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Research article

The role of dorsal root ganglia PIM1 in peripheral nerve injury-induced neuropathic pain

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ABSTRACT

Neuropathic pain induced by peripheral nerve injury is a complex and chronic state that is accompanied by poor quality of life. However, whether PIM1 (proviral integration site 1) contributes to the development of nociceptive hypersensitivity induced by nerve injury remains unknown. The present study was designed to investigate the effects of PIM1 on spinal nerve ligation (SNL) induced pain hypersensitivity. Here, we found that PIM1 positive neurons in the dorsal root ganglion (DRG) were colocalized with nociceptive neuronal markers CGRP, IB4 and substance P and were upregulated after SNL surgery. Knockdown PIM1 in the DRG by AAV5-shPIM1 alleviated SNL-induced pain hypersensitivity. In neuroblastoma cells (neuro-2a), PIM1 regulated the expression of CXCR4 phosphorylated at ser339 (pCXCR4) as well as the CXCL12/CXCR4 pathway. In the DRG tissues, we found that PIM1 was co-expressed with CXCR4, and knockdown of PIM1 attenuated pCXCR4 (ser339) protein expression but had little effect on total CXCR4 protein expression after SNL surgery. These findings suggest that PIM1 contributes to nerve injury-induced nociceptive hypersensitivity. Based on these findings and the characteristics of PIM1, we speculate that PIM1 might be a viable therapeutic target for the treatment of neuropathic pain in the near future.

1. Introduction

Neuropathic pain is a pathological responses to dangerous noxious stimuli that is induced by a lesion or disease of the somatosensory nervous system. The prevalence of neuropathic pain in the general population is approximately 7%–8% [1,2]. The classic symptoms, including spontaneous pain, allodynia, hyperalgesia and others, cause patients who suffer neuropathic pain to live poor-quality lives [3]. According to previous studies, the injured nervous system exhibited inappropriately responses to innocuous and noxious stimulation through various mechanisms. DRGs play an important role in signal transmission from the peripheral nervous system to the central nervous system. Sustained excitability of sensory neurons in the DRGs may be one of the reasons for the symptoms induced by neuropathic pain [4,5]. Therefore, understanding how abnormal neuronal activities arise in DRG neurons may result in changing the treatment strategy from symptomatic relief to specific therapeutic remedies during the development of neuropathic pain.

The PIM family of proto-oncogenes consists of three members: PIM1, PIM2 and PIM3 [6]. As a serine/threonine protein kinase, PIM1 has been involved in the processes of numerous biological functions [7]. Previous studies have shown that a high level of PIM1 might be a tumor biomarker for certain types of solid tumors, which suggests that PIM1 may be a promising therapeutic target for malignant tumors [8]. Under the stimulation of several mediators, PIM1 is notably increased and involved in subsequent biological processes. For example, PIM1 inhibition blocks RANKL-induced NF-κB activation and NFATc1 expression during osteoclastogenesis [9]. Furthermore, several studies have found that PIM1 was not only involved in the synthesis of catecholamine in PC12 cell lines but also implicated in long-term potentiation (LTP) in the hippocampus [10,11]. A recent study also found that PIM1 may become a novel therapeutic target for Alzheimer's disease because of its roles in lowering Aβ levels [12]. Interestingly, PIM1 regulates CXCL12/CXCR4 pathway signaling transduction through modulating CXCR4 surface expression by phosphorylating the CXCR4 C-terminus at serine 339 [13–15]. These attractive findings suggest that

Abbreviations: DRG, dorsal root ganglion; SNL, spinal nerve ligation; PIM1, proviral integration site 1; CGRP, calcitonin gene-related peptide; IB4, isolectin B4; SP, P substance; CXCR4, C-X-C chemokine receptor type 4

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PIM1 may be involved in the development of neuropathic pain induced by the abnormal excitability of DRG neurons.

In this study, we first characterized the expression and distribution patterns of PIM1 in the DRG after SNL-induced neuropathic pain. Then, we investigated whether knockdown of PIM1 in the DRGs could alleviate the nociceptive hypersensitivities induced by SNL. Finally, we explored the potential mechanisms by which PIM1 is involved in the development of neuropathic pain.

2. Material and methods

2.1. Animals

C57BL/6 mice (male, 6–8 weeks old) were obtained from the Animal Experimentation Center of the Second Military Medical University and were housed on a standard 12 h light/dark cycle. All animals had access to water and food pellets ad libitum and were acclimated for one week before behavior measurement to minimize the variability of behavioral outcomes. This experiment was approved by the Institutional Animal Care and Use Committee of Changhai Hospital. During the behavioral test, the experimenters were blinded to the treatment of the groups.

2.2. SNL-induced neuropathic pain model

A SNL-induced neuropathic pain model was carried out as described previously [16]. Briefly, the L5 transverse process was removed after the mice were anesthetized with sevoflurane, and the fourth lumbar spine nerve was ligated with 7-0 silk sutures and transection at the distal site. The surgical procedure for the sham group was identical to that of the SNL group but without ligation or transection of the nerve.

2.3. Immunofluorescence and microscopy

As described previously [16,17], first, mice were perfused via the ascending aorta with 0.01 M phosphate-buffered saline (PBS, pH 7.4) and 4% paraformaldehyde in 0.01 M PBS after being deeply anesthetized with sevoflurane. After the lumbar DRG tissues were postfixed in 4% paraformaldehyde and dehydrated in 30% sucrose in 0.01 M PBS, the DRG tissues were sectioned at a thickness of 15–20 μm. The sections were incubated overnight at 4 °C with primary anti-PIM1 plus one of the following primary antibodies if necessary: anti-NeuN, anti-glutamine synthetase (GS), anti-calcitonin gene-related peptide (CGRP), biotinylated isolectin B4 (IB4), anti-P substance (SP) and anti-CXCR4 antibody after blockade with 5% donkey serum and 0.3% Triton X-100 at 37 °C. The sections were then incubated with a mixture of corresponding secondary antibodies for 2 h at room temperature. All images were observed using a Leica DMI4000 fluorescence microscope and captured with a DFC365FX camera (Leica, Germany). The details of antibody information are listed in Supplementary Table 1.

2.4. Western blotting

As described previously [16], mice were sacrificed under sevoflurane anesthesia, and bilateral L4 and ipsilateral L3 DRGs, as well as the ipsilateral L4 spinal cord, were collected and rapidly frozen in liquid nitrogen. Then, the tissues or cell lines to be used later were homogenized with ice-cold RIPA lysis buffer. The supernatants were harvested after centrifugation at 1000 rpm for 15 min at 4 °C. Equal amounts of protein were heated at 99 °C for 5 min and loaded onto a 4%–12% BeyoGel™ SDS-PAGE Precast Gel after quantification. The separated proteins were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% nonfat milk, the membranes were incubated with specific primary antibodies, including rabbit anti-PIM1 (1:1000, 54523S), rabbit anti-CXCR4 (1:1000, ab124824), rabbit anti-pCXCR4 (1:1000, bs-12256R) and rabbit anti-α-tubulin (1:2000, 2125S), at 4 °C overnight under gentle agitation. Then, the membranes were incubated with the corresponding anti-rabbit secondary antibody (1:2000, Jackson). Protein bands were detected with an enhanced chemiluminescence (ECL) western blot kit and visualized by a ChemiDoc XRS System with Image Lab software (Bio-Rad). The intensity of blots was quantified via densitometry using ImageJ Software. Target protein expression was normalized to the α-tubulin signal.

2.5. Plasmid construction and virus production

A lentivirus plasmid containing mouse full-length PIM1 cDNA (plenti-PIM1) or EGFP under the control of a cytomegalovirus (CMV) promoter was constructed by OBiO Technology Corporation. The PIM1 shRNA (targeted sequence: GCGGAGATATTCCGTTTGA) or the scramble shRNA under the control of a U6 promoter was designed and synthesized by Genepharma Corporation. The adeno-associated virus (AAV) viral particles, including AAV5-shPIM1 and AAV5-scramble, were packaged by OBiO Technology Corporation (Shanghai, China).

2.6. DRG microinjection

As described previously [16,17], after the mice were anesthetized, a midline incision in the lower lumbar back region was made, and the lumbar articular process was exposed and removed. Then, the exposed DRG was injected with a viral solution (0.5–1 μl) through a glass micropipette connected to a Hamilton syringe. The glass micropipette remained at the injection site for 10 min to avoid leakage of the microinjection. The surgical field was irrigated with sterile saline, and the skin incision was closed with clips. After that, the behavior of the mice that received the DRG microinjection was tested; mice that showed signs of paresis or other abnormalities were excluded.

2.7. Behavioural analysis

A mechanical behavioral test was carried out as described previously [16,17]. Mice were placed in a transparent glass chamber on an elevated mesh platform. Calibrated von Frey filaments (0.07 g and 0.4 g) were applied to the plantar surface of the hind paw for approximately 1 s, and each stimulation was repeated 10 times for both hind paws with 5-min intervals. The frequency of paw withdrawal in 10 trials was used as an indication of the amount of paw withdrawal. A thermal behavioral test was performed by a Model 336 Analgesic Meter as described previously [16]. In response to the heat from the light, the light beam was turned off if the animal withdrew its foot. The length of time between the start of the light beam and the foot withdrawal was regarded as the paw withdrawal latency. Each test was repeated five times at 5-min intervals for both hind paws on each side. To avoid tissue damage to the hind paw, a cut-off time of 20 s for withdrawal was applied.

2.8. Cell culture, plasmids transfection and confocal immunofluorescence

Mouse neuroblastoma cells (neuro-2a) were obtained from Shanghai Institutes of Biological Sciences and were cultured in DMEM containing 10% FBS at 37 °C in a humidified incubator with 5% CO_2. The plasmids (including pLenti-PIM1 or AAV5-shPIM1), as well as control plasmids, were transfected into neuro-2a cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The proteins were extracted to detect the levels of PIM1, CXCR4 and pCXCR4 (ser339) 48 h after plasmid transfection. Furthermore, to detect the internalization of CXCR4, neuro-2a cells were cultured in the plate, which was specifically used for confocal microscopy. The PIM1-specific inhibitor SMI-4a (80 μM, Selleckchem) was precultured with neuro-2a 1 h before CXCL12 (10 nM, ab240836) stimulation. To carry out confocal immunofluorescence, neuro-2a cells

were fixed with 4% paraformaldehyde for 20 min at 3 h after CXCL12 stimulation. After that, these fixed cells were blocked in the plate with PBS containing 5% donkey serum and 0.3% Triton X-100 for 2 h at room temperature. Then, the fixed cells were incubated with rabbit anti-CXCR4 (1:1000, ab124824) overnight at 4 °C. The fixed cells were then incubated with donkey anti-rabbit IgG conjugated with Alexa Fluor 488 (1:500, Jackson). Before measurement, the samples were treated with Vectashield plus 4′, 6-diamidino-2-phenylindole (DAPI) mounting medium (Vector Laboratories). All images were obtained by a Leica SP8 laser scanning confocal microscope (Leica, Germany).

2.9. Statistical analysis

All of the results are given as the mean ± SD after the normality was assessed by the Kolmogorov–Smirnov test. Student's *t*-test was used to analyze the statistical significance for two groups. Statistical comparisons of differences among three or more groups were made with a one-way analysis of variance followed by Tukey's multiple comparison test. The changes in the behavioral testing over time among groups were tested using two-way ANOVA with repeated measures, followed by a LSD post hoc test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Distribution of PIM1 in the neuron of lumbar dorsal root ganglia

To explore the roles of PIM family genes during the development of neuropathic pain, we first examined the mRNA variations of PIM family genes (PIM1, PIM2 and PIM3) in the DRGs 7 days after SNL. According to our results, PIM1 mRNA was remarkably upregulated when compared with the Sham group (Supplementary Fig. 1). Based on this finding, we focused on the roles of PIM1 in DRG tissues during the development of neuropathic pain. First, we examined the distribution pattern of PIM1 in the lumbar DRG of mice. In this study, we found that NeuN (neuronal marker)-positive cells, but not GS (marker for satellite glial cells)-positive cells, were co-expressed with PIM1 in the DRGs (Fig. 1A-B). This finding indicates that PIM1 is mainly expressed in the neurons of DRG. To further confirm the characteristics of PIM1-positive neurons, we carried out double labeling for PIM1-positive neurons and classical pain-related neuronal markers: CGRP, SP and IB4. As shown in Fig. 2A-C, PIM1 was co-expressed with these neuronal biomarkers (CGRP, IB4 and SP) which involved in the development of neuropathic pain in the DRG tissues.

3.2. Upregulation of PIM1 in injured DRG following SNL-induced neuropathic pain

To investigate the roles of PIM1 expressed in lumbar DRG neurons, we examined the alteration of PIM1 protein levels in DRGs after SNL surgery. As expected, PIM1 protein in the ipsilateral L4 DRGs was upregulated approximately 1.8-fold, 1.5-fold and 2.3-fold at 3 days, 7 days and 14 days after SNL, respectively, when compared with 0 days (Fig 3A&B). Sham surgery did not alter the basal expression of PIM1 in the ipsilateral L4 DRGs following surgery (Fig 3A&B). Then, we quantified the percentage of PIM1-positive neurons in the L4 DRGs 7 days after Sham or SNL surgery by immunofluorescence. Similar to our results above, the mean fluorescence intensity of each neuron was 1.5-fold greater after SNL surgery than that of the Sham group (Fig 3C&D).

3.3. PIM1 inhibition attenuated SNL-induced pain hypersensitivity

The results (Fig 3E&F) demonstrated that PIM1 upregulation in the injured DRGs (7 days post SNL) was inhibited after AAV5-shPIM1 microinjection. Based on this result, we measured behavioral changes following. As expected, the withdrawal frequency in response to mechanical stimulation was increased remarkably after SNL surgery, while microinjection of AAV5-shPIM1, but not AAV5-scramble, attenuated SNL-induced paw withdrawal frequency to mechanical stimuli (Fig 3G&H). In addition, the paw withdrawal latencies to thermal stimulation were improved dramatically after PIM1 knockdown in the AAV5-shPIM1 group when compared with the AAV5-scramble group (Fig. 3I). These results suggest that PIM1 inhibition in the injured DRGs alleviated SNL-induced pain hypersensitivities.

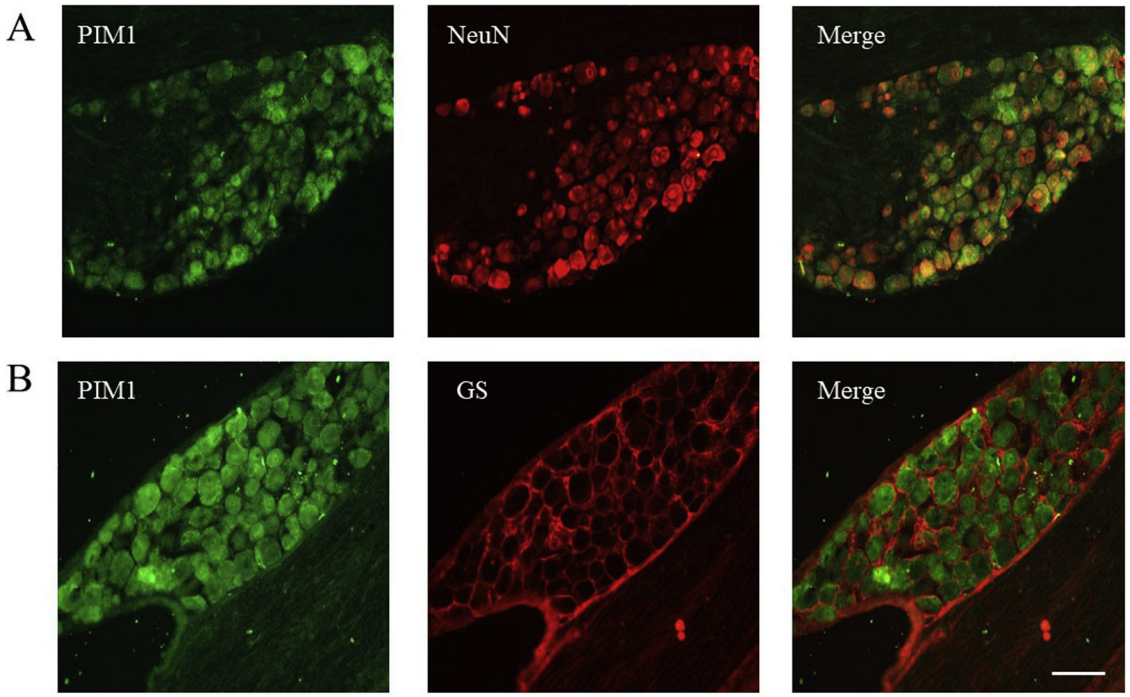

Fig. 1. PIM1 is mainly expressed in dorsal root ganglion (DRG) neurons of mice. (A). PIM1 is co-expressed with NeuN in DRG neurons. (B). PIM1 is not co-labeled with GS, which represents satellite glial cells. Scale bar: 100 μm.

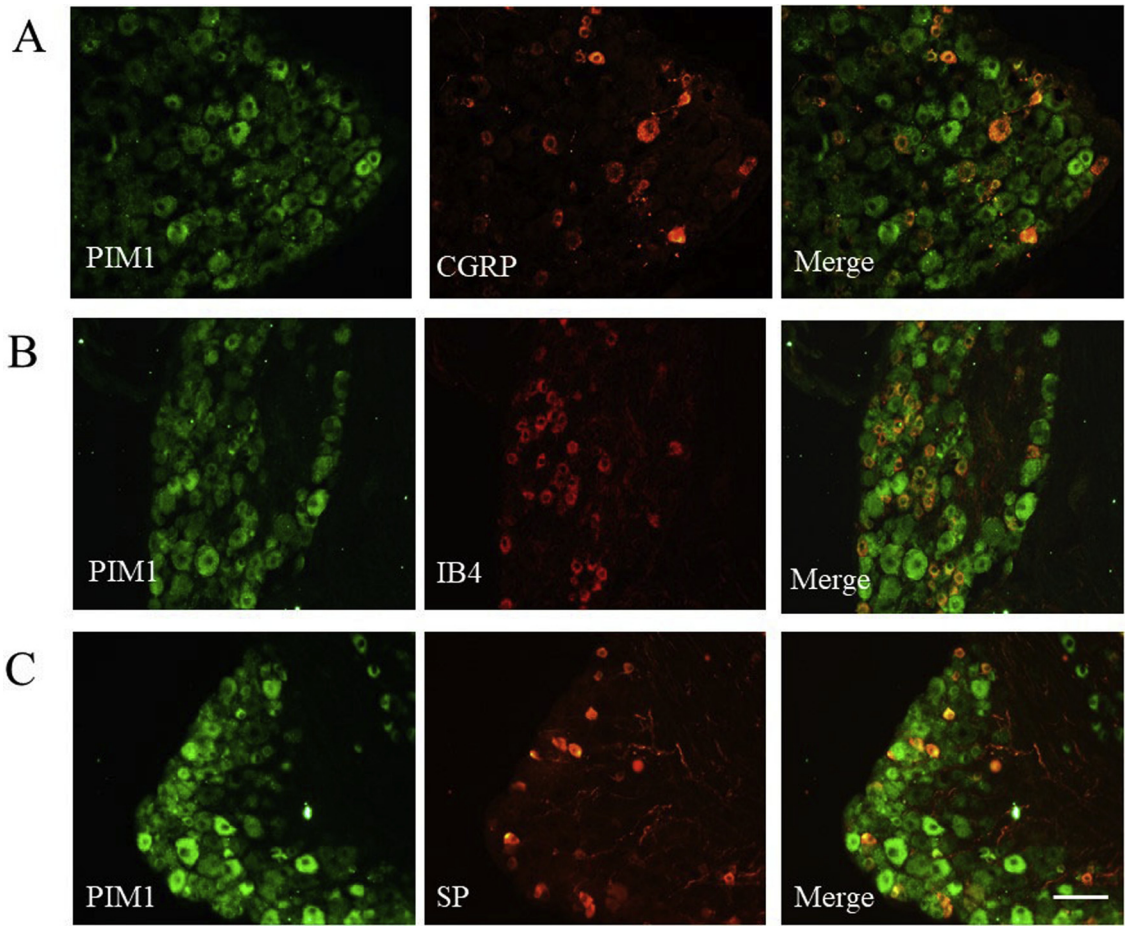

Fig. 2. Co-expression of PIM1 with distinct DRG neuronal markers, CGRP, IB4 and SP, in the DRG neurons. Double immunofluorescent staining showed that (A). PIM1 is co-expressed with CGRP in DRG neurons. (B). PIM1 is co-expressed with IB4 in DRG neurons. (C). PIM1 is co-expressed with SP in DRG neurons. Scale bar: 100 μm.

3.4. PIM1 inhibition blocks the CXCL12/CXCR4 pathway by reducing phosphorylated CXCR4 expression

In this study, we first used neuro-2a cell lines to investigate the relationship between PIM1 and CXCR4. As shown in Fig. 4A&B, PIM1 overexpression increased pCXCR4 levels (phosphorylated at ser339), which may be responsible for cell surface CXCR4 expression, while pCXCR4 (ser339) was downregulated after PIM1 knockdown (Fig. 4C& D). However, neither overexpression nor knockdown of PIM1 affected CXCR4 protein expression (Fig. 4A-D). These findings suggested that PIM1 may be involved in cell surface CXCR4 expression. Furthermore, we used confocal immunofluorescence to validate the roles of PIM1 in the CXCL12/CXCR4 pathway. As Fig. 4E demonstrates, recombinant CXCL12 binds to surface CXCR4 and results in significant internalization, as observed by CXCR4 staining. However, in the presence of the PIM1-specific inhibitor SMI-4a, the internalization of CXCR4 was significantly reduced. These findings suggest that PIM1 may participate in the CXCL12/CXCR4 pathway. As shown in Fig. 5A, approximately 25.6% of PIM1-positive neurons were colabeled with CXCR4, which suggests an interactive relationship between PIM1 and CXCR4 in the DRGs tissues of mice. According to Fig. 5B-E, the levels of CXCR4 and pCXCR4 (ser339) protein were increased significantly 7 days after SNL. Similar to our results mentioned above, knockdown of PIM1 in the injured DRGs had little effect on CXCR4 protein expression, while the level of pCXCR4 (ser339) was decreased dramatically.

4. Discussion

Peripheral nerve injury induces long-term pain hypersensitivity in a mouse model that mimics trauma-induced neuropathic pain, which has been observed in the clinic frequently [18]. Exploring the potential mechanism of pain hypersensitivity may provide novel therapeutic targets for neuropathic pain treatment. After peripheral injury, primary sensory neurons in the DRGs demonstrate maladaptive molecular changes that result in peripheral sensitization and are critical for the onset and maintenance of chronic pain [19]. Therefore, DRG neurons become effective targets for safer therapeutic approaches to chronic pain states induced by inflammation, cancer, neuropathy and other conditions [20–22].

PIM1 is a serine/threonine kinase and a member of the proto-oncogene PIM kinase family that is involved in cell survival, differentiation, and proliferation [23,24]. Until recently, PIM1 had gained much attention due to its high expression in a variety of cancers [6]. However, Ramon et al. found that hippocampus PIM1 protein was induced and enriched in activated neurons after LTP-producing stimulation, suggesting that PIM1 inhibition may become a novel therapeutic strategy for Alzheimer's disease [11,12]. These studies suggest that PIM1-positive neurons may have some relationship with pain hypersensitivity.

In this study, we found that PIM1 was mainly located in the small neurons of DRG tissue, which mediate pain hypersensitivity, but not in satellite glial cells. Furthermore, PIM1 was co-expressed with classical protein markers that represent specific pain-related neurons in the DRGs. These results provide strong evidence for the hypothesis that

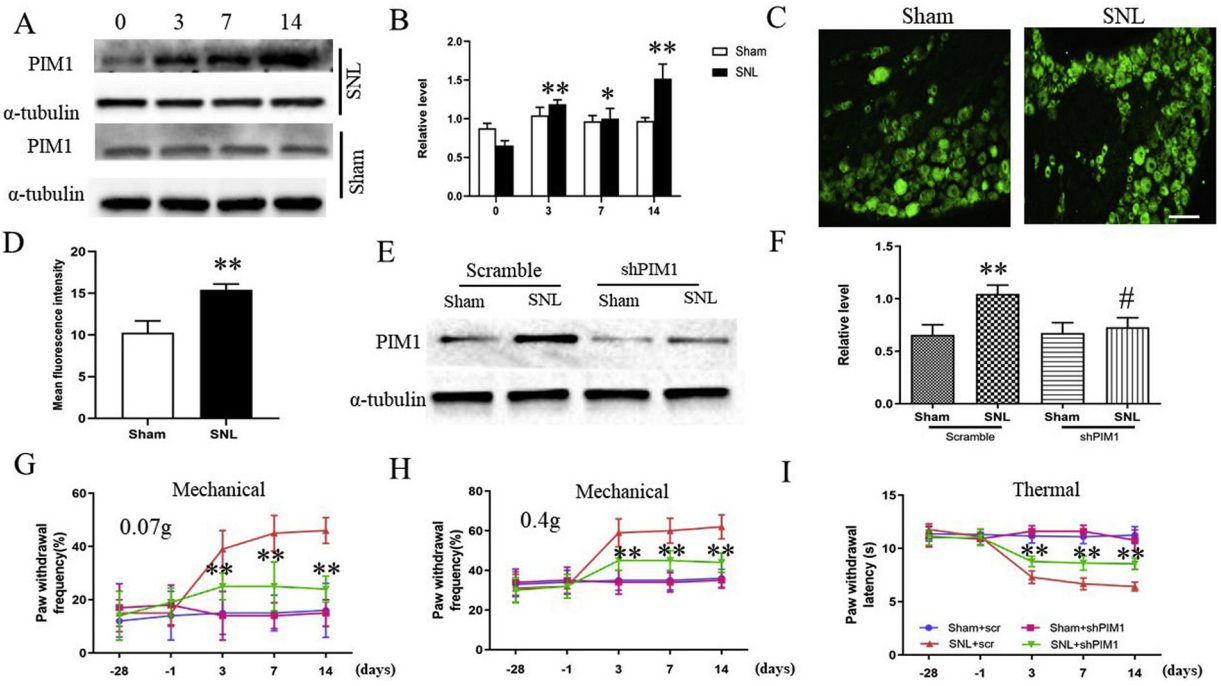

Fig. 3. PIM1 was involved in SNL-induced pain hypersensitivity. (A). Typical Western blot images representing PIM1 protein expression in the ipsilateral L4 DRG after Sham/SNL surgery at different time points. (B). The intensity analysis showed that PIM1 was increased significantly after SNL surgery, while Sham surgery did not alter the basal expression of PIM1. $^{**}P < 0.01$ vs. the corresponding control group (0 day), $^{*}P < 0.05$ vs. the corresponding control group (0 day). (C). Representative images of PIM1-positive neurons in lumbar DRGs at 7 days after Sham or SNL surgery. (D). The mean fluorescence intensity of PIM1 in DRG neurons after Sham/SNL surgery. $^{**}P < 0.05$ vs. Sham group. (E). Representative images that reflected the knockdown efficiency of AAV5-shPIM1 microinjection. (F). A statistical graph of intensity analysis reflecting the efficiency of PIM1 knockdown. $^{**}P < 0.01$ vs. Sham plus Scramble group, $^{\#}P < 0.05$ vs. SNL plus Scramble. (G–I). Paw withdrawal responses to 0.07 g/0.4 g von Frey filament or thermal stimulation on the ipsilateral side of all mice before or after SNL surgery. n = 10 mice/group, $^{**}P < 0.01$ vs. SNL plus Scramble group.

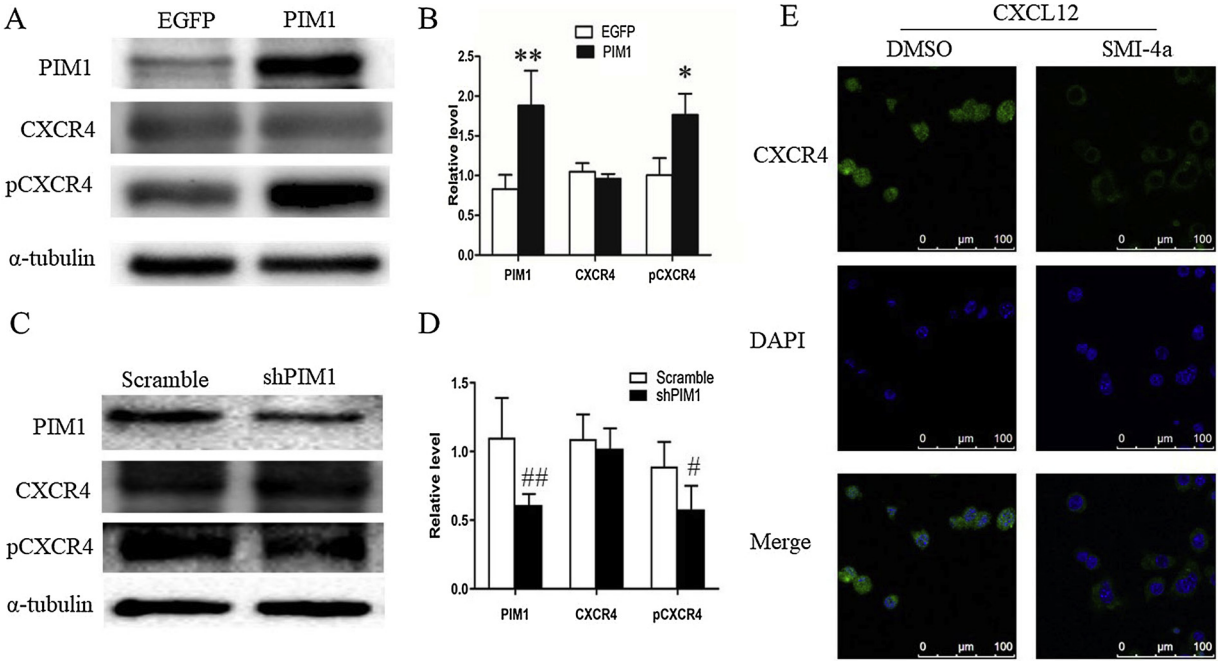

Fig. 4. PIM1 regulates the CXCL12/CXCR4 pathway by phosphorylating CXCR4 at ser339 in cell lines. (A–D). PIM1 overexpression increased pCXCR4 (ser339) protein levels, while PIM1 knockdown decreased pCXCR4 (ser339) protein levels. $^{**}P < 0.01$ vs. EGFP group, $^{*}P < 0.05$ vs. EGFP group, $^{\#\#}P < 0.01$ vs. Scramble group, $^{\#}P < 0.05$ vs. Scramble group. (E). Representative images of confocal immunofluorescence on CXCR4, which represent the activation of the CXCL12/CXCR4 pathway.

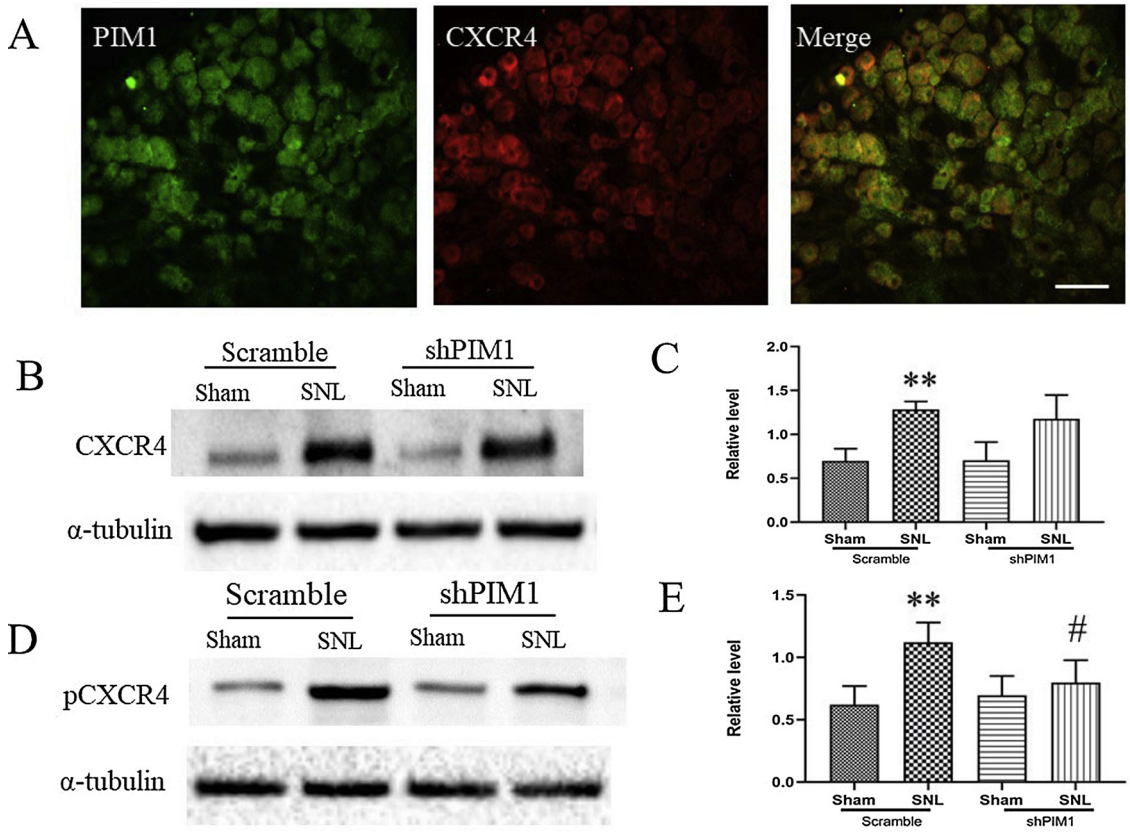

Fig. 5. PIM1 knockdown attenuated pCXCR4 (ser339) levels in the injured DRG tissues after SNL. (A). Double immunofluorescent staining showed that approximately 25.6% of PIM1-positive neurons were co-labeled with CXCR4. (B–C). Knockdown of PIM1 in injured DRGs induced by SNL has little effect on CXCR4 expression. (D–E). Knockdown of PIM1 in injured DRGs induced by SNL attenuated the upregulation of pCXCR4 (ser339). $^{**}P < 0.01$ vs. Sham plus Scramble group, $^{\#}P < 0.05$ vs. SNL plus Scramble group.

PIM1 may participate in the development of neuropathic pain induced by peripheral nerve injury. As our results demonstrated, after SNL surgery, DRG PIM1 was significantly increased after SNL surgery, while inhibition of PIM1 in the injured DRGs alleviated the hyperalgesia induced by SNL surgery. However, how does PIM1 inhibition in DRGs improve SNL-induced hyperalgesia?

Given that PIM1 exerts its function by phosphorylating many target genes, we speculated that many mechanisms may account for the protective roles of PIM1 blockade. Interestingly, according to the study by Schwaller et al, PIM1 was regarded as a regulator of the CXCL12/CXCR4 pathway by phosphorylating CXCR4 at ser339, which is responsible for CXCR4 surface expression [14]. However, inhibition of CXCR4 phosphorylation impaired CXCR4 surface expression, which subsequently blocked the CXCL12/CXCR4 pathway [25]. Considering that the contributions of CXCL12/CXCR4 mediate the $Ca2^{+}$ response to neuropathic pain in DRG tissue [26,27], we proposed that PIM1 may participate in the development of neuropathic pain by modulating CXCR4 phosphorylation at ser339.

As shown in Fig. 5A, PIM1 was co-expressed with CXCR4 in DRG tissue, which suggested the possibility that PIM1 mediates SNL-induced hyperalgesia by interacting with CXCR4. This speculation was first verified in a neuronal cell line. PIM1 is the key regulator of surface CXCR4 expression through modulating phosphorylated CXCR4 levels at ser339, while neither overexpression nor knockdown of PIM1 effects the levels of CXCR4 protein. Similar to these findings, CXCR4 and phosphorylated CXCR4 (ser339) were upregulated significantly in the injured DRGs after SNL, while knockdown of PIM1 in the injured DRGs dramatically reduced the level of phosphorylated CXCR4 (ser339). These results suggest that knockdown of PIM1 attenuated pain hypersensitivity through blocking the CXCL12/CXCR4 pathway by reducing CXCR4 surface expression.

Interestingly, we still found that PIM1 was involved in the stabilization of NF-κB signaling through modulation of p65 phosphorylation (ser276), which was consistent with previous findings [28,29]. Inhibition of PIM1 by SMI-4a impaired proinflammatory cytokine production by downregulating p65 phosphorylation (ser276). These may partly account for the phenomenon that SMI-4a administration intrathecally improved withdrawal frequencies in response to mechanical stimulation.

5. Conclusion

In summary, we have identified the expression and distribution of PIM1 in DRG neurons and clarified the protective roles of PIM1 inhibition in injured DRGs in SNL-induced pain hypersensitivity. Considering the crystallographic structure of PIM1 and the phenotypic alternations when PIM1 is knocked-out in the mouse [30,31], PIM1 might be a viable therapeutic target for the treatment of neuropathic pain in the near future.

Conflict of interest

The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neulet.2019.134375.

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