Research Paper

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Shp-1 dephosphorylates TRPV1 in dorsal root ganglion neurons and alleviates CFA-induced inflammatory pain in rats

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Abstract

Transient receptor potential vanilloid 1 (TRPV1) receptors are expressed in nociceptive neurons of rat dorsal root ganglions (DRGs) and mediate inflammatory pain. Nonspecific inhibition of protein-tyrosine phosphatases (PTPs) increases the tyrosine phosphorylation of TRPV1 and sensitizes TRPV1. However, less is known about tyrosine phosphorylation's implication in inflammatory pain, compared with that of serine/threonine phosphorylation. Src homology 2 domain-containing tyrosine phosphatase 1 (Shp-1) is a key phosphatase dephosphorylating TRPV1. In this study, we reported that Shp-1 colocalized with and bound to TRPV1 in nociceptive DRG neurons. Shp-1 inhibitors, including sodium stibogluconate and PTP inhibitor III, sensitized TRPV1 in cultured DRG neurons. In naive rats, intrathecal injection of Shp-1 inhibitors increased both TRPV1 and tyrosine-phosphorylated TRPV1 in DRGs and induced thermal hyperalgesia, which was abolished by pretreatment with TRPV1 antagonists capsazepine, BCTC, or AMG9810. Complete Freund's adjuvant (CFA)–induced inflammatory pain in rats significantly increased the expression of Shp-1, TRPV1, and tyrosine-phosphorylated TRPV1, as well as the colocalization of Shp-1 and TRPV1 in DRGs. Intrathecal injection of sodium stibogluconate aggravated CFA-induced inflammatory pain, whereas Shp-1 overexpression in DRG neurons alleviated it. These results suggested that Shp-1 dephosphorylated and inhibited TRPV1 in DRG neurons, contributing to maintain thermal nociceptive thresholds in normal rats, and as a compensatory mechanism, Shp-1 increased in DRGs of rats with CFA-induced inflammatory pain, which was involved in protecting against excessive thermal hyperalgesia.

Keywords: Src homology 2 domain-containing tyrosine phosphatase 1, Transient receptor potential vanilloid 1, Inflammatory pain, Tyrosine phosphorylation

1. Introduction

The transient receptor potential vanilloid 1 (TRPV1) receptor, a nonselective cation channel activated by capsaicin, heat (>43°C), and protons (pH < 6)[3,4] transduces noxious heat stimuli in nociceptive dorsal root ganglion (DRG) neurons and mediates complete Freund's adjuvant (CFA)–induced inflammatory pain.[5] The activation of TRPV1 is regulated by its phosphorylation status that depends on balanced actions of protein kinases and protein phosphatases.[7] Based on their substrate specificity, these protein kinases/phosphatases are further divided into protein serine/threonine kinases/phosphatases, protein-tyrosine kinases (PTKs)/phosphatases, and dual-specificity kinases/phosphatases.[24] The serine/threonine phosphorylation of TRPV1 has been extensively studied; protein serine/threonine kinases, including protein kinase A (PKA), protein kinase C (PKC), Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II), protein kinase D1 (PKD1), and cyclin-dependent protein kinase 5 (Cdk5), phosphorylate TRPV1 at specific residues and sensitize TRPV1[7,27]; however, protein serine/threonine phosphatases such as protein phosphatase 2B (PP2B) dephosphorylate TRPV1 and mediate TRPV1 desensitization.[20,22]

Tyrosine phosphorylation is important for TRPV1 activation as well. The PTK Src increases TRPV1 tyrosine phosphorylation at Tyr200, leads to trafficking of TRPV1 to the plasma membrane, and thus enhances TRPV1 responses in DRG neurons.[12,29] Complete Freund''s adjuvant–induced inflammatory pain is relieved by TRPV1 antagonism or Src deficiency.[5,18,21,28] Nonspecific protein-tyrosine phosphatase (PTP) inhibition also increases the tyrosine phosphorylation of TRPV1.[12,29] Src homology 2 domain-containing tyrosine phosphatase 1 (Shp-1) is a key PTP that dephosphorylates TRPV1. It abolishes increased TRPV1 tyrosine phosphorylation by Src in cultured HEK-293 cells,[29] whereas Shp-1 mutant mice exhibit systemic inflammation and thermal hyperalgesia.[9] Shp-1 exists in the central nervous system,[23] but its localization in DRGs has not been reported nor has its potential role in inflammatory pain. In this study, we examined the expression pattern of Shp-1 in rat DRG neurons and investigated its modulation over TRPV1 and CFA-induced inflammatory pain.

2. Materials and methods

2.1. Animals

Adult male Sprague–Dawley rats (160-180 g) were provided by the Department of Laboratory Animal Sciences, Peking

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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University Health Science Center. All animal experimental procedures followed the Guidelines of the Committee for Research and Ethical Issues of International Association for the Study of Pain[32] and were approved by the Animal Care and Use Committee of Peking University Health Science Center. Rats were housed under a 12-hour alternating light/dark cycle with food and water available ad libitum.

2.2. Lentivirus packaging

pMig plasmid harboring human Shp-1 was a kind gift from Dr Toshiaki Kawakami (La Jolla Institute for Allergy and Immunology, La Jolla, CA). The sequence coding Shp-1 was amplified from pMig plasmid with polymerase chain reaction (PCR) and cloned into a lentivirus (LV) packaging vector GV287 (GeneChem, Shanghai, China) with an ubiquitin promoter. The lentiviral vectors were constructed, and the lentiviruses (LV-Shp-1 and LV-GFP) were packaged by Shanghai Jikai Gene Chemistry Technology Inc.[16] LV-GFP without Shp-1 insertion was used as a control.

2.3. Drug preparation and application

As a stock solution, PTP inhibitor III (α-bromo-4-(carboxymethoxy)-acetophenone; Merck Millipore) and BCTC (N-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl) tetrahydropyrazine-1(2H)-carbox-amide, Biomol) were dissolved at 500 mmol/L and 20 mg/mL in dimethyl sulfoxide (DMSO), respectively. AMG9810 (Tocris, Bristol, United Kingdom) and capsaicin (Sigma-Aldrich) were dissolved at 100 mmol/L and 10 mmol/L in ethanol, respectively, and capsazepine (Sigma-Aldrich, St Louis, MO) was dissolved at 100 mmol/L in methanol. The stock solution was freshly diluted in normal saline (NS) or external bath solution; to prepare the vehicle control, in the stock solution, the above drugs were replaced by DMSO, ethanol, or methanol, respectively.

Sodium stibogluconate (SSG; Merck Millipore) was freshly dissolved and diluted in NS, pipette solution, or external solution. Complete Freund's adjuvant (Sigma-Aldrich) was freshly diluted in incomplete Freund's adjuvant (Sigma-Aldrich) to prepare 25% CFA.

2.4. Behavior test for heat-induced paw withdrawal latencies

The rat was adapted to the test environment for 15 minutes before experiments. Focused radiant heat was applied onto the left hind paw of the rat,[15] and the latency before the rat withdrew its paw from the heat was taken as the paw withdrawal latency (PWL). The test was repeated 3 times at a minimal 5-minute interval, and the average of PWL values was calculated for each rat. To avoid tissue damage, a cutoff time of 30 seconds was imposed.

2.5. Establishment of CFA-induced inflammatory pain

Rats were anaesthetized with 2.5% isoflurane. For the CFA rat, 100 μL CFA was injected into the plantar of left hind paw, whereas for the control rat, an equal volume of NS was injected instead.[17]

2.6. Intrathecal delivery of drugs or lentivirus

Rats were anaesthetized with sodium pentobarbital (50 mg/kg). Intrathecal catheter implantation was performed as previously described.[17] A guide cannula was used to puncture the dura at the cauda equina level, and a PE-10 catheter was then implanted to the lumbar enlargement level (3.5-4 cm rostral than the cannula) at the subarachnoid space through the cannula. The outer part of the PE-10 catheter was plugged and fixed onto the skin between the rat ears. A 5-day recovery was given before further experimental procedures. The drug or LV solution was injected with a Hamilton syringe connecting to the PE-10 catheter.

2.7. Western blot detection of Shp-1 and TRPV1 proteins in rat DRGs

L4 and L5 DRGs were sonicated in the lysis buffer (in millimole per liter): Tris 100, NaCl 150, ethylenediaminetetraacetic acid (EDTA) 5, phenylmethanesulfonyl fluoride ([PMSF], Sigma-Aldrich) 1, and 1% Triton X-100 (Sigma-Aldrich), and the pH was adjusted to 7.4. After centrifugation, the supernatants were collected to determine the protein concentration. The protein samples were separated on 10% SDS-PAGE gels and then transferred to polyvinylidene fluoride membranes. After blocking with 5% nonfat milk, the membranes were incubated overnight at 4°C with mouse monoclonal anti–Shp-1 antibody (1:1000; BD Transduction Laboratories, Franklin Lake, NJ), mouse monoclonal anti-phosphotyrosine antibody 4G10 (1:1000; Merck Millipore), or rabbit polyclonal anti-TRPV1 antibody (1:500; Santa Cruz Biotechnology, Dallas, TX). The signal was detected with SuperSignal West Pico (Pierce). Blots were scanned with CanoScan LiDE 110 controlled by the software MP Navigator EX 4.00 (Canon, Inc, Tokyo, Japan), and band densities were detected and compared with the software Quantity One 4.6.2 (Bio-Rad, Hercules, CA).

2.8. Immunoprecipitation

Immunoprecipitation was performed as previously described with modifications.[30] After dissection, L4 and L5 DRGs were mechanically homogenized in the lysis buffer (inmillimole per liter): Tris 100, NaCl 150, EDTA 5, PMSF 1, Na-pervanadate (Sigma-Aldrich) 1, and 1% Triton X-100, and the pH was adjusted to 7.4. After centrifugation at 12,000 rpm for 10 minutes at 4°C, supernatants were collected to determine the protein concentration. Proteins (500 to 700 μg) were incubated with 4 μg mouse monoclonal anti–Shp-1 antibody, rabbit polyclonal anti-TRPV1 antibody, or normal mouse/rabbit IgG control (Sigma-Aldrich) overnight, followed by incubation of 40 μL prewashed protein A/G beads for 4 h at 4°C. The immunoprecipitants were separated by SDS-PAGE gel and probed by anti–Shp-1 (1:1000), anti-TRPV1 (1:500), or mouse monoclonal anti-phosphotyrosine antibody 4G10 (1:1000).

2.9. Immunofluorescence detection of colocalization of Shp-1 and TRPV1 in DRG neurons

DRG sections and immunofluorescence were obtained and performed as previously described.[11,28] Over-anesthetized rats were perfused with NS, followed by 4% paraformaldehyde in 100 mmol/L phosphate buffer. L4 and L5 DRGs were removed, post-fixed in 4% paraformaldehyde for 6 hours, and then dehydrated in graded ethanol series (70% to 100%). After cleaning in xylene for 40 minutes, the tissues were embedded in paraffin wax, finally cut in 8-μm thick serial longitudinal sections, and mounted on gelatin (Sigma-Aldrich type II)-coated glass

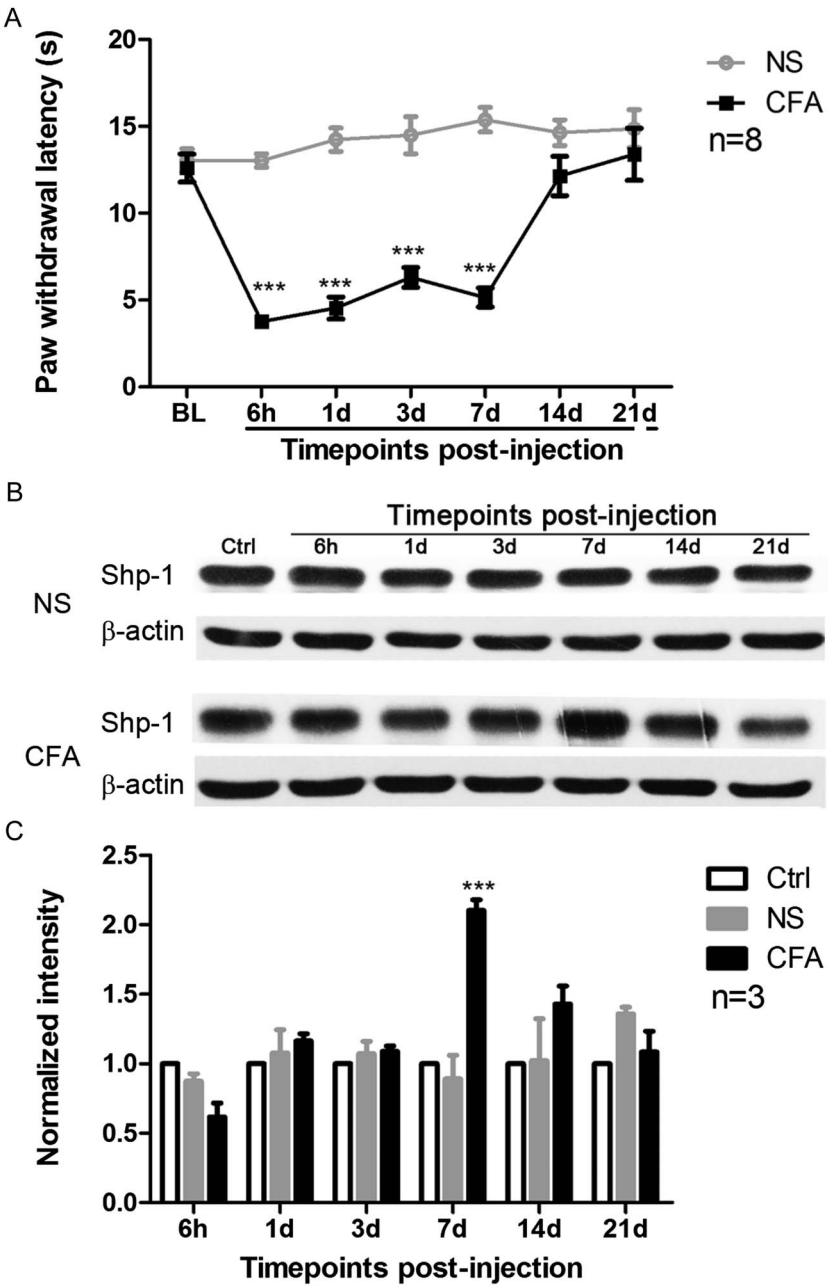

Figure 1. Increased Shp-1 protein levels in ipsilateral L4 and L5 DRGs in rats with CFA-induced inflammatory pain. (A) Thermal hyperalgesia induced by intraplantar injection of CFA in rats. A significant decrease in paw withdrawal latencies was observed from 6 hours to 7 days after CFA injection. (B and C) β-actin served as a loading control. Shp-1 protein expression levels in normal saline (NS) group or CFA group were standardized with that in the naive group (Ctrl). Shp-1 proteins increased at 7 days in the inflammatory pain rats compared with those in the NS rats. Data were represented as mean ± SEM, n = 8 in the behavior test and n = 3 in the Western blot experiment; ***$P < 0.001$ vs NS group (2-way ANOVA followed by Bonferroni posttests). BL, basal latency.

slides. After deparaffinization and graded hydration, the paraffin sections were blocked in 5% bovine serum albumin and then incubated with anti–Shp-1 (1:250) and anti-TRPV1 (1:500) antibodies overnight at 4°C, followed by incubation with Alexa Fluor 568–conjugated goat anti-mouse IgG and Alexa Fluor 488–conjugated goat anti-rabbit IgG (both from Invitrogen Life Technologies) for 1 h at room temperature. A fluorescent microscope (Leica DMI4000 B) controlled by Leica Application Suite V4.2 was used for image capture, and the images were then analyzed with Image-Pro Plus software (Media Cybernetics Co, Rockville, MD).

2.10. Culture of acutely dissociated DRG neurons

L4 and L5 left DRGs of adult rats were dissected and cultured as previously described.[31] Dorsal root ganglions were digested with type I_A collagenase (Sigma-Aldrich) at 3 mg/mL for 45 minutes, followed by 0.25% wt/vol trypsin for 10 minutes at 37°C. The trypsin digestion was terminated with Dulbecco's minimum essential medium containing 10% fetal bovine serum. The DRGs were mechanically dissociated by a fire-polished Pasteur pipette in fresh Dulbecco's minimum essential medium containing 10% fetal bovine serum. The cell suspension was seeded in poly-D-lysine (Sigma-Aldrich)–coated glass coverslips. Cells were cultivated in

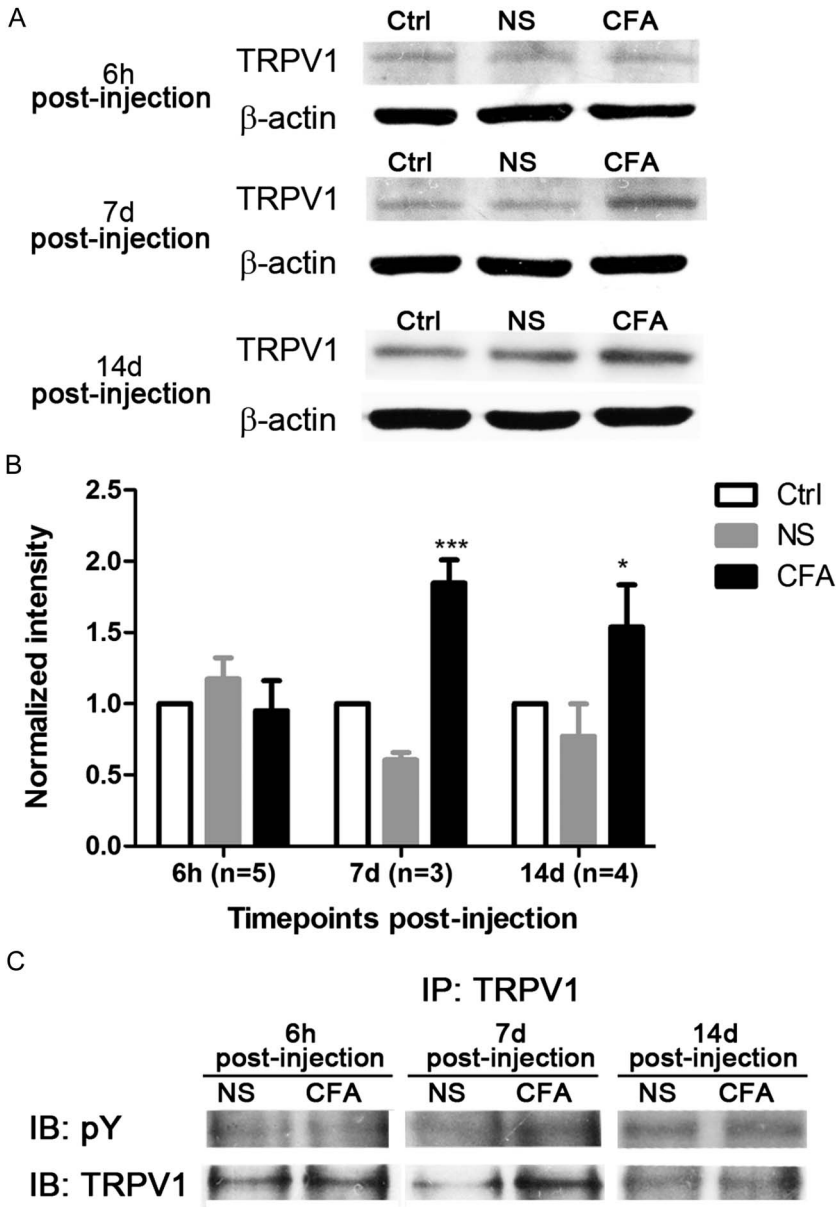

Figure 2. Both TRPV1 and tyrosine-phosphorylated TRPV1 increased in the ipsilateral L4 and L5 DRGs in rats with CFA-induced inflammatory pain. (A and B) β-actin served as a loading control. TRPV1 protein expression levels in normal saline (NS) group or CFA group were standardized with that in the naive group (Ctrl). TRPV1 protein levels increased at 7 and 14 days in the inflammatory pain rats compared with those in the NS rats. Data were represented as mean ± SEM, n = 3 to 5; ***$P < 0.001$, *$P < 0.05$ (2-way ANOVA followed by Bonferroni posttests). (C) The tyrosine phosphorylation of TRPV1 increased at 7 and 14 days in rats with CFA-induced inflammatory pain compared with that in the NS rats. Results were representatives of 4 individual trials.

5% CO_2 incubator at 37°C for 3 hours before the whole-cell patch-clamp recording or the calcium imaging experiments as below.

2.11. Calcium imaging analysis of capsaicin-evoked responses in DRG neurons

The cultured DRG neurons were used for calcium imaging. The bath solution contained (in millimole per liter) NaCl 135, $CaCl_2$ 2, KCl 5.4, $MgCl_2$ 1, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 5, and glucose 5.5. The pH was adjusted to 7.4 with NaOH solution. According to the modified method,[2] the acutely dissociated DRG neurons were loaded with 5 μmol/L Fura-2 AM and 0.02% Pluronic F-127 (both were from Invitrogen Life Technologies) in external bath solution for 20 minutes. Then, neurons were washed twice, followed by incubation and recovery in external bath solution for 60 minutes. After 1-minute recording as baseline, the responses of neurons to capsaicin (5 μmol/L) were detected. The bound/unbound calcium ratio (F340/F380) was monitored with a fluorescent microscope (Olympus IX81, Tokyo, Japan) equipped with a Andor iXon 897 EMCDD camera with a monochromator alternating between 340 and 380 nm of wavelength (Lambda DG-4; Sutter Instruments Co, Novato, CA). The microscope was controlled by MetaFluor software (Universal Imaging, Downington, PA).

2.12. Whole-cell patch-clamp recording of TRPV1 currents in DRG neurons

The cultured DRG neurons were used for patch-clamp recordings. As described,[12] the external bath solution was identical to that in the calcium imaging experiment, and the pipette solution

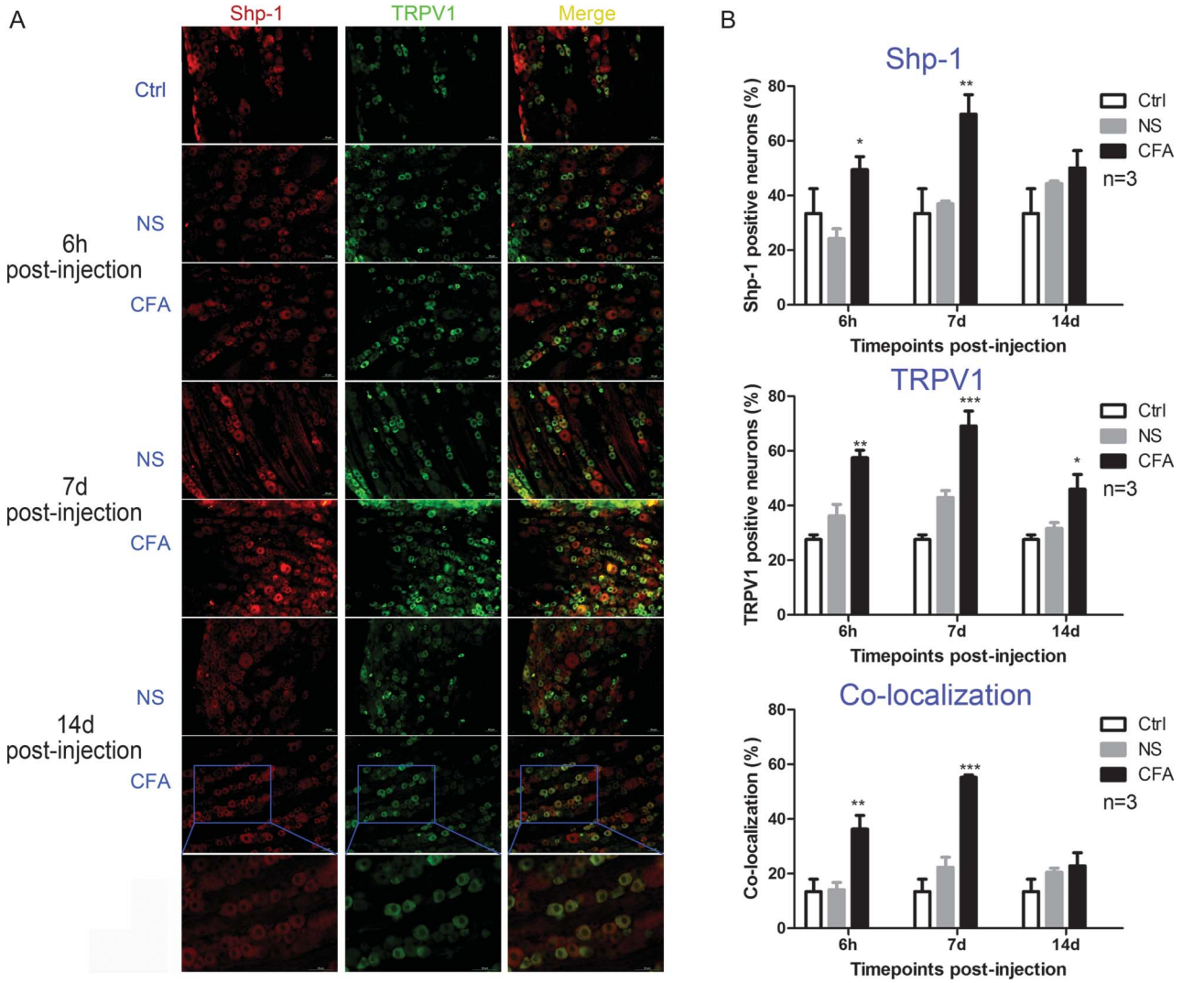

Figure 3. Increased colocalization of Shp-1 and TRPV1 in L4 and L5 DRG neurons of rats with inflammatory pain. (A) Representative images of immunofluorescent double staining of Shp-1 and TRPV1. Shp-1 (red), TRPV1 (green), and their double (yellow) staining existed in DRG neurons in naive rats (Ctrl) and the NS-/CFA-injected rats. Results were representatives of 3 individual trials. Scale bar: 50 μm. (B) Quantification analysis: Shp-1, TRPV1, and their double staining positive neurons increased significantly at 6 hours and 7 days in rats with CFA-induced inflammatory pain compared with those in NS rats, while TRPV1-positive neurons also increased at 14 days. Data were represented as mean ± SEM, n = 3; ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ (2-way ANOVA followed by Bonferroni posttests).

contained (in millimole per liter) CsCl 30, Cs-aspartate 100, ethylene glycol tetraacetic acid 5, $MgCl_2$ 1, HEPES 5, Na_2-ATP 5, and Na_2-GTP 0.4, and the pH was adjusted to 7.2 with CsOH. The filled patch pipette had a resistance of 4 to 7 MΩ, and the series resistance was compensated to above 75%. The neurons were patched at −70 mV when recording the capsaicin-induced TRPV1 currents. Capsaicin (0.3 μmol/L) was applied for 2 seconds to elicit the inward TRPV1 currents, followed by washout with the external bath solution. Only 1 neuron on each coverslip was recorded to avoid cross-drug contamination. EPC-10 amplifier and Patch master software (HEKA Electronics Inc, Bellmore, NY) were used, and all experiments were conducted at room temperature.

2.13. Statistical analysis

Data were presented as mean ± SEM and analyzed using unpaired *t* test, paired *t* test, 1-way analysis of variance (ANOVA) followed by Tukey posttests, or 2-way ANOVA followed by Bonferroni posttests. GraphPad Prism 5 was used for data analysis. Statistically significant difference was considered at $P < 0.05$.

3. Results

3.1. Increased Shp-1 expression in DRGs of rats with inflammatory pain

To explore the presence of Shp-1 in the DRG of naive rats and its changes in pain, we first established the rat model of inflammatory pain with intraplantar injection of CFA. After CFA injection, rats exhibited thermal hyperalgesia within 6 hours, which lasted 7 days (**Fig. 1A**). The PWLs decreased significantly in CFA rats compared with those in control rats at 6 hours, 1, 3, and 7 days (time points: $F_{6,98} = 13.67$, $P < 0.001$; groups: $F_{1,98} = 178.3$, $P < 0.001$; time points × groups: $F_{6,98} = 13.20$, $P < 0.001$; $P < 0.001$ at 6 hours, 1, 3, and 7 days; 2-way ANOVA followed by Bonferroni posttests), and recovered gradually from 14 to 21 days.

Shp-1 protein expression levels were detected with Western blotting in ipsilateral L4 and L5 DRGs at 6 hours, 1, 3, 7, 14, and 21

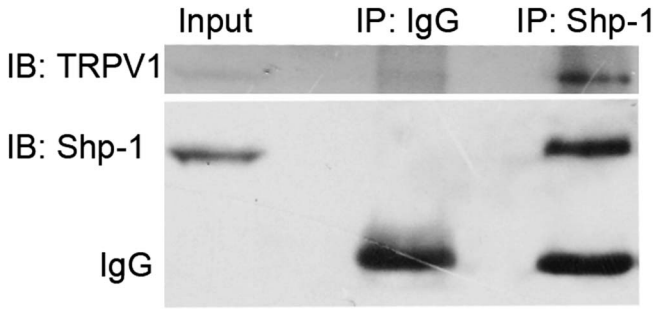

Figure 4. Coimmunoprecipitation of Shp-1 and TRPV1 in L4 and L5 DRGs. Proteins (500-700 μg) from DRG lysates were used for Shp-1 immunoprecipitation. Total proteins (50-70 μg) were used as the "input" positive control, while normal mouse IgG immunoprecipitation was performed as the negative control, to demonstrate the specificity of antibodies. The "IgG" bands were used as the loading control. Both Shp-1 and TRPV1 were captured with anti–Shp-1, indicating their interaction. Results were representatives of 3 individual trials.

days after NS or CFA injection (**Fig. 1B, C**). Shp-1 protein expression increased significantly at 7 days ($P < 0.001$) in the CFA-injected rats compared with that in the NS control rats (time points: $F_{5,36} = 6.523$, $P < 0.001$; groups: $F_{2,36} = 8.405$, $P < 0.01$; time points × groups: $F_{10,36} = 7.936$, $P < 0.001$; 2-way ANOVA followed by Bonferroni posttests).

3.2. Increased protein expression and tyrosine phosphorylation of TRPV1 in DRGs of rats with inflammatory pain

To investigate the possible role of Shp-1 in TRPV1 modulation in inflammatory pain, the protein expression and tyrosine phosphorylation of TRPV1 were examined in ipsilateral L4 and L5 DRGs at 6 hours, 7, and 14 days after NS or CFA injection. **Figure 2A** and **Figure 2B** shows that total TRPV1 protein expression increased significantly at 7 days ($P < 0.001$) and 14 days ($P < 0.05$) in CFA rats compared with that in NS rats (groups: $F_{2,27} = 9.306$, $P < 0.001$; time points: $F_{2,27} = 0.2983$, $P = 0.7445$; groups by time points: $F_{4,27} = 4.889$, $P < 0.01$; 2-way ANOVA followed by Bonferroni posttests). Analysis on the immunoprecipitated TRPV1 with anti-phosphotyrosine antibody 4G10 also revealed increased tyrosine phosphorylation of TRPV1 in the CFA group at 7 and 14 days (**Fig. 2C**).

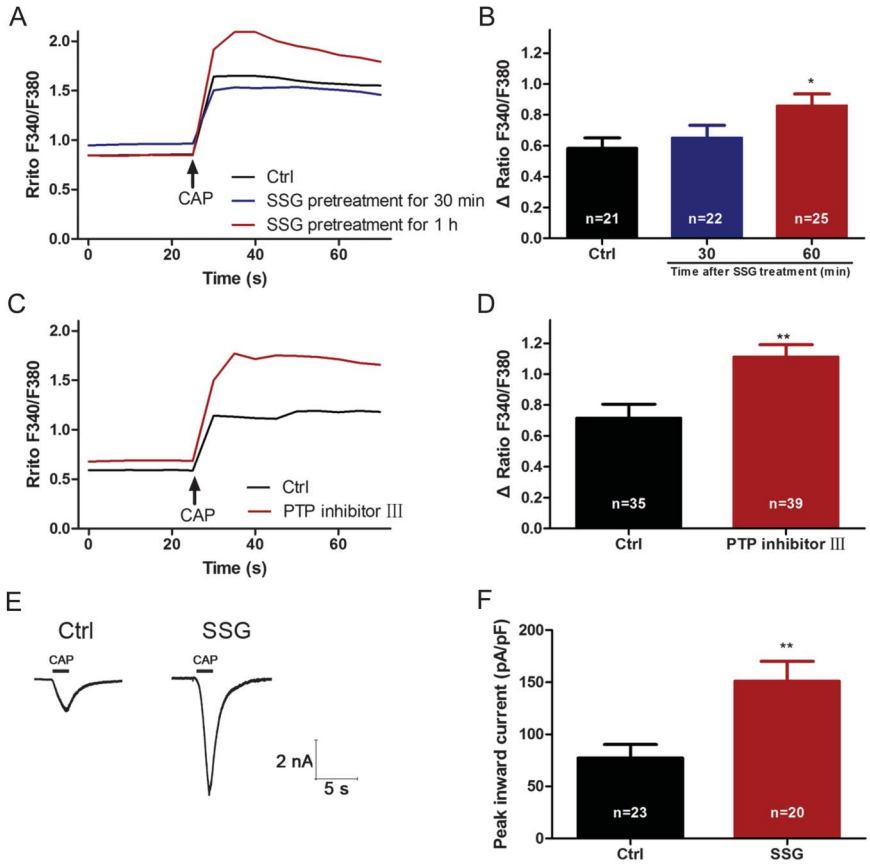

Figure 5. Sensitization of TRPV1 responses to capsaicin (CAP) by pretreatment with Shp-1 inhibitors in cultured DRG neurons. (A and B) The trace represented the response of a single neuron to capsaicin, and the statistical results showed the maximum increase of ratio F340/F380 induced by capsaicin with or without extracellular pretreatment with the Shp-1 inhibitor SSG in calcium imaging experiment. Sodium stibogluconate significantly enhanced capsaicin responses in 60 minutes. Data were represented as mean ± SEM, n = 21 in the vehicle control group (Ctrl), n = 22 (30 minutes) or 25 (60 minutes) in SSG groups; $^{*}P < 0.05$ vs control group (1-way ANOVA followed by Tukey posttests). (C and D) The trace represented the response of a single neuron to capsaicin, and the statistical results showed the maximum increase of ratio F340/F380 induced by capsaicin with or without extracellular pretreatment with the Shp-1 inhibitor PTP inhibitor III in calcium imaging experiment. PTP inhibitor III significantly enhanced capsaicin responses in 60 minutes. Data were represented as mean ± SEM, n = 35 in vehicle control group (Ctrl) and n = 39 in PTP inhibitor III group; $^{**}P < 0.01$ vs control group (unpaired *t* test). (E and F) The trace represented the response of a single neuron to capsaicin, and the statistical results showed peak amplitudes normalized to cell capacitance with or without intracellular application of the Shp-1 inhibitor SSG in whole-cell patch-clamp recording experiments. Sodium stibogluconate immediately enhanced capsaicin-induced currents. Data were represented as mean ± SEM, n = 23 in vehicle control group (Ctrl) and n = 20 in SSG group; $^{**}P < 0.01$ vs control group (unpaired *t* test).

3.3. Increased colocalization and coimmunoprecipitation of Shp-1 and TRPV1 in DRG neurons of rats with inflammatory pain

Double immunofluorescent staining of Shp-1 and TRPV1 was detected in ipsilateral L4 and L5 DRG neurons at 6 hours, 7 and 14 days after NS or CFA injection (**Fig. 3**). In naive rats, there were 33.4% ± 9.1% Shp-1–positive staining neurons, 27.6% ± 1.7% TRPV1-positive staining neurons, and 13.4% ± 4.5% Shp-1 and TRPV1 double staining neurons. Six hours and 7 days after CFA injection, Shp-1–positive neurons (49.5% ± 4.7% vs 24.2% ± 3.6%, $P < 0.05$ at 6 hours; 69.8% ± 7.1% vs 37.0 ± 1.0%, $P < 0.01$ at 7 days; groups: $F_{2,18} = 11.75$, $P < 0.001$; time points: $F_{2,18} = 2.212$, $P = 0.1384$; time points × groups: $F_{2,18} = 1.718$, $P = 0.1898$), TRPV1-positive neurons (57.5% ± 2.7% vs 36.1% ± 4.3%, $P < 0.01$ at 6 hours; 69.1% ± 5.5% vs 43.0% ± 2.5%, $P < 0.001$ at 7 days; groups: $F_{2,18} = 60.19$, $P < 0.001$; time points: $F_{2,18} = 8.477$, $P < 0.01$; time points × groups: $F_{2,18} = 2.879$, $P = 0.0526$), and Shp-1 and TRPV1 double staining neurons (36.3 ± 5.0 vs 14.1% ± 2.6%, $P < 0.01$ at 6 hours; 55.2% ± 0.9% vs 22.3% ± 3.7%, $P < 0.001$ at 7 days; groups: $F_{2,18} = 34.21$, $P < 0.001$; time points: $F_{2,18} = 7.382$, $P < 0.01$; time points × groups: $F_{2,18} = 5.944$, $P < 0.01$) all increased significantly (2-way ANOVA followed by Bonferroni posttests). At 14 days, TRPV1-positive neurons still increased significantly (46.0% ± 5.4% vs 31.7% ± 2.1%, $P < 0.05$, 2-way ANOVA followed by Bonferroni posttests), although its colocalization with Shp-1 did not.

Coimmunoprecipitation was used to study possible interactions between Shp-1 and TRPV1 at the protein level (**Fig. 4**). Shp-1 proteins were immunoprecipitated by the anti–Shp-1 antibody, together with TRPV1 proteins. Both Shp-1 and TRPV1 were detected in the DRG lysates as a positive control ("input" sample), but not in the normal mouse IgG-negative control ("IgG" sample), demonstrating the specificity of the antibody. It indicated that Shp-1 and TRPV1 bound to each other in DRG neurons.

3.4. Shp-1 inhibition sensitized TRPV1 functions in cultured DRG neurons

We next examined the functional significance of interactions between Shp-1 and TRPV1 with calcium imaging. Capsaicin, a specific TRPV1 agonist, activates TRPV1 and induces calcium influx and inward currents in DRG neurons. Extracellular treatment with SSG, a specific Shp-1 inhibitor, at 10 μg/mL for 60 minutes significantly increased the capsaicin-induced calcium influx in DRG neurons compared with the vehicle control ($P < 0.05$ at 60 minutes, 1-way ANOVA followed by Tukey posttests, **Fig. 5A, B**). The incubation time with SSG affected the capsaicin responses significantly

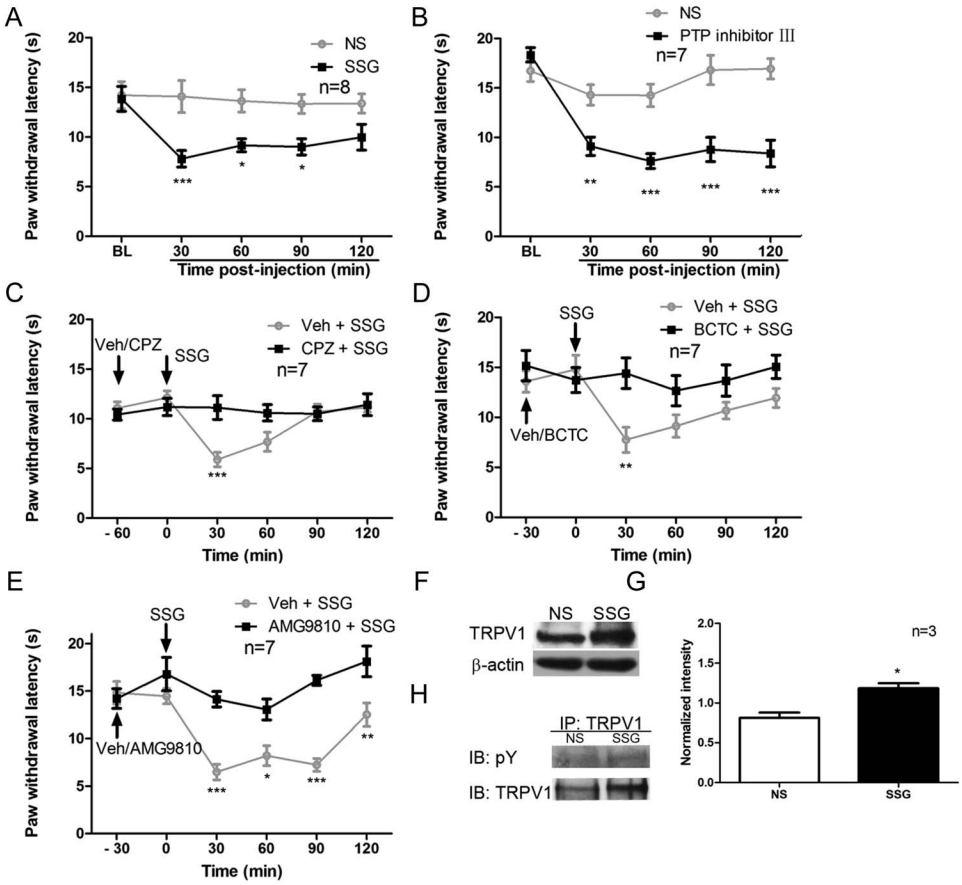

Figure 6. Shp-1 inhibition induced thermal hyperalgesia through a TRPV1-dependent manner in naive rats. (A) Intrathecal injection of the Shp-1 inhibitor SSG induced significant decrease in paw withdrawal latencies (PWLs) from 30 to 90 minutes in naive rats. (B) Intrathecal injection of the Shp-1 inhibitor PTP inhibitor III induced significant decreases in PWLs from 30 minutes in naive rats. (C–E) Pretreatment with TRPV1 antagonists capsazepine (CPZ), BCTC, or AMG9810 abolished SSG-induced thermal hyperalgesia, respectively. Data were represented as mean ± SEM, n = 7 to 8; ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ vs normal saline (NS) group or vehicle (Veh) group (2-way ANOVA followed by Bonferroni posttests). (F and G) SSG injection (without CPZ pretreatment) significantly increased TRPV1 protein expression in ipsilateral DRGs compared with that after NS injection. β-actin served as a loading control. Data were represented as mean ± SEM, n = 3; *$P < 0.05$ vs NS group (unpaired *t* test). (H) The tyrosine-phosphorylated TRPV1 also increased compared with NS injection. Results were representatives of 3 individual trials. BL, basal latency.

($F_{2,65} = 3.891$, $P < 0.05$). Another specific Shp-1 inhibitor, PTP inhibitor III, showed similar effects. It also increased capsaicin-induced calcium influx significantly after extracellular application at 200 μmol/mL for 60 minutes (**Fig. 5C, D**, $P < 0.01$, unpaired t test). With whole-cell patch-clamp recording, SSG added into the pipette solution at 1 μg/mL immediately (within 10 seconds) amplified the capsaicin-induced current density in SSG-treated neurons compared with that in the vehicle-treated neurons ($P < 0.01$, unpaired t test, **Fig. 5E, F**). These results suggested that intracellular inhibition of Shp-1 sensitized TRPV1 functions.

3.5. Shp-1 inhibition induced thermal hyperalgesia in naive rats through TRPV1 sensitization

We next studied the impact of Shp-1 and TRPV1 interactions on pain behaviors in naive rats (**Fig. 6**). The Shp-1 inhibitor SSG (10 μg/mL, 20 μL) or NS (20 μL) was intrathecally injected to naive rats at the lumbar enlargement level before PWL measurement. From 30 to 90 minutes after the SSG injection, thermal hyperalgesia developed, ie, PWLs reduced significantly compared with those after the NS injection (groups: $F_{1,70} = 28.02$, $P < 0.001$; time points: $F_{4,70} = 2.467$, $P = 0.0526$; groups × time points: $F_{4,70} = 1.855$, $P = 0.1280$; $P < 0.001$ at 30 minutes, $P < 0.05$ at 60 and 90 minutes; 2-way ANOVA followed by Bonferroni posttests, **Fig. 6A**). Another specific Shp-1 inhibitor, PTP inhibitor III, showed similar effects (**Fig. 6B**). Intrathecal injection of PTP inhibitor III (1 mmol/L, 20 μL) reduced PWLs significantly from 30 minutes (groups: $F_{1,60} = 59.47$, $P < 0.001$; time points: $F_{4,60} = 11.03$, $P < 0.001$; groups × time points: $F_{4,60} = 7.007$, $P < 0.001$; $P < 0.01$ at 30 minutes and $P < 0.001$ from 60 to 120 minutes; 2-way ANOVA followed by Bonferroni posttests). These results indicated that Shp-1 inhibition could induce thermal hyperalgesia in naive rats.

To test whether the effect of Shp-1 inhibition was dependent on the activation of TRPV1, the TRPV1 specific antagonist capsazepine (16 μg, 10 μL) was intrathecally injected 60 minutes before the SSG injection. Sodium stibogluconate did not change basal PWLs in naive rats but almost completely prevented the thermal hyperalgesia induced by the Shp-1 inhibition (groups: $F_{1,72} = 5.466$, $P < 0.05$; time points: $F_{5,72} = 4.598$, $P < 0.01$; groups × time points: $F_{5,72} = 4.513$, $P < 0.01$; $P < 0.001$ at 30 minutes; 2-way ANOVA followed by Bonferroni posttests, **Fig. 6C**). Similar effects were observed in experiments with other 2 TRPV1 speicfic antagonists BCTC (300 nmol, 10 μL) and AMG9810 (45 μg, 10 μL). Sodium stibogluconate –induced thermal hyperalgesia was prevented by both BCTC (groups: $F_{1,72} = 14.31$, $P < 0.001$; time points: $F_{5,72} = 2.909$, $P < 0.05$; groups × time points: $F_{5,72} = 1.942$, $P = 0.0978$; $P < 0.01$ at 30 minutes; 2-way ANOVA followed by Bonferroni posttests, **Fig. 6D**) and AMG9810 (groups: $F_{1,72} = 56.06$, $P < 0.001$; time points: $F_{5,72} = 9.494$, $P < 0.001$; groups × time points: $F_{5,72} = 4.958$, $P < 0.001$; $P < 0.001$ at 30 and 90 minutes, $P < 0.05$ at 60 minutes and $P < 0.01$ at 120 minutes; 2-way ANOVA followed by Bonferroni posttests, **Fig. 6E**).

Thirty minutes after the injection of the Shp-1 inhibitor SSG or NS to naive rats, the tyrosine phosphorylation and protein expression of TRPV1 were examined in acutely dissected L4 and L5 DRGs. Compared with those in the NS group, TRPV1 proteins increased significantly in the SSG group ($P < 0.05$, unpaired t test, **Fig. 6F, G**). The tyrosine phosphorylation of TRPV1 also increased after Shp-1 inhibition through analysis of the immunoprecipitated TRPV1 with anti-phosphotyrosine antibody 4G10 (**Fig. 6H**).

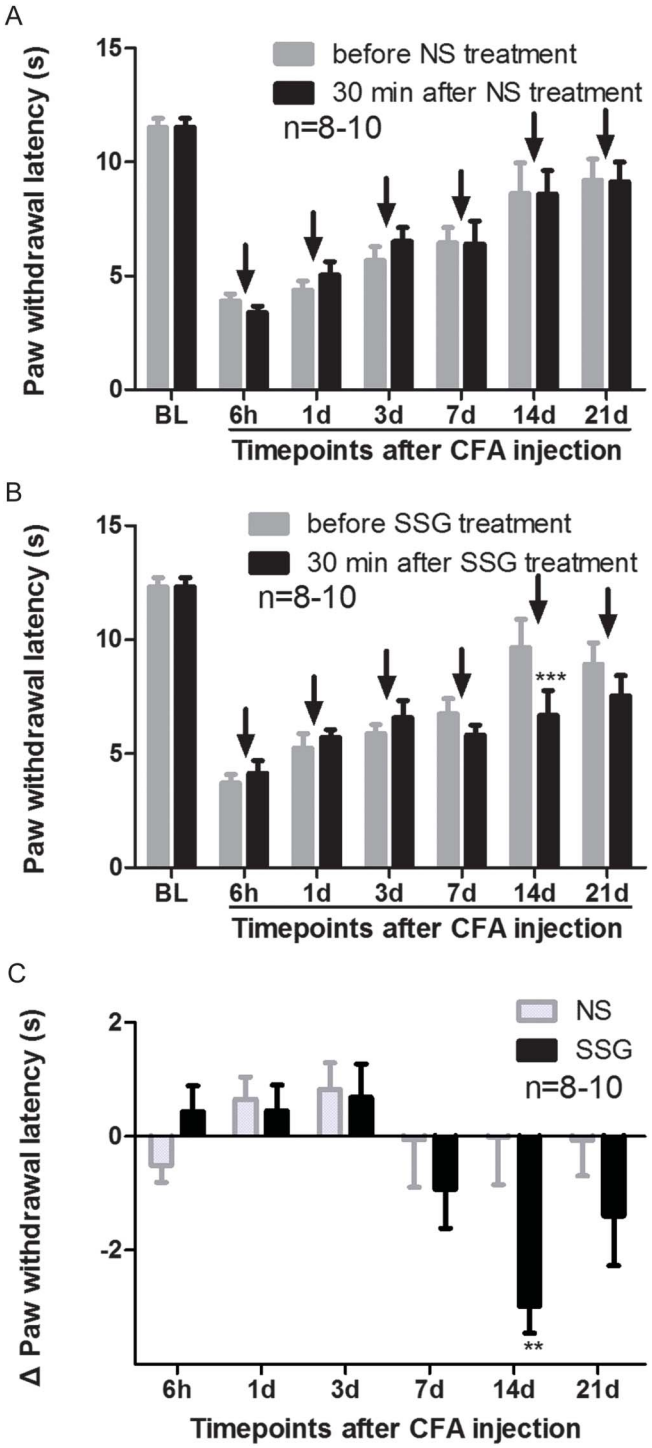

Figure 7. Intrathecal injection of the Shp-1 inhibitor SSG aggravated CFA-induced inflammatory pain in rats. (A) Normal saline injection did not affect CFA-induced inflammatory pain at any time points. (B) At 14 days, 30 minutes after SSG injection, rats showed more severe thermal hyperalgesia compared with that before SSG injection. (C) At 14 days, the paw withdrawal latencies decreased significantly 30 minutes after SSG treatment compared with those after the NS treatment (Δ PWL = $PWL_{after} - PWL_{before}$). Data were represented as mean ± SEM, n = 8 to 10; ***$P < 0.001$ (paired t test), **$P < 0.01$ (2-way ANOVA followed by Bonferroni posttests). BL, basal latency.

These results suggested that Shp-1 inhibited the tyrosine phosphorylation and protein expression of TRPV1 in DRGs to maintain the thermal nociceptive threshold in naive rats.

3.6. Antinociceptive effects of Shp-1 on inflammatory pain

Finally, we observed the effects of Shp-1 inhibition or overexpression on CFA-induced thermal hyperalgesia. The Shp-1 inhibitor SSG (10 μg/mL, 20 μL) or NS (20 μL) was intrathecally injected at the time points of 6 hours, 1, 3, 7, 14, and 21 days after CFA injection in rats. Thermal hyperalgesia was tested before and 30 minutes after SSG or NS injection at each time point. The PWLs did not change after NS injection (**Fig. 7A**) but significantly decreased after SSG injection at 14 days compared with those before the injection ($P < 0.001$, paired t test, **Fig. 7B**). We further calculated the change of PWL values after NS or SSG injection ($\Delta PWL = PWL_{after} - PWL_{before}$) at each time point and found that the PWLs decreased significantly after the SSG injection compared with those after the NS injection at 14 days (groups: $F_{1,96} = 4.767$, $P < 0.05$; time points: $F_{5,96} = 3.895$, $P < 0.01$; groups × time points: $F_{5,96} = 2.427$, $P < 0.05$; $P < 0.01$ at 14 days; 2-way ANOVA followed by Bonferroni posttests, **Fig. 7C**). Taken together, Shp-1 inhibition aggravated thermal hyperalgesia in CFA-induced inflammatory pain.

To overexpress Shp-1 in L4 and L5 DRGs of rats, LV-Shp-1 (1×10^7 TU) was injected intrathecally, with LV-GFP (green fluorescent protein, 1×10^7 TU) as a control. Seven days after the injection, the green fluorescence of GFP expressed by LV-Shp-1 or LV-GFP was directly observed in left L4 and L5 DRG neurons, indicating a low, stable, and efficient infection of the LV vector (**Fig. 8A**). Shp-1 proteins increased significantly in the LV-Shp-1 group compared with those in the LV-GFP group ($P < 0.05$, unpaired t test, **Fig. 8B**). Twenty-five percent CFA was injected to the left hind paws of rats to establish a mild inflammatory pain model. Paw withdrawal latencies showed a significant decrease from 30 to 120 minutes after CFA injection compared with those after the NS injection ($P < 0.001$, **Fig. 8C**). In addition, the thermal hyperalgesia in LV-Shp-1 rats was significantly alleviated compared with that in the LV-GFP control rats ($P < 0.05$ at 60 minutes, **Fig. 8D**). There was significant difference among groups ($F_{3,92} = 41.09$, $P < 0.001$), time points ($F_{3,92} = 12.53$, $P < 0.001$) and group × time interactions ($F_{9,92} = 3.158$, $P < 0.01$). These results further suggested that Shp-1 could prevent the development of CFA-induced inflammatory pain.

4. Discussion

4.1. Shp-1 inhibited the activation of TRPV1 and maintained thermal nociceptive thresholds in normal rats

We observed the existence of Shp-1 proteins in rat DRG neurons (**Figs. 1 and 3**). Shp-1 colocalized with and bound to TRPV1 in L4

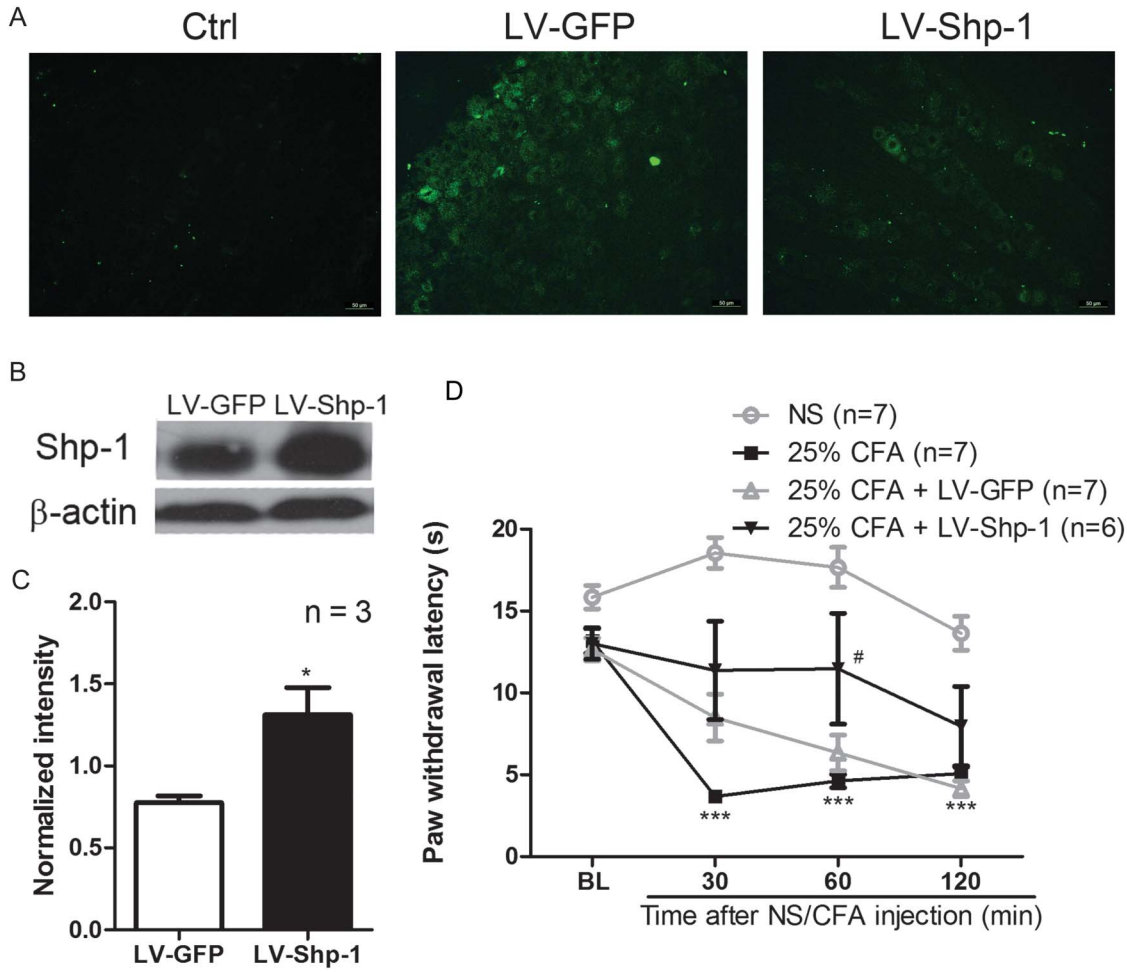

Figure 8. Overexpression of Shp-1 after intrathecal injection of LV-Shp-1 decreased CFA-induced inflammatory pain in rats. (A) Lentivirus infected L4 and L5 DRG neurons and expressed green fluorescent proteins 7 days after intrathecal injection. Strong fluorescence was observed in both LV-Shp-1 and LV-GFP groups, but not in naive rats (Ctrl). Results were representatives of 3 individual trials. (B and C) Shp-1 protein expression significantly increased in DRGs in LV-Shp-1 group compared with that in LV-GFP group. β-actin served as a loading control. Data were represented as mean ± SEM, n = 3; *$P < 0.05$ vs NS group (unpaired t test). (D) Shp-1 overexpression alleviated thermal hyperalgesia at 60 minutes in inflammatory pain rats after intraplantar injection of 25% CFA. Data were represented as mean ± SEM, n = 6 to 7; ***$P < 0.001$ NS group vs CFA group, #$P < 0.05$ LV-Shp-1 group vs LV-GFP group (2-way ANOVA followed by Bonferroni posttests). BL, basal latency.

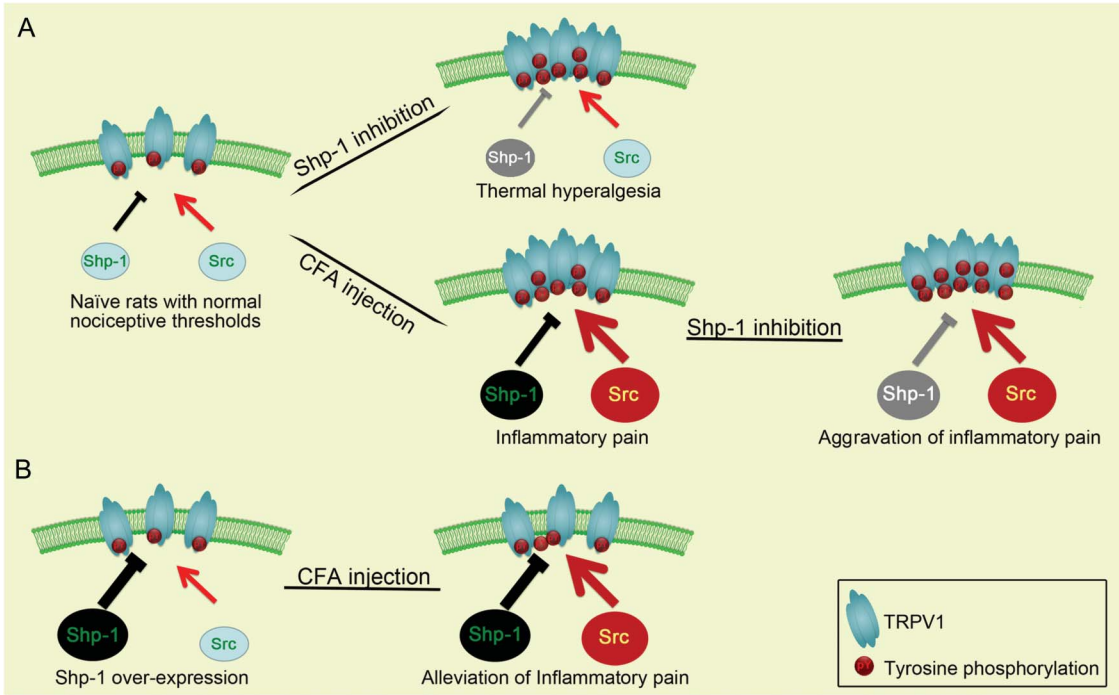

Figure 9. A schematic diagram of the protective role of the protein-tyrosine phosphatase Shp-1 in rat DRG neurons in inflammatory pain. (A) In naive rats, the balanced action of Shp-1 and the protein-tyrosine kinase Src determined TRPV1 at a normal level of tyrosine phosphorylation as well as normal nociceptive thresholds. In the upper panel, when Shp-1 was inhibited, the phosphorylation and protein expression of TRPV1 increased, and the thermal hyperalgesia was induced. In the lower panel, when CFA was injected into the hind paws of rats to induce inflammation, the overwhelming Src activation induced more tyrosine phosphorylation of TRPV1, and thus inflammatory pain happened. At the same time, Shp-1 was also upregulated to limit the inflammatory pain, but it was not dominant compared with the Src activation. When Shp-1 was further inhibited, inflammatory pain was aggravated. (B) When Shp-1 was overexpressed before the CFA injection, the tyrosine phosphoration of TRPV1 was inhibited, and thus the CFA-induced inflammatory pain was alleviated.

and L5 DRG neurons (**Figs. 3 and 4**), indicating its possible modulation over TRPV1. Application of Shp-1 inhibitors increased capsaicin responses in DRG neurons (**Fig. 5**) and induced thermal hyperalgesia in naive rats (**Fig. 6A, B**), which was abolished with pretreatment of TRPV1 antagonists (**Fig. 6C–E**). These results suggested that Shp-1 was involved in the maintenance of thermal nociceptive thresholds through inhibiting the activation of TRPV1 in DRG neurons in normal rats.

In a previous study, Shp-1 mutant mice exhibited systemic inflammation and thermal hyperalgesia,[9] but there were no further studies on the underlying mechanisms of the thermal hyperalgesia that may be attributed to changed ion channel functions during systemic inflammation, such as TRPV1, P2X3, acid-sensing ionic channels, ATP-sensitive potassium channel (K_{ATP}), voltage-gated sodium channels, Kv7, etc.[14,19] In this study, intrathecal injection of Shp-1 inhibitors induced thermal hyperalgesia in naive rats, which was completely abolished by pretreatment of TRPV1 antagonists (**Fig. 6A–E**), implying the dependence of Shp-1 in nociception on TRPV1 activation.

4.2. Shp-1 dephosphorylated TRPV1 and suppressed TRPV1 protein expression in DRG neurons

Thirty minutes after the application of SSG, tyrosine phosphorylation of TRPV1 increased in L4 and L5 DRGs (**Fig. 6H**). The PTK Src phosphorylated TRPV1 at Tyr200 and sensitized TRPV1[12,29]; nonspecific PTPs inhibition also increased the tyrosine phosphorylation and activation of TRPV1.[12,29] Tyr200 was near the cytosolic N-terminus of TRPV1, and Shp-1 was a non-receptor PTP expressed in the cytoplasma,[1] making it rational that in this study, intracellular application of SSG augmented capsaicin responses more rapidly and required a lower concentration (**Fig. 5E, F**) than extracellular application (**Fig. 5A, B**). In cultured DRG neurons, TRPV1 was sensitized 60 minutes after extracellular application of the Shp-1 inhibitor SSG but immediately after intracellular application (**Fig. 5**).

The expression level of TRPV1 proteins was also upregulated in L4 and L5 DRGs 30 minutes after intrathecal injection of the Shp-1 inhibitor SSG (**Fig. 6F, G**). The rapid increase of protein levels most possibly resulted from decreased protein degradation but not increased protein synthesis. The phosphorylation may protect against degradation of TRPV1 proteins. Tyrosine phosphorylation was important for TRPV1 trafficking to plasma membranes[10,12,29] and increased the plasma membrane TRPV1 expression within 10 minutes.[10,25,29] When Tyr200 of TRPV1 was mutated, Src kinases failed to phosphorylate TRPV1, resulting in TRPV1 translocation from the plasma membrane to the cytoplasma, with the proteasome-dependent degradation of TRPV1 increased and total TRPV1 protein expression decreased.[25] In general, tyrosine phosphorylation of TRPV1 promotes its trafficking to the plasma membrane and prevents TRPV1 degradation. In our experiment, the increase of tyrosine phosphorylation of TRPV1 induced by Shp-1 inhibition may enhance TRPV1 trafficking to the membrane surface, suppress the proteasome-dependent degradation of TRPV1, and finally increase protein expression levels of TRPV1 in DRGs. In other words, Shp-1 and PTKs such as Src may exert balanced regulation over the membrane trafficking of TRPV1, which is important for the physiological metabolism and normal protein expression of TRPV1 in DRG neurons of naive rats. This interesting phenomenon deserves further investigation.

4.3. Shp-1 alleviated CFA-induced inflammatory pain

CFA induced inflammatory pain within 7 days, which recovered from 14 days (**Fig. 1**). The tyrosine phosphorylation and protein expression of TRPV1 increased in DRGs after CFA injection (**Fig. 2**). Antagonism of TRPV1 inhibited CFA-induced inflammatory pain.[28] Shp-1 inhibition sensitized TRPV1 in cultured L4 and L5 DRG neurons and aggravated CFA-induced inflammatory pain at 14 days (**Figs. 5 and 7**). However, overexpression of Shp-1 in L4 and L5 DRG neurons decreased inflammatory pain induced by 25% CFA (**Fig. 8**). These results suggested that the dephosphorylation of TRPV1 by Shp-1 protected against inflammatory pain.

Shp-1 proteins and their colocalization with TRPV1 increased in L4 and L5 DRG neurons after CFA injection (**Figs. 1 and 3**), which seemed contradictory to the increase of tyrosine phosphorylation of TRPV1 at the same time (**Fig. 2**) and to Shp-1's protective role in chronic pain through dephosphorylating TRPV1 (**Figs. 5–8**). As we all know, tyrosine phosphorylation of proteins was dynamically and reversibly modulated by PTKs and PTPs. The PTK Src contributed to inflammatory pain through phosphorylating and sensitizing TRPV1.[12,21,29] And, a number of studies investigated the interaction between Src and Shp-1. In some cells, Shp-1 activated Src, but in others like HEK-293 cells, Shp-1 did not have such effects.[8,24,26] However, Src activated Shp-1 and increased the opportunity of its phosphorylated substrates to be dephosphorylated by Shp-1.[6,8] When inflammation was induced, the function of Src and Shp-1 might be enhanced simultaneously. They might form an auto-balancing system in CFA inflammatory pain rats to prevent excessive hyperalgesia. Within 14 days after CFA injection, the phosphorylation of TRPV1 by Src might overcome the dephosphorylation by Shp-1; therefore, the tyrosine phosphorylation of TRPV1 increased in DRGs and hyperalgesia happened in rats; after that, the function of Src decreased and Shp-1 helped recover from hyperalgesia. When the balance between Shp-1 and Src was broken, nociceptive thresholds of rats would change. As shown in our results, inhibition of Shp-1 with SGG treatment enhanced tyrosine phosphorylation of TRPV1 (**Fig. 6H**) and induced thermal hyperalgesia (**Fig. 7**), whereas overexpression of Shp-1 alleviated thermal hyperalgesia (**Fig. 8**). Therefore, the increase of Shp-1 in DRGs actually protected against Src's effects as a compensatory mechanism. This compensatory mechanism was also reported when Shp-1 served as an autoinhibitory molecule and protected against TNF-α–induced endothelial inflammation; TNF-α increased Shp-1 activity and protein expression, while Shp-1 inhibited TNF-α–induced inflammation.[13] It may imply a universal auto-balancing system when phosphatases like Shp-1 respond to microenvironmental changes, including that in nociception.

We summarize the hypothesis from this study in a schematic diagram as **Fig. 9**. Of course, more experiments should be performed to explore the balance between Shp-1 and Src in inflammatory pain, although we focused on the protective role of Shp-1 here.

5. Conclusions

In conclusion, Shp-1 dephosphorylated TRPV1 and inhibited TRPV1 activation in DRG neurons, contributing to the maintenance of normal thermal nociceptive thresholds. The increase of Shp-1 in DRGs may be involved in the protection against thermal hyperalgesia in CFA-induced inflammatory pain rats as a compensatory mechanism.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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References

[1] Alonso A, Sasin J, Bottini N, Friedberg I, Friedberg I, Osterman A, Godzik A, Hunter T, Dixon J, Mustelin T. Protein tyrosine phosphatases in the human genome. Cell 2004;117:699–711.
[2] Anand U, Otto WR, Anand P. Sensitization of capsaicin and icilin responses in oxaliplatin treated adult rat DRG neurons. Mol Pain 2010;6:82.
[3] Bevan S, Geppetti P. Protons: small stimulants of capsaicin-sensitive sensory nerves. Trends Neurosci 1994;17:509–12.
[4] Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 1997;389:816–24.
[5] Cortright DN, Szallasi A. Biochemical pharmacology of the vanilloid receptor TRPV1. An update. Eur J Biochem 2004;271:1814–19.
[6] Ferjoux G, Lopez F, Esteve JP, Ferrand A, Vivier E, Vely F, Saint-Laurent N, Pradayrol L, Buscail L, Susini C. Critical role of Src and SHP-2 in sst2 somatostatin receptor-mediated activation of SHP-1 and inhibition of cell proliferation. Mol Biol Cell 2003;14:3911–28.
[7] Ferrer-Montiel A, Garcia-Martinez C, Morenilla-Palao C, Garcia-Sanz N, Fernandez-Carvajal A, Fernandez-Ballester G, Planells-Cases R. Molecular architecture of the vanilloid receptor. Insights for drug design. Eur J Biochem 2004;271:1820–6.
[8] Frank C, Burkhardt C, Imhof D, Ringel J, Zschornig O, Wieligmann K, Zacharias M, Bohmer FD. Effective dephosphorylation of Src substrates by SHP-1. J Biol Chem 2004;279:11375–83.
[9] Hudson CA, Christophi GP, Cao L, Gruber RC, Massa PT. Regulation of avoidant behaviors and pain by the anti-inflammatory tyrosine phosphatase SHP-1. Neuron Glia Biol 2006;2:235–46.
[10] Jeske NA, Patwardhan AM, Henry MA, Milam SB. Fibronectin stimulates TRPV1 translocation in primary sensory neurons. J Neurochem 2009; 108:591–600.
[11] Jiang YQ, Xing GG, Wang SL, Tu HY, Chi YN, Li J, Liu FY, Han JS, Wan Y. Axonal accumulation of hyperpolarization-activated cyclic nucleotide-gated cation channels contributes to mechanical allodynia after peripheral nerve injury in rat. PAIN 2008;137:495–506.
[12] Jin X, Morsy N, Winston J, Pasricha PJ, Garrett K, Akbarali HI. Modulation of TRPV1 by nonreceptor tyrosine kinase, c-Src kinase. Am J Physiol Cell Physiol 2004;287:C558–63.
[13] Koch E, Pircher J, Czermak T, Gaitzsch E, Alig S, Mannell H, Niemeyer M, Krotz F, Wornle M. The endothelial tyrosine phosphatase SHP-1 plays an important role for vascular haemostasis in TNFα-induced inflammation in vivo. Mediators Inflamm 2013;2013:279781.
[14] Linley JE, Rose K, Ooi L, Gamper N. Understanding inflammatory pain: ion channels contributing to acute and chronic nociception. Pflugers Arch 2010;459:657–69.
[15] Liu J, Liu FY, Tong ZQ, Li ZH, Chen W, Luo WH, Li H, Luo HJ, Tang Y, Tang JM, Cai J, Liao FF, Wan Y. Lysine-specific demethylase 1 in breast cancer cells contributes to the production of endogenous formaldehyde in the metastatic bone cancer pain model of rats. PLoS One 2013;8:e58957.

[16] Liu M, Yang H, Fang D, Yang JJ, Cai J, Wan Y, Chui DH, Han JS, Xing GG. Upregulation of P2X3 receptors by neuronal calcium sensor protein VILIP-1 in dorsal root ganglions contributes to the bone cancer pain in rats. PAIN 2013;154:1551–68.
[17] Lu Y, Sun YN, Wu X, Sun Q, Liu FY, Xing GG, Wan Y. Role of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor subunit GluR1 in spinal dorsal horn in inflammatory nociception and neuropathic nociception in rat. Brain Res 2008;1200:19–26.
[18] Luo H, Xu IS, Chen Y, Yang F, Yu L, Li GX, Liu FY, Xing GG, Shi YS, Li T, Han JS, Wan Y. Behavioral and electrophysiological evidence for the differential functions of TRPV1 at early and late stages of chronic inflammatory nociception in rats. Neurochem Res 2008;33:2151–8.
[19] Mathie A. Ion channels as novel therapeutic targets in the treatment of pain. J Pharm Pharmacol 2010;62:1089–95.
[20] Mohapatra DP, Nau C. Regulation of Ca^{2+}-dependent desensitization in the vanilloid receptor TRPV1 by calcineurin and cAMP-dependent protein kinase. J Biol Chem 2005;280:13424–32.
[21] Nagae M, Hiraga T, Wakabayashi H, Wang L, Iwata K, Yoneda T. Osteoclasts play a part in pain due to the inflammation adjacent to bone. Bone 2006;39:1107–15.
[22] Novakova-Tousova K, Vyklicky L, Susankova K, Benedikt J, Samad A, Teisinger J, Vlachova V. Functional changes in the vanilloid receptor subtype 1 channel during and after acute desensitization. Neuroscience 2007;149:144–54.
[23] Poole AW, Jones ML. A SHPing tale: perspectives on the regulation of SHP-1 and SHP-2 tyrosine phosphatases by the C-terminal tail. Cell Signal 2005;17:1323–32.
[24] Roskoski RJ. Src kinase regulation by phosphorylation and dephosphorylation. Biochem Biophys Res Commun 2005;331:1–14.
[25] Shimizu T, Shibata M, Toriumi H, Iwashita T, Funakubo M, Sato H, Kuroi T, Ebine T, Koizumi K, Suzuki N. Reduction of TRPV1 expression in the trigeminal system by botulinum neurotoxin type-A. Neurobiol Dis 2012;48:367–78.
[26] Somani AK, Bignon JS, Mills GB, Siminovitch KA, Branch DR. Src kinase activity is regulated by the SHP-1 protein-tyrosine phosphatase. J Biol Chem 1997;272:21113–19.
[27] Wang Y. The functional regulation of TRPV1 and its role in pain sensitization. Neurochem Res 2008;33:2008–12.
[28] Yu L, Yang F, Luo H, Liu FY, Han JS, Xing GG, Wan Y. The role of TRPV1 in different subtypes of dorsal root ganglion neurons in rat chronic inflammatory nociception induced by complete Freund's adjuvant. Mol Pain 2008;4:61.
[29] Zhang X, Huang J, McNaughton PA. NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. EMBO J 2005;24:4211–23.
[30] Zhao XT, Qian YK, Chan AW, Madhavan R, Peng HB. Regulation of ACh receptor clustering by the tyrosine phosphatase Shp2. Dev Neurobiol 2007;67:1789–801.
[31] Zheng Q, Fang D, Liu M, Cai J, Wan Y, Han JS, Xing GG. Suppression of KCNQ/M (Kv7) potassium channels in dorsal root ganglion neurons contributes to the development of bone cancer pain in a rat model. PAIN 2013;154:434–48.
[32] Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. PAIN 1983;16:109–10.