

Up-regulation of CXCR4 expression contributes to persistent abdominal pain in rats with chronic pancreatitis

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Abstract

Background: Pain in patients with chronic pancreatitis is critical hallmark that accompanied inflammation, fibrosis, and destruction of glandular pancreas. Many researchers have demonstrated that stromal cell-derived factor 1 (also named as CXCL12) and its cognate receptor C-X-C chemokine receptor type 4 (CXCR4) involved in mediating neuropathic and bone cancer pain. However, their roles in chronic pancreatic pain remain largely unclear.

Methods: Chronic pancreatitis was induced by intraductal injection of trinitrobenzene sulfonic acid to the pancreas. Von Frey filament tests were conducted to evaluate pancreas hypersensitivity of rat. Expression of CXCL12, CXCR4, Nav1.8, and pERK in rat dorsal root ganglion was detected by Western blot analyses. Dorsal root ganglion neuronal excitability was assessed by electrophysiological recordings.

Results: We showed that both CXCL12 and CXCR4 were dramatically up-regulated in the dorsal root ganglion in trinitrobenzene sulfonic acid-induced chronic pancreatitis pain model. Intrathecal application with AMD3100, a potent and selective CXCR4 inhibitor, reversed the hyperexcitability of dorsal root ganglion neurons innervating the pancreas of rats following trinitrobenzene sulfonic acid injection. Furthermore, trinitrobenzene sulfonic acid-induced extracellular signal-regulated kinase activation and Nav1.8 up-regulation in dorsal root ganglia were reversed by intrathecal application with AMD3100 as well as by blockade of extracellular signal-regulated kinase activation by intrathecal U0126. More importantly, the trinitrobenzene sulfonic acid-induced persistent pain was significantly suppressed by CXCR4 and extracellular signal-regulated kinase inhibitors.

Conclusions: The present results suggest that the activation of CXCL12–CXCR4 signaling might contribute to pancreatic pain and that extracellular signal-regulated kinase-dependent Nav1.8 up-regulation might lead to hyperexcitability of the primary nociceptor neurons in rats with chronic pancreatitis.

Keywords

Dorsal root ganglion, chronic pancreatitis, CXCR4, Nav1.8, chronic pain

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Introduction

Persistent pain is the major feature of chronic pancreatitis (CP) that results from persistent inflammation, fibrosis, and destruction of the glandular pancreas. It becomes a major public health problem worldwide.^{1,2} An increasing number of evidence has demonstrated that the up-regulation of cytokines and chemokines is involved in the generation of neuronal hyperexcitability, which contributes to inflammatory pain and hyperalgesia.^{3–6} As a member of the CXC family, stromal cell-derived factor 1 α (also named as CXCL12) is seen

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constitutively expressed in the peripheral and central nervous system.^{7–10} CXCL12 exerts its biological functions by binding to the G-protein-coupled receptors CXCR4. It has been shown that bone cancer-induced pain was involved in up-regulation of the expression level of CXCL12 and CXCR4 in both dorsal root ganglion (DRG) and the spinal cord.^{11,12} More recent research has showed that CXCL12/CXCR4 signaling contributed to the development and maintenance of neuropathic pain following spared nerve injury in rats.¹³ However, whether CXCL12/CXCR4 signaling is involved in pancreatic hyperalgesia remains unknown.

Voltage-gated sodium channels play key roles in cellular excitability and are essential for the generation of action potentials (APs). In primary sensory neurons, sodium channel Nav1.8 mostly contributes to the enhanced excitability and produces majority of currents during AP upstroke. Accumulating data showed that Nav1.8 channels were up-regulated in several visceral pain models.^{14–16} A recent research has showed that CXCL12 could directly modulate neuronal excitability by the activation of CXCR4.¹⁷ Furthermore, earlier studies have observed that chemokines (including CCL2 and CXCL1) could directly induce Nav1.8 up-regulation in the DRGs and thus excite the primary nociceptive neurons.¹⁸ Considering all of these data, we hypothesized that the modulating effect of CXCL12/CXCR4 signaling on neuronal excitability was in part due to the regulation of Nav1.8, which in turn contributes to the generation of pain. However, the link between CXCL12/CXCR4 signaling and up-regulation of Nav1.8 was not clear.

Extracellular signal-regulated kinase (ERK), as a member of mitogen-activated protein kinases (MAPKs), is a key molecule that transduce extracellular stimulus into intracellular.^{19,20} Serving as key nociceptive signals in glial cells, ERK is activated and mediated neuron sensitization under pain conditions. Several experiments have shown that ERK are prone to be phosphorylated after the activation of some chemokine receptors.²¹ Therefore, it is reasonable to speculate that the CXCL12/CXCR4 signaling might mediate pain sensitization via activating ERK in DRGs under chronic pancreatic conditions.

In the present study, we first examined the expression of CXCR4 and CXCL12 in DRGs of rats with CP induced by intraductal trinitrobenzene sulfonic acid (TNBS) injection. Then, we investigated the role of CXCR4 in the neuronal excitability and Nav1.8 expression by applying the selective inhibitors of CXCR4 and pERK. Finally, we investigated whether blocking CXCR4 signaling pathway could alleviate the inflammatory pain behaviors. The present findings might provide evidence to target CXCR4 signaling pathway as a potential therapeutic strategy for the treatment of pain in patients with CP.

Materials and methods

Animals

All experiments were performed on male adult Sprague-Dawley rats, weighing from 200 to 220 g. Animals were housed with free access to standard laboratory diet and fresh water under controlled conditions (07:00 h, 19:00 h lighting, temperature $24 \pm 2^\circ\text{C}$). The experimental protocol was approved by the Institutional Animal Care and Use Committee of Soochow University. All procedures were conducted in accordance with the guidelines of the International Association for the Study of Pain. Abdominal operation was conducted under chloral hydrate (360 mg/kg body weight, i.p.) anesthesia, and all necessary efforts were taken to minimize the suffering. After tissue harvest, rats were sacrificed by decapitation.

Induction of CP and cell labeling

As described in detail previously,^{22,23} CP was evoked by intraductal injection of TNBS, and 1,1-dioleil-3,3,3,3-tetramethylindocarbocyanine methanesulfonate (DiI) was injected into the pancreas before TNBS injection to label pancreas-specific DRG neurons for patch-clamp recordings.

Von Frey filament measurements

Von Frey filament testing was used to evaluate pain behavior of rats as described previously.²² In brief, the belly of rats was shaved, and areas specified for stimulation were spotted in relation to the pancreas before testing. Rats were housed in plastic cages with mesh floor and adapted for 30 min before testing. All kinds of filaments (Stoelting) were applied to the spotted abdominal area 10 times each for 1–2 s in ascending order, with a 10-s interval after application. A response was considered positive when the rat had withdrawal response (lift its belly). The data were expressed as percentages of the numbers of positive responses in 10 times of stimulation with each filament for each rat. All tests were performed in a blinded manner.

Real-time quantitative polymerase chain reaction for mRNAs

Total RNA was extracted from T9–T13 DRGs from control and TNBS-injected rats with Trizol (Invitrogen). cDNA was synthesized from total RNA using an Omniscript RT kit 50 (QIAGEN) following the supplier's instructions. The sequences of the primers for CXCL12, CXCR4, and β -actin (as an internal control) used in quantitative polymerase chain reaction were as follows: CXCL12 forward: CGATTCTTTGAGAGCC ATGTC, CXCL12 reverse: TTAAGGCTTTGTCCAG

GTACTCT; CXCR4 forward: CTCTGAGGCGTTTG GTGCT, CXCR4 reverse: TGCCCACTATGCCAGT CAAG, β -actin forward: TCAGGTCATCACTATCG GCA, β -actin reverse: GGCATAGAGGTCTTTACG GAT. Control reaction was carried out in the absence of cDNA templates. The Ct value was defined as the cycle number at which fluorescence intensity reached a certain threshold where amplification of each target gene was within the linear region of the reaction amplification curves. The relative expression level for each target gene was normalized by Ct value of β -actin using a $2^{-\Delta\Delta Ct}$ relative quantification method as described previously.²⁴

Drugs

To evaluate the role of CXCR4 in the development of mechanical pain sensitivity, AMD3100 (5 μ g/10 μ l, Sigma) or vehicle was intrathecally injected once daily for one week starting from two weeks after TNBS injection. To evaluate the role of ERK, an isoform of MAPK, U0126 (Sigma, 10 μ g dissolved in 10 μ l DMSO), was administered through intrathecal injection 10 min prior to behavior test in TNBS rats.

Western blotting

Rats were sacrificed by decapitation after behavioral testing. The T9-T13 DRGs of control or CP rats were obtained and homogenized in a RIPA lysis buffer containing protease inhibitors (Applygen Technologies Inc., China). Protein concentrations of the lysate were determined using a BCA Protein Assay kit (Thermo Scientific, Rockford, IL, USA). Protein samples were heated for 10 min at 75°C with SDS-PAGE sample buffer, and equal amounts of protein were then separated by 10% separation gels. The resolved proteins were subsequently transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) followed by the incubation with 5% non-fat milk (Bio-Rad, CA, USA) in PBS with 0.05% Tween 20 (PBST) for at least 2 h at room temperature. Then, the membranes were incubated with primary antibodies at 4°C overnight. Primary antibodies used in this experiment included CXCL12 (1:500), CXCR4 (1:1,000), and p-ERK (1:200). These primary antibodies were purchased from Santa Cruz. Nav1.8 (1:500) were purchased from Alomone. β -actin (1:2000, Abcam) was used as a loading control.

Dissociation of DRG neurons

Isolation of DRG neurons from adult rats was performed as described previously.²² In brief, bilateral T9-T13 DRGs were dissected out and incubated in dissecting solution containing collagenase D (1.5–1.8 mg/mL; Roche, Indianapolis, IN, USA) and trypsin (1.2 mg/mL;

Sigma-Aldrich) for 90 min at 34.5°C. DRGs were then washed and transferred to the dissecting solution containing DNase (0.5 mg/mL; Sigma-Aldrich). A single-cell suspension was obtained by repeated trituration through flame-polished glass pipettes. Cells were then plated onto acid-cleaned glass coverslips for patch-clamp recordings.

Patch-clamp recordings

As described previously, coverslips containing adherent DRG neurons were put in a chamber (~1 mL volume), attached to the stage of an inverting microscope (IX70 Olympus, Tokyo, Japan), and continuously superfused (1.5 mL/min) at a room temperature with normal external solution. Recording pipettes were pulled from borosilicate glass tubing using a horizontal puller (P-97, Sutter Instruments) and typically had a resistance of 5–8 M Ω when filled with normal pipette solution. Recordings were performed with an EPC10 amplifier and the program Patchmaster (HEKA Elektronik, Lambrecht, GER).

Statistical methods

All data in the present study were expressed as mean \pm SEM or as percentage. Statistical analyses were conducted using OriginPro 8 (OriginLab, USA). Normality of all data was checked before further analyses. Significance of difference was determined by two-sample or pair-sample *t*-test, one-way repeated measures ANOVA followed by Tukey post hoc test, or two-way ANOVA followed by Tukey post hoc test. A *p* value less than 0.05 was considered statistically significant.

Results

Up-regulation of CXCR4 and CXCL12 in DRGs of rats with CP

Pancreatic injection of TNBS significantly enhanced the expression of CXCR4 in the DRGs both at mRNA and protein levels when compared with the saline-treated control rats. The CXCR4 mRNA level was increased by ~1.6 folds after TNBS injection (Figure 1(a), **p* < 0.05, *n* = 4 for each group). Meanwhile, expression levels of CXCR4 proteins were also examined. The relative densitometry of CXCR4 expression was 0.56 ± 0.042 (*n* = 4) for control rats and 0.87 ± 0.015 (*n* = 4) for TNBS group. TNBS injection also significantly increased expression levels of CXCR4 (Figure 1(b), **p* < 0.05). Because of the increase of receptors often due to high expression of its ligand, we next sought to determine the level of CXCL12 expression in the DRG under CP pain

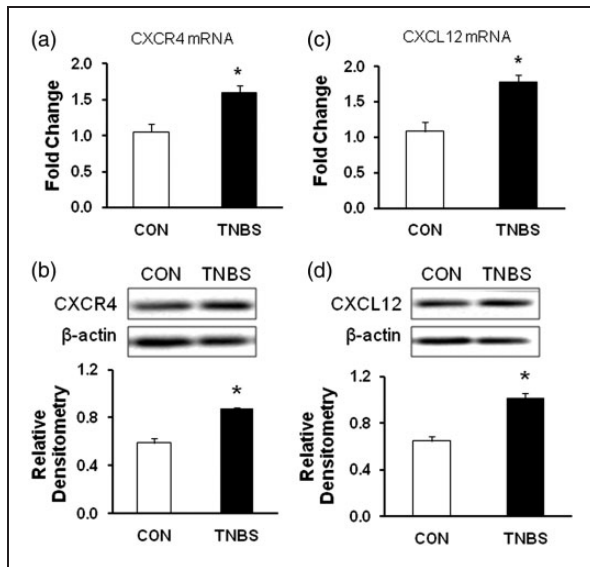


Figure 1. TNBS injection led to up-regulation of CXCR4 and CXCL12 in DRGs. (a) TNBS injection significantly increased CXCR4 mRNA expression levels of pancreas-projected DRG ($n = 4$ for each group, $*p < 0.05$). (b) TNBS injection markedly enhanced CXCR4 protein expression of pancreas-projected DRG ($n = 4$ for each group, $*p < 0.05$). (c) TNBS injection significantly increased CXCL12 mRNA expression levels of pancreas-projected DRG ($n = 4$ for each group, $*p < 0.05$). (d) TNBS injection markedly enhanced CXCL12 protein expression levels of pancreas-projected DRG ($n = 4$ for group, $*p < 0.05$).

state. The CXCL12 mRNA level was increased by ~ 1.7 folds after TNBS injection (Figure 1(c), $*p < 0.05$). TNBS injection also significantly increased expression of CXCL12 at protein levels (Figure 1(d), $*p < 0.05$, $n = 4$ for each group). The relative densitometry of CXCL12 expression was 0.61 ± 0.034 ($n = 4$) for control rats and 1.09 ± 0.051 ($n = 4$) for TNBS group, respectively.

Relief of TNBS-induced pancreatic hyperalgesia by intrathecal injection of AMD3100, a selective antagonist of CXCR4

We then determined whether CXCL12/CXCR4 signaling is involved in pancreatic hyperalgesia induced by TNBS injection. AMD3100, a selective antagonist of CXCR4, was administrated intrathecally. Injection (i.t.) of AMD3100 at $5 \mu\text{g}$ significantly reduced nociceptive responses in CP rats (Figure 2(a), $*p < 0.05$, $n = 8$). However, the solvent of AMD3100 has little effect on pain behavior of CP rats (Figure 2(b), $n = 8$). The effect of AMD3100 at doses of $5 \mu\text{g}$ lasted for at least 4 h (Figure 2(c), $*p < 0.05$, $n = 8$). In addition, AMD3100 has little effect on age-matched healthy rats (Figure 4(d), $n = 8$).

Inhibition of the TNBS-induced hyperexcitability of DRG neurons by AMD3100

In the present study, we determined whether enhanced excitability of pancreas-specific DRG neurons was mediated by CXCL12/CXCR4 signaling. Pancreas-specific DRG (including T9-T13 DRGs) neurons were identified by the fluorescent dye DiI, which was injected into the pancreas (Figure 3(a) to (c)). Similar to our previous reports,²⁵ the DRG neurons were hyperexcited after TNBS injection. The mean value of resting membrane potential (RP) threshold was -50.92 ± 0.72 for control group, -46.29 ± 0.71 for TNBS group, and -48.43 ± 0.91 for TNBS + AMD3100 group, respectively. The absolute value of RP was significantly decreased in the TNBS-treated group. AMD3100 treatment did not significantly change the absolute value of RP when compared to TNBS group (Figure 3(d), $**p < 0.01$ vs. CON; $n = 15$ for each group). The absolute value of AP threshold was also significantly increased in the TNBS-treated group when compared to the saline control and rats receiving AMD3100 (Figure 3(e), $**p < 0.01$ vs. CON; $\#p < 0.05$ vs. TNBS, $n = 15$ for each group). The mean value of AP threshold was -23.57 ± 0.72 for control group, -29.49 ± 1.33 for TNBS group, and -22.76 ± 1.35 for TNBS + AMD3100 group, respectively. Meanwhile, TNBS injection also significantly decreased rheobase of pancreas-specific DRG neurons when compared to saline control and rats receiving AMD3100 (Figure 3(f), $**p < 0.01$ vs. control; $\#p < 0.05$ vs. TNBS, $n = 15$ for each group). The mean value of rheobase was $82.85 \pm 12.44 \text{ pA}$ for control group, $33.89 \pm 8.03 \text{ pA}$ for TNBS group, and $-74.67 \pm 6.33 \text{ pA}$ for TNBS + AMD3100 group, respectively. In addition, numbers of APs evoked by 300 and 500 pA ramp current stimulation was determined (Figure 4). Examples of APs evoked by 300 and 500 pA ramp current stimulation in saline control, TNBS, and TNBS + AMD3100 group (Figure 4(a) and (c)). Bar graphs showed that the average number of APs evoked by 300 and 500 pA ramp current stimulation in the TNBS-treated group was significantly increased when compared to the saline control and AMD3100-treated group (Figure 4(b) and (d), $**p < 0.01$ vs. control; $\#p < 0.05$ vs. TNBS, $n = 15$ for each group). The mean number of APs evoked by 300 pA ramp current stimulation was 5.1 ± 1.4 for control rats, 9.5 ± 1.7 for TNBS group, and 5.8 ± 0.9 for TNBS + AMD3100 group, respectively. The mean number of APs evoked by 500 pA ramp current stimulation was 9 ± 2.3 for control rats, 19.1 ± 3.1 for TNBS group, and 10.1 ± 1.0 for TNBS + AMD3100 group, respectively.

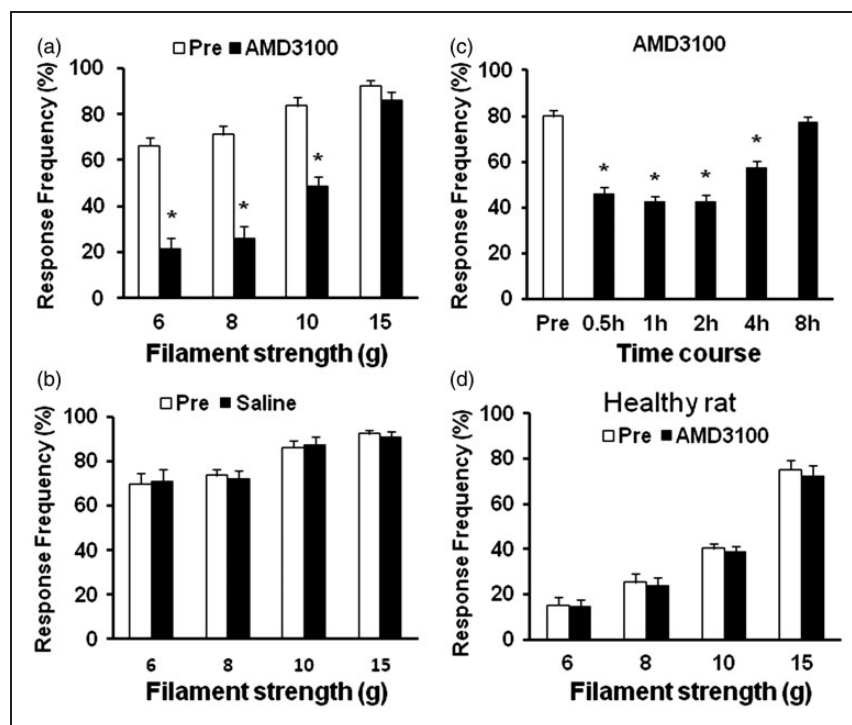


Figure 2. Antagonism of CXCR4 reduced pancreatic pain behaviors. (a) Administration of CXCR4 antagonist AMD3100 (5 μ g, intrathecal, i.t.) remarkably attenuated response frequency of CP rats to VFF stimulation (* $p < 0.05$, compared with pretreatment (Pre), $n = 8$, Dunn's post hoc test following Friedman ANOVA). (b) Administration of solvent saline had no effect on response frequency of CP rats to VFF stimulation. (c) Time course of effect of AMD3100 on mechanical hyperalgesia. The antinociceptive effect lasted for at least 4 h (* $p < 0.05$, compared with Pre; $n = 8$, Dunn's post hoc test following Friedman ANOVA). (d) Administration of AMD3100 did not produce any effect on response frequency in age-matched healthy control rats ($n = 8$ for each group).

Suppression of Nav1.8 and phosphorylated ERK expression by a CXCR4 antagonist

Since the previous study showed that the up-regulation of Nav1.8 expression following peripheral inflammation was mediated by CXCL12–CXCR4 signaling¹⁷ and there was no alteration in Nav 1.7 expression in CP rats (data not shown), the present study was focused on the expression of Nav1.8. We showed that TNBS injection enhanced Nav1.8 expression and that AMD3100 treatment reversed TNBS-induced up-regulation of Nav1.8 in pancreas DRGs (Figure 5(a) and (b), * $p < 0.05$ vs. control; # $p < 0.05$ vs. TNBS). The relative densitometry of Nav1.8 expression was 1.6 ± 0.23 ($n = 4$) for control rats, 2.6 ± 0.15 ($n = 4$) for TNBS group, and 1.7 ± 0.09 ($n = 4$) for TNBS + AMD3100 group, respectively. Because it has been suggested that ERK, a subfamily of MAPK, is a common intracellular downstream messenger of CXCL12–CXCR4 signaling,^{26–28} examination of the roles of ERK is also performed. As shown in Figure 5(c), TNBS injection enhanced pERK expression and that treatment with AMD3100 remarkably reduced the expression levels of p-ERK in the DRGs of TNBS

rats (* $p < 0.05$ vs. control; # $p < 0.05$ vs. TNBS, $n = 4$ for each group).

Suppression of Nav1.8 expression by U0126, an inhibitor of ERK

In order to determine whether the activation of ERK involved in modulation of expression of Nav1.8, U0126, an inhibitor of ERK was used.²⁰ The relative densitometry of Nav1.8 expression was 2.6 ± 0.15 ($n = 4$) for TNBS rats and 1.7 ± 0.09 ($n = 4$) for TNBS + U0126 group, respectively. Results showed that U0126 treatment for seven days significantly decreased high expression of Nav1.8 induced by TNBS treatment (Figure 6(a) and (b), * $p < 0.05$ vs. control; # $p < 0.05$ vs. TNBS). As expected, U0126 significantly decreased high expression of p-ERK induced by TNBS treatment (Figure 6(c), * $p < 0.05$ vs. control; # $p < 0.05$ vs. TNBS, $n = 4$ for each group).

Attenuation of TNBS-induced pancreatic hyperalgesia by intrathecal injection of U0126

Since we have demonstrated that ERK participated in modulation of expression of Nav1.8, we next examined

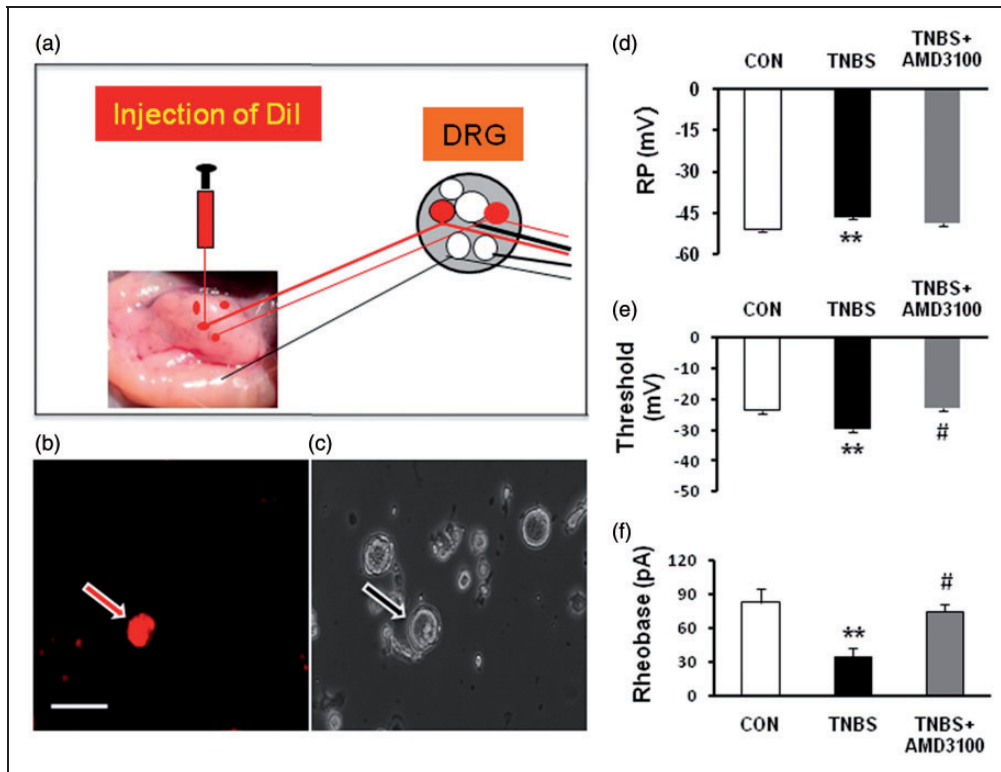


Figure 3. Reversal of TNBS-induced hyperexcitability of pancreas-specific DRG neurons by CXCR4 antagonist. (a) A cartoon showing that Dil was injected into the pancreas. (b) Example of a Dil-labeled dorsal root ganglion (DRG) neuron after acute isolation (arrow). (c) Phase image of the same DRG neuron labeled by Dil. Bar = 50 μ m. (d–f) Histograms exhibiting the effect of intrathecal application of AMD3100 on resting membrane potential (RP, D), the action potential (AP) threshold (e) and AP rheobase (f) of DRG neurons from control, TNBS–, and TNBS+AMD3100-treated rats (** $p < 0.01$ vs. CON; # $p < 0.05$ vs. TNBS, $n = 15$ for each group).

whether ERK signaling involved in pancreatic hyperalgesia induced by TNBS injection. U0126 was administered intrathecally. Injection of U0126 at 10 μ g significantly reduced nociceptive responses in CP rats (Figure 7(a), * $p < 0.05$ vs. pre, $n = 8$). However, the solvent of normal saline has little effect on pain behavior of CP rats (Figure 7(b)). The effect of U0126 at doses of 10 μ g lasted for 2 h (Figure 7(c), * $p < 0.05$ vs. Pre, $n = 8$). In addition, U0126 has little effect on age-matched healthy rats (Figure 7(d), $n = 8$).

Discussion

In the previous study, we have demonstrated that CP induced by TNBS caused hyperexcitability of pancreas-specific DRG neurons.^{22,29} However, which signal pathway is possible for cell body hyperexcitability in the DRG is not well elucidated. In the present work, we showed that CXCL12 and CXCR4 were up-regulated in DRGs following TNBS injection. According to a previous work, under physiological state, DRG neurons can constitutively secrete CXCL12 at a very low level.³⁰ In CP condition, we showed that both of CXCL12 and CXCR4

expression were enhanced. Inhibition of CXCL12-CXCR4 signaling suppressed CP-induced mechanical allodynia. In a rat model of antiretroviral toxic neuropathy, CXCL12 and CXCR4 expression at mRNA levels was enhanced at 7 and 14 days following injection of antiretroviral drug 2, 3-dideoxycytidine.³¹ All of these suggest that CXCL12-CXCR4 plays an important role in mediating pain hypersensitivity.

The detailed mechanisms of CXCL12-CXCR4 signaling have yet to be further investigated. A previous study showed that the expression of CXCL12 was mostly enhanced in the non-neuronal cells of DRGs.³¹ Similarly, a more recent report has shown that SGCs surrounding the DRG neurons were the source of CXCL12, evidenced by co-localization of CXCL12 and glutamine synthetase in the DRGs in the rat model of neuropathic pain.³² Given above lines of evidence, we speculated that the release of CXCL12 from SGCs under pancreatic inflammatory conditions might lead to the development of intraganglionic inflammation, by which DRG neuronal hyperexcitability was maintained. CXCL12, a selective cognate receptor for CXCR4, were overexpressed under CP state that

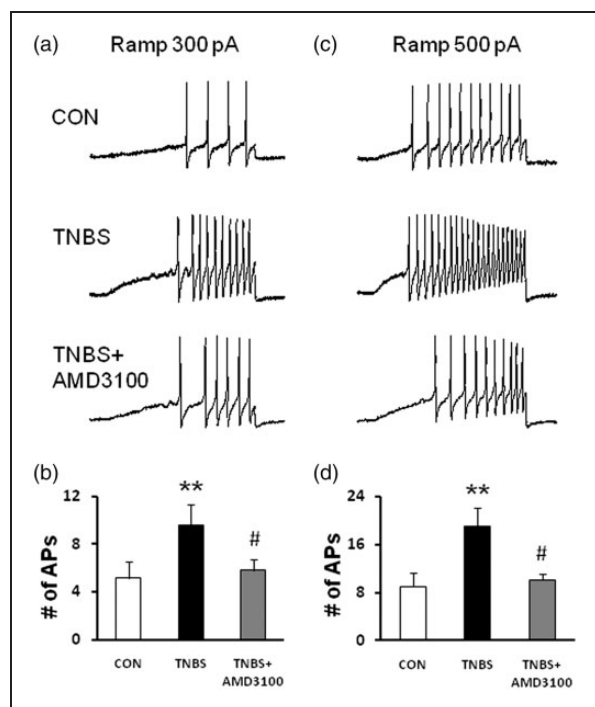


Figure 4. Reversal of TNBS-induced increase in numbers of AP evoked by ramp current stimulation of pancreas-specific DRG neurons by CXCR4 antagonist. (a) Representative traces of 300 pA ramp current-evoked APs in the DRG neurons harvested from saline- and TNBS- and TNBS + AMD3100-treated rats. (b) Histograms exhibiting the effect of AMD3100 on numbers of APs induced by 300 pA ramp current in DRG neurons from control, TNBS-, and TNBS+AMD3100-treated rats ($n = 15$ for each group, $**p < 0.05$ vs. CON; $\#p < 0.05$ vs. TNBS). (c) Representative traces of 500 pA ramp current-evoked APs in the DRG neurons harvested from saline- and TNBS- and TNBS+AMD3100-treated rats. (d) Histograms exhibiting the effect of AMD3100 on numbers of APs induced by 500 pA ramp current in DRG neurons from control, TNBS-, and TNBS+AMD3100-treated rats ($n = 15$ for each group, $**p < 0.05$ vs. CON; $\#p < 0.05$ vs. TNBS).

satisfied the enhanced expression of CXCR4. It is becoming clear that, through producing and releasing CXCL12, the activated glial cells not only enhanced neuronal sensitization but also facilitated glial activation in return. Such positive feedback loops, which compose of glial–glial interactions, depend on both perseverant release of chemokines from glial cells and persistent activation of chemokine receptors on themselves or others. In our research, CXCL12 enhanced calcium concentration of SGCs (data not shown), further supporting the above hypothesis.

Although the mechanisms underlying the up-regulation of both CXCL12 and CXCR4 have to be investigated in the future, the functional nature of the increased CXCR4 receptor expression was identified by

our behavioral pharmacology assays in which treatment with AMD3100 significantly reversed the TNBS-induced CP pain behaviors. Moreover, previous report showed that treatment with AMD3100 also significantly reduced tonic discharges by restoration of rheobase value to normal level, suggesting a maintaining role of CXCL12–CXCR4 signaling in the primary nociceptor hyperexcitability.¹⁷ Since the decrease of rheobase value may reflect changes in persistent Na⁺ conductance,^{33,34} the roles of TTX-resistant voltage-gated sodium channel α subunits Nav1.8, which are selectively expressed in the DRG neurons, should be further investigated. Since injection of TNBS enhanced Nav1.8 but not Nav1.7 (data not shown) expression in pancreatic DRGs, therefore, Nav1.8 expression was further investigated. In our present study, we demonstrated that intrathecal injection CXCR4 inhibitor (AMD3100) reversed the up-regulation of Nav1.8 sodium channels in DRGs and hyperexcitability of DRG neurons. Although previous studies demonstrated that the increased expression of Nav1.8 might underlie the enhanced excitability of the DRG neurons, we did not have enough evidence to make the connection in the present study. Future experiments are definitely needed to measure TTX-resistant sodium currents in control and TNBS-treated rats or to detect neuronal excitability after treatment of PF-01247324, a selective inhibitor of Nav1.8. Since intrathecal injection CXCR4 inhibitor (AMD3100) reversed the up-regulation of Nav1.8 sodium channels in DRGs and hyperexcitability of DRG neurons, thus we proposed a functional link between CXCL12–CXCR4 signaling and expression of Nav1.8 that maintains DRG neuronal hyperexcitability under chronic pancreatic pain state. Of note is that voltage-gated potassium channels^{25,35} and other channels³⁶ might be also involved in the development of chronic pancreatic hyperalgesia. Whether sensitization of these channels is mediated by CXCL12–CXCR4 signaling needs to be further studied.

The mechanism by which CXCL12–CXCR4 signaling sensitizes Nav1.8 activity remains largely unknown. In the present study, treatment of AMD3100 not only significantly suppressed Nav activity but also greatly reduced the phosphorylation of ERK, indicating an involvement of ERK in the CXCL12–CXCR4 signaling pathways. Our finding that intrathecal injection of U0126 (an ERK inhibitor) diminished the level of pERK and expression of Nav1.8 further confirmed the role of ERK in this pathway. The previous study has showed that phosphorylation level of ERK was important for expression of Nav1.8 channels.³⁷ CXCL12–CXCR4 activation has also been demonstrated to modify multiple intracellular signal transduction pathways, such as ERK and PI3K pathways.^{26–28} These results are consistent with our present study in which intrathecal injection of U0126 reversed TNBS-induced

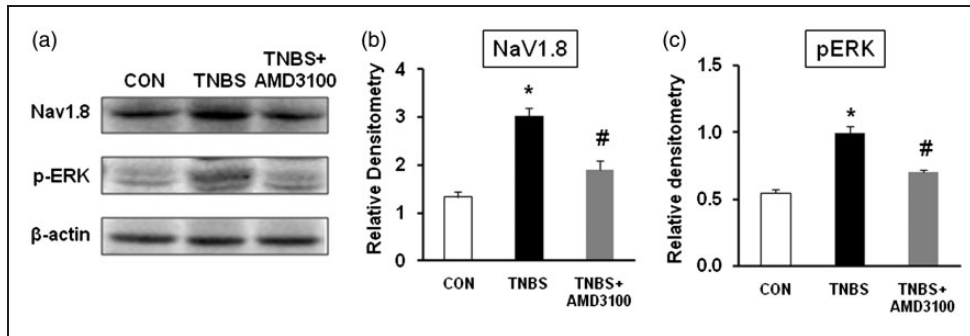


Figure 5. Blockade of TNBS-induced phosphorylation of ERK and up-regulation of Nav1.8 protein expression in pancreas DRG by intrathecal application of AMD3100. (a) Representative photomicrograph bands showing expression of Nav1.8 and p-ERK in the pancreas DRGs from control rats, TNBS rats, and rats receiving consecutive 7-day intrathecal injection of AMD3100. (b) Western blots showing that AMD3100 reversed the TNBS-induced up-regulation of Nav1.8 in pancreas-related DRG ($n = 4$ for each group, $*p < 0.05$ vs. CON; $\#p < 0.05$ vs. TNBS). (c) Western blots showing that AMD3100 reversed the TNBS-induced pERK activation in pancreas-related DRG ($n = 4$ for each group, $*p < 0.05$ vs. CON; $\#p < 0.05$ vs. TNBS).

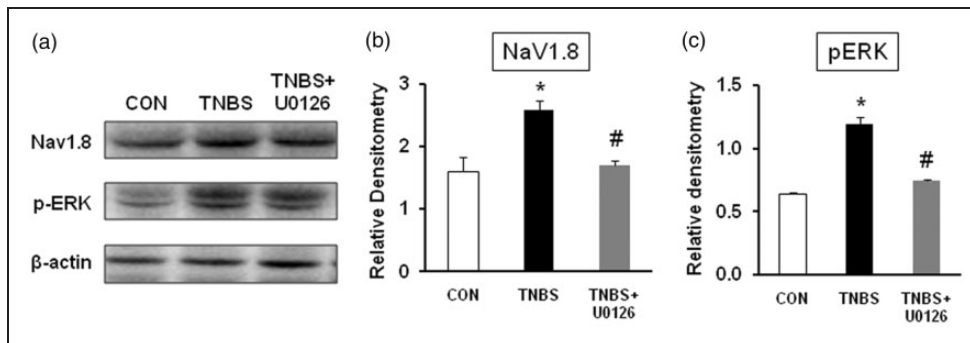


Figure 6. Blockade of TNBS-induced phosphorylation of ERK and up-regulation of Nav1.8 protein expression in pancreas-related DRG by intrathecal application of U0126. (a) Representative photomicrograph bands showing expression of Nav1.8 and p-ERK in the pancreas DRGs from control, TNBS-treated rats, and rats receiving consecutive 7-day intrathecal injection of U0126 at 10 μ g. (b) Western blots showing that U0126 treatment reversed the TNBS-induced up-regulation of Nav1.8 in pancreas-related DRG ($n = 4$ for each group, $*p < 0.05$ vs. CON; $\#p < 0.05$ vs. TNBS). (c) Western blots showing that U0126 treatment reversed the TNBS-induced pERK activation in pancreas-related DRG ($n = 4$ for each group, $*p < 0.05$ vs. CON; $\#p < 0.05$ vs. TNBS).

mechanical hypersensitivity and reduced expression level of Nav1.8 in the DRGs. These findings indicate that CXCL12-CXCR4 pathway might be involved in up-regulation of Nav1.8 expression via an ERK-dependent pathway, thus contributing to hyperexcitability of the DRG neurons that mediate chronic pancreatic pain hypersensitivity. Several other lines of evidence also support this hypothesis. For examples, up-regulation of Nav1.8 expression by chemokine CCL2 stimulation has been reported in acutely isolated DRG neurons from normal healthy rats.^{18,38,39} The Nav1.8 expression level was increased by TNF- α treatment in rat DRG neurons.⁴⁰ However, the underlying mechanism was unlikely mediated by the release of CXCL12 from the SGCs. The chemokine CCL2 and pro-inflammatory mediators such as TNF α and

IL-10 were thought to be released from DRG neurons that act on their respective receptors.^{18,38-41} Therefore, we hypothesized that two regulating pathways are likely involved in regulation of Nav1.8 activities under peripheral inflammatory and/or injury conditions. One may be initiated by neuronal activity-dependent autocrine-autoreceptor signaling pathway, i.e., TNF α -TNFR signaling pathways within DRG neurons, as reported previously.⁴⁰ The other may be initiated by SGC-neuronal pattern mediated by CXCL12-CXCR4 pathways, as described by our and previous studies.¹⁷ The first one might be involved in the early induction process, while the SGC-neuronal pattern may be critical in maintaining chronic pain and pancreatic inflammation as well. However, these require to be determined by future studies.

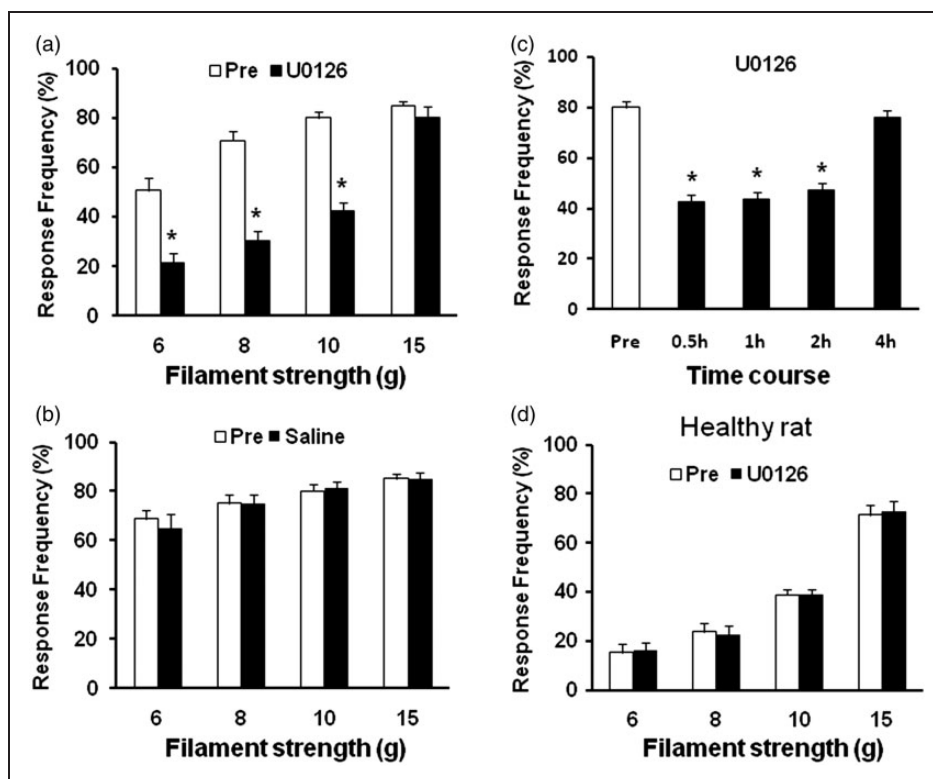


Figure 7. Treatment with ERK inhibitor U0126 suppressed pancreatic pain behaviors. (a) Administration of ERK inhibitor U0126 (i.t., 10 μ g) significantly attenuated response frequency of CP rats to VFF stimulation (* p < 0.05, compared with pretreatment (Pre), Dunn's post hoc test following Friedman ANOVA, n = 8). (b) Administration of solvent DMSO had no effect on response frequency of CP rats to VFF stimulation. (c) Time course of effect of ERK inhibitor U0126 (10 μ g) on mechanical hyperalgesia. The antinociceptive effect lasted for at least 2 h (* p < 0.05, compared with Pre, Dunn's post hoc test following Friedman ANOVA, n = 8). (d) Administration of U0126 did not produce any effect on response frequency in age-matched healthy control rats (n = 8 for each group).

In conclusion, the present study demonstrates for the first time that TNBS-induced CP significantly enhances both CXCL12 and CXCR4 expression in the pancreatic DRGs. The activation of CXCL12-CXCR4 signaling induces an ERK-dependent up-regulation of Nav1.8, thus maintaining DRG neuronal hyperexcitability and persistent pancreatic pain hypersensitivity under CP.

Author Contributions

Hong-Yan Zhu and Xuelian Liu contributed equally to this work. Hong-Yan Zhu and Xuelian Liu performed experiments, analyzed data, prepared figures, and draft the manuscript. Xiuhua Miao and Di Li analyzed data and prepared figures. Shusheng Wang analyzed data, prepared figures, and drafted the manuscript. Guang-Yin Xu designed and supervised the experiments, and edited the manuscript.

Declaration of Conflicting Interests

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