Research Article

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Reduced capsaicin-induced mechanical allodynia and neuronal responses in the dorsal root ganglion in the presence of protein tyrosine phosphatase non-receptor type 6 overexpression

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Robin Vroman[1,2], Shingo Ishihara[1,3], Spencer Fullam[1], Matthew J Wood[1], Natalie S Adamczyk[1], Nolan Lomeli[1], Fransiska Malfait[2], Anne-Marie Malfait[1,3], Rachel E Miller[1,3], and Adrienn Markovics[1,4]

Abstract

Transient Receptor Potential Vanilloid 1 (TRPV1) is a nonselective cation channel expressed by pain-sensing neurons and has been an attractive target for the development of drugs to treat pain. Recently, Src homology region two domain-containing phosphatase-1 (SHP-1, encoded by *Ptpn6*) was shown to dephosphorylate TRPV1 in dorsal root ganglia (DRG) neurons, which was linked with alleviating different pain phenotypes. These previous studies were performed in male rodents only and did not directly investigate the role of SHP-1 in TRPV-1 mediated sensitization. Therefore, our goal was to determine the impact of *Ptpn6* overexpression on TRPV1-mediated neuronal responses and capsaicin-induced pain behavior in mice of both sexes. Twelve-week-old male and female mice overexpressing *Ptpn6* (Shp1-Tg) and their wild type (WT) littermates were used. *Ptpn6* overexpression was confirmed in the DRG of Shp1-Tg mice by RNA in situ hybridization and RT-qPCR. *Trpv1* and *Ptpn6* were found to be co-expressed in DRG sensory neurons in both genotypes. Functionally, this overexpression resulted in lower magnitude intracellular calcium responses to 200 nM capsaicin stimulation in DRG cultures from Shp1-Tg mice compared to WTs. *In vivo*, we tested the effects of *Ptpn6* overexpression on capsaicin-induced pain through a model of capsaicin footpad injection. While capsaicin injection evoked nocifensive behavior (paw licking) and paw swelling in both genotypes and sexes, only WT mice developed mechanical allodynia after capsaicin injection. We observed similar level of TRPV1 protein expression in the DRG of both genotypes, however, a higher amount of tyrosine phosphorylated TRPV1 was detected in WT DRG. These experiments suggest that, while SHP-1 does not mediate the acute swelling and nocifensive behavior induced by capsaicin, it does mediate a protective effect against capsaicin-induced mechanical allodynia in both sexes. The protective effect of SHP-1 might be mediated by TRPV1 dephosphorylation in capsaicin-sensitive sensory neurons of the DRG.

Keywords

capsaicin, nociceptors, pain, src homology region 2 domain-containing phosphatase 1, transient receptor potential vanilloid 1

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Introduction

Transient Receptor Potential Vanilloid 1 (TRPV1) is a nonselective cation channel expressed on a subset of unmyelinated C and medium diameter myelinated Aδ nociceptors.[1] TRPV1 has been an attractive drug target for the

[1]Department of Internal Medicine, Division of Rheumatology, Rush University Medical Center, Chicago, IL, USA
[2]Department of Biomolecular Medicine, Center for Medical Genetics, Ghent University, Ghent, Belgium
[3]Chicago Center on Musculoskeletal Pain, Chicago, IL, USA
[4]Department of Orthopedic Surgery, Rush University Medical Center, Chicago, IL, USA

Corresponding Author:
Adrienn Markovics, Department of Orthopedic Surgery, Rush University Medical Center, 1735 W Harrison St, Chicago, IL 606123801, USA.
Email: Adrienn_Markovics@rush.edu

treatment of various types of pain, and both TRPV1 agonists and antagonists entered clinical trials. However, due to the physiological effects of TRPV1 on core body temperature and noxious heat sensation,[2] the development of TRPV1 agonists and antagonists has been challenged by serious on-target adverse effects such as hyperthermia and impaired cutaneous noxious heat sensation.[3,4] One of the major mechanisms of TRPV1 inhibition is dephosphorylation by protein phosphatases.[5] Interestingly, eliminating a serine phosphorylation site in TRPV1 by CRISPR/Cas9 editing reduced pain without affecting physiological TRPV1 functions in an inflammatory pain model,[6] raising the possibility that modulating TRPV1 phosphorylation may offer an attractive strategy for targeting TRPV1 for analgesia.

Src homology region two domain-containing phosphatase 1 (SHP-1) is an intracellular protein tyrosine phosphatase expressed mainly in hematopoietic cells.[7,8] It plays a negative regulatory role in immune cell signaling[9] and recently, its potential modulatory role in autoimmunity gained interest.[10,11] A protective effect of SHP-1 has been proposed by others in different rodent models of pain. For example, SHP-1 has been shown to alleviate Complete Freund adjuvant (CFA)-induced thermal hyperalgesia.[12] It was also reported that SHP-1 dephosphorylates (i.e., deactivates) TRPV1 in dorsal root ganglia (DRG) and delays the development of cancer-induced bone pain in mice.[13] However, these previous studies were performed only in male rodents and did not directly investigate the role of SHP-1 in TRPV-1 mediated sensitization. Therefore, the objective of this study was to determine the impact of genetically enhanced SHP-1 expression on TRPV1-mediated neuronal responses and capsaicin-induced swelling and pain behavior in mice of both sexes. While selective SHP-1 inhibitors are commercially available, selective SHP-1 activators are lacking. Hence, SHP-1 transgenic mice (Shp1-Tg) with genetically enhanced SHP-1 expression were used to uncover the effect of increased SHP-1 activity.

Materials and methods

Animals

A total of 121 WT and 126 Shp1-Tg twelve- to fourteen-week-old mice were used for the study (n = 74 WT female, n = 47 WT male, n = 73 Shp1-Tg female, n = 53 Shp1-Tg male). Animals were housed with food and water ad libitum and kept at a 12-h light cycle. Transgenic mice over-expressing the SHP-1 gene *Ptpn6* (Shp1-Tg) on a BALB/c genetic background and their wild type (WT) littermates were used for the experiments. The generation of Shp1-Tg mice has been described elsewhere.[10] Mice were acclimatized to handling and to behavioral equipment for 3 days prior to the behavior studies. All animal experiments were approved by the Institutional Animal Care and Use Committee at Rush University Medical Center (IACUC #23-032).

Drugs and chemicals

E-Capsaicin (04-621-00), Hank`s Balanced Salt Solution (HBSS, 14,025-092), DMEM/F12 nutrient mixture (10-090-CV), and papain (NC9212788) were purchased from Fisher Scientific. Calbryte 520 a.m. (20,650) was purchased from AAT Bioquest. Collagenase (C5138), DNase (10,104,159,001), poly-L-lysine (P1274), laminin (L2020), Tween 80 (P4780), fetal bovine serum (FBS, SH30071.03, HyClone), N2 (17,502-048), DMSO (D4540), penicillin-streptomycin (30-001-CI, MediaTech), ethanol and formaldehyde were purchased from Millipore-Sigma. SDS-polyacrylamide gels (10%, 4,561,034), PVDF membrane (1,620,177), blotting grade blocker (170-6404) and ECL Clarity Western blot substrate (170-5060) were purchased from Bio-Rad. BCA protein assay kit (23,227) was purchased from ThermoFisher Scientific. Protein A/G Plus agarose beads (sc-2003) were purchased from Santa Cruz Biotechnology.

Isolation of dorsal root ganglia

Briefly, mice were sacrificed at twelve weeks of age, and the lumbar region of the spinal column was carefully dissected out as previously described.[14] The spine was cut in half in the sagittal plane one vertebra at a time and the spinal cord was removed. L3-5 DRGs were identified using a dissection microscope and excised with a micro dissection tool. These isolated DRGs were used for the following applications: 1) For RT-qPCR, bilateral L3-L5 DRGs of n = 6 naïve female WT and n = 5 naïve female Shp1-Tg mice were used. 2) For RNA in situ hybridization, bilateral L3-L5 DRGs of n = 3 naïve female WT and n = 3 naïve female Shp1-Tg mice were used. 3) For DRG culture and *in vitro* calcium imaging, bilateral L3-L5 DRGs of n = 6 female WT, n = 6 male WT, n = 7 female Shp1-Tg and n = 6 male Shp1-Tg mice were used. Capsaicin-induced neuronal activation was analyzed on n = 16 coverslips total (3-4 coverslips/genotype/sex).

RT-qPCR

Ptpn6 (the gene encoding SHP-1) gene expression was determined in the DRG of naive female mice (n = 6 WT, n = 5 Shp1-Tg, twelve-week-old) by RT-qPCR. Briefly, total RNA was extracted with Direct-Zol RNA Mini-Prep Plus (Zymo Research, Irvine, CA) and reverse transcribed using the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA). cDNA was amplified using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) in a CFX Connect Real-Time PCR Detection System (Bio-Rad). Measured ΔCq values were normalized to the actin b (*Actb*) gene. Relative expression between Shp1-Tg and WT samples was calculated using CFX Manager Software (Bio-Rad) and illustrated as ΔΔCq values relative to zero. *Ptpn6* primer sequences were as follows:

Forward: 5′-TCT CAG TCA GGG TGG ATG AT-3′
Reverse: 5′-CCT GCT GCT GCG TGT AAT A-3′

DRG RNA in situ hybridization

Bilateral L3-L5 DRGs were collected from naïve female WT and Shp1-Tg mice (n = 3 WT, n = 3 Shp1-Tg, twelve-week-old), fixed in 4% PFA, transferred to 30% sucrose solution for cryoprotection, embedded in OCT and cryo-sectioned onto slides at 12 µm. During sectioning slides were kept within the cryostat at −20°C before storage at −80°C. RNA in situ hybridization was performed using ACD BioTechne RNAscope Multiplex Fluorescent v2 Assay, described in detail elsewhere.[15] *Scn10a* (426,011-C2 (probe for the gene encoding voltage-gated sodium channel $Na_V1.8$)), *Trpv1* (313,331-C3), *Ptnp6* (450,081-C1) probes and ACD Bio-Techne DAPI were used. ACD Bio-Techne positive and negative control probes were conducted prior to start of work. Negative controls were included on every slide. All imaging was performed using a Fluoview FV10i confocal microscope at ×10 and ×60 magnification (Olympus Fluoview FV10-ASW Ver.04.02). Multiple planes of focus were captured, and the optimally focused image was chosen for processing and analysis. Laser intensity was used at ≤9.9% throughout. Images were processed and quantified using Fiji software v2.9.0. For mouse DRGs, three sections per mouse were quantified and averaged. First, the total number of neuronal cells was identified using both the nuclei staining with DAPI and the phase contrast channel. Each cell was then assessed for the expression of each probe, and labeled as single, double, triple expression, or no expression. Positive signal was determined when two or more positive 'dots' per cell were found.

DRG culture and calcium imaging

Bilateral L3-L5 DRGs were collected from 12-week-old naïve Shp1-Tg (n = 7 female, n = 6 male) and WT (n = 6 female, n = 6 male) mice and pooled for enzymatic digestion using collagenase type IV (2 mg/mL), papain (26 U/ml), and DNase (1 mg/mL), as described.[16] Dissociated DRG cells were plated on poly-L-lysine and laminin-coated glass coverslips in 6-well plates and cultured in F12 medium supplemented with 1 × N2, 0.5% FBS, penicillin and streptomycin (100 µg/ml and 100 U/ml), at 37°C and 5% CO_2. Cultured DRG neurons were used for *in vitro* calcium imaging in two independent experiments (n = 2-3 coverslips per genotype/sex/experiment). Neuronal responses were evaluated on 4-day old DRG cultures by *in vitro* calcium imaging using Calbryte 520 a.m. (excitation: 393 nm, emission: 515 nm, FITC filter set) calcium indicator dye, using a standard protocol.[17] Following 20 min of Calbryte loading (4 µM in 2 mL of balanced salt solution [NaCl (140 mM), HEPES (10 mM), $CaCl_2$ (2 mM), $MgCl_2$ (1 mM), Glucose (10 mM), KCl (5 mM)]), cells were rinsed and left untouched for 30 min to recover in balanced salt solution before being placed in an imaging chamber with a continuous flow system. Baseline neuronal activity was recorded (0.5 Hz) for 20 s, then vehicle (0.4 % DMSO) or 200 nM capsaicin was applied to the imaging chamber. Neuronal activity was recorded for a total of 300 s. Neuronal responses were visualized under an inverted microscope (Zeiss Axio Observer D1). DRG cultures from WT and Shp1-Tg mice were evaluated on the same day. Neuronal response was quantified as the change in fluorescent intensity at a given time point compared to baseline fluorescence (ΔF/F0) using Image J software. Positive neuronal response was defined as maximum ΔF/F0 above 0.75. High responders were differentiated from low responders by applying a cutoff value ΔF/F0 > maximum ΔF/F0 x 0.5. Response amplitude of the positive neuronal responses were calculated for both genotypes.

Basal mechanical sensitivity

Fourteen-week-old naïve female and male WT and Shp1-Tg mice (n = 10 WT female, n = 8 WT male, n = 10 Shp1-Tg female, n = 13 Shp1-Tg male) were habituated to the von Frey apparatus for four consecutive days. Basal mechanical sensitivity of the left hind paw was determined using calibrated von Frey filaments.[16] Briefly, mice were placed on a perforated metal grid with plexiglass cubicles. A set of six von Frey fibers (Stoelting Touch Test Sensory Evaluator Kit) were applied to the plantar surface of the hind paw until they bowed. The force that resulted 50% paw withdrawal threshold was calculated as previously described.[18]

Basal thermal sensitivity

Twelve- to fourteen-week-old naïve WT and Shp1-Tg mice of both sexes (n = 8 female WT, n = 8 female Shp1-Tg, n = 6 male WT, n = 5 male Shp1-Tg) were habituated to the Hot Cold Plate Analgesia Meter (IITC Life Science Inc.) for three consecutive days. Basal thermal sensitivity was determined as the response latency (seconds) of the first nocifensive behavior observed such as lifting, shaking, or licking of the hind paw. Temperature settings were 51°C starting and 55°C cutoff temperature, with 1°C/minute increases. The measurement was terminated at the time of the first appearance of nocifensive behavior.

Capsaicin-induced acute pain behavior

Twelve- to fourteen-week-old naïve WT and Shp1-Tg mice of both sexes (n = 31 female WT, n = 30 female Shp1-Tg, n = 27 male WT, n = 29 male Shp1-Tg) were habituated to a plexiglass chamber and to the von Frey apparatus for three consecutive days. Mice were injected with 12 µg capsaicin[19] or vehicle (80% saline, 10% ethanol, 10% Tween 80) in the left footpad (10-20 µL) using a Hamilton syringe (n = 14-17/treatment group). Capsaicin-induced paw licking, as a

measure of nocifensive behavior, was recorded for 5 min in a transparent plexiglass chamber immediately after footpad injection and quantified as the time spent with paw licking during the first 5 min after injection.[20] After that, mice were returned to their home cage and allowed to rest until 4 h after footpad injection (to recover weight bearing on the injected paw), when mechanical allodynia was determined using calibrated von Frey filaments and the up-down staircase method.[16] Five hours after footpad injection, the thickness of the injected paw was determined using a caliper. The investigator was not blinded to the *in vivo* experimental groups, due to the obvious swelling and redness of the paw following capsaicin footpad injection.

Immunoprecipitation and Western blot

For immunoprecipitation, ipsilateral L3-L5 DRGs of $n = 10$ female WT and $n = 10$ female Shp1-Tg mice were used (12 weeks of age) in two independent experiments. WT and Shp1-Tg female mice were injected with 12 µg capsaicin in the left footpad ($n = 5$/genotype/experiment). Ipsilateral L3-L5 DRGs were harvested 4 hours after footpad injection and a whole cell lysate was prepared using RIPA buffer containing protease and phosphatase inhibitors. The protein concentrations were determined using a BCA Protein Assay kit as described previously.[10] For immunoprecipitation, 300 µg cell lysates were incubated with anti-phospho-tyrosine antibody (#8954, Cell Signaling Technology) or normal rabbit IgG (#2729, Cell Signaling Technology) at 4°C overnight. Protein A/G Plus agarose beads were added to the lysate-antibody mixture and incubated for 4 h. Samples were washed and eluted in reducing sample buffer, followed by boiling for 5 min. As input, total TRPV1 protein expression was detected in 10 µg whole cell lysates of both genotypes. Proteins were separated on 10% SDS-polyacrylamide gels, blotted onto PVDF membranes, then blocked with Tris-buffered saline + 0.05% Tween-20 containing 3% blotting-grade blocker. The membrane was probed with anti-TRPV1 antibody (NBP1-97,417, Novus Biologicals) at 4°C overnight, followed by goat anti-rabbit HRP-conjugated secondary antibody (31,466, ThermoFisher Scientific). Clarity ECL Western Blotting Substrate was used to generate chemiluminescence signals, which were detected by X-ray films.

Statistical analysis

Results are presented as the mean ± SEM. RT-qPCR data were analyzed with Mann-Whitney test. RNA in situ hybridization data were compared with unpaired Student`s t test. The number of responding neurons was analyzed with Fisher`s exact test; response amplitudes were compared with Mann-Whitney test. Data obtained *in vivo* (time spent with paw licking, paw thickness, mechanical allodynia, baseline mechanical and thermal sensitivity) were analyzed with Mann-Whitney test. Paw withdrawal threshold data were logarithm transformed prior to statistical analysis. Analyses were carried out using GraphPad Prism v9.3.1 software. A p-value of less than 0.05 was considered statistically significant.

Results

Ptpn6 is overexpressed in the DRG of Shp1-Tg mice and co-expressed with Trpv1 *in nociceptors*

First, we aimed to confirm SHP-1 (encoded by the *Ptpn6* gene) overexpression in the DRG of Shp1-Tg mice by RT-qPCR. We found significantly higher *Ptpn6* expression in the DRG of Shp1-Tg mice compared to WT mice, when normalizing the measured ΔCq values to the actin b (*Actb*) housekeeping gene (Figure 1(a)).

Next, we sought to determine if *Ptpn6* and *Trpv1* are both expressed in the nociceptors of the L3-L5 DRG, allowing for the potential modulation of TRPV1 function by SHP-1. The expression of *Trpv1* (*Trpv1* probe, green) and *Ptpn6* (*Ptpn6* probe, yellow) were visualized in nociceptive sensory neurons (*Scn10a* probe encoding $Na_V1.8$, dark blue) of the DRG in both genotypes by RNA in situ hybridization. We found that some of the *Scn10a* positive sensory neurons were also positive for the *Ptpn6* and *Trpv1* probes, indicating co-expression of *Ptpn6* and *Trpv1* in the DRG sensory neurons of both genotypes (representative overlay images of DRGs from $n = 3$ naïve female WT mice and $n = 3$ naïve female Shp1-Tg mice are shown in Figure 1(b)–(c)). We observed pronounced *Ptpn6* expression in *Scn10a* negative cells as well (Figure 1(b)–(c)), which might be due to abundant *Ptpn6* expression by hematopoietic cells [8] that can be present in the DRG. Representative images with individual probes from both genotypes are shown in Supplemental Figure 1. Overall *Ptpn6* expression in the DRGs was quantified by normalizing the average number of *Ptpn6* dots per 60X field of view. *Ptpn6* was found to be elevated in the DRG of Shp1-Tg mice compared to WT mice (Figure 1(d)), confirming the results observed by RT-qPCR. The proportion of *Ptpn6*, *Trpv1* and *Scn10a* positive neurons are illustrated in Figure 1(e)–(f).

Reduced capsaicin-induced neuronal responses in the DRG of Shp1-Tg mice

To evaluate the capsaicin-induced neuronal activation in the L3-L5 DRGs of WT and Shp1-Tg mice, neuronal responses were recorded *in vitro* in cultured DRG neurons from both genotypes and sexes, using calcium imaging (Figure 2). The number of responding neurons and the total number of neurons analyzed are summarized in Figure 2(c). We found that upon setting a low threshold $\Delta F/F0 > 0.75$ to differentiate responders from non-responders, a similar proportion of neurons showed activation in both genotypes in response to capsaicin (WT: 17%, Shp1-Tg: 19%). However, applying a high threshold $\Delta F/F0 >$ maximum $\Delta F/F0 \times 0.5$ to differentiate high responders from low responders, we found a

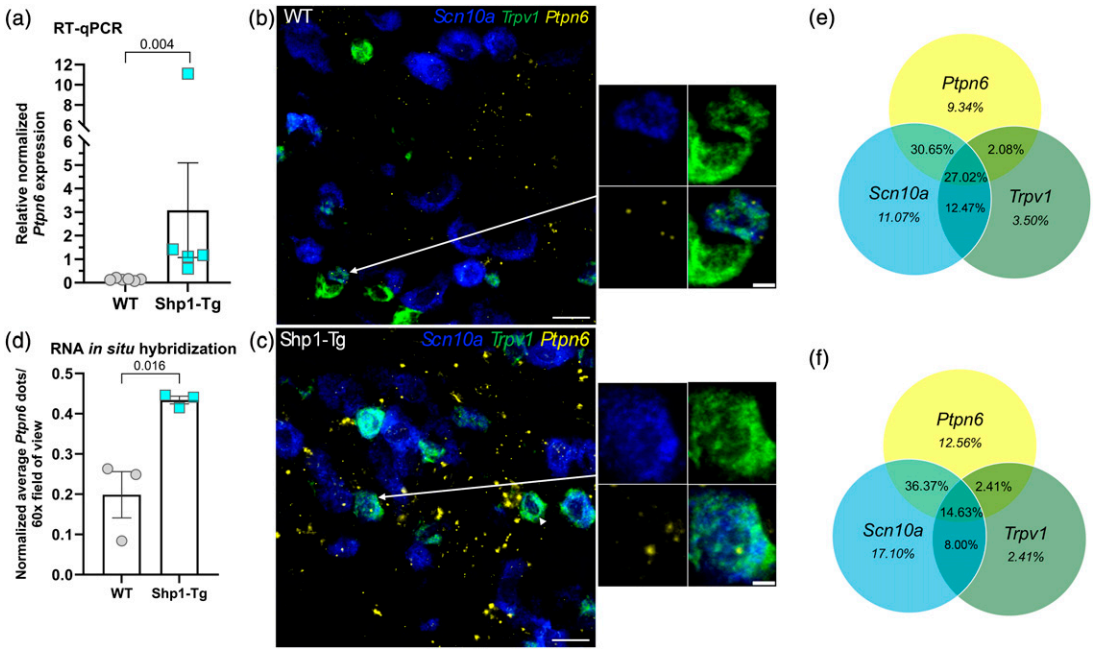


Figure 1. *Ptpn6*, *Trpv1* and *Scn10a* expression in the DRG. (a) *Ptpn6* gene (encoding SHP-1) expression in the DRG of WT and Shp1-Tg mice, quantified by RT-qPCR (twelve-week-old naïve females, $n = 5$ WT, $n = 6$ Shp1-Tg, Mann Whitney test). b-c: Overlay of representative RNA *in situ* hybridization images of the DRG of twelve-week-old $n = 3$ WT (b) and $n = 3$ Shp1-Tg female mice (c). Probes: blue-*Scn10a*, green-*Trpv1*, yellow-*Ptpn6*. Arrowhead and arrows are pointing to cells positive to *Ptpn6*, *Trpv1* and *Scn10a*. Scale bar: 25 μm, 5 μm for zoomed-in highlight. (d) *Ptpn6* expression quantified based on fluorescence by RNA *in situ* hybridization (twelve-week-old naïve female, $n = 3$ WT, $n = 3$ Shp1-Tg, unpaired t-test). Data are expressed as mean ± SEM. e-f: % expression of *Ptpn6*, *Trpv1* and *Scn10a* in the DRG neurons of WT (e) and Shp1-Tg (f) mice.

significantly lower percentage of high responding neurons in the DRG of Shp1-Tg mice compared to WTs (WT: 11%, Shp1-Tg: 6%). The amplitude of positive neuronal responses did not differ significantly between genotypes (Supplemental Figure 2). Representative neuronal responses are illustrated in Figure 2. Vehicle (DMSO) administration did not elicit neuronal responses (not shown).

Genetically enhanced SHP-1 expression did not affect basal mechanical sensitivity

Basal mechanical sensitivity was determined in naïve female and male WT and Shp1-Tg mice using von Frey filaments and the 50% paw withdrawal threshold values were log transformed for analysis. We did not detect a statistically significant difference in baseline mechanical sensitivity between genotypes in neither sex (Supplemental Figure 3).

Genetically enhanced SHP-1 expression did not affect basal thermal sensitivity

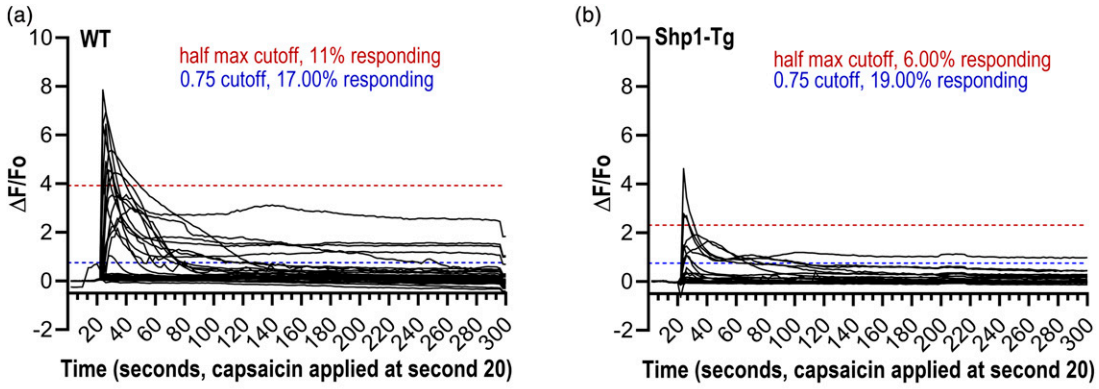
Basal thermal sensitivity was determined by increasing temperature hot plate in naïve WT and Shp1-Tg mice of both sexes. We did not detect statistically significant differences in the latency of the first nocifensive behavior between genotypes (Supplemental Figure 4).

Genetically enhanced SHP-1 expression did not affect capsaicin-induced paw licking and paw swelling

12-to fourteen-week-old female and male WT and Shp1-Tg mice were used for behavioral testing. As a measure of capsaicin-induced acute nocifensive behavior, we monitored the time spent with paw licking in the first 5 minutes after capsaicin or vehicle footpad injection. WT and Shp1-Tg mice of both sexes spent significantly more time with paw licking in response to capsaicin than to vehicle (Figure 3(a)–(d)). Male mice of both genotypes appeared to spend less time with paw licking in response to capsaicin compared to females, however the difference was not statistically significant (WT capsaicin female vs WT capsaicin male: $p = .278$; Shp1-Tg capsaicin female vs Shp1-Tg capsaicin male: $p = .067$). The thickness of the injected paw was determined 5 h after hind paw injection. Capsaicin injection led to paw swelling indicated by increased paw thickness compared to vehicle in both genotypes (Figure 3(e)–(h)).

Shp1-Tg mice show reduced capsaicin-induced mechanical allodynia

To detect mechanical allodynia as a result of vehicle or capsaicin injection, we determined the paw withdrawal threshold with von Frey filaments 4 h after capsaicin or



(c)

High threshold for response ($\Delta F/F_0$ > half max (maximum $\Delta F/F_0$ x 0.5))			
Genotype	**No. neuronal responses/total no. neurons**	**% Responding**	**P value***
WT	68/640	11	0.02
Shp1-Tg	26/405	6	

Low threshold for response ($\Delta F/F_0$ > 0.75)			
Genotype	**No. neuronal responses/total no. neurons**	**% Responding**	**P value***
WT	110/640	17	0.41
Shp1-Tg	78/405	19	
*Fisher`s exact test was used to compare the number of responding and non-responding neurons between genotypes.			

Figure 2. Capsaicin induced neuronal responses of the DRG *in vitro*. Representative capsaicin-induced responses of cultured DRG neurons from twelve-week-old WT (a) and Shp1-Tg (b) mice (n = 7-9 coverslips/genotype, n = 6 female WT, n = 6 male WT, n = 7 female Shp1-Tg, n = 6 male Shp1-Tg). (c) Number of neuronal responses and total number of neurons analyzed.

vehicle footpad injections. We compared the effect of vehicle to the effect of capsaicin on paw withdrawal threshold in WT and in Shp1-Tg mice. Capsaicin induced a significant drop in the 50% withdrawal threshold in both female and male WT mice compared to their respective vehicle administration (Figure 4(a) and (c)). Remarkably, neither female nor male Shp1-Tg mice exhibited significant mechanical allodynia induced by capsaicin compared to vehicle (Figure 4(b) and (d)).

Reduced tyrosine phosphorylation of TRPV1 in the DRG of Shp1-Tg mice

To further investigate the observed protective effect on mechanical allodynia, we compared the tyrosine phosphorylation status of TRPV1 in the L3-L5 DRG of a separate cohort of WT and Shp1-Tg mice 4 h after injection with capsaicin (Figure 5). Total tyrosine phosphorylated proteins were immunoprecipitated from tissue lysates of both genotypes and immunoblotted with an anti-TRPV1 antibody. 10 µg tissue lysate without immunoprecipitation from both genotypes served as input for total TRPV1 immunoblotting. We detected a similar amount of total TRPV1 in WT and Shp1-Tg DRG lysates (lane one and 2). In contrast, while we observed a band according to tyrosine phosphorylated TRPV1 in the lysate of WT mice (lane 3), we did not detect tyrosine phosphorylated TRPV1 in the lysate of Shp1-Tg mice (lane 4). To confirm the specificity of the immunoprecipitation, in an independent Western blot assay, protein lysate from WT DRG was incubated with normal rabbit IgG. As expected, we did not detect any specific band after immunoprecipitation with IgG (Supplemental Figure 5, lane 3). A specific band according to phosphorylated TRPV1 was present in the WT sample (Supplemental Figure 5, lane 1), independently confirming the results seen in Figure 5.

Discussion

The aim of this study was to investigate the effect of genetically enhanced *Ptpn6* expression on TRPV1-mediated neuronal responses to capsaicin in the DRG as well as on TRPV1-mediated swelling and pain behavior using WT and Shp1-Tg mice. Here we confirm that *Ptpn6* and *Trpv1* are both expressed in the nociceptors of the L3-L5 DRG. In addition, we detected reduced neuronal responses to capsaicin in the DRGs of Shp1-Tg mice. Basal mechanical and thermal sensitivity was not affected by SHP-1 overexpression in naïve mice. Capsaicin footpad injection led to nocifensive behavior and swelling in both genotypes and sexes, while reduced capsaicin-evoked mechanical allodynia was observed in the presence of genetically enhanced *Ptpn6* expression. Furthermore, reduced tyrosine phosphorylated TRPV1 was detected in the DRG of Shp1-Tg mice.

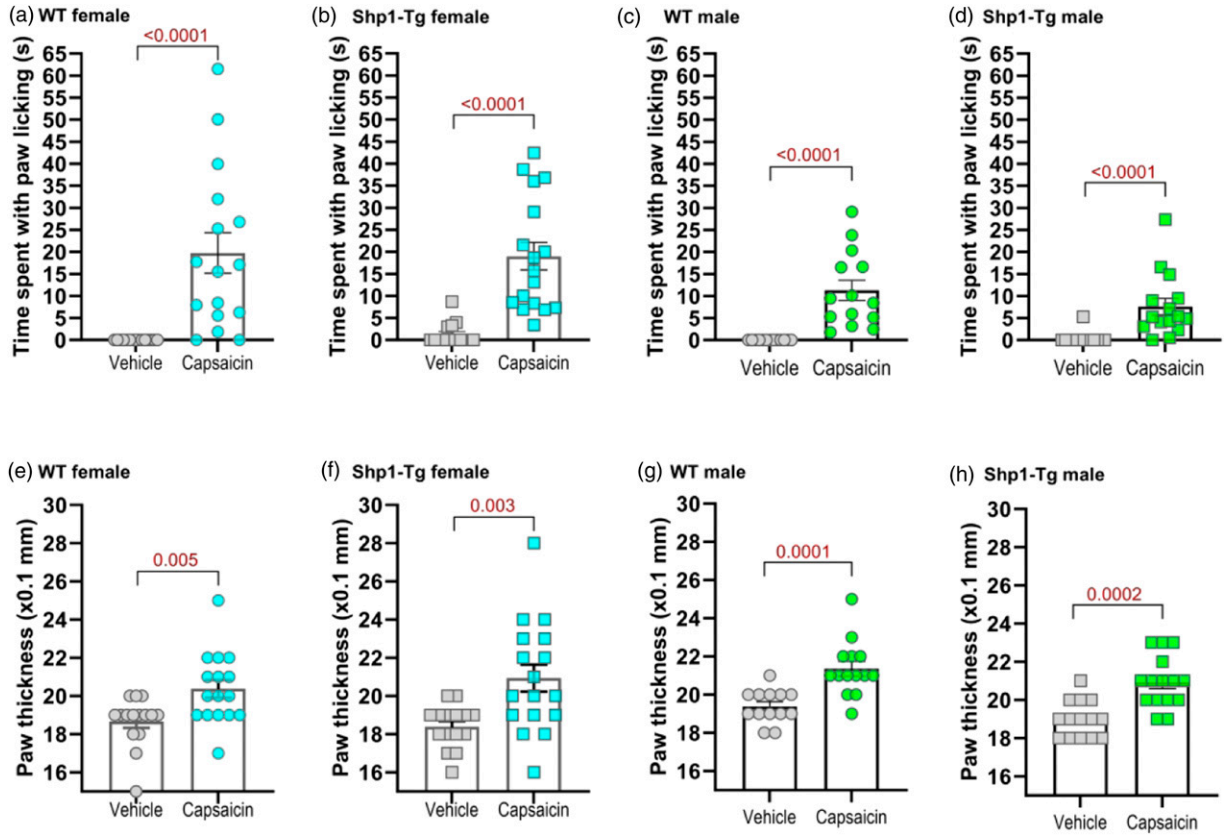


Figure 3. Capsaicin-induced acute nocifensive behavior and paw swelling in WT and Shp1-Tg mice. a-d: Time spent with paw licking in twelve- to fourteen-week-old female (a-b) and male (c-d) mice. e-h: Paw thickness in twelve- to fourteen-week-old female (e-f) and male (g-h) mice after capsaicin or vehicle footpad injection (n = 31 female WT, n = 27 male WT, n = 30 female Shp1-Tg, n = 29 male Shp1-Tg, mean ± SEM, Mann-Whitney test). p-values lower than 0.05 are indicated in red font.

Our results support previous findings that demonstrated a protective role of SHP-1 in other types of inflammatory pain models. For example, SHP-1 alleviated CFA-induced inflammatory pain in rats[12] by counteracting the tyrosine phosphorylation of TRPV1 by Src (a non-receptor protein tyrosine kinase) in the L4-L5 DRG of rats. SHP-1-TRPV1 interaction in DRG neurons has also been reported by Liu *et al.*,[13] who found that Programmed Death Ligand-1 (PD-L1) diminished bone cancer-induced pain in mice through suppressing TRPV1 function, and this effect was mediated by SHP-1 in the DRG. However, when the role of SHP-1 in the central nervous system was examined, SHP-1 was found to mediate pain through effects independent of TRPV1,[21] and *Shp1* knockdown by siRNA in the spinal cord alleviated CFA-induced inflammatory pain.[22] These contradictory data on the role of SHP-1 in pain can be explained by TRPV1-independent mechanisms and different levels of the pain pathway affected. Our findings indicate reduced mechanical allodynia after capsaicin injection in Shp1-Tg mice, outlining a protective role of SHP-1 in TRPV1-mediated sensitization.

Phosphorylation plays an important role in the modulation of TRPV1 activity. Src can phosphorylate TRPV1 at Tyr200, leading to TRPV1 trafficking to the plasma membrane and an increase of TRPV1 responses in the DRG.[23,24] Interestingly, many of the substrates of Src can be subject to dephosphorylation by SHP-1.[25] SHP-1 has been shown to abolish increased TRPV1 tyrosine phosphorylation by Src in cultured HEK-293 cells.[24] SHP-1 might also counteract NGF-induced TRPV1 sensitization, as it was reported that NGF binding to its receptor, TrkA, initiates a signaling cascade in which Src kinase phosphorylates TRPV1 in a single tyrosine residue, promoting its traffic to the plasma membrane and contributing to NGF-induced sensitization.[24]

We found a similar total TRPV1 protein expression in the L3-L5 DRG of both WT and Shp1-Tg mice, while there was much less tyrosine phosphorylated TRPV1 in the DRG of capsaicin-injected Shp1-Tg mice. Therefore, we hypothesize that the reduced mechanical allodynia is conveyed by the enhanced dephosphorylation of TRPV1 by SHP-1 in the DRG.

In this study, Shp1-Tg mice appeared to be protected from capsaicin-induced mechanical allodynia, while acute nocifensive behavior and swelling developed similarly in both

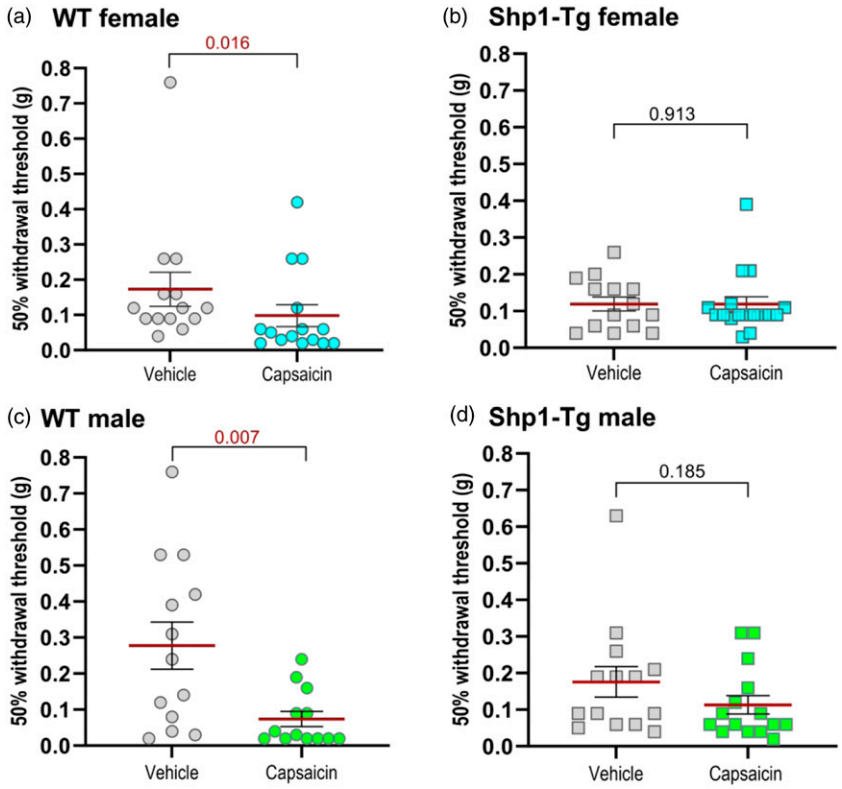


Figure 4. Capsaicin-induced mechanical allodynia in WT and Shp1-Tg mice. Paw withdrawal threshold after capsaicin or vehicle administration in twelve- to fourteen-week-old WT female (a), Shp1-Tg female (b), WT male (c) and Shp1-Tg male (d) mice (n = 31 female WT, n = 27 male WT, n = 30 female Shp1-Tg, n = 29 male Shp1-Tg, mean ± SEM, Mann-Whitney test). p values lower than 0.05 are indicated in red font.

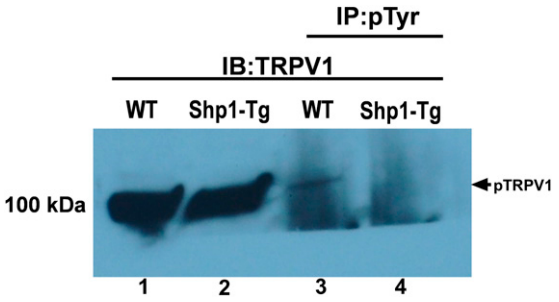


Figure 5. Tyrosine phosphorylation status of TRPV1 in the DRG. Lane 1-2: Immunoblot of DRG protein extracts from twelve-week-old female WT (n = 10) and female Shp1-Tg (n = 10) mice, probed with anti-TRPV1. Lane 3-4: immunoblot of immunoprecipitated tyrosine phosphorylated proteins from DRG protein extracts of WT and Shp1-Tg mice, probed with anti-TRPV1. Representative of n = 2 experiments (n = 10 female WT, n = 10 female Shp1-Tg mice).

genotypes. It is possible that in Shp1-transgenic mice, *Ptpn6* overexpression did not affect all modalities of the capsaicin-sensitive sensory neurons equally. For example, immediate capsaicin-induced TRPV1 activation might be unaffected, leading to acute pain sensation (and nocifensive behavior) and neurogenic inflammation (redness, swelling). However, a genetically enhanced SHP-1 activity and increased dephosphorylation of TRPV1 could diminish TRPV1 sensitization and protect from capsaicin-induced mechanical allodynia. Our interesting finding might suggest a novel strategy for TRPV1 modulation by dephosphorylation, which might overcome the adverse effects of TRPV1 antagonists in the future.

Strengths of our study include the investigation of capsaicin-induced nocifensive behavior in both sexes using a transgenic mouse strain with genetically enhanced SHP-1 expression. Limitations of our study include that SHP-1 overexpression is systemic in Shp1-Tg mice. Furthermore, capsaicin-induced thermal hypersensitivity was not investigated due to limited numbers of transgenic mice. Future experiments will explore capsaicin-induced thermal hypersensitivity in WT in Shp1-Tg mice. Also, since SHP-1 has other substrates than TRPV1 in sensory afferents, we cannot exclude the effect of SHP-1 overexpression on other substrates in capsaicin-induced mechanical allodynia[26,27] and will investigate it in future studies. Another limitation is that the investigator was not blinded to the *in vivo* experimental groups, due to the obvious swelling and redness of the paw following capsaicin footpad injection.

We conclude that systemic SHP-1/*Ptpn6* overexpression leads to decreased capsaicin-induced mechanical allodynia, by a mechanism involving TRPV1 dephosphorylation by SHP-1. Our results warrant further investigation into the potential benefit of TRPV1 modulation by SHP-1 on pain related to TRPV1 sensitization and in disease-specific models such as murine models of osteoarthritis.

Appendix

Abbreviations

DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DRG	Dorsal root ganglia
FBS	Fetal bovine serum
HBSS	Hank`s Balanced Salt Solution
NGF	Nerve growth factor
PD-L1	Programmed death ligand 1
PKA	Protein kinase A
Ptpn6	Protein Tyrosine Phosphatase Non-Receptor Type 6
Scn10a	Sodium Voltage-Gated Channel Alpha Subunit 10
SHP-1	Src homology region two domain-containing phosphatase 1
Tg	transgenic
TrkA	Tropomyosin receptor kinase A
TRPV1	Transient receptor potential vanilloid 1
WT	wild type

Author contributions

RV: Data acquisition, data analysis, manuscript editing
SI: Data acquisition
SF: Data acquisition
MW: Data acquisition, data analysis
NA: Data acquisition
NL: Data acquisition
FM: Manuscript editing
AMM: Conceptualization, manuscript editing
REM: Conceptualization, data analysis, manuscript editing
AM: Conceptualization, data acquisition, data analysis, manuscript drafting and editing

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ORCID iD

Adrienn Markovics https://orcid.org/0000-0003-4913-845X

Supplemental Material

Supplemental material for this article is available online.

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