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Sensitization of small-diameter sensory neurons is controlled by TRPV1 and TRPA1 association

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Abstract

Unique features of sensory neuron subtypes are manifest by their distinct physiological and pathophysiological functions. Using patch-clamp electrophysiology, Ca²⁺ imaging, CGRP release assay from tissues, protein biochemistry approaches and behavioral physiology on pain models, this study demonstrates the diversity of sensory neuron pathophysiology is due in part to subtype-dependent sensitization of TRPV1 and TRPA1. Differential sensitization is influenced by distinct expression of inflammatory mediators, such as prostaglandin E₂ (PGE₂), bradykinin (BK) and nerve growth factor (NGF) as well as multiple kinases, including protein kinase A (PKA) and C (PKC). However, the co-expression and interaction of TRPA1 with TRPV1 proved to be most critical for differential sensitization of sensory neurons. We identified N- and C-terminal domains on TRPV1 responsible for TRPA1-TRPV1 (A1–V1) complex formation. Ablation of A1–V1 complex with dominant negative peptides against these domains substantially reduced sensitization of TRPA1, as well as BK- and CFA-induced hypersensitivity. These data indicate that often occurring TRP channel complexes regulate diversity in neuronal sensitization and may provide a therapeutic target for many neuro-inflammatory pain conditions.

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Competing interests:

All authors declare that they have no competing interests.

Keywords

TRPV1; TRPA1; sensory neurons; pain; sensitization

Introduction:

Sensory ganglia such as dorsal root (DRG), trigeminal (TG) and nodose/jugular complex (ND/JG) contain unique subsets of sensory neurons that demonstrate distinct transcription profiles (1–3), markers (4–6), biochemical makeups (7), electrophysiological signatures (4, 8) and innervation sites (9). Variation in these features results in functional variation in naïve animals (8, 9). Many pathologic conditions trigger sensitization of sensory neurons through changes in neurotrophic factors (10), cytokines (11, 12), chemokines (12) and other mediators (13, 14). Differential involvement of sensory neuron subgroups during pathologic conditions indicates that these subgroups undergo unique sensitization processes (9). This possibility has not been investigated in detail, and potential mechanisms for sensory neuron type-dependent sensitization are largely unknown.

The presumed sensory neuron type-dependent sensitization is most likely multi-layered, as activation of receptors by endogenous ligands and the induction of signaling pathways leading to transient or long-lasting sensitization of sensory neurons are not enough to explain subgroup-specific differences in sensitization (9, 14). In the present study, we investigated whether activation of protein kinases produce similar or differential sensitization of transient potential receptor V1 (TRPV1) and/or A1 (TRPA1)-positive DRG sensory neurons. Next, we evaluated whether co-expression of TRPA1 and TRPV1 in sensory neurons informs their sensitization levels. Finally, since it is known that TRPA1-TRPV1 (A1–V1) complexes in mice (15–18) and humans (19) influences small-diameter nociceptor function, we examined the role of A1–V1 complexes in differential sensitization of small-diameter sensory neuron subgroups.

Materials and Methods:

Mouse reporter lines

All animal experiments conformed to APS's Guiding Principles in the Care and Use of Vertebrate Animals in Research and Training, and to protocols approved by the University Texas Health Science Center at San Antonio (UTHSCSA) Animal Care and Use Committee (IACUC).

All experiments were performed on 8–12-week-old male mice. Following reporter mouse lines were used to delineate small-diameter sensory neurons in this study: TRPV1 KO (stock: 003770), TRPA1 KO (stock: 006401), Rosa26^{LSL-tTomato/+} (stock: 007914) and vGLUT3^{cre/+} (stock: 028534; Jackson Laboratory; Bar Harbor, ME); TRPV1-GFP (GENSAT; UC Davis, CA); CGRP^{cre/+}-ER; MrgprA3^{cre/+} and MrgprD-GFP. *Cre*-recombinase was induced by 3 (every second day) i.p. injections of 100mg/kg tamoxifen in corn oil. *Cre*-recombination occurred within 2–3 weeks post last injection of tamoxifen.

Plasmids

Original expression plasmids contained TRPV1 (accession number NM031982) in pcDNA3 (Thermo-Fisher Scientific; Waltham, MA, USA) and TRPA1 (NM177781) in pcDNA5/FRT (Thermo-Fisher Scientific; Waltham, MA, USA) (15, 20). Deletions in TRPV1 were generated using PCR and resulting mutated TRPV1 constructs were sub-cloned into pcDNA5/FRT. Fragments of TRPV1 cDNA representing different domains were generated by PCR and sub-cloned into pECFP-N1 (BD Biosciences Clontech, Mountain View, CA, USA) to generate fusion proteins with CFP tagging on C-terminals. Every plasmid was fully sequenced to validate TRPV1 deletions and domain-CFP fusion constructs. Construction of all plasmids were outsourced to TOP Gene Technologies, Inc. (Montreal, Quebec, Canada)

Expression in CHO cells and primary DRG neuronal culture

TRPV1 and TRPA1 was always co-transfected with GFP or RedFP for visualization purposes. For Ca^{2+} -imaging and electrophysiology experiments, plasmid constructs were delivered into CHO cells using PolyFect (Qiagen, Valencia, CA) according to manufacturers' protocols. Cells were used within 24–48 h after transfection. For biochemistry experiments, Lipofectamine 2000 Transfection Reagent (Thermo-Fisher Scientific; Waltham, MA, USA) was employed for high-efficiency delivery of plasmids into CHO cells according to manufacturers' protocols. Expression constructs were delivered into DRG neurons using Nucleofector II (Lonza; Cologne, Germany) and Basic Neuron SCN Nucleofector Kit (Lonza; Cologne, Germany) according to detailed manufacturer's protocol. DRG neurons were subjected to experimental procedures within 24–48 h after transfection. Sensory neuronal culture from L3–L5 DRGs of naïve or reporter mice were prepared and maintained in DMEM with 2% fetal bovine serum as previously described (15). Experiments were carried out with post-plating times specified in the text.

CGRP-release assay

Capsaicin (CAP: Fluka, St. Louis, MO) stock prepared in 100% ethanol, mustard oil (MO: 10 M stock solution; Sigma, St. Louis, MO) were diluted in Hank's buffer. 2 hind paw skins, 6 DRG or 1 spinal cord were used per well. CGRP-release experiments were carried out at 37°C. Tissues were washed once with Hank's buffer and then soaked in Hank's buffer for 30 min to equilibrate. The supernatant was collected for measurement of baseline CGRP release after 15 mins in Hank's. Then, tissues were pre-treated for 15 min with agonists and/or antagonists. PBDu (10 μ M) and 8Br-cAMP (10 μ M) pre-treatment was used to activate PKC and PKA, respectively. Pre-treatment solution with agonists or antagonists was substituted with fresh Hank's buffer containing CAP, MO or other TRPA1 or TRPV1 activators. Tissues were exposed for 2 min with TRP activator, and solution was replaced. Tissues were maintained for additional 18 min in Hank's buffer. The total evoked CGRP release was measured by pooling the 2 min MO/CAP (or other TRP activator) exposure sample with an 18 min vehicle post-exposure sample. Biopsies were only used once and only exposed to one treatment condition. The CGRP radio-immune assay was conducted essentially as described (21) with primary antibody against CGRP (final dilution, 1:1,000,000; kindly donated by Dr. Michael J. Iadarola (NIDCR/NIH, Bethesda, MD). CGRP release data was normalized by weight of biopsies. Data was presented as % of release above baseline.

Co-immunoprecipitation (Co-IP) and Western Blotting

CHO in 6-cm plates and or DRG cultures in 3-cm plates were treated and prepared for homogenization and isolation of crude plasma membrane fractions, as described previously (22). Crude plasma membrane homogenates are quantified for protein concentration by Bradford analysis. Equal samples underwent co-immunoprecipitation (Co-IP; 100–200µg total protein) or gross homogenate WB analysis (10µg total protein). Samples isolated for Co-IP were diluted to 500 µl with homogenization buffer and incubated with appropriate antibodies: anti-TRPV1 (Oncogene, San Diego, CA) or anti-TRPA1 (Neuromics, Edina, MN). Co-IP complexes were pulled down with protein agarose-A (Sigma-Aldrich, St. Louis, MO), and resolved by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Gels were transferred to polyvinylidene difluoride (PVDF, EMD Millipore, Billerica, MA), blocked in 5% non-fat milk in Tris buffered saline with 0.1% Tween-20 non-ionic detergent, and incubated 18 h with appropriate primary antibody. Anti-rabbit or anti-mouse secondary antibodies (GE Healthcare Life Sciences, Piscataway, NJ) were applied to rinsed blots, incubated at room temperature (RT) for 1 h, and then blots were rinsed again. Blots were incubated with enhanced chemo-luminescence solution (GE Healthcare Life Sciences), exposed to X-ray film and developed for analysis. All films were scanned, and immune-reactive bands were quantified using Image J shareware.

Electrophysiology

Recording: Marker-positive or negative DRG neurons for recording (or Ca^{2+} -imaging) were selected randomly. For some experiments, before patch clamp recording or Ca^{2+} -imaging loading with Fura-2AM, DRG cells were stained for 0.5–4 hr at 37°C with IB4 Alexa Fluor-488, -594 or -647 (1:1000; Thermo-Fisher Scientific, Waltham, MA). Whole-cell and single-channel inside-out patch clamp recording was conducted essentially as described (18, 23). Data were acquired using an Axopatch 200B amplifier and analyzed with pCLAMP10.2 software (Molecular Devices, Sunnyvale, CA). Recording data were filtered at 0.5–5 kHz and sampled at 2–20 kHz depending on current kinetics. Data were rejected when R_s changed >20% during recording, leak currents were >100pA, or input resistance was < 100 MΩ. Currents were considered positive when their amplitudes were 5-fold bigger than displayed noise (in root mean square). Drugs were applied by a fast, pressure-driven and computer controlled 4-channel system (ValveLink8; AutoMate Scientific, San Francisco, CA) with quartz application pipettes. Data was accumulated from 2–4 independent mouse DRG neuronal cultures. Quantitative Ca^{2+} imaging was performed as previously described (23). Details of experiments presented in the Supplementary Material and Method section.

Solutions: For whole-cell recording from CHO cells and DRG neurons, standard external solution (SES) contained (in mM): 140 NaCl, 5 KCl, 2 CaCl_2 , 1 MgCl_2 , 10 D-glucose and 10 HEPES, pH 7.4; and the standard pipette solution (SIS) contained (in mM): 140 KCl, 1 MgCl_2 , 1 CaCl_2 , 10 EGTA, 10 D-glucose, 10 HEPES, pH 7.3, 2.5 ATP and 0.2 GTP. For single-channel inside-out recording from CHO cells, the bath solution (SES-SCh) consisted of (in mM): 100 K-gluconate, 4 KCl, 1 MgCl_2 , 1 EGTA, 10 D-glucose and 10 Hepes (pH 7.3); and the pipette solution (SIS-SCh) was (mM): 100 Na-gluconate, 10 NaCl, 1 MgCl_2 , 2 CaCl_2 , 10 D-glucose and 10 Hepes (pH 7.3).

Protocols and data analysis: Whole-cell currents from DRG neurons were recorded using GAP-free protocol at $V_h = -60\text{mV}$ (15). Current magnitudes were measured from data generated by these recordings. Whole-cell currents from CHO cells were recorded using episodic protocol. CHO cell kept at 0 mV, and then voltage was ramped from -100mV to $+60\text{mV}$ during 300 msec. This ramp protocol was cycled every 3 secs. First 4 cycles were used to generate baseline I-V plot, and then I-V plot was generated every 3 secs when CAP or MO was applied to CHO cells for 30 or 60 secs, respectively. Baseline I-V plot was subtracted from I-V plot in presence of CAP or MO (15). Maximal currents at $V = -60\text{mV}$ was measured (15).

Single-channel currents were recorded using Gap-free protocol at $V_h = -60\text{mV}$ (18). Single-channel currents were filtered with an 8-pole, low pass Bessel filter at 0.1 kHz, since dwelling time of the TRPA1 and TRPV1 single-channel currents were >0.5 sec. Single-channel unitary current (i) was determined from the best-fit Gaussian distribution of amplitude histograms. Open probability (P_o) was calculated using pCLAMP9.2 software (Molecular Devices, Sunnyvale, CA) by normalizing NP_o for the total number of estimated channels (N) in the patch. In order to increase accuracy in measurement of P_o , only patches containing less than 3 channels were used (18).

Ca²⁺ imaging

Data was collected and analyzed with NIS-elements software (Nikon Instruments, Melville, NY). The experiments were performed in SES solution on CHO or DRG cells loaded with calcium-sensitive, cell permeable dye, Fura-2 AM (2 μM ; Molecular Probes, Carlsbad, CA). The net changes in Ca^{+2} influx were calculated by subtracting the basal $[\text{Ca}^{+2}]_i$ (mean value collected for 60 s prior to agonist addition) from the peak $[\text{Ca}^{+2}]_i$ value achieved after exposure to the agonists. Ratio-metric data were converted to $[\text{Ca}^{+2}]_i$ (nm) as previously described (22, 23).

Behavior experiments

Nociception measurement (i.e. nocifensive behavior): On the day of the experiment, CAP was dissolved in 5% Tween and 5% DMSO; MO, BK and PGE₂ was diluted or dissolved in saline. Vehicle controls contained the appropriate solution for each compound. The right hindpaw of mice received an intra-plantar (ipl) injection (10 μl) of selected concentrations of drugs. The duration (in secs) that the animal spent flinching the injected hind paw over a 5 min period was collected by an observer blinded to treatment allocation. For the sensitization experiments, the animals were either injected with a mix of inflammatory mediator and CAP/MO/other activator, or first, pre-injected with 10 μl of inflammatory mediators (or the appropriate vehicle as control), and then after some time past with CAP/MO/other activator. In any case, nociception was measured immediately upon application of CAP/MO/other activator.

Pain models: Inflammatory model was generated with Complete Freund Adjuvant (CFA; CFA/Saline=1:1) (24). CFA was given intraplantar (ipl) in 10 μl volumes via a 28-gauge needle inserted through the lateral footpad just under the skin to minimize tissue damage. Control/vehicle is saline. Neuropathic model was generated by placing loosely constrictive

ligatures around the common sciatic nerve (i.e. chronic constriction injury; CCI) (25). Control is sham surgery that does not include manipulation with sciatic nerve as described before (25).

Heat, cold and mechanical nociception and hypersensitivity

measurements: Mice were tested for baseline (BL) responses prior to establishing inflammatory or neuropathic models. For all behavior experiments, observers were blinded to the treatment conditions and genotypes of the animals. We also used randomized designs for behavioral experiments and tested animals in small groups each time. Mechanical stimulus-induced nociception of mouse paw was measured with a plantar electronic von Frey Aesthesiometer (Ugo Basile) (26). Heat-induced nociception of mouse paw was assessed with Hargreaves' apparatus (Ugo Basile) (26). Cold-induced nociception in mice was measured using a modified IITC incremental hot/cold plate (modified IITC Life Sciences, which is capable ramp temperature as low as -10°C) (26). The thresholds (mechanical or temperature) were manifested by escape reflexes of mice.

Statistics

GraphPad Prism 7.0 (GraphPad, La Jolla, CA) was used for statistical analyses. Data in the figures are mean $\pm$ standard error of the mean, with "n" referring to the number of analyzed cells and the number of tissues or animals per group. *In vitro* experiments were performed at least in duplicate. Critical *in vivo* experiments were also performed twice. Differences between groups were assessed by unpaired *t*-test or regular 1-way ANOVA with Tukey's post-hoc tests. Differences between groups with two variables were assessed by 2-way ANOVA with Bonferroni's post-hoc tests. A difference is accepted as statistically significant when $p < 0.05$. Interaction F ratios, and the associated p values are reported.

Results:

Sensory neuron-type dependent sensitization of TRPA1 responses

Through a combination of single-cell sequencing (1, 27) and electrophysiology (4), seven small sensory neuron subgroups were identified using a variety of reporter mice (Figure 1A). S1–S3 groups represent peptidergic sensory neurons, S4–S6 are non-peptidergic neurons, and S7 are non-nociceptive C-fiber low-threshold mechano-receptors (C-LTMR) (Figure 1A). Medium-to-large mouse DRG sensory neurons do not respond to TRPA1 or TRPV1 agonists at concentrations used in this study (4). Sequencing data indicate that vGLUT3⁺ sensory neurons (i.e. S7) have TRPA1 mRNA (1); however, no TRPA1 responses were recorded from these neurons (4). Therefore, S7 and medium-to-large sensory neuron groups were not considered further in this study.

All sensory neuron subgroups express substantial levels of protein kinase C epsilon (PKC ϵ), and regulatory and catalytic subunits of cAMP-dependent kinase (PKA) (1, 27). Since these kinases are known to be involved in sensitization of TRP channels (28, 29), we investigated the role of PKA and PKC in TRPA1 sensitization in different sensory neuron subgroups. In naïve mice, MrgprA3⁺ neurons (S1), which co-express TRPA1 and TRPV1 (4), MO-evoked Ca²⁺ influx was potentiated by activation of PKC but not PKA (1-way ANOVA; F(2,

44)=16.09; $P<0.0001$; $n=15-16$; Figures 1B, 1C). In TRPV1⁺/CGRP⁻ neurons (S6), which express TRPV1 at high levels and TRPA1 at low levels (4), MO responses were substantially sensitized by activation of PKC as well as PKA (1-way ANOVA; $F(2, 22)=12.85$; $P=0.0002$; $n=7-11$; Figure 1D, 1E). In MrgprD⁺ neurons (S4 and S5), expressing high levels of TRPA1 with no TRPV1, MO-evoked Ca²⁺ influx ($n=13-28$; Fig 1F) and current (I_{MO} ; Figure 1G) were unaffected by PKA or PKC activation (1-way ANOVA; $F(2, 61)=0.42$; $P=0.65$).

TRPA1 could potentially be sensitized by other kinases in MrgprD⁺ neurons, so we utilized CFA to broadly stimulate multiple kinases and then evaluated sensitization. MrgprD⁺ neurons from CFA treated animals exhibited no TRPA1 sensitization, and additional PKC activation did not potentiate TRPA1 responses (t -test; $t=0.048$ $df=11$; $P=0.96$; $n=6-7$; Figure 1F). Besides S1 and S6 groups, TRPA1 only co-expresses with TRPV1 in a subset of MrgprA3⁻/V1-GFP⁺ sensory neurons (S3), which exhibit weak IB4 labeling (4). S3 neurons showed weak TRPA1-mediated responses, and TRPA1 was not potentiated by either PKA or PKC (1-way ANOVA; $F(2, 67)=1.1$; $P=0.32$; $n=7-13$; Figure 1H). Interestingly, CFA treatment dramatically increased MO responses in these neurons (2-way ANOVA; $n=9-16$; **** $p<0.0001$; Figures 1H, 1I). Additionally, PKA and PKC activation after CFA treatment did not further sensitize TRPA1 responses (1-way ANOVA; $F(2, 60)=0.06$; $P=0.94$; $n=9-16$; Figure 1H). Together, the presented data indicate that TRPA1 sensitization by kinases is sensory neuron type-dependent and may be influenced by co-expression with TRPV1.

Sensitization of TRPA1 in small-diameter sensory neurons is TRPV1-dependent

Based on insights from the previous experiments (Figure 1), we examined whether TRPA1 sensitization depends on co-expression with TRPV1. To do so, MrgprD⁺ neurons were transfected with TRPV1 expressing plasmid. TRPV1 plasmids were delivered into MrgprD⁺ neurons using Nucleofector II and Basic Neuron SCN Nucleofector Kit, since neither PolyFect nor Lipofectamine 2000 transfection reagents were able carrying plasmids into sensory neurons. In MrgprD⁺ neurons containing TRPV1, TRPA1 was slightly potentiated with PKA activation (1 way-ANOVA; $P=0.22$), and substantially with PKC (1-way ANOVA; $F(2, 30)=14.87$; $P<0.0001$; $n=11$; Figures 2A, 2B). Because culturing sensory neurons has been shown to alter their biochemical make up (30, 31), CGRP-release assay was used to evaluate TRPV1 dependence on TRPA1 sensitization in DRG, hind paw and spinal cord tissues from WT and TRPV1 KO mice (32). CAP and MO-evoked CGRP release both showed greatest effect in the spinal cord (Figure S1A). In naïve animals, MO (10mM)-evoked CGRP release in the spinal cord was substantially reduced by TRPV1 ablation (1-way ANOVA; *** $p<0.001$; $n=3-4$; Figure 2C) (15). CFA injection in hind paw significantly sensitized the MO-evoked CGRP release from spinal cord (2-way ANOVA; *** $p<0.001$); and, as expected, the effect of CFA on TRPA1 sensitization was dramatically reduced in TRPV1 KO animals (1 way-ANOVA; $F(3, 8)=136.5$; $P<0.0001$; $n=3-4$; Figure 2C). Moreover, TRPA1 sensitization in central terminals of the spinal cord became non-significant in the absence of TRPV1 (2-way ANOVA, $P=0.99$, Figure 2C).

Next, we evaluated whether the TRPV1-dependent sensitization of TRPA1 is observed *in vivo*. First, using TRPV1 and TRPA1 KO mice, we demonstrated that CAP (1 μ g) and MO (0.001%; 10mM) nociception is specifically mediated by TRPV1 and TRPA1, respectively

(1-way ANOVA; $F(8, 33)=100.8$; $P<0.0001$; $n=5-6$; Figure S1B). Furthermore, in naïve animals, MO-evoked nociception was attenuated in TRPV1 KO mice, while CAP-evoked nociception was independent of TRPA1 presence (1-way ANOVA; * $p<0.05$; Figure S1B). Sensitization of TRPA1 was achieved by several relevant mediators, including prostaglandin E₂ (PGE₂), bradykinin (BK), nerve growth factors (NGF) and CFA (Figures 2, S2). PGE₂ receptor 1 is present in S1–S6 neuronal subgroups and PGE₂ receptor 4 is present in all sensory neuron subgroups (1). MO-evoked nociception was substantially potentiated with PGE₂ (2-way ANOVA; $F(6, 42) = 12.37$; $P<0.0001$; $n=4-6$; Figure 2D and 1-way ANOVA $F(3, 14)=134.8$; $P<0.0001$; $n=4-6$; Figure 2E), and the PGE₂-induced potentiation persisted in TRPV1 KO animals (Figures 2D, 2E). These data along with that shown in Figures 1B, 1D, 1H and 2A imply that PGE₂ and PKA-induced sensitization of TRPA1 is independent of TRPV1 co-expression. Moreover, PKA and possibly PGE₂-induced sensitization of TRPA1 occur only in TRPV1⁺/CGRP[−] neurons (S6).

BK receptor 2 is in S3 neurons co-expressing TRPA1 and TRPV1 and is at low levels in MrgprD⁺ (1). BK sensitizes MO-induced nociception and this sensitization is TRPV1 dependent (2-way ANOVA; $F(15, 96)=23.26$; $P<0.0001$; $n=5-6$; Figures 2F, 2G). Moreover, MO-induced nociception was blocked by TRPA1, but not TRPV1 antagonist (Figure S2B). Interestingly, blocking TRPV1 with CZP did not reduce the BK promoted sensitization of MO-evoked nociception (Figure S2B). This implies that physical interaction between TRPA1 and TRPV1, regardless of TRPV1 activation, may be the most important factor in TRPV1-dependency of TRPA1 sensitization.

The NGF receptor, tyrosine receptor kinase A (TrkA), is expressed in CGRP⁺ neurons (1), induces the tyrosine kinase pathway, which is distinct from PGE₂ and BK G-protein coupled receptor signaling pathway, and is a potent sensitizer of sensory neurons (33, 34). Like BK, NGF is a potent sensitizer of MO-evoked nociception, and, similarly, this effect requires TRPV1 expression (2-way ANOVA; $F(3, 8)=35.9$; $P<0.0001$; $n=4$; Figure S2A).

Finally, we evaluated the effect of CFA on TRPA1 sensitization, which initiates the accumulation of many inflammatory mediators. CFA strongly sensitized MO-induced nociception, and this robust sensitization showed the pronounced dependency on TRPV1 (2-way ANOVA; $F(6, 42)=10.02$; $P<0.0001$; $n=6$; Figures 2H, 2I). Altogether, the presented data identify that TRPA1 sensitization is dependent on types of inflammatory mediators, kinases and TRPV1 co-expression.

Sensitization of TRPV1 in small-diameter sensory neurons is TRPA1-dependent

In naïve animals, CAP-evoked responses in sensory neurons and *in vivo* nociception are independent of TRPA1 co-expression (Figure S1B) (15). We further evaluated whether TRPV1 sensitization is regulated by TRPA1 co-presence. PGE₂ was used to significantly sensitize CAP-evoked nociception. Ablation of TRPA1 substantially reduced the level of TRPV1 sensitization (2-way ANOVA; $F(15, 78)=7.28$; $P<0.0001$; $n=5-6$; Figure S2C). Regulation of CFA-induced TRPV1 sensitization was also TRPA1 dependent (2-way ANOVA; $F(10, 78)=11.12$; $P<0.0001$; $n=4-10$; Figure S2D). Overall, unlike CAP responses in naïve animals, TRPV1 sensitization is dependent on TRPA1 co-expression.

Identification of interaction domains for the TRPA1-TRPV1 complex

To identify interaction domains for A1–V1 complex formation, we generated a set of plasmids containing deletions in N-terminal, C-terminals and transmembrane regions of TRPV1. Then, the plasmids containing mutated TRPV1 (WT TRPV1 size is 90kDa (18, 35, 36)) were co-expressed with wild-type TRPA1 (WT TRPA1 size is 120 kDa (18, 28, 35, 36)) in CHO cells. We used Lipofectamine 2000 transfection approach for biochemical experiments, which unlike PolyFect, allows to deliver plasmids into at least 50% of CHO cells. Co-immuno-precipitation (co-IP) assay showed that deletions of the 100–240 amino acids (aa) region of TRPV1 reduced co-IP and A1–V1 interaction (Figures 3A, 3B); while deletion of the 200–320aa TRPV1 region ablated co-IP and A1–V1 interaction (Figures 3A, 3B). Moreover, co-ablation of the 100–240aa with the 684–790aa region led to almost entire A1–V1 complex disruption (Figures 3A, 3B). In contrast, co-ablation of the 200–320 aa domain with the 684–790aa region does not produce additional A1–V1 complex disruption (Figures 3A, 3B). Deletion of only 684–790aa domain of TRPV1 produced a slight, non-significant reduction of A1–V1 co-IP (1-way ANOVA; $F(10, 22)=22.52$; $n=3$; $P<0.0001$; Figure 3B). The region containing the TRP-box is located within the 684–790aa region (37). These data indicate that A1–V1 interaction domains (IDs) are located within the N-terminal 100–320aa as well as C-terminal 684–790aa regions of TRPV1.

To more precisely identify IDs, we co-expressed TRPV1, TRPA1 as well as plasmids encoding different domains of TRPV1 in CHO cells. For these electrophysiology experiments, plasmids were delivered into CHO cells using PolyFect, but more effective Lipofectamine 2000 reagent produces like PolyFect results (i.e. current magnitudes and effects) as was evaluated for data presented in Figure 3C. Since in naïve conditions, MO responses are smaller in the presence of TRPV1 (Figure S2B) (15), disruption of the A1–V1 complex by ID encoding peptides will act as dominant-negative constructs, increasing MO response. MO-activated current (I_{MO}) was increased by co-expression with 180–320aa and 140–260aa regions, but not the combinations of 140–220&260–320aa or 140–180&220–320aa regions (1-way ANOVA; $F(4, 29)=5.9$; $P=0.0014$; $n=5–11$; Figure 3C). I_{MO} was also significantly larger with co-expression of 684–750aa and 684–720&750–790aa regions, non-significantly increased by the 684–790aa region, and unchanged by 720–790aa regions (1-way ANOVA; $F(6, 129)=7.35$; $P<0.0001$; $n=5–9$; Figures 3D, 3E). These data indicate that the primary IDs are in the N-terminal 220–260aa and C-terminal 684–720aa regions of TRPV1. Plasmids containing combined 220–260aa and 684–720aa regions were very effective in disrupting the A1–V1 complex and produced I_{MO} levels like those generated in TRPA1-independent cells (1-way ANOVA; $F(2, 22)=5.5$; $P=0.011$; $n=5–8$; Figure S3A). To support the presented data, we examined whether IDs co-expression with TRPA1 and TRPV1 in CHO cells could physically disrupt the complex. Co-expression of N- or C-terminal regions alone with TRPA1 and TRPV1 did not effectively disrupt assemblage of TRPA1 and TRPV1 into a complex (Figure 4A). However, the combined plasmid containing both N- and C-terminal IDs successfully and fully disrupted the A1–V1 complex (Figure 4A).

To verify the effects of IDs were not due to direct inhibition of TRPV1 or TRPA1, we co-expressed TRPV1 with plasmids containing the 180–320aa or 684–750aa regions and

showed no change in responsiveness of TRPV1 (1-way ANOVA; $F(2, 17)=0.91$; $P=0.42$; $n=5-9$; Figure 3F). Similarly, these regions did not change TRPA1 responsiveness (1-way ANOVA; $F(2, 15)=1.7$; $P=0.22$; $n=5-8$; Figure 3G). Moreover, plasmids containing both N- and C-terminal IDs did not impact TRPV1 (t-test; $t=0.51$ $df=13$; $P=0.61$; $n=6-9$; Figure S3B) or TRPA1-mediated currents (t-test; $t=1.1$ $df=11$; $P=0.28$; $n=5-8$; Figure S3C). To summarize, data on Figures 3, 4A and S3 indicate that specific regions of mouse TRPV1 (i.e. 220–260aa and 684–720aa) act as IDs that could be used for disrupting the A1–V1 complex without affecting responses from TRPA1 and TRPV1 homo-tetramers.

Modulation of TRPA1-mediated responses in sensory neurons by dominant-negative IDs

In this section, all experiments were performed on sensory neurons from naïve animals to validate efficiency of IDs in disruption of the A1–V1 complex. First, we investigated the effectiveness of plasmids expressing dominant-negative IDs in complex ablation and/or modulation of TRPA1 responses in sensory neurons. Expression plasmids containing both N- and C-terminal IDs transfected into MrgprD[−] sensory neurons reduced I_{MO} significantly (1-way ANOVA; $F(2, 28) = 4.2$; $P=0.02$; $n=8-13$; Figure 4B). Control plasmids expressing a scrambled sequence did not affect I_{MO} . In contrast, disruption of the complex showed no effect on TRPV1-mediated responses (t-test; $t=0.63$ $df=12$; $P=0.54$; $n=7-9$; Figure 4C). These results confirm disruption of the A1–V1 complex in sensory neurons co-expressing both TRPV1 and TRPA1, since separation of TRPA1 from TRPV1 within the complex reduces TRPA1-mediated current in sensory neurons (15, 16).

We next evaluated whether cell-permeable peptide encoding N- and C-terminal IDs could be an effective tool for disruption of the A1–V1 complex. Cell-permeability of ID peptides was achieved by myristoylation from the N-terminus (16). Unlike a scrambled peptide, cell-permeable IDs concentration-dependently inhibited MO-evoked Ca^{2+} influx into MrgprD[−] DRG neurons (1-way ANOVA; $F(3, 159) = 3.22$; $P=0.0243$; $n=15-63$; Figure 4D). In a similar fashion, ID peptides reduced I_{MO} in MrgprD[−] DRG neurons (t-test; $t=3.057$ $df=12$; $P=0.0100$; $n=8$; Figures 4E, 4F). Altogether, these data show that ID encoding plasmids and cell-permeable peptides are effective in disrupting the A1–V1 complex, and consequently, reducing TRPA1-mediated responses in sensory neurons expressing both TRPV1 and TRPA1. The data also show that the validated IDs could be used for studies of A1–V1 complex roles in sensory neuron sensitization.

Co-presence of the TRPA1-TRPV1 complex and homomers in sensory neurons

Sensory neuron subgroups, such as MrgprA3⁺ and MrgprA3[−]/TRPV1-GFP⁺, contain TRPA1 and TRPV1 channels at different levels. This implies that sensory neurons might have channels in both heteromeric and homomeric forms; however, the relative ratios are unknown, and it is also not clear how disruption of the complex affects this relative ratio. Recently, agonists activating either the A1–V1 complex or the TRPV1 or TRPA1 homomers were reported (38). These agonists have the potential to be used for assessing homomer and heteromer co-presence in sensory neurons. We validated the specificity of these agonists in expression systems, sensory neurons and tissue biopsies. Recording in inside-out single-channel configuration from TRPA1 and/or TRPV1 expressing CHO cells showed that the TRPA1-specific agonist Propylparaben (Prop; 100 μ M) activates patches containing TRPA1

only (respond to MO; 100 μ M), but not TRPV1 only (respond to CAP 100nM) or both TRPA1 and TRPV1 (respond to both CAP and MO) (Figure S4A). Prop is about 5-fold less potent than MO (100 μ M). Prop also shows specificity for the TRPA1 homomer at <1.5mM but activates the A1–V1 containing patches at >1.5mM (data not shown). Prop (400 μ M) activates DRG neurons in WT and TRPV1 KO, but not TRPA1 KO mice (Figures S4D, S4E). Prop also produces larger Ca²⁺ influx in DRG neurons (t-test; t=2.913 df=73; P=0.0047; n=28–47; Figure S4D); and calcitonin gene related peptide (CGRP) release from spinal cord biopsies of TRPV1 KO than WT (1-way ANOVA; F (2, 16) = 10.04; P=0.0015; n=5–7; Figure S4E). In the TRPA1 KO spinal cord slices, Prop (400uM) did not evoke CGRP release (Figure S4E).

The A1–V1 complex-specific agonist quercinitol (Quer; 100 μ M) was active on patches containing both TRPA1 and TRPV1, but not TRPA1 or TRPV1 alone (Figures S4B, S4C). Quer shows about 4-fold less potency than MO. It is specific at <800 μ M, but at >800 μ M it activates patches containing TRPA1 alone (data not shown). Also, Quer (400 μ M) activates cultured DRG neurons from WT, but not TRPV1 KO mice (t-test; t=6.423 df=76; P<0.0001; n=28–47; Figure S4D). Similarly, Quer (200 μ M) did not evoke CGRP release from spinal cord slices of TRPV1 KO mice (n=4; Figure S4F). Thus, agonists for the A1–V1 complex and TRPA1 homomer show specificity at given concentrations. These essential tools can distinguish heteromeric complexes from homomers (*in vitro*, *in vivo*), and assess the dynamics of complex formation in pathological conditions.

We next examined co-presence of the complexes and TRPA1 homomers in sensory neurons. Cultured MrgprD⁺ DRG neurons were activated sequentially by Quer (400uM) and then Prop (400 μ M), or vice versa. Approximately 93% of responsive neurons were activated by both agonists, but [Ca²⁺]_i rise ratios (i.e. Quer/Prop) vary from cell to cell (Figure S5A). This method does not precisely establish the relative amount of complex to TRPA1 homomer, since efficacy of these agonists are different. However, it does indicate the co-presence of A1–V1 complexes and TRPA1 homomers in sensory neurons. TRPV1 KO increased TRPA1 homomeric responses (by Prop) in cultured DRG neurons (Figure S4D) and central terminals in spinal cord (Figure S4E), while the plasma membrane fraction levels of TRPA1 protein are equal in WT and TRPV1 KO DRG neurons (Figures S5B, S5C). Similarly, the disruption of complexes with ID peptides leads to an increased Prop response (t-test; t=2.222 df=54; P= 0.0305; n=20–36; Figure S5D). These data imply that TRPV1 KO or complex disruption leads to re-distribution of TRPA1 from complexes to homomers. Moreover, increased prop responses $\approx$ 70–80% upon TRPV1 KO (Figures S4D, S4E) or complex disruption (Figure S5C) suggest that in naïve animals, the average ratio of the complex to TRPA1 homomer in DRG neurons could be estimated as approximately 1:1. To test potential alteration in heteromeric-to-homomeric ratio under conditions of inflammation, we showed that CFA-induced inflammation increased Quer (0.6 mM)-evoked CGRP release in spinal cord slices (t-test; t=2.661 df=6; P=0.0375; n=4; Figure S5E). Overall, TRP homomers and complexes co-present in sensory neurons, and A1–V1 complex disruption increased homomers. Moreover, inflammation increased intrinsic sensitization of the complex.

Regulation of PKC-mediated TRPA1 sensitization by disrupting the TRPA1-TRPV1 complex with ID peptides

Since PKC activation effectively sensitizes MO responses in MrgprA3⁺ neurons, we recorded I_{MO} from mrgprA3⁺ neurons treated with vehicle or PDBu with or without ID peptides. The A1–V1 complex in sensory neurons were ablated with cell-permeable peptides, but this complex disruption can also be achieved by dialyzing cells with ID peptide delivered with a recording pipette after whole-cell configuration is established. ID-peptide substantially inhibited TRPA1 sensitization through PKC activation (2-way ANOVA; $F(3, 21)=21.02$; $P<0.0001$; $n=6-8$; Figure 5A). Accordingly, BK-induced sensitization of I_{MO} was inhibited by ID peptides in a subset of MrgprA3⁻/TRPV1-GFP⁺ DRG neurons (S3; Figure 1A), which express BK receptor 2 (2-way ANOVA; $F(5, 41)=12.26$; $P<0.0001$; $n=7-10$; Figures 5B, 5C). These results show that ID peptides can be utilized to achieve inhibition of PKC-mediated sensory neuron sensitization.

Regulation of nociception and inflammatory hypersensitivity by disruption of the TRPA1-TRPV1 complex with ID peptides

Blockage of TRPV1 and TRPA1 affects nociception (39, 40). It is unknown whether homomeric and/or heteromeric states of TRPA1 and TRPV1 channels regulate baseline nociception, so we examined the role of ID peptide in the regulation of nociception in naïve mice. Heat-induced nociception was reduced (i.e. paw withdrawal latency is increased) by TRPV1 ablation as reported previously (40) (2-way ANOVA; $F(5, 29)=8.9$; $P<0.0001$; $n=6-8$; Figure 5D). ID peptide delivered into both hind paw and spinal cord did not change heat-induced nociception in WT or TRPV1 KO mice (Figure 5D). Cold-induced nociception was also impaired in TRPV1 KO animals but was not affected by administration of ID peptide (2-way ANOVA; $F(5, 18)=102$; $P<0.0001$; $n=4-9$; Figure 5E). Finally, mechanical stimuli-induced nociception was not affected by either TRPV1 ablation or complex disruption by ID peptides (2-way ANOVA; $F(5, 18)=0.93$; $P=0.48$; $n=5-9$; Figure 5F). These data indicate that the TRPV1 channel, but not complex formation, regulates baseline thermal nociception in naïve animals.

We next examined whether ID-peptide can regulate inflammatory mediator-induced hypersensitivity. Inflammatory mediator BK induced heat hypersensitivity, which was significantly reduced by administration of ID peptides in hind paw (1-way ANOVA; $F(2, 14)=73.1$; $P<0.0001$; $n=4-6$; 2 trials; Figure 6A). TRPV1 ablation eliminated BK-induced heat hypersensitivity, and as expected, ID-peptides did not additionally contribute in the TRPV1 KO (Figure 6A). In TRPA1 KO mice, BK produced heat hypersensitivity but not to the same severity as in WT animals. The hypersensitivity was not reversed by ID peptides, since the animals are lacking A1–V1 complexes (1-way ANOVA; $F(2, 6)=26.9$; $P=0.001$; $n=4-6$; Figure 6A). BK also induced cold hypersensitivity, and it was significantly reversed by ID peptide administration into the hind paw (1-way ANOVA; $F(2, 8)=17.1$; $P=0.0013$; $n=4-6$; Figure 6B). TRPV1 ablation did not alter BK-induced cold hypersensitivity, but lacking complexes in the KO mouse line nullified the effect of ID peptides (1-way ANOVA; $F(2, 6)=32.62$; $P=0.0006$; $n=4-6$; Figure 6B). TRPA1 KO reversed BK cold hypersensitivity, and ID peptide showed no difference (1-way ANOVA; $F(2, 6)=0.89$; $P=0.46$; $n=4-6$; Figure 6B). Finally, ID peptide successfully reduced BK-induced mechanical hypersensitivity in

WT mice (1-way ANOVA; $F(2, 11)=80.2$; $P<0.0001$; $n=4-6$; Figure 6C). In control experiments, BK mechanical hypersensitivity was abolished in TRPV1 KO mice, while ID peptides did not add to this effect (1-way-ANOVA; $F(2, 7)=5.4$; $P=0.04$; $n=4-6$; Figure 6C). TRPA1 KO surprisingly did not influence the development of BK-induced mechanical hypersensitivity, and predictably negated the action of ID peptide (1-way ANOVA; $F(2, 6)=26.9$; $P=0.001$; $n=4-6$; Figure 6C). Presented data clearly demonstrate that unlike baseline nociception, BK-induced hypersensitivity for all tested modalities can be significantly reversed by hind paw injection of ID peptides. Moreover, in control experiments lacking TRPA1 or TRPV1 in sensory neurons, ID peptide was ineffective.

To test the effect of ID peptide in a pathologically relevant pain condition, inflammatory pain was generated by injection of CFA (24), and neuropathic model was chronic constriction injury (CCI) (25). Schematic of CFA model generation and ID peptide hind paw or spinal cord administration is illustrated in Figure 7A. Spinal cord injection of ID, but not scramble peptides significantly reversed CFA-triggered heat hypersensitivity (1-way-ANOVA; $F(3, 18)=53.4$; $P<0.0001$; $n=5-7$; Figure 7B). Local hind paw administration of ID-peptides slightly reversed CFA heat hypersensitivity; however, it was not statistically significant (data not shown). CFA mechanical hyperalgesia was substantially reversed by local (1-way ANOVA; $F(3, 16)=67.19$; $P<0.0001$; $n=5-7$; Figure 7C) and spinal injection of ID peptides (1-way ANOVA; $F(3, 15)=112.3$; $P<0.0001$; $n=5-7$; Figure 7C).

For CCI model, ID peptide was used 7d-post CCI surgery, when hypersensitivity reached maximum values (25). Schematic of CCI model generation and ID peptide hind paw or spinal cord administration is illustrated in Figure 7D. At 7d post CCI, heat hypersensitivity was established (1-way-ANOVA; $F(3, 18) = 45.77$; $P<0.0001$; $n=5-6$; Figure 7E); and vehicle (PBS), scramble peptide or ID-peptide were administered. Spinal cord injection of ID did not alter CCI-induced heat hypersensitivity ($P=0.98$; Figure 7E). Established CCI-induced mechanical hyperalgesia (1-way ANOVA; $F(3, 18) = 116.8$; $P<0.0001$; $n=5-7$; Figure 7F) was also not significantly reversed by either local ($P=0.97$; Figure 7F) or spinal injection of ID peptides ($P=0.44$; Figure 7C). These results indicate that ID peptides are capable of attenuating inflammatory, but not nerve damage-induced hypersensitivity. Additionally, ID peptides are more effective in the spinal cord compared to local sites.

Discussion:

The diversity of sensory neuron subtypes allows for substantial flexibility in discerning sensory signals from environmental stimuli: physical and chemical. Accordingly, sensory neuron subgroups have distinct transcriptomes, biochemical make up, electrophysiology profiles and functions in naïve animals and humans (1, 4, 9, 27, 41). Pain conditions trigger plasticity of sensory neurons via transcriptional, translational and post-translational mechanisms (42). Previously, it was not clear whether these changes were sensory neuron type-dependent and what might define their dependency. The present study demonstrates that transient sensitization of TRPA1 and TRPV1 channels in a variety of small-sized nociceptors is sensory neuron subgroup specific and is defined by the type of inflammatory mediator or kinase as well as the co-expression of TRPA1 and TRPV1.

The focus of this study was on four subgroups of nociceptors. The first is MrgprA3⁺, which are itch sensing neurons (43) and represent polymodal nociceptors (44). These neurons are not sensitized by PKA or PGE₂, even though the PKA and PGE₂ receptors are present. Moreover, TRPA1, TRPV1 and Tmem100, an adaptor protein that can contribute to the A1–V1 complex formation, are all present on these neurons (1). The lack of PGE₂ and PKA sensitization could be explained by the absence of A-kinase anchoring protein 79/150 (AKAP-5) in this subgroup (1). Interestingly, recent single-cell data suggest that there are two types of MrgprA3⁺ DRG neurons: a first MrgprA3⁺ subgroup has both TRPV1 and TRPA1, while a second MrgprA3⁺ subgroup, which is represented by lesser number of neurons, express only TRPV1 (27). Physiological meaning of these two distinct population of MrgprA3⁺ DRG neurons is unknown. The second subgroup is MrgprD⁺ DRG neurons, which are itch sensing neurons (45) and also encode C-mechano-heat nociceptors (46). Recent single-cell RNA-sequencing data (27) and electrophysiology profiling of DRG sensory neurons (4) showed that there are two distinct MrgprD⁺ subgroups (S4 and S5) representing C-mechano-heat and C-mechano-sensitive nociceptors (46). One of these groups (S4) express TRPA1 (4, 46), but neither expresses TRPV1 or Tmem100 (1, 16). These neurons are not transiently sensitized by inflammatory mediators; however, recent reports show TRPA1-positive MrgprD⁺ neurons promote neuropathic pain (47). This implies that MrgprD⁺ neurons could be more important for nerve damage compared to inflammatory pain. In chronic nerve injury pain conditions, TRPA1 could undergo up-regulation that leads to long-term sensitization in MrgprD⁺ neurons, which is independent of TRPV1 (47). This may explain that disruption of TRPV1-TRPA1 complex with ID peptides did not reverse neuropathic pain induced by CCI (Figures 7D–7F). The third subgroup (S6) is the TRPV1⁺/CGRP[−] neurons. The function of these neurons is not clear. They express TRPV1 at the highest level of all subgroups and PIEZO2 at the lowest level (1, 27). Data on CAP-responsive nerve fibers in primates imply that these neurons may be mechano-insensitive (48). TRPA1 in this subgroup undergoes substantial sensitization by all inflammatory mediators and kinases used in the study. These data indicate that S6 neurons may play a critical role in inflammatory pain. This was previously shown using the inflammatory postoperative pain model (49). The fourth group is MrgprA3[−]/TRPV1⁺ (S3), which accounts for the largest population of polymodal nociceptors. These neurons do not have TRPM8 expression, which is present in the S2 group, and have slight IB4 labeling (4, 27). S3 neurons have TRPV1 with low expression of TRPA1 (1). In this subgroup, TRPA1 is substantially sensitized by CFA, and this potentiation depends on TRPV1. PKC and PKA do not sensitize these neurons, pointing to the possibility that TRPA1 is sensitized by activation of certain tyrosine kinases in this subgroup. To demonstrate such a possibility, we showed that NGF-induces sensitization of TRPA1 and requires TRPV1 co-expression. Overall, these data suggest that sensory neurons could be divided into groups predominantly contributing to neuropathic pain (MrgprD⁺) and groups primarily involved in inflammatory pain (MrgprA3⁺, CGRP[−]/TRPV1⁺ and MrgprA3[−]/TRPV1⁺). In this group involved in inflammatory pain, ID peptide effects on reversing hypersensitivity are most pronounced.

The assembly of TRP channels in complexes is documented in many types of cells in animals (50–54) as well as humans (19). This assembly determines the unique biophysical (55) and regulatory properties of TRP channels (52, 54). Multiple studies have demonstrated

that TRPA1 and TRPV1 form a complex in sensory neurons with distinct biophysical properties (15, 18, 56–58). Despite these advances, the role of TRP complexes in pathophysiological conditions has been under-studied and remains a large gap in our understanding of this important field. The present study shows that modeling inflammatory pain conditions through a variety of inflammatory mediators and adjuvants leads to substantial modulation of TRPA1 and TRPV1 within complex compared to the homomeric states of these channels. It was previously demonstrated that alteration of the A1–V1 complex by removal of Tmem100 led to a reduction of sensitization by loosening the bond between TRPA1 and TRPV1 in sensory neurons (16). Preventing association of Tmem100 to the V1–A1 complex also reduced mechanical hypersensitivity in inflammatory as well as neuropathic pain conditions (16). Disruption of the V1–A1 complex with cell-permeable ID peptide, representing two distinct association domains on TRPV1, also results in attenuation of inflammatory, but nerve damage-induced hypersensitivity. Moreover, ablation of the complex in spinal cord central terminals produced more pronounced anti-hypersensitivity than disruption of the complex in hind paws. It means ID-peptide is lesser effective at the peripheral compared to spinal site. This implies that the reduction of sensitization occurs by attenuating neurotransmission at pre-synaptic levels more than by lowering receptor potentials in peripheral terminals. It was shown that thermal hyperalgesia could be inhibited by blocking TRPV1 and TRPA1 at the periphery or spinal levels. In contrast, spinal, but not peripheral, blockage of TRPV1 or TRPA1 inhibits mechanical hypersensitivity (59).

Although disrupting the A1–V1 complex by using Tmem100 KO mice produces effective anti-hypersensitivity in inflammation and nerve injury-induced pain models (16), ablation of the complex with ID peptide does not produce statistically significant anti-hypersensitivity in CCI pain model (Figures 7D–7F). This could be explained by wider expression patterns for Tmem100 and the possible impact on interactions of other proteins (41). Tmem100 might also modulate TRPA1 homomerization (16), and the MrgprD⁺ TRPA1 homomer was recently reported to be involved in neuropathic pain (47). Ablation of the A1–V1 complex by ID peptides provides two distinct benefits over targeting Tmem100. First, Tmem100 expression levels in human DRG are relatively low. Second, ablation or inhibition of Tmem100 could lead to significant side effects since it is critically important in other tissues (60, 61).

Alteration of sensitization levels within the A1–V1 complex could occur in two ways: activity-dependent and activity-independent. Activity-dependent interactions between TRPA1 and TRPV1 channels often require sequential stimulation of TRPA1 and TRPV1 (21, 57, 58, 62). For instance, TRPA1 or TRPV1 activate upon stimulation of G-protein receptors with mediators (63, 64); this leads to Ca²⁺ influx, which affects the other channel since both channels are gated by intracellular Ca²⁺ (65, 66). Activity-independent interaction between channels occurs within the complex, where physical binding is the key event (15, 17, 18, 38, 51). To determine which is the primary mechanism of activity, TRPA1 was co-expressed with inactive TRPV1 lacking the “pore forming domain” (V1 PL; deletion of the region between transmembrane domains 5 and 6 (629–647aa). V1 PL does not respond to CAP, protons or temperature (67). These two channels form complex, and like TRPV1, V1 PL can bind and co-IP with TRPA1 in CHO cells (Figures S6A, S6B). Expression levels of TRPV1 and V1 PL are equal and do not alter TRPA1 levels as monitored by WB in cell

lysates (Figure S6A). V1 PL co-expression with TRPA1 (even at 1:5 ratio) results in suppression of I_{MO} (10 μ M) (Figure S6C) (15). This indicates that TRPA1 interacts with V1 PL, and modulation of TRPA1 within the complex is TRPV1 activity-independent. The primary requirement of TRPA1 regulation by TRPV1 is the binding of TRPA1 and TRPV1.

In conclusion, sensory neuron type-dependent sensitization could provide a way to specifically target hypersensitivity in defined pain conditions. It is well documented that directly blocking TRPA1 or TRPV1 leads to dysregulation of the body temperature control system (68, 69). Therapeutic strategies based on disrupting the V1–A1 complex are especially promising, since it does not disrupt the normal function of either channel, potentially circumventing the significant side-effects of a direct blockade.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations:

ID	interaction domain
CAP	capsaicin
MO	mustard oil
Prop	Propylparaben
Quer	quercinitol
CCI	chronic constriction injury
CFA	complete Freund's adjuvant
BK	bradykinin
PGE₂	prostaglandin E2
NGF	nerve growth factor
CGRP	calcitonin gene related peptide
RIA	radio-immune assay
TRPV1	transient receptor potential V1
TRPA1	transient receptor potential A1

DRG	dorsal root ganglia
TG	trigeminal ganglia (TG)
ND/JG	nodose/jugular ganglion complex
CHO cells	Chinese hamster ovary cells

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Figure 1: Sensitization of TRPA1-mediated responses in subsets of small DRG neurons

A: Schematic representation of sensory neuron markers in 7 (S1–S7) groups of small, unmyelinated mouse DRG neurons. Weak IB-4 staining is marked with a pale orange box. Sensory neuron markers are noted within each box. Sensitization of mustard oil (MO)-evoked $[Ca^{2+}]_i$ rise in MrgprA3⁺ (**B**), TRPV1⁺/CGRP⁻ (**D**), MrgprD⁺ (**F**) and MrgprA3⁻/TRPV1⁺/IB4⁺ (**H**) DRG neuronal subsets. Sensitization was induced by 10 min treatment with PKC activator, phorbol 12,13-dibutyrate (1 μ M; PDBu), or PKA activator, 8-Bromo-cAMP (1 μ M; 8Br-cAMP). Representative MO-evoked Ca^{2+} imaging traces recorded from vehicle (Veh), PDBu or 8Br-cAMP pre-treated DRG neurons that belong to MrgprA3⁺ (**C**) and TRPV1⁺/CGRP⁻ (**D**) subsets. **G:** Representative I_{MO} (25 μ M) traces recorded from MrgprD⁺ neurons of naive mice pre-treated with vehicle or PDBu. **I:** Representative I_{MO} traces recorded from MrgprA3⁻/TRPV1⁺/IB4⁺ (S3) neurons cultured from mice injected with vehicle or CFA.

Figure 2: In vitro and in vivo sensitization of TRPA1 is TRPV1-dependent

A: PDBu or 8Br-cAMP induced sensitization of MO (10 μ M)-evoked $[Ca^{2+}]_i$ in MrgprD⁺ subset transfected with TRPV1 and RFP. **B:** Representative MO-evoked Ca^{2+} imaging traces recorded from vehicle, PDBu or 8Br-cAMP pre-treated MrgprD⁺ DRG neurons transfected with TRPV1. **C:** MO (10mM)-evoked CGRP release from spinal cord of WT and TRPV1 KO mice 24hr post-injection of vehicle or CFA. **D** and **E:** MO (50mM)-evoked nociception in WT and TRPV1 KO mice injected with vehicle or PGE₂ (1 μ g). MO and PGE₂ were co-injected into the paw and nociception was measured every 5 min (**D**) for 15 min (**E**). **F** and **G:** MO-evoked nociception in WT and TRPV1 KO mice injected with vehicle or BK (1 μ g). MO and BK were co-injected into the paw and nociception was measured every 5 min (**F**) for the first 15 min post-injection (**G**). **H** and **I:** MO-evoked nociception in WT and TRPV1 KO mice injected with vehicle or CFA. MO was injected in paw 24hr-post CFA administration in paw. Nociception was measured every 5 min (**H**) for the first 15 min post-injection (**I**).

Figure 3: Identification of TRPV1 domains involved in TRPA1-TRPV1 interaction.

A: Unaltered (WT) or mutated TRPV1 channels were co-expressed (1:1) with WT TRPA1 in CHO cells and then Co-IPed with TRPA1. Membrane lysate (Lys) or Co-IP were probed with anti-TRPV1. **B:** The percent of Co-IP relative to Lys band densities. **C:** I_{MO} density in CHO cells co-expressing TRPV1 and TRPA1 and indicated fragments (1:1.5 ratio) from the N-terminal domain of TRPV1. **D:** Same paradigm as panel C but co-expressing fragments from the C-terminal domain of TRPV1. **E:** Representative I-V traces for I_{MO} recorded from cells co-expressing TRPV1, TRPA1 and indicated fragments. **F:** I_{MO} density in CHO co-expressing TRPA1 and indicated TRPV1 fragments (1:5 ratio). **G:** I_{CAP} (100nM) density in CHO co-expressing TRPV1 and indicated TRPV1 fragments (1:5 ratio).

Figure 4: Modulation of TRPA1-mediated responses in sensory neurons of naïve mice with plasmids or cell-permeable peptides encoding interaction domains (IDs).

A: Co-IP of TRPV1 and TRPA1 from CHO cells co-transfected with plasmids containing 220–260V1 (N-term), 220–260&684–720V1 (N+C-term) or scrambled (Scram) sequences fused to CFP. TRPA1:TRPV1:domain ratio is 1:1:10. **B:** I_{MO} in MrgprD⁻ DRG neurons transfected with constructs indicated below X-axis. **C:** I_{CAP} in TRPV1⁺ DRG neurons transfected with constructs indicated below X-axis. **D:** MO-evoked Ca^{2+} influx into MrgprD⁻ DRG neurons pre-treated for 30–60 min with indicated below X-axis cell-permeable peptides (220–260 and 684–720aa for TRPV1; ID-pept). **E:** I_{MO} in MrgprD⁻ DRG neurons pre-treated for 30–60 min with vehicle (PBS) or ID-pept (20 μ g/ml each). **F:** Representative I_{MO} traces for the panel E.

Figure 5. Regulation of TRPA1 sensitization and nociception in naïve mice by TRPA1-TRPV1 interaction counteracting peptide

A: I_{MO} in MrgprA3⁺ DRG neurons sensitized for 10 min with PDBu and pre-treated for 30–60 min with ID-pept (20μg/ml each). **B:** I_{MO} in a subset of MrgprA3⁻/TRPV1-GFP⁺ DRG neurons sensitized for 10 min with BK (100ng/ml) and pre-treated for 30–60 min with ID-pept. Scram is the scrambled version. **C:** Representative I_{MO} traces for panel **B**. **D:** Heat-induced nociception in WT and TRPV1 KO mice after injection of vehicle or ID-pept (20μg each) in both spinal cord and hind paw. **E:** Cold-induced nociception in WT and TRPV1 KO mice after injection of vehicle or ID-pept in both spinal cord and hind paw. **F:** Mechanical stimuli-induced nociception in WT and TRPV1 KO mice after injection of vehicle or ID-pept in both spinal cord and hind paw. Pre-treatments with ID-pept and mouse lines are indicated below the X-axis.

Figure 6. Inhibition of bradykinin-induced hypersensitivity with TRPA1-TRPV1 complex disrupting ID peptides

Effect of ID-pept (Pep; 20µg each) on BK (1µg)-induced heat (A), cold (B) and mechanical (C) hypersensitivity in WT, TRPV1 KO and TRPA1 KO mice. Nociception was measured 10 min after BK injection into hind paw.

Figure 7. Effects of local and spinal delivery of ID peptides on CFA- and CCI-induced hypersensitivity

A: Schematic of experiments on panels *B* and *C*. **B:** Effect of spinal cord injection of vehicle (PBS), ID-pept (20 μ g) or scrambled peptide (20 μ g; Scrm) at 1d post-CFA-induced heat hypersensitivity. **C:** Effect of local (i.e. hind paw) and spinal injection of vehicle, ID-pept or Scrm on 1d post-CFA-induced mechanical hypersensitivity/threshold. Heat and mechanical hypersensitivity on panels *B* and *C* reflect measurements at 1h post-injection of vehicle, ID-pept or Scrm into spinal cord or hind paw. **D:** Schematic of experiments on panels *E* and *F*. **E:** Effect of spinal cord injection of vehicle (PBS), ID-pept (20 μ g) or scrambled peptide (20 μ g; Scrm) at 7d post-CCI-induced heat hypersensitivity. **F:** Effect of local (i.e. hind paw) and spinal injection of vehicle, ID-pept or Scrm on 7d post-CCI-induced mechanical hypersensitivity/threshold. Heat and mechanical hypersensitivity on panels *E* and *F* reflect measurements at 1h post-injection of vehicle, ID-pept or Scrm into spinal cord or hind paw.