operated rats were subjected to a similar procedure but without nerve isolation and ligation. After surgery, posture was closely monitored before subsequent experiments.

Isolation and culture of primary microglia. Primary rat microglia were isolated and cultured according to a previously reported method with minor modifications (24). Briefly, the rats were sacrificed by an intraperitoneal injection of pentobarbital sodium (150 mg/kg; Merck, Darmstadt, Germany). The spinal cord from lumbar enlargements was removed, finely minced and subsequently digested using a neural tissue dissociation kit (Miltenyi Biotec, Auburn, CA, USA). The cells were filtered using a 70-μm nylon cell strainer (BD Biosciences, San Jose, CA, USA) and seeded in a T-75 flask coated with poly-L-lysine containing Dulbecco's modified Eagle's medium (DMEM)/nutrient mixture F-12, 20% fetal bovine serum, and 1% penicillin/streptomycin (all from Invitrogen, Carlsbad, CA, USA) under 5% CO2 conditions at 37˚C. The medium was replenished every 2 days.

Reverse transcription quantitative PCR (RT-qPCR). Total RNA was extracted from each sample using an miRNeasy mini kit (Qiagen, Shanghai, China). cDNA was generated using M-MLV reverse transcriptase (Clontech, Palo Alto, CA, USA) and reverse transcribed using a TaqMan miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Gene expression was detected with an RT-qPCR assay using SYBR Premix Ex Taq™ II commercial kit (Takara, Dalian, China). Relative gene expression was quantified through a comparison with the internal reference gene GAPDH or U6 snRNA using the 2^ΔΔCt method.

Intrathecal catheter implantation. The rats in this study were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg) before implantation of the intrathecal catheter. After separation of the occipital muscles and exposure of the cisternal membrane, the polyethylene catheter was inserted into the cisterna magna and tested by injecting lidocaine in order to paralyze the bilateral hind limbs. The wound was closed, and the catheter was fixed under the skin. The rats were allowed to recover for 3 days prior to subsequent experiments.

Intrathecal miR administration. The miR-218 inhibitor and scrambled control (GenePharma, Shanghai, China) were administered to rats using the pre-implanted intrathecal catheter. Briefly, 5 μg miR-218 inhibitor diluted in 10 μl sterile PBS was administered intrathecally once daily for 4 days after CCI. Lumbar enlargement (L4-L5) was removed in this study for biological detection.

Pain threshold assessment. Clinical symptoms associated with neuropathic pain were evaluated through the paw withdrawal threshold (PWT) in response to mechanical allodynia and paw withdrawal latency (PWL) in response to radiant heat, as described previously (25-27). Briefly, the rats were placed in a box with a metal mesh floor. The hind paws were probed with the electronic Von Frey filament (IITC, Woodland Hills, CA, USA). The time of hind paw withdrawal in response to the stimuli was recorded, and data were obtained based on three latency measurements. For PWL measurement in response to heat, the rats were placed in a perspex box with a radiant heat source focused on the plantar surface of the hind paw. Three measurements were recorded for each rat at an interval of 10 min. Heat irradiation was administered for 10 sec with a cut-off time of 20 sec to avoid tissue damage.
Western blot analysis. Equivalent amounts of protein (50 µg) from different samples were separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5%) and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 3.0% non-fat milk in Tris-buffered saline (TBS) at 37°C for 1 h. The membranes were blotted with anti-SOCS3 (sc-9023), anti-STAT3 (sc-482), anti-phosphorylated STAT3 (sc-135649) and anti-GAPDH (sc-25778) antibodies (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and incubated at 4°C overnight. After washing with TBS Tween-20, the membrane was incubated with horseradish peroxidase conjugated with secondary antibodies (1:2,000; bs-0295-HRP; Bioss, Beijing, China) for 1 h and subjected to an enhanced chemiluminescence detection system (Amersham, Little Chalfont, UK). The grey value of protein bands was quantified with Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Dual-luciferase reporter assay. The 3′-untranslated regions (3′-UTRs) of wild-type (WT) and mutant-type (MT) SOCS3 in the putative miR-218 binding sites were amplified and sub-cloned into pGL3 vectors (Promega, Madison, WI, USA). The pGL3 vectors were co-transfected with or without 50 nM miR-218 mimics or miR-218 inhibitor into human embryonic kidney 293 (293) cells using Lipofectamine 2000 transfection reagent (Invitrogen). The 293 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and maintained in DMEM (Invitrogen) plus 10% fetal calf serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen), and maintained in a humidified incubator containing 95% air/5% CO₂ at 37°C. After co-incubation for 48 h, the transfected cells were harvested, and luciferase activity was detected using a dual-luciferase reporter system (Promega) according to the manufacturer’s instructions.

Data analysis. All data are reported as the means ± standard deviation. Data were processed using SPSS version 11.5 (SPSS Inc., Chicago, IL, USA). Statistical differences were analyzed by a one-way analysis of variance, followed by a Bonferroni post-hoc test. A p-value <0.05 was considered to indicate a statistically significant difference.

Results

Expression of miR-218 is persistently increased in rats with CCI. To investigate the involvement of miR-218 in the regulation of neuropathic pain, we determined the expression patterns of miR-218 in rats with CCI through RT-qPCR analysis. The results demonstrated that miR-218 expression was significantly upregulated in the spinal cord of rats with CCI relative to that of the sham-operated group on post-operative days 1, 3, 7 and 14 (Fig. 1A). We also detected the expression profile of miR-218 in primary microglia isolated from the spinal cord. As shown in Fig. 1B, miR-218 expression was persistently upregulated in the microglia of the rats with CCI compared with the sham-operated group. Our data indicated that miR-218 may be involved in neuropathic pain development.

Neuropathic pain symptoms in rats with CCI are markedly attenuated by treatment with miR-218 inhibitor. As miR-218 was persistently expressed in CCI rats, we suggest that down-regulation of miR-218 attenuates neuropathic pain development. To test this hypothesis, we intrathecally administrated rats with CCI with miR-218 inhibitor. The results showed that miR-218 expression was significantly downregulated in the spinal cord by miR-218 inhibitor (Fig. 2A). The pain behavior analysis showed that the PWT (Fig. 2B) and PWL (Fig. 2C) significantly increased relative to that of the rats with CCI which were treated with miR-scrambled control. These results suggest that down-regulation of miR-218 attenuated neuropathic pain development.

Downregulation of miR-218 significantly inhibits inflammation in rats with CCI. To verify the role of the miR-218 inhibitor in neuropathic pain, we determined the effect of miR-218 inhibitor on neuroinflammation in rats with CCI. The results showed that the expression levels of IL-1β (Fig. 3A), TNF-α (Fig. 3B), and IL-6 (Fig. 3C) were significantly increased in rats with CCI compared with those in the sham-operated group, and the levels were significantly decreased by miR-218 inhibitor treatment, as detected through RT-qPCR analysis. The data indicated that downregulation of miR-218 inhibited neuroinflammation in CCI rats.

miR-218 directly targets the 3′-UTR of SOCS3. To explore the potential mechanism of miR-218 in regulating neuropathic pain, we sought to determine the potential target gene of miR-218 that may target genes and may be involved in regulating neuropathic pain. Bioinformatics analysis revealed that SOCS3, which is a critical mediator of inflammation signaling, was the predicted
target gene of miR-218 (Fig. 4A). To validate the association between miR-218 and SOCS3, we performed a dual-luciferase reporter assay to determine the interaction between miR-218 and the 3'-UTR of SOCS3. The results showed that treatment with miR-218 mimics significantly reduced the luciferase activity in WT 3'-UTR of SOCS3-transfected cells relative to that induced by miR-scrambled control treatment (Fig. 4B). Furthermore, miR-218 mimics did not have a marked effect on luciferase activity in the MT 3'-UTR of SOCS3-transfected cells. These results demonstrate that miR-218 directly targeted the 3'-UTR of SOCS3.

Downregulation of miR-218 upregulates the expression of SOCS3 and inhibits STAT3 activation in rats with CCI in vivo. To verify the regulatory effect of miR-218 on SOCS3 expression, we detected the mRNA and protein expression of SOCS3 in rats with CCI which were treated with miR-218 inhibitor or scrambled control. *p<0.01 vs. sham; **p<0.05; ***p<0.01 vs. CCI + miR-scrambled.
negatively regulated by SOCS3. As shown in Fig. 5C and D, the phosphorylation of STAT3 (p-STAT3) in rats with CCI was considerably downregulated by miR-218 inhibitor. The data imply that downregulation of miR-218 increased SOCS3 expression, leading to inactivation of STAT3 signaling. Downregulation of miR-218 suppresses microglia activation and STAT3 signaling activation in primary microglia in vitro. To verify the role of the miR-218 inhibitor in inhibiting neuroinflammation, we detected its effect on microglial activation in vitro. Stimulation of primary microglia with IL-6 significantly increased the expression levels of p-STAT3 (Fig. 6) and integrin alpha M (ITGAM), a microglial cell marker (Fig. 7A). The expression levels of p-STAT3 and ITGAM were markedly suppressed by miR-218 inhibitor treatment. Furthermore, the downstream target genes of STAT3 signaling, cyclooxygenase 2 (COX2) (Fig. 7B) and chemokine (C-C motif) ligand 2 (CCL2) (Fig. 7C), were also significantly downregulated by miR-218 inhibitor. These results suggest that downregulation of miR-218 inhibits microglial activation and STAT3 signaling in vitro.

Discussion

Although various pharmacological treatments have been developed for neuropathic pain, their effects remain limited (28). In the present study, we demonstrated that the enhanced expression of miR-218 contributed to the reduced expression of SOCS3. Treatment of rats with CCI with miR-218 inhibitor considerably improved neuropathic pain behavior. We suggest that the underlying mechanism is the inhibitory effect on the JAK/STAT3 inflammatory signaling induced by SOCS3, which was upregulated by miR-218 inhibitor.

Previous studies have suggested that the JAK/STAT3 signaling pathway is involved in neuropathic pain: for example, Tsuda et al. (29) demonstrated that the JAK/STAT3 signaling pathway mediated spinal astrocyte proliferation and played an important role in maintaining neuropathic pain in rats. The phosphorylation of STAT3 has been reported to rapidly increase in the microglial cells of the dorsal spinal cord after peripheral nerve injury, and treatment with a JAK2 inhibitor blocked STAT3 signaling and attenuated neuropathic pain development (6). Similarly, treatment with a STAT3 inhibitor has been shown to inhibit the STAT3 signaling pathway, thereby specifically alleviating neuropathic pain behavior in rats (30). Moreover, intrathecal administration of aspirin-triggered lipoxin A4 significantly suppressed the activation of STAT3 signaling and proinflammatory cytokine production, and also attenuated mechanical allodynia in rats with CCI (31). In the present study, we demonstrated that miR-218 inhibitor significantly inhibited the STAT3 signaling pathway and attenuated pain behavior by upregulating SOCS3 expression. Our data

![Figure 5. Treatment with miR-218 inhibitor enhanced suppressor of cytokine signaling 3 (SOCS3) expression. (A) mRNA and (B) protein expression levels of SOCS3 in the spinal cords of rats with chronic constriction injury (CCI), as detected through RT-qPCR or western blot analysis, respectively. *p<0.05 vs. sham; **p<0.05 vs. CCI + miR-scrambled. (C) Expression levels of total signal transducer and activator of transcription 3 (STAT3) and p-STAT3 in the spinal cord of rats with CCI as detected by western blot analysis with the indicated antibodies. (D) Relative protein expression levels in the western blot images were quantified using Image-Pro Plus 6.0 software. "p <0.01 vs. sham; *p<0.05 vs. CCI + miR-scrambled.](image-url)
are consistent with the findings of Dominguez et al (8), who demonstrated that overexpression of SOCS3 abolished the JAK/STAT3 signaling pathway, inhibited spinal cord neuroinflammation, and improved pain behavior after peripheral nerve injury. Various studies have demonstrated that SOCS3 has a negative regulatory effect on the JAK/STAT3 signaling pathway. SOCS3 silencing has been shown to induce the phosphorylation of STAT3 and uncontrolled proliferation in myeloid leukemia cells (32). Overexpression of STAT3 suppressed the continuous activation of the JAK/STAT3 signaling pathway in colorectal carcinoma cells, inhibited cell growth, and induced apoptosis in vitro and in vivo (33). These findings confirm that the STAT3 signaling pathway is inhibited by SOCS3.

Previous research has demonstrated that SOCS3 expression is regulated by various miRs. Silencing of miR-203 has been reported to upregulate SOCS3 expression and increase chemosensitivity in breast cancer cells (34). Also, miR-19a has been proposed as an enhancer of JAK/STAT signaling, due to its inhibitory effects on SOCS3 expression (35). miR let-7 inhibited STAT3 activation by upregulating SOCS3 expression in pancreatic cancer cells (36). Xu et al (37) reported that miR-221 targeted and inhibited SOCS3 expression to heighten the effects of anti-hepatitis C virus on IFN-α. SOCS3 inhibition by miR-185 was also shown to inhibit β-cell dysfunction in cases of diabetes (38). In the present study, for the first time to the best of our knowledge, we demonstrated that miR-218 directly targeted the 3′-UTR of SOCS3 and regulated SOCS3 expression, suggesting that miR-218 is a novel miR which regulates SOCS3 expression.

To date, various studies have used miRs to treat neuropathic pain in animal models. miR-23 has been noted to attenuate neuropathic pain by silencing NADPH oxidase 4 (39). Increased expression of miR-195 was induced in the spinal microglia of rats with spinal nerve ligation, and overexpression of miR-195 contributed to increased mechanical and cold hypersensitivity; conversely, suppression of miR-195 reduced mechanical and cold sensitivity (40). Further study has suggested that miR-195 targets autophagy-related gene 14 to regulate autophagy activation and neuroinflammation in spinal microglia in vivo and in vitro (40). miR-21 expression has been found to be highly upregulated in the injured dorsal root ganglion neurons, and intrathecal administration of miR-21 inhibitor significantly

**Figure 6.** Treatment with miR-218 inhibitor inhibited signal transducer and activator of transcription 3 (STAT3) activation in microglia. (A) Western blot analyses of the protein expression level of p-STAT3 in microglia treated with miR-218 inhibitor and interleukin (IL)-6 (50 ng/ml) for 24 h. (B) Relative protein expression in western blot images were quantified using Image-Pro Plus 6.0 software. **p<0.01 vs. PBS; *p<0.01 vs. IL-6 + miR-scrambled.

**Figure 7.** Treatment with miR-218 inhibitor inhibited microglial activation and signal transducer and activator of transcription 3 (STAT3) downstream gene expression in microglia. RT-qPCR analysis of the mRNA expression levels of (A) microglial integrin alpha M (ITGAM), (B) cyclooxygenase 2 (COX2), and (C) chemokine (C-C motif) ligand 2 (CCL2). Cells were treated with miR-218 inhibitor with interleukin (IL)-6 (50 ng/ml) for 24 h. **p<0.01 vs. PBS; *p<0.01 vs. IL-6 + miR-scrambled.
attenuated mechanical allodynia and thermal hyperalgesia in cases of neuropathic pain of rats (41). Sakai et al reported that miR-7a was the most noticeably reduced miR in the injured dorsal root ganglion neurons and was involved in regulating neuropathic pain development by targeting the β2 subunit of the voltage-gated sodium channel (42). Intrathecal administration of miR-96 alleviated neuropathic pain inCCI rats by inhibiting Nav1.3 expression in dorsal root ganglion neurons (43). Overexpression of miR-183 reduced the expression of Nav1.3 and brain-derived neurotrophic factor and attenuated spinal nerve ligation-induced mechanical allodynia (44). Recently, Tan et al (45) reported that miR-155 inhibitor suppressed nuclear factor-κB and p38 mitogen-activated protein kinase signaling activation by upregulating SOCS1 expression. Taken together, these findings suggest that specific miRs are potential targets and therapeutics for the treatment of neuropathic pain. In the present study, we demonstrated that miR-218 was upregulated in the spinal cord and microglia in rats with CCI, and treatment with miR-218 inhibitor significantly reduced neuroinflammation and neuropathic pain development. Targeting specific miRs is a potential treatment for neuropathic pain. It has also been previously noted that spinal microglial activation contributed to the excessive secretion of proinflammatory cytokines and greater pain (46-49). The suppression of neuroinflammation mediated by microglia attenuates neuropathic pain (50,51). In this study, we demonstrated that miR-218 inhibitor significantly inhibited microglial activation and inflammation in vitro. The data further confirmed that miR-218 inhibitor treated neuropathic pain by regulating inflammation.

Our data demonstrated that miR-218 was extensively involved in neuropathic pain. SOCS3 expression upregulated by miR-218 inhibitor partially attenuated neuropathic pain and neuroinflammation by inhibiting the STAT3 signaling pathway. We thus propose that upregulating SOCS3 expression by targeting miR-218 is a promising and effective treatment for neuropathic pain.

References


