SHORT COMMUNICATION

Nocistatin sensitizes TRPA1 channels in peripheral sensory neurons

Luca Avenali, Oli Abate Fulas, Julia Sondermann, Pratibha Narayanan, David Gomez-Varela, and Manuela Schmidt
Max Planck Institute of Experimental Medicine, Somatosensory Signaling and Systems Biology Group, Goettingen, Germany

ABSTRACT

The ability of sensory neurons to detect potentially harmful stimuli relies on specialized molecular signal detectors such as transient receptor potential (TRP) A1 ion channels. TRPA1 is critically implicated in vertebrate nociception and different pain states. Furthermore, TRPA1 channels are subject to extensive modulation and regulation - processes which consequently affect nociceptive signaling. Here we show that the neuropeptide Nocistatin sensitizes TRPA1-dependent calcium influx upon application of the TRPA1 agonist mustard oil (MO) in cultured sensory neurons of dorsal root ganglia (DRG). Interestingly, TRPV1-mediated cellular calcium responses are unaffected by Nocistatin. Furthermore, Nocistatin-induced TRPA1-sensitization is likely independent of the Nocistatin binding partner 4-Nitrophenylphosphatase domain and non-neuronal SNAP25-like protein homolog 1 (NIPSNAP1) as assessed by siRNA-mediated knockdown in DRG cultures. In conclusion, we uncovered the sensitization of TRPA1 by Nocistatin, which may represent a novel mechanism how Nocistatin can modulate pain.

KEYWORDS

NIPSNAP1; nociception; Nocistatin; peripheral sensory neurons; sensitization; TRPA1

Introduction

Transient receptor potential (TRP) A1 channels play a fundamental role in vertebrate nociception and pain. TRPA1 is highly expressed in peripheral sensory neurons (specifically in nociceptors) where it serves as a primary detector of noxious stimuli comprising a wide variety of exogenous and endogenous molecules with pro-inflammatory and pro-algesic properties. Beyond direct activation by its ligands, TRPA1 function can be modulated. Modulators of TRPA1 are diverse and include protein kinase A and phospholipase C signaling cascades, growth factors as well as inflammatory mediators like lipid metabolites, chemokines and proteases. Many of these have been implicated in phenomena of hypersensitivity which, in turn, may contribute to clinically-relevant chronic pain conditions. Even the TRPA1 agonist mustard oil (MO) itself evokes sensitization of TRPA1-mediated nociceptive signaling in vitro and pain in vivo. In addition, several proteins physically interact with TRPA1 thereby altering its function. We recently characterized AnxA2 as a TRPA1-associated protein that limits TRPA1 membrane abundance and appears to regulate its activity both in vitro and in vivo. Tmem100 is yet another modulator of protein complexes harboring TRPA1 and TRPV1 (commonly known as the vaniloid receptor). Tmem100 has been shown to associate with TRPA1 and TRPV1 in sensory neurons where it potentiates TRPA1 activity in a TRPV1-dependent manner.

Given the crucial role of TRPA1 in nociception and pain, further exploration of molecular mechanisms regulating TRPA1 is highly relevant to understanding its function in both, physiological and pathological conditions. Here, we provide evidence that the neuropeptide Nocistatin sensitizes TRPA1-mediated cellular calcium responses in DRG neurons. Our results propose TRPA1 as a hitherto unrecognized molecular target of Nocistatin in the context of pain transmission.

Results

Nocistatin specifically sensitizes TRPA1 responses in sensory neurons

Nocistatin is a biologically active neuropeptide implicated in nociceptive signaling and pain. Nocistatin...
was initially discovered as a functional antagonist of Nociceptin-induced hyperalgesia and allodynia. In addition, Nocistatin also exerts numerous biological effects per se, such as modulation of inflammatory pain in the rodent formalin model. Within this context, Nocistatin has been proposed to alter neurotransmission in the dorsal horn of the spinal cord (i.e. in the sensory processing areas of the spinal cord). Since the formalin model also involves sensory neurons of the peripheral nervous system and TRPA1 channels expressed in nociceptors play a crucial role for formalin-induced inflammatory pain and hypersensitivity, we asked whether Nocistatin might affect TRPA1 physiology.

We prepared acute cultures from mouse DRG neurons and investigated the consequences of Nocistatin application on TRPA1-mediated cellular calcium influx by ratiometric calcium imaging. Of note, in vivo Nocistatin was described to elicit bimodal effects on chronic pain behaviors, i.e., low doses (picomolar range) are analgesic whereas high doses (nanomolar range) are pro-algesic. In contrast, careful in vitro studies have not reported this bidirectionality of Nocistatin function. Since increasing doses of Nocistatin between 1 \( \mu M \) up to 100 \( \mu M \) efficiently diminished inhibitory synaptic transmission in spinal cord slices, we used 10 \( \mu M \) Nocistatin in our experiments. First, we observed that application of Nocistatin did not elicit any apparent calcium-influx into DRG cultures (Fig. 1A). However, when Nocistatin was co-administered with MO, we measured a significant increase in the number of responders to 25 \( \mu M \) MO (24.4 \( \pm \) 2.2%) compared to vehicle-treated controls (11.5 \( \pm \) 2.2%; Fig. 1A and 1B), while response magnitudes were similar (Fig. 1B). The total number of TRPA1-expressing neurons was unaffected by Nocistatin as revealed by comparable results to saturating 1 \( \mu M \) CAPS after washout of Nocistatin at the end of each experiment (i.e., application of CAPS without Nocistatin, please see materials and methods for details; Vehicle: 15.9 \( \pm \) 4.3 % vs. Nocistatin: 17.5 \( \pm \) 6.0 % responders; response amplitude: Vehicle: 0.67 \( \pm \) 0.21 vs. Nocistatin: 0.49 \( \pm \) 0.13; 2-tailed Student’s t-test). These findings reveal a certain degree of specificity for Nocistatin-induced sensitization of TRPA1 activity to MO in sensory neurons.

Taken together, these data propose TRPA1 as a potential target of Nocistatin function in nociceptors.

**NIPSNAP1 knock-down does not alter Nocistatin-induced TRPA1 sensitization**

A compelling study uncovered the physical association of Nocistatin and NIPSNAP1. NIPSNAP1 is abundantly expressed in the brain, spinal cord and in peripheral sensory neurons. Accordingly, it has very recently also been shown to be implicated in pain. Nipsnap1-deficient mice exhibit deficits in formalin-, CFA- and carrageenan-induced inflammatory pain paradigms. In addition, Nocistatin functionally interacts with NIPSNAP1 which, in turn, is required for a specific type of Nocistatin-mediated allodynia. In light of these findings, we asked whether NIPSNAP1 might be necessary for the modulation of TRPA1 by Nocistatin. In order to test this, we intended to study the consequences of NIPSNAP1 knock-down for the Nocistatin-induced TRPA1 sensitization via calcium imaging in DRG cultures. First, we assessed the efficacy of siRNA-mediated NIPSNAP1 knock-down at both mRNA and protein level in cultured DRG neurons. Quantitative RT-PCR demonstrated a decrease of the relative expression of NIPSNAP1 mRNA by approx. 78% in controls vs. 37.6 \( \pm \) 3.4% upon NIPSNAP1 siRNA (Fig. 2A). The significant reduction of NIPSNAP1 mRNA was manifested in a discernible decrease of NIPSNAP1-expressing neurons as assessed by immunocytochemistry (NIPSNAP1-positive neurons: 65.8 \( \pm \) 2.7% in controls vs. 37.6 \( \pm \) 3.4% upon NIPSNAP1 siRNA transfection; \( p = 0.0019 \); 2-tailed Student’s t-test; Fig. 2B). In accordance with a recent study on NIPSNAP1 and its role in inflammatory pain, we detected NIPSNAP1 expression in vehicle-treated controls (Fig. 1D). In addition, the total number of TRPV1-expressing neurons and response amplitudes were unaffected by Nocistatin as revealed by comparable results to saturating 1 \( \mu M \) CAPS after washout of Nocistatin at the end of each experiment (i.e., application of CAPS without Nocistatin, please see materials and methods for details; Vehicle: 15.9 \( \pm \) 4.3 % vs. Nocistatin: 17.5 \( \pm \) 6.0 % responders; response amplitude: Vehicle: 0.67 \( \pm \) 0.21 vs. Nocistatin: 0.49 \( \pm \) 0.13; 2-tailed Student’s t-test). These findings reveal a certain degree of specificity for Nocistatin-induced sensitization of TRPA1 activity to MO in sensory neurons. To further explore Nocistatin function, we evaluated the consequences of NIPSNAP1 knock-down for the Nocistatin-mediated allodynia.21 In light of these findings, we asked whether NIPSNAP1 might be necessary for the modulation of TRPA1 by Nocistatin. In order to test this, we intended to study the consequences of NIPSNAP1 knock-down for the Nocistatin-induced TRPA1 sensitization via calcium imaging in DRG cultures. First, we assessed the efficacy of siRNA-mediated NIPSNAP1 knock-down at both mRNA and protein level in cultured DRG neurons. Quantitative RT-PCR demonstrated a decrease of the relative expression of NIPSNAP1 mRNA by approx. 78% in controls vs. 37.6 \( \pm \) 3.4% upon NIPSNAP1 siRNA (Fig. 2A). The significant reduction of NIPSNAP1 mRNA was manifested in a discernible decrease of NIPSNAP1-expressing neurons as assessed by immunocytochemistry (NIPSNAP1-positive neurons: 65.8 \( \pm \) 2.7% in controls vs. 37.6 \( \pm \) 3.4% upon NIPSNAP1 siRNA transfection; \( p = 0.0019 \); 2-tailed Student’s t-test; Fig. 2B). In accordance with a recent study on NIPSNAP1 and its role in inflammatory pain, we detected NIPSNAP1 expression in vehicle-treated controls (Fig. 1D). In addition, the total number of TRPV1-expressing neurons and response amplitudes were unaffected by Nocistatin as revealed by comparable results to saturating 1 \( \mu M \) CAPS after washout of Nocistatin at the end of each experiment (i.e., application of CAPS without Nocistatin, please see materials and methods for details; Vehicle: 15.9 \( \pm \) 4.3 % vs. Nocistatin: 17.5 \( \pm \) 6.0 % responders; response amplitude: Vehicle: 0.67 \( \pm \) 0.21 vs. Nocistatin: 0.49 \( \pm \) 0.13; 2-tailed Student’s t-test). These findings reveal a certain degree of specificity for Nocistatin-induced sensitization of TRPA1 activity to MO in sensory neurons.
small- and medium-sized sensory neurons of DRG (Fig. 2C). Furthermore, NIPSNAP1 is present in nociceptors as revealed by co-label with Peripherin, a marker for small nociceptive neurons (Fig. 2C).

Having established NIPSNAP1 expression and knock-down in cultured DRG neurons, we then turned to examine Nocistatin-induced TRPA1-sensitization in NIPSNAP1 siRNA-transfected DRG cultures using the same protocol as described above in Figure 1. Unexpectedly, we did not observe any difference in calcium responses between control (AllStar Negative control, Qiagen) and NIPSNAP1 siRNA-transfected cultures (Fig. 2D and 2E). Nocistatin-induced sensitization of TRPA1 was equally prominent upon NIPSNAP1 knock-down as in controls. Further, TRPA1-mediated calcium influx to MO was comparable among conditions.

These data indicate that NIPSNAP1 is probably not essential for TRPA1-sensitization by Nocistatin.

**Discussion**

In this study we describe Nocistatin as a novel modulator of TRPA1. Our results reveal the sensitization of TRPA1-mediated calcium influx by Nocistatin, which is likely independent of NIPSNAP1.

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**Figure 1.** Nocistatin specifically sensitizes TRPA1-mediated calcium response in DRG neurons. (A) Representative averaged traces of ratiometric calcium imaging in a population of cultured DRG neurons (1 day in vitro, DIV) upon application of indicated stimuli. Averaged changes in intracellular calcium levels are represented by the ratio of the fluorescence intensity at 340nm over 380nm. Traces represent averaged changes in intracellular calcium from a population of neurons (n > 20 neurons in this example) including responding neurons and non-responders of both conditions. As a quality control for neuronal health, only neurons responding to 50 μM MO at the end of the experiment are considered. Note that application of 10 μM Nocistatin or Vehicle (N/V) alone (first stimulus) does not elicit apparent calcium changes. (B,C) Quantification of the percentage of responding neurons and mean amplitude of neuronal calcium changes upon stimulation with (B) 10 μM Nocistatin or Vehicle (N/V) along with 25 μM MO (percentage of responding neurons: V: 11.5 ± 2.4 %; N: 24.4 ± 2.2 %; p = 0.017, 2-tailed Student’s t-test), and (C) upon application of 50 μM MO at the end of the experiment (ns, 2-tailed Student’s t-test). (D) Quantification of the percentage of responding neurons and mean amplitude of neuronal calcium changes upon stimulation with 10 μM Nocistatin or Vehicle (N/V) along with 0.1 μM Capsaicin (CAPS). Nocistatin neither affects the percentage of responders nor the mean amplitude of responses (ns, 2-tailed Student’s t-test). For each condition we analyzed n > 300 neurons from N = 3 independent cultures. All data are represented as mean ± SEM.
Figure 2. NIPSNAP1 knock-down does not affect Nocistatin-induced TRPA1 sensitization. (A, B) The extent of NIPSNAP1 knock-down (KD) after transfection with NIPSNAP1 siRNA as measured by (A) qPCR (relative Nipsnap1 mRNA levels: 0.22 ± 0.05 compared to GAPDH used as reference, i.e. approx. 78 % knock-down, p = 0.0041; 2-tailed Student’s t-test) and (B) immunocytochemistry (p = 0.0019; 2-tailed Student’s t-test; ≥700 neurons analyzed). N = 2–3 independent cultures each. (C) Representative images and quantification of immunohistochemistry on cryosections of mouse DRG co-labeled for NIPSNAP1 and Peripherin. White arrows highlight neurons co-expressing NIPSNAP1 and Peripherin. Scale bar, 50 μm. ≥12000 neurons analyzed. (D, E) DRG cultures were transfected with NIPS-NAP1-siRNA or AllStar Negative control (Qiagen) and used for ratiometric calcium imaging 3 days later (3 DIV). Quantification of the percentage of responding neurons of neuronal calcium changes upon stimulation with 10 μM Nocistatin or Vehicle (N/V) along with (D) 25 μM MO (p = 0.0354, one way ANOVA followed by Fisher’s LSD test) or (E) 50 μM MO at the end of the experiment (ns, one way ANOVA followed by Fisher’s LSD test). Mean response amplitudes to either MO concentration were not altered among conditions (data not shown). For each condition we analyzed a minimum of n ≥ 150 neurons from N = 5 independent cultures. Please note that presented values cannot be compared to values shown in Figure 1 due to differences in culture conditions and transfection (experiments were performed 1 DIV in Figure 1, while 3 DIV after transfection in this figure). All data are represented as mean ± SEM.
Nocistatin was originally discovered as a functional antagonist of the pro-nociceptive peptide Nociceptin/orphanin FQ (N/OFQ). It is produced from the same precursor protein as N/OFQ, but has opposite effects on pain transmission. Nocistatin counteracts N/OFQ-induced allodynia and hyperalgesia, and bi-directionally modulates inflammatory pain responses in vivo in a dose-dependent manner, i.e. Nocistatin may elicit both analgesic as well as pro-nociceptive effects. Despite numerous investigations on Nocistatin and its role in pain, the specific molecular mechanisms of its action are not yet clearly defined. Among proposed targets are N-methyl-D-aspartate (NMDA) receptors and inhibitory glycine receptors in the dorsal horn of the spinal cord. We report here that Nocistatin modulates TRPA1 channels in peripheral sensory neurons of DRG. By means of calcium imaging we revealed that co-application of Nocistatin and the TRPA1 agonist MO significantly enhanced TRPA1-mediated calcium responses in cultured sensory neurons. These results are in line with previous experiments on pro-nociceptive effects of Nocistatin such as the facilitation of nociceptive flexor reflexes as well as nocifensive behaviors in the rat formalin model of inflammatory pain. Intriguingly, TRPA1 is crucially implicated in inflammatory pain and hypersensitivity of the formalin model. The pro-nociceptive action of Nocistatin in these pain paradigms has so far been attributed to attenuation of inhibitory neurotransmission in the spinal cord and/or facilitation of neurotransmitter release from a population of capsaicin-sensitive (and therefore TRPV1-expressing) peripheral sensory neurons. It is noteworthy that a large subset of capsaicin-sensitive TRPV1-positive sensory neurons also expresses TRPA1 channels. However, despite the apparent co-expression of both channels, cellular responses to TRPV1 activation were unaffected by Nocistatin in our experiments arguing for a certain degree of specificity of the observed TRPA1 sensitization by Nocistatin. Therefore, we propose TRPA1 as a hitherto unknown target of Nocistatin in the peripheral nervous system. It is tempting to speculate that TRPA1 channels contribute to Nocistatin functions in pain transmission in vivo. Hence, it would be exciting to test this hypothesis on Trpa1-deficient mice subjected to inflammatory pain paradigms followed by application of Nocistatin in future studies.

Mechanistically, pharmacological action of Nocistatin may consist of direct or indirect modulation of TRPA1 biophysical properties. In our study, we used similar concentrations of Nocistatin as reported elsewhere in vitro and did not observe cellular calcium influx upon Nocistatin application on its own. Therefore, Nocistatin is unlikely to directly gate TRPA1 channels. Alternatively, Nocistatin could alter TRPA1 membrane expression or modulate intracellular signaling pathways/proteins thereby indirectly controlling TRPA1 activity. Nocistatin is known to physically bind NIPSNAP1, which appears to be required for the inhibition of N/OFQ-induced allodynia by Nocistatin. Interestingly, studies in different cellular systems provided evidence that members of the NIPSNAP family regulate the function of ion channels. For example, NIPSNAP1 associates with TRPV6 channels in mouse liver cells, which results in a dramatic inhibition of channel function. In addition, NIPSNAP2 has been shown to modulate L-type calcium channels and concomitantly downstream CREB signaling in a neuronal cell line. In light of these findings we hypothesized that Nocistatin-mediated TRPA1 sensitization might require NIPSNAP1, yet our experiments in NIPSNAP1-siRNA-treated DRG cultures did not provide supportive evidence for this assumption. While we cannot disregard the possibility that remaining NIPSNAP1 expression (i.e., approx. 22% on mRNA level, Fig. 2A) might be sufficient to drive Nocistatin-mediated TRPA1 sensitization, these new data may indicate that NIPSNAP1 is likely dispensable for Nocistatin-induced TRPA1 sensitization. Nocistatin and NIPSNAP1 influence nociception and pain synergistically (as reported elsewhere) as well as possibly independently. Indeed, evidence for independent actions of NIPSNAP1 and Nocistatin was previously suggested. A very recent study on the involvement of NIPSNAP1 in inflammatory pain proposes that exacerbation of inflammatory pain upon constitutive deletion of NIPSNAP1 could be independent of Nocistatin. In this context, further studies should investigate Nocistatin-induced TRPA1 sensitization in Nipsnap1-deficient mice to unambiguously confirm our observation.
Taken together, we identified Nocistatin as a modulator of TRPA1 physiology—a mechanism which potentially contributes to some of Nocistatin’s effects on pain in vivo.

**Material and methods**

**Reagents**

Mustard oil (AITC), capsaicin and DMSO were purchased from Sigma-Aldrich. Nocistatin (bovine, bNST) was purchased from Tocris. Growth factors were purchased from R&D Systems, and cell culture reagents from Life Technologies (Gibco) except poly-D-lysine (Millipore) and papain (Worthington).

**Immunocytochemistry**

Cultured DRG were fixed for 20 minutes in 4% PFA and blocked with 5 % donkey serum (Dianova) and 0.4% Triton X-100 in PBS for 1 h at room temperature, followed by incubation with primary antibody against NIPSNAP1 (rabbit; 1:100; Abcam) overnight at 4°C in 1% donkey serum and 0.1% Triton X-100 in PBS. Cells were then washed 5 times with PBS and incubated with anti-rabbit Alexa488 (donkey; Life Technologies) secondary antibodies (1:250) for 2 h at room temperature in 1% donkey serum and 0.1% Triton X-100 in PBS. Cells were washed again 5 times with PBS before mounting with SlowFade Gold reagent (Life Technologies).

**Immunohistochemistry**

Mice (ages 6–12 weeks) were sacrificed by CO2 inhalation. DRG were carefully dissected, collected in 4% PFA/PBS, and fixed overnight at 4°C. After overnight cryoprotection in 30% sucrose/PBS tissues were frozen in optimal cutting temperature compound (Sakura), sectioned as step serial sections with a cryostat at 10 μm width, mounted on SuperFrost Plus slides, and stored at −80°C. Frozen slides were thawed at room temperature for 30 minutes, washed 3 times in PBS with 0.4% Triton X-100 (PBT), blocked for 30 minutes in PBT containing 5% donkey serum (Dianova), and incubated overnight at 4°C with primary antibodies diluted in antibody solution (1% donkey serum and 0.1% Triton X-100 in PBS). Sections were stained with anti-NIPSNAP1 (rabbit; 1:200; Abcam) and 1:100 anti-Peripherin (chicken; 1:100; Abcam). The next day, after washing 5 times in PBS, corresponding secondary fluorescent antibodies (anti-rabbit Alexa488 (donkey; 1:250; Life Technologies) and anti-chicken Alexa555 (donkey; 1:250; Life Technologies)) were applied and incubated for 2 h at room temperature in antibody solution. Sections were then washed 5 times in PBT and mounted in SlowFade Gold reagent (Life Technologies).

**Image acquisition and analysis of immunostainings**

Digital images of stained cultures and DRG cryosections were obtained at an epifluorescence microscope (Zeiss Axio Observer Z1). Images for all experimental groups were taken using identical acquisition parameters. All groups to be compared were processed in parallel using the same culture or tissue preparation. Raw images were analyzed by using NIH ImageJ essentially as described previously. Briefly, cells were considered positive for their labeled protein (NIPSNAP1, Peripherin) if the mean fluorescence intensity (measured in arbitrary units, AU) was higher than the mean background fluorescence plus 3 times the standard deviation measured from at least 5 random unstained cells for the immunocytochemistry, and 10 random unstained cells for immunohistochemistry. Only for presentation purposes were brightness, contrast and levels of matched images adjusted in Adobe Photoshop (Adobe).

**Culture of mouse DRG neurons**

Preparation and culture of DRG neurons from adult (7–10 weeks of age) C57B6/J mice was performed as described previously. Untransfected neurons were grown for 16–24 h before being used for experiments. Transfection of neurons was achieved by nucleofection of siRNA into freshly isolated DRG neurons using the P3 Primary Cell 4D Nucleofector X Kit with the 4D-Nucleofector X Unit according to the manufacturer’s instructions (Lonza AG). For NIPSNAP1 knock-down a final concentration of 500 nM of NIPSNAP1 siRNA (Mix of 4 siRNA, Qiagen) or control siRNA (AllStar Negative control, Qiagen) were used to transfect cells. After nucleofection, neurons were allowed to recover in RPMI medium for 10 minutes at 37°C before plating in growth medium. Two hours after transfection half of the growth medium was exchanged with fresh medium and neurons were grown 60–72 h.
**RNA isolation and quantitative PCR (qPCR)**

Total RNA extraction was performed on DRG neurons 72 h after nucleofection with NIPSNAP1 siRNA or control siRNA (AllStar Negative control, Qiagen) using NucleoSpin RNA XS (Macherey-Nagel) according to the manufacturer’s instruction. 250 ng of total RNA were then used for first-strand cDNA synthesis using QuantiTect reverse transcription kit (Qiagen). Genomic DNA (gDNA) was eliminated adding gDNA wipe-out buffer and incubating the mix at 42°C for 6 minutes in a thermal cycler (Bio-Rad T100). Reverse transcription (RT) master mix (Quantiscript RT buffer, RT primer mix, Quantiscript reverse transcriptase) was then added, followed by incubation at 42°C for 30 minutes and 95°C for 3 minutes to finally obtain cDNA. NIPSNAP1 gene expression was assessed by real-time qPCR using the SYBR green system (Power SYBR Green PCR Master Mix; Life Technologies) in a LightCycler 480 instrument (Roche). The melting curve analysis of amplification was performed consisting of 1× HBSS (1.3 mM calcium) supplemented with 10 mM HEPES. A working solution of the ratiometric calcium indicator dye Fura-2 AM was prepared by resuspending 50 μg of Fura-2 AM cell permeant (Life Technologies) in 50 μl DMSO, adding 50 μl Pluronic F-127 (Life Technologies) and then diluting the mix 1:200 in imaging buffer. Cells were incubated with the dye for 30–60 minutes at 37°C, according to the manufacturer’s recommendations. Cells were then washed again 3 times prior to imaging. Fura-2 fluorescence was measured by alternating illumination at 340/380 nm. Fluorescence intensity was measured at 510 nm for both excitation wavelengths and the intracellular calcium concentration expressed as the A340/A380 ratio. All experiments were conducted at room temperature. For experiments examining the effect of Nocistatin on TRPA1-mediated responses the following protocol was used: 2 minutes application of 10 μM Nocistatin/vehicle, followed by washing with imaging buffer and application of 10 μM Nocistatin/vehicle in 25 μM MO for 4 minutes. After 5 minutes of washing 50 μM MO was applied for 2 minutes. Experiments testing the influence of Nocistatin on TRPV1-mediated responses: 2 minutes application of 10 μM Nocistatin/vehicle, followed by application of 10 μM Nocistatin/vehicle in 0.1 μM capsacin for 4 minutes. After 5 minutes of washing 1 μM capsacin was applied for 2 minutes. As nucleofection may compromise neuronal health, we introduced criteria to determine the health of the culture: Cultures were considered healthy and included in the analysis if (i) the majority of neuronal somata displayed sharp borders and (ii) a stable baseline could be determined with fluctuations less than 20% of the average baseline before the addition of the first stimulus.

For data analysis, threshold of activation was set at 20% above the baseline obtained from averaging 5 time points immediately before addition of each stimulus. All experimental groups to be compared were processed in parallel using the same culture preparation. At least 2 coverslips from 3 independent culture preparations were analyzed per experimental paradigm. The two-tailed unpaired Student’s t-test was used to evaluate statistical significance comparing 2 conditions, one-way ANOVA was used to compare 3 or more conditions. All values refer to mean ± SEM; p denotes the significance and refers to the respective control in each experimental group if not stated otherwise.

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