Histamine-mediated potentiation of transient receptor potential (TRP) ankyrin 1 and TRP vanilloid 4 signaling in submucosal neurons in patients with irritable bowel syndrome

Balemans, D; Aguilera-Lizarraga, J; Florens, M V; Jain, P; Denadai-Souza, A; Viola, M F; AGUIAR ALPIZAR, Yeranddy; Van Der Merwe, S; Vanden Berghe, P; Talavera, K; Vanner, S; Wouters, M M & Boeckxstaens, G E (2019) Histamine-mediated potentiation of transient receptor potential (TRP) ankyrin 1 and TRP vanilloid 4 signaling in submucosal neurons in patients with irritable bowel syndrome. In: American journal of physiology. Gastrointestinal and liver physiology, 316 (3) , p. G338-G349 -G349.

DOI: 10.1152/ajpgi.00116.2018
Handle: http://hdl.handle.net/1942/30194

Histamine-mediated potentiation of TRPA1 and TRPV4 signaling in submucosal neurons in IBS patients

Running head: Histamine-mediated TRPA1 and TRPV4 sensitization in IBS

Balemans D[1]*, Aguilera-Lizarraga J[1]*, Florens MV[1], Jain P[1], Denadai-Souza A[1], Viola MF[1], Alpizar YA[2,3], Van Der Merwe S[4], Vanden Berghe P[1], Talavera K[2,3], Vanner S[5], Wouters MM[1], Boeckxstaens GE[1]

1 KU Leuven Department of Chronic Diseases, Metabolism and Ageing; Translational Research Center for Gastrointestinal Disorders, Leuven, Belgium.

2 KU Leuven Department of Cellular and Molecular Medicine, Laboratory of Ion Channel Research and TRP channel Research Platform (LICR), Leuven, Belgium

3 VIB Center for Brain and Disease Research, KU Leuven, Belgium

4 KU Leuven Department of KU Leuven Department of Chronic Diseases, Metabolism and Ageing; Hepatology, University Hospital Leuven, Leuven, Belgium

5 Gastrointestinal Diseases Research Unit (GIDRU), Kingston General Hospital, Queen's University, Kingston, Canada

*These authors contributed equally to this work.

Correspondence address:

Boeckxstaens GE, PhD, MD

KU Leuven Department of Chronic Diseases, Metabolism and Ageing; Translational Research Center for Gastrointestinal Disorders, Leuven, Belgium

Herestraat 49, 3000 Leuven, Belgium

E-mail: guy.boeckxstaens@kuleuven.be

Tel: +32 16 33 02 37; Fax: +32 16 33 07 23

Abstract

Previously, we showed histamine-mediated sensitization of TRP vanilloid 1 (TRPV1) in patients with irritable bowel syndrome (IBS). Sensitization of TRP ankyrin 1 (TRPA1) and TRP vanilloid 4 (TRPV4) are also involved in aberrant pain perception in preclinical models of somatic pain. Here, we hypothesize that in parallel with TRPV1, histamine sensitizes TRPA1 and TRPV4, contributing to increased visceral pain in patients with IBS. Rectal biopsies were collected from IBS patients and healthy subjects (HS) to study neuronal sensitivity to TRPA1 and TRPV4 agonists (cinnamaldehyde and GSK1016790A) using intracellular Ca^{2+} imaging. In addition, the effect of supernatants of rectal biopsies on IBS patients and HS was assessed on TRPA1 and TRPV4 responses in murine dorsal root ganglia (DRG) sensory neurons. Finally, we evaluated the role of histamine and histamine 1 receptor (H_1R) in TRPA1 and TRPV4 sensitization. Application of TRPA1 and TRPV4 agonists evoked significantly higher peak amplitudes and percentage of responding submucosal neurons in biopsies of IBS patients compared to HS. In HS, pretreatment with histamine significantly increased the Ca^{2+} responses to cinnamaldehyde and GSK1016790A, an effect prevented by H_1R antagonism. IBS supernatants, but not of HS, sensitized TRPA1 and TRPV4 on DRG neurons. This effect was reproduced by histamine and prevented by H_1R antagonism. We demonstrate that the mucosal microenvironment in IBS contains mediators, such as histamine, which sensitize TRPV4 and TRPA1 via H_1R activation, most likely contributing to increased visceral pain perception in IBS. These data further underscore H_1R antagonism as potential treatment for IBS.

Key words: TRP channels; sensitization; histamine 1 receptor, visceral hypersensitivity

New and Noteworthy

We provide evidence for histamine-mediated TRPA1 and TRPV4 sensitization in IBS via histamine 1 receptor activation, most likely contributing to increased visceral pain perception. Our results reveal a general role of sensory TRP channels as histamine effectors in the pathophysiology of IBS, and provide novel mechanistic insights into the therapeutic potential of H_1R antagonism in IBS.

Introduction

Irritable bowel syndrome (IBS) is the most frequently diagnosed disorder by gastroenterologists worldwide, affecting over 10% of the western population (19). IBS is a functional gastrointestinal disorder characterized by recurrent abdominal pain or discomfort associated with altered defecation pattern in the absence of an organic cause (33). Visceral hypersensitivity (VHS) or aberrant pain perception is present in up to 60% of the patients and represents one of the hallmarks of IBS (9, 25, 30). The underlying pathophysiological mechanism of VHS is however not fully understood.

Upregulation and/or sensitization of nociceptors, in particular of transient receptor potential (TRP) channels, is recognized to play a major role in somatic pain (29, 31). For example, potentiation of TRP vanilloid 1 (TRPV1), TRP vanilloid 4 (TRPV4) and TRP ankyrin 1 (TRPA1) induces mechanical and thermal hyperalgesia in mice treated with the chemotherapeutic drug paclitaxel (15). Furthermore, TRPV4 sensitization and upregulation in trigeminal sensory neurons was described in an inflammatory model of temporomandibular joint pain (14). In parallel to their role in somatic pain perception, altered TRP channel function is also recognized as an important mechanism underlying aberrant visceral pain (20). Especially TRPV1, the capsaicin receptor, has repeatedly been shown to be involved in VHS. TRPV1 expression is increased in preclinical models of VHS (1, 32), while sensitivity to colorectal distention is decreased by TRPV1 antagonists (41, 42) and reduced in *Trpv1* knock-out mice (23).

Similar to TRPV1, evidence is accumulating supporting an important role for TRPV4 in visceral pain. TRPV4 can be activated by mechanical force, osmotic pressure or innocuous temperature (27-34°C). Intracolonic infusion of supernatants from IBS biopsies, but not from

healthy subjects (HS), induced VHS in mice, while knockdown of TRPV4 inhibited this hypersensitivity (13). Furthermore, human serosal nociceptor mechanosensitivity is attenuated by application of the TRPV4 antagonist HC067047, further underscoring the potential role of TRPV4 in visceral pain perception (27). Along the same line, TRPA1 is suggested to play a role in visceral pain in preclinical models (7, 10, 16). TRPA1 is activated by cold, pungent compounds such as allyl isothiocyanate (AITC), and mechanical distention. Intracolonic administration of AITC in rodents results in an increased visceromotor response, which is absent in *Trpa1* knock-out mice (7, 10, 16). Taken together, these data demonstrate that TRPA1 and TRPV4 are involved in VHS.

Altered TRP channel function can be induced by several pro-inflammatory factors, including mast cell mediators that play an important role in IBS (3, 5, 20). Previously, we showed that sensitization of TRPV1 in rectal submucosal neurons of IBS patients was produced by the mast cell mediator histamine via activation of histamine 1 receptor (H_1R) (4, 44). Furthermore, treatment of IBS patients with the H_1R antagonist ebastine resulted in significant improvement of abdominal pain (44) suggesting that H_1R-mediated sensitization of TRPV1 and possibly other TRP channels underlies increased abdominal pain in IBS. In line with this, histamine can also sensitize TRPV4 both *in vitro* and *in vivo* resulting in VHS in mice (12). Although submucosal neurons are not involved in visceral pain perception, it should be emphasized that visceral afferent sensory neurons reside in the same environment and thus will be exposed to the same environmental triggers. Therefore, evaluation of submucosal neurons in biopsies indirectly support a role for neuronal sensitization in IBS. To date, human data supporting a role for histamine-driven TRPA1 and TRPV4 potentiation in IBS patients is however lacking.

Based on the data above, we hypothesized that histamine-induced TRPA1 and TRPV4 sensitization, in parallel to TRPV1, could be involved in VHS in patients with IBS, thereby explaining the previously reported beneficial effect of the H_1R antagonist ebastine in IBS (44). To test this, we compared the response of IBS and HS rectal submucosal neurons to TRPA1 and TRPV4 agonists. Moreover, we assessed the ability of histamine and IBS biopsy supernatants to sensitize these TRP channels in primary cultured murine dorsal root ganglion (DRG) neurons.

Materials and Methods

Study subjects: Healthy subjects (HS, n=38, median age=24 years, IQR=23-47, 21F) were recruited by public advertisement, were free of abdominal symptoms, had no history of gastrointestinal disease, no previous gastrointestinal surgery and were not on gastrointestinal medication. IBS patients (n=39, median age=31 years, IQR=24-53, 30F) were recruited from the outpatient clinic of the University Hospital Leuven and had to fulfill the ROME III criteria for IBS. All participants were invited to undergo a proctoscopy to collect rectal biopsies. Not every subject participated to all sub-protocols described below. Ethical committee of the University Hospitals Leuven approved the protocols (ref. S55484). Informed consent was obtained from all participants.

Rectal proctoscopy and biopsy preparation: During the rectal proctoscopy, biopsies were taken by experienced endoscopists using standard biopsy forceps (single-use biopsy forceps without pin; Onis, Lasne, Belgium). After collection, biopsies were immediately immersed in ice-cold (4°C) Krebs solution (in mM: 120.9 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 11.5 glucose, 14.4 $NaHCO_3$ and 1.2 NaH_2PO_4) previously oxygenated (95% oxygen/5% carbon dioxide), and kept on ice for transport. Biopsies were subsequently carefully stretched and pinned flat in a

Sylgard-lined Petri dish and dissected under a stereomicroscope while continuously perfused with oxygenated (95% oxygen/5% carbon dioxide) ice-cold Krebs solution. The inner submucous layer was carefully removed from the mucosa using watchmaker's forceps. Then, the tissue was gently stretched and pinned flat in a special recording chamber (own design) in which in and outflow volume could be tightly controlled.

Ca^{2+} imaging of human submucosal neurons: Submucosal plexuses were loaded with 1 µM Fluo-4 AM (Molecular Probes, Invitrogen, Merelbeke, Belgium) to perform intracellular Ca^{2+} imaging as previously described (17, 44). Next, the recording chamber was mounted onto an upright Zeiss Examiner microscope equipped with a 20× (NA 1) water dipping lens and coupled to a monochromator (Poly V) and cooled CCD camera (Imago QE) both from TILL Photonics (Gräfelfing, Germany). A gravity-fed perfusion system ensured continuous and constant perfusion (1 mL/min) of the preparation with 95% oxygen/5% carbon dioxide-gassed Krebs solution (at room temperature) and excess solution was removed via a peristaltic suction pump, which kept the experimental volume constant (3 mL). Fluo-4 was excited at 475 nm, and its fluorescence emission was collected at 525/50 nm. Images were acquired at 2 Hz and collected by TillVision software (TILL Photonics, Oberhausen, Germany). Data were analyzed by custom-written macros in IGOR PRO (Wavemetrics, Lake Oswego, Oregon, USA). Neurons within ganglia were selected based on Fluo-4 signal and morphology (big round shape, surrounded by glial cells and enclosed within nerve fibers). These were only included in the analysis when a sharp Ca^{2+} response was displayed after perfusion with high-K^{+} concentration (75nM) (Figure 1A, B). Thus, any other intra-ganglionic non-neuronal cell was excluded. Moreover, neurons over- or under-lapped by a nerve fiber or blood vessels were excluded to avoid non-specific Ca^{2+}. Regions of interest were drawn over each

neuron, fluorescence intensity was normalized to the basal fluorescence at the onset of the recording for each region of interest and peaks were analyzed. Background auto-fluorescence and the bleaching was thereof corrected using a Runge-Kutta iterative deconvolution algorithm assuming monoexponential fluorescence decay. Fluorescence intensities were normalized and expressed as a ΔF/F0 ratio (F0 = baseline fluorescence) and percentage of responsive neurons.

The responses of HS and IBS submucosal neurons to the TRPA1 agonist cinnamaldehyde (CA; 10 nM and 1 µM; Sigma-Aldrich, Diegem, Belgium) and to the TRPV4 agonist GSK1016790A (0.1 and 1 nM; Sigma-Aldrich) were compared. The perfusion rate was 1 mL/min for 5 seconds. In addition, we evaluated the effects of 10min and overnight pre-incubation with 10-100 µM histamine (Sigma-Aldrich) on the responses of HS submucosal neurons to CA (10 nM) and GSK1016790A (0.1 nM). Finally, we also evaluated the TRPA1- and TRPV4-mediated responses after pre-incubation with 10 µM histamine combined with 1 µM pyrilamine (Sigma-Aldrich) in HS submucosal neurons.

Animals: All animal experiments were carried out in accordance to the European Community Council guidelines and were approved by the local ethics committee of the KU Leuven (ECD P157/2014). Ten- to 12-week-old male mice were used in all experiments. C57Bl6 mice were purchased from Janvier Lab (France) and *Hrh1* knock-out (KO) mice from Oriental Bioservice, INC (Kyoto, Japan). In addition, *Trpv1* knock-out mice were obtained from The Jackson Laboratory (http://jaxmice.jax.org/strain/003770.html). *Trpv1*/*Trpv4* double KO mice were obtained from an in-house breeding program. All knockout mice were backcrossed at least 10 times in the C57Bl/6 background. Mice were housed under identical conditions, with a

maximum of four animals per cage on a 14/10-hours light/dark cycle and with food and water ad libitum.

Ca^{2+} imaging of murine DRG neurons: The lumbosacral (L5-S2) dorsal root ganglia (DRG) from 3 to 4 adult mice were bilaterally excised under a dissection microscope. The ganglia were washed in 10% fetal calf serum Neurobasal A medium (basal medium) and then incubated (95% air, 5% CO_2) at 37°C in a mix of collagenase of 1 mg/mL (Gibco, Gent, Belgium) and dispase of 2.5 mg/mL (Gibco) for 45 min. Digested ganglia were gently washed twice with basal medium and mechanically dissociated in B27-supplemented (2%) Neurobasal A medium (Invitrogen, Gent, Belgium) containing GDNF of 2 ng/mL (Invitrogen, Gent, Belgium), NT4 of 10 ng/mL (Peprotech, London, UK), 100 µg/mL penicillin/streptomycin (Invitrogen, Gent, Belgium) and Glutamax (Invitrogen) (complete medium). Neurons were seeded on poly-L-ornithine or poly-D-lysine/laminin-coated glass coverslips and cultured for 12-18 h at 37°C. Cultured DRG neurons were subsequently loaded with 2 µM Fura-2AM for 20 min at 37°C.

DRG neurons were exposed to 10 µM CA before and after acute application (10 min) of histamine (10 µM). These experiments were repeated in *Hrh1* knock-out mice or in the presence of the H_1R antagonist pyrilamine (1 µM) in wild-type (WT) mice. TRPA1-expressing neurons were identified by application of 300 µM CA at the end of the protocol. The response of DRG neurons to GSK1016790A (1 µM) was determined before and after acute (10 min) incubation with 10 µM histamine. In other experiments, DRG neurons were incubated overnight with vehicle (Krebs) or 10/100 µM histamine and exposed to 1 µM GSK1016790A. These experiments were performed in the presence of the TRPV1 antagonist SB-366791 or in cells isolated from *Trpv1* knock-out mice, since high doses of GSK1016790A

196 can activate TRPV1. In accordance with TRPA1, these experiments were repeated in *Hrh1*
197 knock-out mice or in the presence of pyrilamine (1 µM) in WT mice.
198 Finally, rectal biopsies from HS and IBS patients were overnight (ON) incubated in RPMI
199 (Lonza, Verviers, Belgium) supplemented with fetal calf serum (10%) (Pan Biotech,
200 Aidenbach, Germany), Penicillin/Streptomycin (1%) (Lonza, Verviers, Belgium) and
201 Amphotericin B/gentamycin (0.2%) (Invitrogen, Gent, Belgium) at 37°C, 5% CO_2. Twenty-four
202 hours later, supernatants were collected and stored in -80°C until murine DRG neurons were
203 incubated ON with 142 µL of these supernatants derived from either HS or IBS patients in
204 the presence or absence of histamine (10 µM for TRPA1 activation or 100 µM for TRPV4
205 activation) with or without pyrilamine (1 µM). Thereafter, we evaluated the responses of
206 these cells to CA (10 µM) and GSK1016790A (1 µM in the presence of 1 µM SB-366791).
207 The intracellular Ca^{2+} measurements were performed using a monochromator-based
208 imaging system consisting of either a Polychome IV monochromator (Till Photonics,
209 Martinsried, Germany) and a Roper Scientific (Tucson, AZ, USA) CCD camera connected to a
210 Zeiss (Oberkochen, Germany) Axiovert 200M inverted microscope, or on an Olympus (Tokyo,
211 Japan) CellMˆ system. The fluorescence intensity was measured during excitation at 340 and
212 380 nm, and the ratio of the fluorescence intensity at both excitation wavelengths
213 (F340/F380) was monitored. Intracellular Ca^{2+} concentrations were determined as previously
214 described (39). Experiments were performed using standard Krebs solution (containing in
215 mM: 120.9 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 11.5 glucose, 14.4 $NaHCO_3$ and 1.2 NaH_2PO_4).
216 To identify neurons in the DRG cultures we applied a Krebs–based solution in which the KCl
217 concentration was increased to 45 mM by iso-osmotic substitution of NaCl. The baseline was
218 monitored for 120 s and the chamber was thereafter superfused with the TRP agonists.

RNA extraction and RT-qPCR: RNA was extracted from mouse DRG which were overnight incubated with histamine (10 – 100 µM) and from 5 – 10 mg human rectal biopsies which were stored in RNA later (Qiagen Benelux, Venlo, The Netherlands), using RNeasy minikit (Qiagen, Hilden, Germany). cDNA of 2 µg total RNA was synthesized using qScript cDNA supermix (Quanta Biosciences, Gaithersburg, Maryland, USA) according to the manufacturer's instructions. RT-qPCR was performed for quantification of neuronal TRPV4 and TRPA1 mRNA expression FastStart Essential DNA Green Master (Roche GmBH, Mannheim, Germany) relative to the housekeeping gene β-actin (primer sequences are listed in Table 2). Wells of an AmpliStar 96 Well LC480 QPCR Plate (Westburg, Leusden, The Netherlands) were loaded with 2.5 µL of each cDNA sample together with 5 µL FastStart Essential DNA Green Master (Roche GmBH, Mannheim, Germany), 0.2 µL oligonucleotides (10 µM) and 2.3 µL RNase Free Water (Applied Biosystems, Halle, Belgium). Gene expression was normalized to the endogenous reference gene β-actin and the relative gene expression was calculated as $2^{-\Delta\Delta Ct}$ (26).

Immunohistochemistry: To evaluate the translocation of TRPA1 and TRPV4 to the membrane upon stimulation, cultured DRG neurons were incubated with 10-100 µM histamine or vehicle for 10 minutes or overnight (for TRPA1 and TRPV4, respectively). Cells were then fixed in PFA 4% for 15 minutes before permeabilization in Triton 0.1% for 10 minutes. After blocking in 5% Donkey serum for 3h, cells were stained with rabbit anti-TRPA1 (1:200, Alomone Labs, Jerusalem, Israel) or rabbit anti-TRPV4 (1:200, Alomone Labs, Jerusalem, Israel) overnight. After washing, cells were incubated with goat anti-rabbit Cy3 (1:500, Jackson ImmunoResearch, West Grove, PA, USA) for 2 hours and DAPI for 15 minutes.

Confocal images were taken with a Zeiss LSM510 confocal microscope in the Cell Imaging Core (CIC), University of Leuven.

Translocation of TRPA1 or TRPV4 to the membrane of the cell was measured with ImageJ, as a ratio of the TRPA1 or TRPV4 fluorescence intensity (expressed as mean gray value) in the membrane area on the TRPA1 or TRPV4 fluorescence intensity in the cytoplasm, both normalized to the background. Total TRPA1 and TRPV4 was quantified combining the TRPA1 or TRPV4 fluorescence intensity in the membrane and in the cytoplasm. Areas of interest were defined using phase contrast images of the cells.

Statistics: All statistical analyses were performed using Graphpad Prism 7.04 (La Jolla, USA). Continuous data were summarized by their mean and standard deviation. When deviations from normality were observed by the use of Shapiro-Wilk normality test, medians and interquartile values were presented. Comparisons between groups were made using a t-test or Wilcoxon rank-sum test, as appropriate. Statistical significance is assumed when $p \leq 0.05$ after Bonferroni correction for multiple testing.

Statistical analyses of the peak F340/380 ratios for the Ca^{2+} imaging experiments were performed after correction for the individual baseline Ca^{2+}, using Graphpad Prism. Values are expressed as means ± SEM from n cells. When deviations from normality were observed by the use of Shapiro-Wilk normality test, medians and interquartile values were presented. Statistical comparisons were performed by a Wilcoxon signed rank test (for 2 groups) or Mann–Whitney U test as appropriate or ANOVA (for more than 2 groups). Categorical data were analyzed by Fischer's exact test.

Results

Study subjects

A total of 39 IBS patients fulfilling the ROME III criteria and 38 HS were included. Age and gender did not differ between IBS patients (n=39, median age=31 years, IQR=24-53, 30F) and HS (n=38, median age=24 years, IQR=23-47, 21F). 18 patients with diarrhea predominant IBS (IBS-D), 7 patients with constipation predominant IBS (IBS-C) and 4 patients with mixed IBS (IBS-M) were included in the IBS group. 10 patients were classified as unsubtyped (IBS-U). Demographic data are summarized in Table 1.

Sensitization of TRPA1 and TRPV4 on human submucosal neurons in IBS

Rectal biopsies of IBS patients and HS were collected to compare the response of submucosal neurons to the TRPA1 agonist CA (10 nM and 1 µM) and the TRPV4 agonist GSK1016790A (0.1 and 1 nM). Application of CA (10 nM and 1 µM) and GSK1016790A (0.1 and 1 nM) induced significantly higher Ca^{2+} responses in submucosal neurons of IBS patients compared to those of HS (Figure 1C, D). Furthermore, exposure to CA (10 nM and 1 µM) and GSK1016790A (0.1 and 1 nM) activated more submucosal IBS patients neurons than HS ones (Figure 1C, D).

Then, to evaluate if the increased response to TRPA1 and TRPV4 agonists resulted from upregulation of *TRPA1* and *TRPV4*, mRNA expression levels of both TRP channels were evaluated in 30 HS and 30 IBS biopsies. No differences in mRNA TRPA1 and TRPV4 expression could be detected (Figure 2). In addition, *TRPA1* and *TRPV4* mRNA expression was not different between IBS subtypes (data not shown). Furthermore, none of the IBS patients with submucosal TRPA1 and/or TRPV4 sensitization (n=6) had increased *TRPA1* and *TRPV4* mRNA expression (data not shown). Taken together, these results suggest that the increased Ca^{2+} response for these TRP channels is due to sensitization rather than to upregulation.

Recently, our group showed that sensitization of TRPV1 in IBS is mediated by the mast cell mediator histamine (44). To determine if histamine is also involved in the sensitization of TRPA1 and TRPV4, the effect of histamine (10 µM) incubation was assessed on the response to CA (10 µM) and GSK1016790A (1 µM). Of note, rectal submucosal neurons of HS pre-treated with 10 µM histamine showed increased amplitude of the Ca^{2+} response and an increased percentage of neurons responding to CA (10 nM) and GSK1016790A (0.1 nM) (Figure 3A, B).

Histamine 1 receptor is implicated in histamine-mediated sensitization of TRPA1 and TRPV4 in human submucosal neurons

As we previously reported clinical improvement in IBS patients receiving H_1R antagonist ebastine (44), we next assessed the involvement of H_1R in histamine-mediated sensitization of TRPA1 and TRPV4 in human submucosal neurons. Of interest, pretreatment with pyrilamine (1 µM) indeed prevented the histamine-induced increase in Ca^{2+} response and number of responding neurons to the respective TRP channel agonists (Figure 3A, C).

Sensitization of TRPA1 and TRPV4 on murine DRG neurons

Although we showed TRP channel sensitization of submucosal neurons in IBS, it should be emphasized that these neurons are not involved in visceral pain perception. Yet, visceral afferent sensory neurons reside in the same environment and thus will be exposed to the same environmental triggers. To test the hypothesis that bioactive mediators in the micro-environment may also affect visceral afferents, we assessed the effect of the supernatants of

biopsies on isolated murine DRG neurons. Overnight incubation of DRG neurons with IBS supernatants of 6 out of 8 patients significantly increased the Ca^{2+} response to CA compared to HS (Figure 4A). Moreover, the number of neurons responding to CA was significantly increased by supernatants of 7 out of 8 patients compared to HS. Similarly, IBS supernatants of 2 of the 8 IBS patients significantly increased the Ca^{2+} response to the TRPV4 agonist GSK1016790A, while the supernatants of 3 IBS patients activated significantly more neurons compared to those of HS (Figure 4B).

In line with the effect of histamine on human submucosal neurons, pre-incubation of murine DRG neurons for 10 min with histamine resulted in an increased Ca^{2+} response and number of responding neurons to CA (Figure 5) compared to vehicle. This effect was not observed for GSK1016790A (data not shown). However, longer incubation (overnight incubation) of DRG neurons with 100 µM, but not 10 µM (data not shown), of histamine resulted in an increased response to GSK1016790A (Figure 6). To confirm that GSK1016790A (1 µM) does not activate TRPV1 (37), even at high doses (1 µM), the experiments were repeated in the presence of the TRPV1 antagonist SB-366791 (1 µM) and in cells isolated from *Trpv1* knock-out mice (Figure 6B, C). Moreover, sensitization of TRPA1 and TRPV4 was absent in *Trpa1*$^{-/-}$ (data not shown) and double *Trpv1*$^{-/-}$*Trpv4*$^{-/-}$ knock-out mice, respectively (Figure 6B, C). In keeping with these findings, overnight incubation of DRG neurons with HS supernatants supplemented with histamine increased the Ca^{2+} response to both TRP agonists (Figure 7A).

To assess if the increased TRPA1- and TRPV4-mediated Ca^{2+} responses after histamine treatment in DRG neurons resulted from upregulation of *Trpa1* and *Trpv4*, we compared their mRNA expression levels in cells incubated 10 min (10 µM, for TRPA1) or overnight (100 µM, for TRPV4) with histamine or vehicle. In line with the results obtained with rectal

biopsies, no differences in mRNA expression could be detected (data not shown). Moreover, 10 min incubation with histamine did not increase translocation or the total amount of TRPA1 (Figure 8). On the other hand, overnight incubation with histamine increased TRPV4 protein expression and translocation to the membrane in DRG neurons (Figure 8), in line with previous studies (12).

Histamine 1 receptor is implicated in histamine-mediated sensitization of TRPA1 and TRPV4 in murine DRG neurons

Next, we investigated if, similar to our results in human submucosal neurons, H_1R is involved in in the histamine-mediated sensitization of TRPA1 and TRPV4 in murine DRG neurons. Therefore, we first tested the effect of the H_1R antagonist pyrilamine on sensitization of TRPA1 and TRPV4 by IBS supernatants in DRG neurons. Pyrilamine (1 μM) prevented TRPA1 and TRPV4 sensitization in DRG neurons pre-treated with IBS supernatants (Figure 7B). Similarly, the sensitizing effect of overnight incubation of DRG neurons with HS supernatants supplemented with histamine (TRPA1: 10 μM; TRPV4: 100 μM) was blocked by pyrilamine (1 μM) for both TRP channels (Figure 7A). In addition, pyrilamine (1 μM) significantly reduced the response to CA and GSK1016790A in DRG neurons pre-treated with histamine for 10min (TRPA1: 10 μM; TRPV4: 100 μM) (Figure 5 and 6) and histamine pre-incubation did not induce TRPA1 or TRPV4 sensitization in DRG neurons lacking H_1R (Figure 5 and 6), confirming the key role of H_1R in this process.

Discussion

In the present study, we provide the first evidence for TRPA1 and TRPV4 sensitization in the rectal submucosal plexus of IBS patients, an effect mediated by the mast cell mediator histamine via activation of H_1R. Moreover, histamine and IBS biopsy supernatants sensitized TRPA1 and TRPV4 on murine DRG neurons via H_1R activation. These results indicate that not only TRPV1 (4, 44) but also TRPA1 and TRPV4 are involved in the pathophysiology of IBS, further underscoring the concept that histamine-mediated TRP channel sensitization is an important mechanism in IBS. Moreover, our data provide further evidence underscoring H_1R antagonism (44) as a novel therapeutic approach for IBS.

Although the exact pathophysiological mechanisms in IBS are still incompletely understood, upregulation of TRP channel expression or altered TRP channel function have been shown to underlie aberrant visceral pain perception in preclinical models (10, 11, 20, 40, 41). Moreover, we recently showed that TRPV1 sensitization plays an important role in VHS in IBS patients (4, 40, 44). However, to date, data supporting the involvement of sensitization of other TRP channels in IBS is lacking. In the present study, we provide evidence for TRPA1 and TRPV4 sensitization in IBS. Application of the TRPA1-agonist CA and TRPV4-agonist GSK1016790A induced significantly higher Ca^{2+} responses in rectal submucosal neurons of IBS patients compared to those of HS. To what extent these data support a role in abnormal pain perception in IBS can be questioned, especially as, to date, submucosal neurons have not been shown to be directly involved in visceral pain transmission. Nevertheless, our findings indicate that the gut microenvironment contains bioactive mediators that significantly affect neural signaling. Afferent nerve endings of nociceptive DRG neurons, transmitting pain signals to the spinal cord, reside in the same “sensitizing” microenvironment as submucosal neurons, and thus may be similarly affected. We indeed

379 recently demonstrated sensitization of murine colonic afferents to mechanical probing by
380 IBS supernatant (4), suggesting pro-nociceptive changes in the gut micro-environment. Along
381 the same line, intracolonic administration of IBS-D biopsy supernatants induced VHS in mice
382 through a TRPV4 dependent mechanism (13). Moreover, increased neuronal excitability of
383 DRG neurons in response to IBS supernatant has been repeatedly reported (8, 13, 22, 38,
384 44). In the present study, we provide additional evidence that IBS supernatant contains
385 mediators not only sensitizing TRPV1 (44), but also TRPA1 and TRPV4, clearly illustrating that
386 pro-nociceptive mediators are released by biopsies collected from IBS patients. We
387 therefore propose that these mediators not only affect the excitability of submucosal
388 neurons in IBS, but also of visceral afferents residing in the same micro-environment. To
389 date, we have only access to human submucosal neurons to unravel the underlying
390 mechanism in IBS patients. Human nociceptive neurons including colonic/rectal afferent
391 nerves and dorsal root ganglia (DRG) from surgical resections can be collected from patients
392 undergoing surgery, however these patients do not suffer from IBS. Thus, these tissues can
393 merely be used to characterize human TRP channels and investigate TRP channel
394 sensitization by inflammatory mediators including histamine. Interestingly, a recent study
395 showed decreased human serosal nociceptor mechanosensitivity after incubation with the
396 TRPV4 antagonist HC067047, further underscoring the role of TRPV4 in human visceral pain
397 perception (27). Taken together, we propose that sensitization of TRPV1, TRPV4 and TRPA1
398 represents one of the mechanisms contributing to aberrant pain signaling in IBS.
399 A plethora of pro-inflammatory mediators induce modulation of TRP channels on peripheral
400 sensory nerve endings leading to increased pain perception (31) but there is increasing
401 evidence that histamine could be particularly important. Recently, we showed that

402 treatment of IBS patients with H_1R antagonist ebastine improved abdominal pain, possibly
403 by blocking histamine-mediated sensitization of TRPV1 (44). Here, we show that histamine is
404 also involved in TRPV4 and TRPA1 sensitization. The observed increase in TRPA1 and TRPV4
405 Ca^{2+} responses reported here can result from either increased synthesis of the TRP channels,
406 translocation of more receptors to the cell membrane or due to phosphorylation and
407 subsequent sensitization. Although it should be emphasized that not only neurons express
408 TRP channels, we failed to show an increase in TRPA1 and TRPV4 mRNA levels in mucosal
409 biopsies from IBS compared to HV. Moreover, mRNA expression levels of TRPA1 and TRPV4
410 were not altered in murine DRG neurons incubated with histamine. In contrast, using
411 immunohistochemistry, we were able to demonstrate that histamine promoted
412 translocation of TRPV4 to the cell membrane of murine DRG neurons, as previously
413 demonstrated (12). Cenac et al. further showed that TRPV4 plasma membrane relocation is
414 mediated via a specific MAPKK pathway. Of interest, we did not observe translocation of
415 TRPA1 in response to histamine, indicating that sensitization of TRPA1 most likely explains
416 TRPA1 potentiation. Sensitization of TRP channels via coupling with G-protein coupled
417 receptors such as histamine receptors (3, 6, 43) has been repeatedly demonstrated in
418 sensory neurons, a mechanism mediated by stimulation of the phospholipase C/protein
419 kinase C signaling pathway with phosphorylation of the TRP channels (12, 24, 34, 35). Of
420 interest, the increased TRPV4 Ca^{2+} response induced by histamine is also dependent on this
421 pathway (12), suggesting that increased TRPV4 signaling might result from both receptor
422 relocation and sensitization.
423 Of interest, we observed that sensitization of TRPV4 requires prolonged incubation with
424 histamine. Indeed, sensitization of TRPA1 and TRPV1 (44) in murine DRG neurons was

425 already induced with 10 µM of histamine after 10 min while only overnight incubation with
426 100 µM histamine sensitized TRPV4. In line with our results, Cenac et al. showed TRPV4
427 sensitization in DRG neurons but only with higher (50 and 100 µM) concentrations of
428 histamine (12). Moreover, potentiation of TRPV4 on murine DRG neurons by other
429 inflammatory mediators was different compared to TRPV1 and TRPA1 sensitization. For
430 example, TRPV4 potentiation by serotonin required a higher dose (12) compared to
431 serotonin-induced TRPV1 sensitization on DRG neurons (36). On the other hand, TRPV4
432 sensitization via protease-activated receptor 2 (PAR-2) was induced after a longer incubation
433 period of PAR-2 agonists (21) compared to TRPV1 (2) and TRPA1 (18). These results suggest
434 that sensitization of TRPV4 requires a longer incubation period and/or a higher
435 concentration of mediators such as histamine compared to TRPV1 (44) and TRPA1, and
436 might explain why only 2 out of 8 IBS supernatants were able to sensitize TRPV4. Further
437 investigation to explain the differences in TRPV1, TRPA1 and TRPV4 sensitization is however
438 warranted.
439 Taken together, our data indicate that the intestinal microenvironment in IBS contains
440 histamine and/or histamine metabolites which, in parallel to TRPV1 (4, 44), sensitizes TRPA1
441 and TRPV4 via H_1R activation, contributing to VHS in IBS. These data further underscore H_1R
442 antagonism as potential treatment for IBS. Stratifying IBS patients for a specific treatment is
443 of particular importance in IBS as the patient population is very heterogeneous and includes
444 patients with different underlying mechanisms. This most likely explains why not all IBS
445 supernatants were able to sensitize TRP channels and why not all IBS patients respond to
446 H_1R antagonism (44). Therefore, identifying an indicator that can predict the therapeutic
447 response to H_1R antagonism would represent a major step forward. Interestingly, a recent

clinical trial showed that urinary histamine concentrations could predict the therapeutic response to low fermentable oligosaccharides, disaccharides and monosaccharides and polyols diet in IBS patients (28). Together with our results, measuring concentrations of histamine and/or its metabolites in patient samples could be helpful in the future to predict whether this patient would respond to H_1R antagonism counteracting TRP channel sensitization.

In summary, we provide evidence for histamine-mediated TRPA1 and TRPV4 sensitization in IBS via H_1R activation, most likely contributing to increased visceral pain perception. These results reveal a general role of sensory TRP channels as histamine effectors in the pathophysiology of IBS, and provide novel mechanistic insights into the therapeutic potential of H_1R antagonism in IBS (44).

Acknowledgements

All authors read and approved the final version of the manuscript.

DB: study concept and design, inclusion and characterization of patients, acquisition of data, analysis and interpretation of data; drafting of the manuscript

DB, JA, MVF, PJ, ADS, YAA: acquisition, analysis and interpretation of data

PVdB, KT, SV: interpretation of data and critical revision of the manuscript for important intellectual content

MMW, GEB: study concept and design, study supervision, obtained funding, critical revision of the manuscript for important intellectual content

470 We thank the Cell and Tissue Imaging Cluster for the use of the NIKON inverted TillVision
471 microscope, epifluorescence microscope BX 41 Olympus and Zeiss LSM880 – Airyscan
472 confocal microscope. Cell and Tissue Imaging Cluster, CIC, Supported by Hercules
473 AKUL/15/37_GOH1816N and FWO G.0929.15 to Prof. Pieter Vanden Berghe, KU Leuven.

474 **Grants**

475 GEB was funded by a governmental grant (Odysseus programme, G·0905·07) of the Research
476 Foundation - Flanders (FWO) and by a KU Leuven university grant (Global Opportunities for
477 Associations GOA 14.011). MMW and YAA are supported by FWO postdoctoral fellowships
478 (1248513N and 12H8217N, respectively). DB, JAL and MVF are supported by an FWO PhD
479 fellowship (1127415N, 11Y2118N and 1110019N, respectively). This work was funded by
480 FWO research grant G·0699·10N to GEB and MMW.

481 **Disclosures**

482 The authors declare no conflicts of interest

Reference List:

1. **Akbar A**, **Yiangou Y**, **Facer P**, **Walters JRF**, **Anand P**, **Ghosh S**. Increased capsaicin receptor TRPV1-expressing sensory fibres in irritable bowel syndrome and their correlation with abdominal pain. *Gut* (2008). doi: 10.1136/gut.2007.138982.
2. **Amadesi S**. Protease-Activated Receptor 2 Sensitizes the Capsaicin Receptor Transient Receptor Potential Vanilloid Receptor 1 to Induce Hyperalgesia. *J. Neurosci.* (2004). doi: 10.1523/JNEUROSCI.5679-03.2004.
3. **Balemans D**, **Boeckxstaens GE**, **Talavera K**, **Wouters MM**. Transient receptor potential ion channel function in sensory transduction and cellular signaling cascades underlying visceral hypersensitivity. *Am. J. Physiol. - Gastrointest. Liver Physiol.* (2017). doi: 10.1152/ajpgi.00401.2016.
4. **Balemans D**, **Mondelaers SU**, **Cibert-Goton V**, **Stakenborg N**, **Aguilera-Lizarraga J**, **Dooley J**, **Liston A**, **Bulmer DC**, **Vanden Berghe P**, **Boeckxstaens GE**, **Wouters MM**. Evidence for long-term sensitization of the bowel in patients with post-infectious-IBS. *Sci Rep* 7: 13606, 2017.
5. **Barbara G**, **Stanghellini V**, **De Giorgio R**, **Cremon C**, **Cottrell GS**, **Santini D**, **Pasquinelli G**, **Morselli-Labate AM**, **Grady EF**, **Bunnett NW**, **Collins SM**, **Corinaldesi R**. Activated Mast Cells in Proximity to Colonic Nerves Correlate with Abdominal Pain in Irritable Bowel Syndrome. *Gastroenterology* 126: 693–702, 2004.
6. **Blackshaw LA**. Transient receptor potential cation channels in visceral sensory pathways. *Br. J. Pharmacol.* (2014). doi: 10.1111/bph.12641.
7. **Brierley SM**, **Hughes PA**, **Page AJ**, **Kwan KY**, **Martin CM**, **O'Donnell TA**, **Cooper NJ**, **Harrington AM**, **Adam B**, **Liebregts T**, **Holtmann G**, **Corey DP**, **Rychkov GY**, **Blackshaw LA**. The Ion Channel TRPA1 Is Required for Normal Mechanosensation and Is Modulated by Algesic Stimuli. *Gastroenterology* (2009). doi: 10.1053/j.gastro.2009.07.048.
8. **Buhner S**, **Braak B**, **Li Q**, **Kugler EM**, **Klooker T**, **Wouters M**, **Donovan J**, **Vignali S**, **Mazzuoli-Weber G**, **Grundy D**, **Boeckxstaens G**, **Schemann M**. Neuronal activation by mucosal biopsy supernatants from irritable bowel syndrome patients is linked to visceral sensitivity. *Exp. Physiol.* (2014). doi: 10.1113/expphysiol.2014.080036.
9. **Camilleri M**, **McKinzie S**, **Busciglio I**, **Low P a**, **Sweetser S**, **Burton D**, **Baxter K**, **Ryks M**, **Zinsmeister AR**. Prospective study of motor, sensory, psychologic, and autonomic functions in patients with irritable bowel syndrome. *Clin. Gastroenterol. Hepatol.* (2008). doi: 10.1016/j.cgh.2008.02.060.
10. **Cattaruzza F**, **Spreadbury I**, **Miranda-Morales M**, **Grady EF**, **Vanner S**, **Bunnett NW**. Transient receptor potential ankyrin-1 has a major role in mediating visceral pain in mice. *AJP Gastrointest. Liver Physiol.* (2010). doi: 10.1152/ajpgi.00221.2009.
11. **Cenac N**, **Altier C**, **Chapman K**, **Liedtke W**, **Zamponi G**, **Vergnolle N**. Transient Receptor Potential Vanilloid-4 Has a Major Role in Visceral Hypersensitivity Symptoms. *Gastroenterology* (2008). doi: 10.1053/j.gastro.2008.05.024.
12. **Cenac N**, **Altier C**, **Motta JP**, **D'Aldebert E**, **Galeano S**, **Zamponi GW**, **Vergnolle N**. Potentiation of TRPV4 signalling by histamine and serotonin: An important mechanism for visceral hypersensitivity. *Gut* 59: 481–488, 2010.
13. **Cenac N**, **Bautzova T**, **Le Faouder P**, **Veldhuis NA**, **Poole DP**, **Rolland C**, **Bertrand J**, **Liedtke W**, **Dubourdeau M**, **Bertrand-Michel J**, **Zecchi L**, **Stanghellini V**, **Bunnett NW**,

Barbara G, **Vergnolle N**. Quantification and potential functions of endogenous agonists of transient receptor potential channels in patients with irritable bowel syndrome. *Gastroenterology* (2015). doi: 10.1053/j.gastro.2015.04.011.

14. **Chen Y**, **Williams SH**, **McNulty AL**, **Hong JH**, **Lee SH**, **Rothfusz NE**, **Parekh PK**, **Moore C**, **Gereau IV RW**, **Taylor AB**, **Wang F**, **Guilak F**, **Liedtke W**. Temporomandibular joint pain: A critical role for Trpv4 in the trigeminal ganglion. *Pain* (2013). doi: 10.1016/j.pain.2013.04.004.
15. **Chen Y**, **Yang C**, **Wang ZJ**. Proteinase-activated receptor 2 sensitizes transient receptor potential vanilloid 1, transient receptor potential vanilloid 4, and transient receptor potential ankyrin 1 in paclitaxel-induced neuropathic pain. *Neuroscience* (2011). doi: 10.1016/j.neuroscience.2011.06.085.
16. **Christianson JA**, **Bielefeldt K**, **Malin SA**, **Davis BM**. Neonatal colon insult alters growth factor expression and TRPA1 responses in adult mice. *Pain* (2010). doi: 10.1016/j.pain.2010.08.029.
17. **Cirillo C**, **Tack J**, **Vanden Berghe P**. Nerve activity recordings in routine human intestinal biopsies. *Gut* (2013). doi: 10.1136/gutjnl-2011-301777.
18. **Dai Y**, **Wang S**, **Tominaga M**, **Yamamoto S**, **Fukuoka T**, **Higashi T**, **Kobayashi K**, **Obata K**, **Yamanaka H**, **Noguchi K**. Sensitization of TRPA1 by PAR2 contributes to the sensation of inflammatory pain. *J. Clin. Invest.* (2007). doi: 10.1172/JCI30951.
19. **Drossman DA**, **Camilleri M**, **Mayer EA**, **Whitehead WE**. AGA technical review on irritable bowel syndrome. *Gastroenterology* (2002). doi: 10.1053/gast.2002.37095.
20. **Gold MS**, **Gebhart GF**. Nociceptor sensitization in pain pathogenesis. *Nat. Med.*: 2010.
21. **Grant AD**, **Cottrell GS**, **Amadesi S**, **Trevisani M**, **Nicoletti P**, **Materazzi S**, **Altier C**, **Cenac N**, **Zamponi GW**, **Bautista-Cruz F**, **Lopez CB**, **Joseph EK**, **Levine JD**, **Liedtke W**, **Vanner S**, **Vergnolle N**, **Geppetti P**, **Bunnett NW**. Protease-activated receptor 2 sensitizes the transient receptor potential vanilloid 4 ion channel to cause mechanical hyperalgesia in mice. *J. Physiol.* (2007). doi: 10.1113/jphysiol.2006.121111.
22. **Hughes PA**, **Harrington AM**, **Castro J**, **Liebregts T**, **Adam B**, **Grasby DJ**, **Isaacs NJ**, **Maldeniya L**, **Martin CM**, **Persson J**, **Andrews JM**, **Holtmann G**, **Ashley Blackshaw L**, **Brierley SM**. Sensory neuro-immune interactions differ between Irritable Bowel Syndrome subtypes. *Gut* 62: 1456–1465, 2013.
23. **Jones RCW**. The Mechanosensitivity of Mouse Colon Afferent Fibers and Their Sensitization by Inflammatory Mediators Require Transient Receptor Potential Vanilloid 1 and Acid-Sensing Ion Channel 3. *J. Neurosci.* (2005). doi: 10.1523/JNEUROSCI.0703-05.2005.
24. **Kajihara Y**, **Murakami M**, **Imagawa T**, **Otsuguro K**, **Ito S**, **Ohta T**. Histamine potentiates acid-induced responses mediating transient receptor potential V1 in mouse primary sensory neurons. *Neuroscience* (2010). doi: 10.1016/j.neuroscience.2009.12.001.
25. **Kuiken SD**, **Lindeboom R**, **Tytgat GN**, **Boeckxstaens GE**. Relationship between symptoms and hypersensitivity to rectal distension in patients with irritable bowel syndrome. *Aliment Pharmacol Ther* 22: 157–164, 2005.
26. **Livak KJ**, **Schmittgen TD**. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* (2001). doi: 10.1006/meth.2001.1262.
27. **McGuire C**, **Boundouki G**, **Hockley JRF**, **Reed D**, **Cibert-Goton V**, **Peiris M**, **Kung V**,

Broad J, **Aziz Q**, **Chan C**, **Ahmed S**, **Thaha MA**, **Sanger GJ**, **Blackshaw LA**, **Knowles CH**, **Bulmer DC**. Ex vivo study of human visceral nociceptors. *Gut* (2018). doi: 10.1136/gutjnl-2016-311629.

28. **McIntosh K**, **Reed DE**, **Schneider T**, **Dang F**, **Keshteli AH**, **De Palma G**, **Madsen K**, **Bercik P**, **Vanner S**. FODMAPs alter symptoms and the metabolome of patients with IBS: A randomised controlled trial. *Gut* (2017). doi: 10.1136/gutjnl-2015-311339.
29. **Mcmahon SB**, **La Russa F**, **Bennett DLH**. Crosstalk between the nociceptive and immune systems in host defence and disease. *Nat. Rev. Neurosci.*: 2015.
30. **Mertz H**, **Naliboff B**, **Munakata J**, **Niazi N**, **Mayer E a.** Altered rectal perception is a biological marker of patients with irritable bowel syndrome. *Gastroenterology* (1995). doi: 10.1016/0016-5085(95)90267-8.
31. **Mickle AD**, **Shepherd AJ**, **Mohapatra DP**. Nociceptive TRP channels: Sensory detectors and transducers in multiple pain pathologies. *Pharmaceuticals*: 2016.
32. **Miranda A**, **Nordstrom E**, **Mannem A**, **Smith C**, **Banerjee B**, **Sengupta JN**. The role of transient receptor potential vanilloid 1 in mechanical and chemical visceral hyperalgesia following experimental colitis. *Neuroscience* (2007). doi: 10.1016/j.neuroscience.2007.05.034.
33. **Ohman L**, **Simrén M**, **Öhman L**. Pathogenesis of IBS: Role of inflammation, immunity and neuroimmune interactions. *Nat Rev Gastroenterol Hepatol* 7: 163–173, 2010.
34. **Premkumar LS**, **Ahern GP**. Induction of vanilloid receptor channel activity by protein kinase C. *Nature* (2000). doi: 10.1038/35050121.
35. **Schmidt M**, **Dubin AE**, **Petrus MJ**, **Earley TJ**, **Patapoutian A**. Nociceptive Signals Induce Trafficking of TRPA1 to the Plasma Membrane. *Neuron* (2009). doi: 10.1016/j.neuron.2009.09.030.
36. **Sugiuar T**. TRPV1 Function in Mouse Colon Sensory Neurons Is Enhanced by Metabotropic 5-Hydroxytryptamine Receptor Activation. *J. Neurosci.* (2004). doi: 10.1523/JNEUROSCI.2639-04.2004.
37. **Thorneloe KS**, **Sulpizio AC**, **Lin Z**, **Figueroa DJ**, **Clouse AK**, **McCafferty GP**, **Chendrimada TP**, **Lashinger ESR**, **Gordon E**, **Evans L**, **Misajet BA**, **DeMarini DJ**, **Nation JH**, **Casillas LN**, **Marquis RW**, **Votta BJ**, **Sheardown SA**, **Xu X**, **Brooks DP**, **Laping NJ**, **Westfall TD**. N-((1S)-1- -3-hydroxypropanoyl)-1-piperazinyl]carbonyl}-3-methylbutyl)-1-benzothiophene-2-carboxamide (GSK1016790A), a Novel and Potent Transient Receptor Potential Vanilloid 4 Channel Agonist Induces Urinary Bladder Contraction and Hyperactivity: Part I. *J Pharmacol Exp Ther* 326: 432–442, 2008.
38. **Valdez-Morales EE**, **Overington J**, **Guerrero-Alba R**, **Ochoa-Cortes F**, **Ibeakanma CO**, **Spreadbury I**, **Bunnett NW**, **Beyak M**, **Vanner SJ**. Sensitization of peripheral sensory nerves by mediators from colonic biopsies of diarrhea-predominant irritable bowel syndrome patients: A role for PAR2. *Am J Gastroenterol* 108: 1634–1643, 2013.
39. **Vriens J**, **Watanabe H**, **Janssens A**, **Droogmans G**, **Voets T**, **Nilius B**. Cell swelling, heat, and chemical agonists use distinct pathways for the activation of the cation channel TRPV4. *Proc. Natl. Acad. Sci.* (2004). doi: 10.1073/pnas.0303329101.
40. **Van Wanrooij SJMM**, **Wouters MM**, **Van Oudenhove L**, **Vanbrabant W**, **Mondelaers S**, **Kollmann P**, **Kreutz F**, **Schemann M**, **Boeckxstaens GE**. Sensitivity testing in irritable bowel syndrome with rectal capsaicin stimulations: role of trpv1 upregulation and sensitization in visceral hypersensitivity? *Am. J. Gastroenterol.* (2014). doi: 10.1038/ajg.2013.371.

41. **Van Den Wijngaard RM**, **Klooker TK**, **Welting O**, **Stanisor OI**, **Wouters MM**, **Van Der Coelen D**, **Bulmer DC**, **Peeters PJ**, **Aerssens J**, **de Hoogt R**, **Lee K**, **de Jonge WJ**, **Boeckxstaens GE**. Essential role for TRPV1 in stress-induced (mast cell-dependent) colonic hypersensitivity in maternally separated rats. *Neurogastroenterol Motil* 21: 1107-e94, 2009.
42. **Winston J**, **Shenoy M**, **Medley D**, **Naniwadekar A**, **Pasricha PJ**. The Vanilloid Receptor Initiates and Maintains Colonic Hypersensitivity Induced by Neonatal Colon Irritation in Rats. *Gastroenterology* (2007). doi: 10.1053/j.gastro.2006.11.014.
43. **Woolf CJ**, **Ma Q**. Nociceptors-Noxious Stimulus Detectors. *Neuron*: 2007.
44. **Wouters MM**, **Balemans D**, **Van Wanrooy S**, **Dooley J**, **Cibert-Goton V**, **Alpizar YA**, **Valdez-Morales EE**, **Nasser Y**, **Van Veldhoven PP**, **Vanbrabant W**, **Van Der Merwe S**, **Mols R**, **Ghesquière B**, **Cirillo C**, **Kortekaas I**, **Carmeliet P**, **Peetermans WE**, **Vermeire SS**, **Rutgeerts P**, **Augustijns P**, **Hellings PW**, **Belmans A**, **Vanner S**, **Bulmer DC**, **Talavera K**, **Vanden Berghe P**, **Liston A**, **Boeckxstaens GE**, **Ghesqui??re B**, **Cirillo C**, **Kortekaas I**, **Carmeliet P**, **Peetermans WE**, **Vermeire SS**, **Rutgeerts P**, **Augustijns P**, **Hellings PW**, **Belmans A**, **Vanner S**, **Bulmer DC**, **Talavera K**, **Vanden Berghe P**, **Liston A**, **Boeckxstaens GE**. Histamine Receptor H1-Mediated Sensitization of TRPV1 Mediates Visceral Hypersensitivity and Symptoms in Patients with Irritable Bowel Syndrome. *Gastroenterology* 150: 875–887.e9, 2016.

Tables:

Table 1: Demographic information from IBS and HS.

	HS	IBS	p value
N	38	39	
Male/Female	17/21 (55% F)	9/30 (77% F)	0.06
Median Age (years)	24	32	0.52
IQR (25 and 75 percentile)	23 – 47	24 – 53	
IBS-D, n (%)	/	18 (46%)	
IBS-C, n (%)	/	7 (18%)	
IBS-M, n (%)	/	4 (10%)	
IBS-U, n (%)	/	10 (26%)	

F = female, HS = healthy subjects, IBS = irritable bowel syndrome, IBS-M = mixed type IBS, IBS-C = constipation predominant IBS, IBS-D = diarrhea predominant IBS, IBS-U = unclassified IBS, IQR = interquartile range. Statistics: Unpaired t-test (Age), Fisher's exact test (Gender).

Table 2: Primer sequences for gene detection by RT-qPCR,

Gene	Protein	Forward (5' -> 3')	Reverse (3' -> 5')
humanTRPV4	TRPV4	GCGAGGTCATTACGCTCTTC	TAGAGGGCTGCTGAGACGAT
human TRPA1	TRPA1	ATTGCGTGCACCACAAATAA	CTGAAAATGCAGCTTGGTGA
mouse TRPV4	TRPV4	TGGAACCAGAACTTGGGCAT	GGACCAACGATCCCTACGAA
mouse TRPA1	TRPA1	ACGAGGCTTTTGAATGAAGGG	CATGCACTCGGGGAGGTATT

Table 2 summarizing the gene, the protein and corresponding primer sequences.

Figure Legends

Figure 1 | TRPA1 and TRPV4 on human submucosal neurons of IBS patients is more sensitive compared to healthy subjects. (A) Representative images of a ganglion loaded with Fluo 4 (left panel) and HuCD immunostaining (right panel), and **(B)** traces of neurons responding to high-K^+. Red and blue squares and arrows in A correspond to the respective response represented by a red and blue line in B. **(C)** Representative traces of the intracellular Ca^{2+} response of human submucosal neurons in biopsies of healthy subjects (HS, blue) and IBS patients (red) to acute application of cinnamaldehyde (CA, 10 nM) and data showing the amplitude of the Ca^{2+} flux and the number of responding neurons (%) to CA in IBS patients (n = 14) and HS (n = 10). **(D)** Representative traces of the intracellular Ca^{2+} response of human submucosal neurons in biopsies of healthy subjects (HS, blue) and IBS patients (red) to acute application of GSK1016790A (GSK, 0.1 nM) and data showing the amplitude of the Ca^{2+} flux and the number of responding neurons to GSK in IBS patients (n=7) and HS (n=10). Data are presented as median + interquartile range (left) and mean + SD (right). *$p < 0.05$, ***$p < 0.001$, Unpaired t-test (left) and Fisher's exact test (right).

Figure 2 | No TRPA1 and TRPV4 mRNA upregulation in rectal biopsy samples of IBS patients. Relative mRNA expression for neuronal TRPA1 and TRPV4 normalized to β-actin in rectal biopsies of HS (n = 30) and IBS patients (n = 30). Mann-Whitney U test. Data are shown as mean + SEM.

Figure 3 | Histamine sensitizes TRPA1 and TRPV4 through H_1R in human submucosal neurons. (A) Representative traces of submucosal neurons in biopsies of HS upon application of CA (10 nM) and GSK1016790A (1 nM) before and after incubation with histamine (10 µM; TRPA1: n = 7; TRPV4: n = 7) in the presence or absence of the H_1R antagonist pyrilamine (1 µM; TRPA1: n = 6; TRPV4: n = 5). Data showing the effect of histamine in the absence **(B)** or presence **(C)** of pyrilamine on the amplitude of the Ca^{2+} response and the percentage of responding neurons. Data are shown as median + interquartile range (Amplitude) and mean + SEM (% responding neurons). *p < 0.05, Wilcoxon signed rank-test (Amplitudes) and Fisher's exact test (% responding neurons).

Figure 4 | Rectal biopsy supernatants from IBS patients sensitize TRPA1 on murine DRG neurons. (A) Effect of overnight incubation of murine DRG neurons with supernatant of cultured rectal biopsies of IBS patients (n = 8) or HS (n = 8) on the Ca^{2+} response (left) and % neurons responding (right) to cinnamaldehyde (10 µM). **(B)** Data showing the effect of overnight incubation with HS (n = 8) or IBS (n = 8) supernatants on the Ca^{2+} response (left) and % neurons responding (right) to GSK1016790A (1 µM). *p < 0.05, ***p < 0.001, Mann-Whitney U test (Amplitudes) or Fisher's exact test (% responding neurons). Data are shown as median + interquartile range.

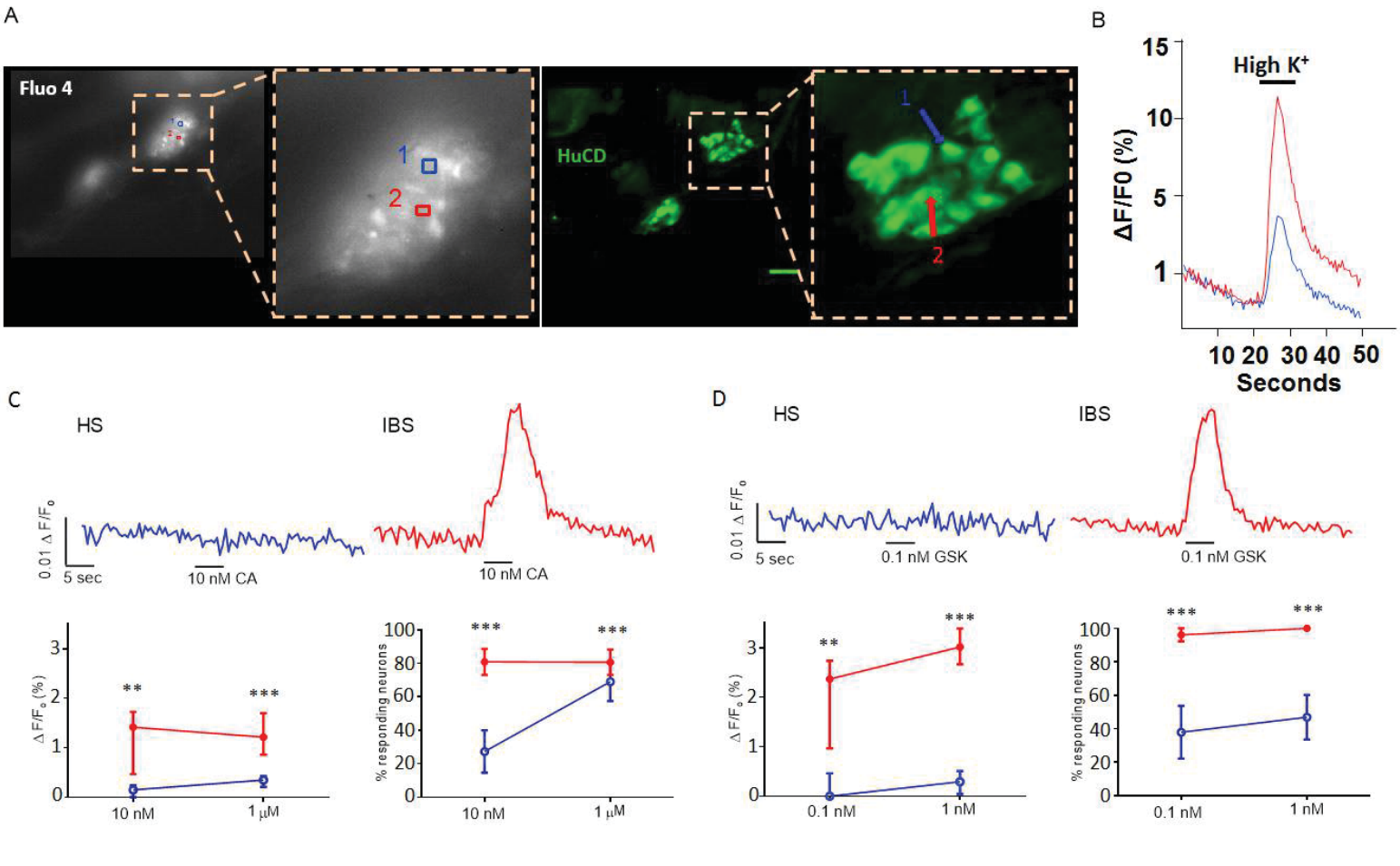
Figure 5 | TRPA1 is sensitized by histamine via H_1R in murine DRG neurons. (A) Representative traces of the effect of histamine and pyrilamine on the Ca^{2+} responses of DRG neurons to 10 µM cinnamaldehyde (CA). Histamine (10 µM) potentiates the effect of CA, an effect that is completely abolished in the presence of the H_1R antagonist pyrilamine (1

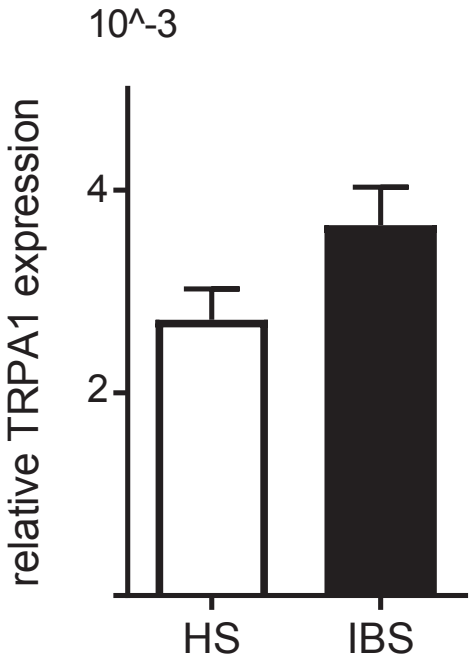
μM). **(B and C)** The effect of histamine, pyrilamine and DRG neurons lacking H_1R (*Hrh1*$^{-/-}$) on the amplitude of the Ca^{2+} response **(B)** and the percentage DRG neurons **(C)** responding to 10 μM CA. Data are shown as mean + SEM. ***p < 0.001; one-way ANOVA with Bonferroni's multiple comparison correction (Amplitudes) and Fisher's exact test (% responding neurons). Pre: CA response prior to incubation, Veh: vehicle, Hist: histamine, Pyr: pyrilamine.

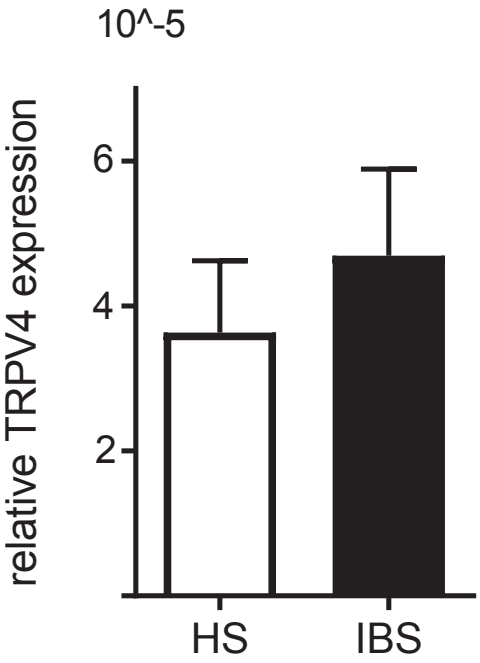
Figure 6 | TRPV4 is sensitized by histamine via H_1R in murine DRG neurons. (A) Representative traces of the effect of histamine and pyrilamine on the Ca^{2+} response of DRG neurons evoked by 1 μM GSK1016790A. The conditions vehicle, histamine, histamine + pyrilamine and *Hrh1*$^{-/-}$ were combined with the TRPV1 antagonist (SB 366791). Overnight incubation with 100 μM histamine potentiates the effect of GSK1016790A, an effect that is blocked in the presence of the H_1R antagonist pyrilamine (1 μM). **(B and C)** The effect of histamine, pyrilamine and DRG neurons lacking H_1R (*Hrh1*$^{-/-}$), TRPV1 (Trpv1$^{-/-}$) and TRPV1V4 (Trpv1$^{-/-}$v4$^{-/-}$) on the amplitude of the Ca^{2+} response **(B)** and the percentage of DRG neurons **(C)** responding to 1 μM GSK1016790A. The conditions vehicle, histamine, histamine + pyrilamine and *Hrh1*$^{-/-}$ were combined with the TRPV1 antagonist (SB 366791). Data are shown as mean + SEM. **p < 0.01 ***p < 0.001; 1-way ANOVA with Bonferroni's multiple comparison correction (Amplitudes) and Fisher's exact test (% responding neurons). Veh: vehicle, Hist: histamine, Pyr: pyrilamine.

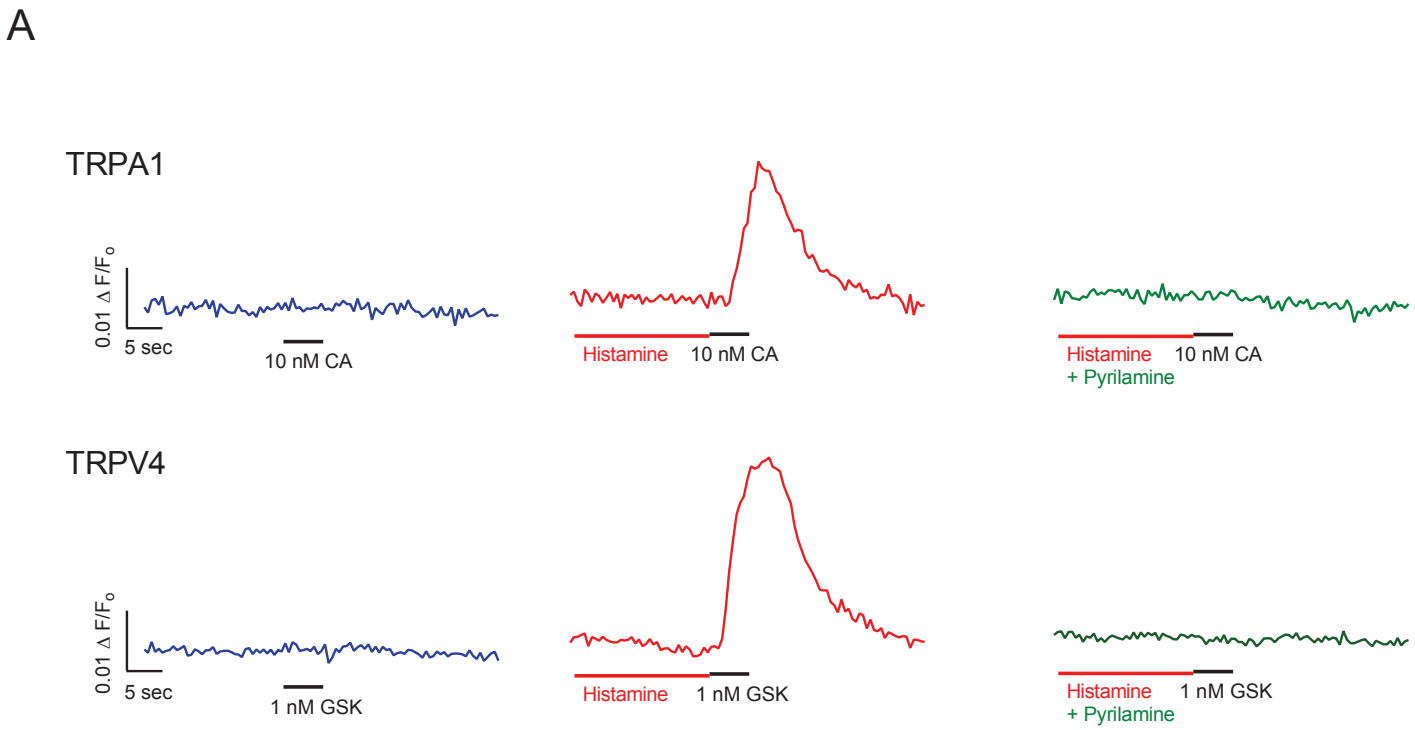
Figure 7 | Sensitization of TRPA1 and TRPV4 by IBS supernatants on DRG neurons is mediated by histamine and the H_1R. (A) Data showing the effect of overnight incubation with HS supernatants (n=8) supplemented with histamine (TRPA1: 10 μM; TRPV4: 100

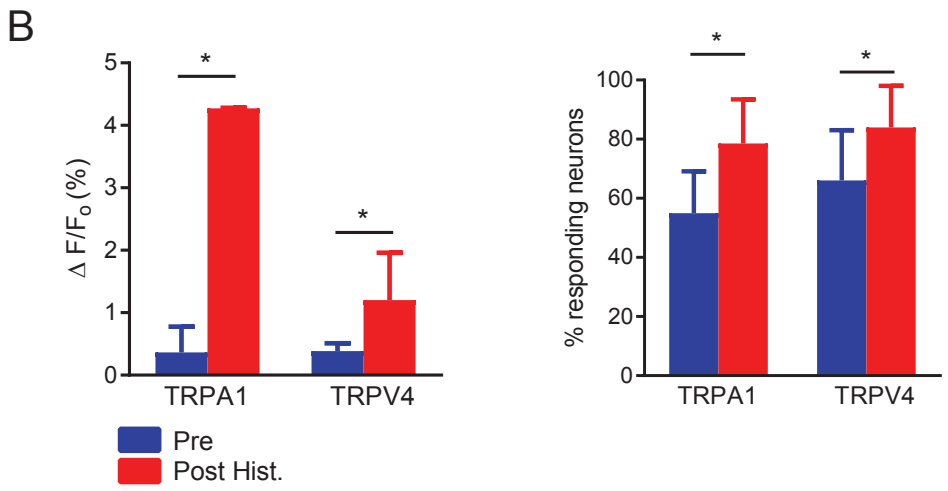
723 µM)(+H) (n = 8) or histamine and pyrilamine (1 µM)(+H+P) (TRPA1: n = 5; TRPV4: n = 3) on
724 the Ca^{2+} response to CA (left panel) and GSK1016790A (right panel). **(B)** Data showing the CA
725 (left panel) and GSK1016790A (right panel)-induced Ca^{2+} response after overnight incubation
726 with supernatant of cultured rectal biopsies of IBS patients (n = 8) in the presence or
727 absence pyrilamine (+P; 1 µM; n = 8). *p < 0.05, **p < 0.01 Mann-Whitney U test
728 (Amplitudes). H = histamine, P = pyrilamine.
729
730 **Figure 8 | (A)** Representative images of murine DRG neurons used to quantify
731 expression/translocation of TRPV4 upon histamine stimulation. **(B)** TRPA1 (n = 19-21) and **(C)**
732 TRPV4 (n = 20-22) quantification of channel translocation to the plasma membrane (upper
733 panels) and total expression in the cell (lower panels) in cultured DRG neurons treated with
734 histamine (TRPA1: 10 µM for 10 min; and TRPV4: 100 µM overnight). Data are shown as
735 mean + SEM. *p < 0.05 with Mann Whitney test or student t-test (as appropriate).

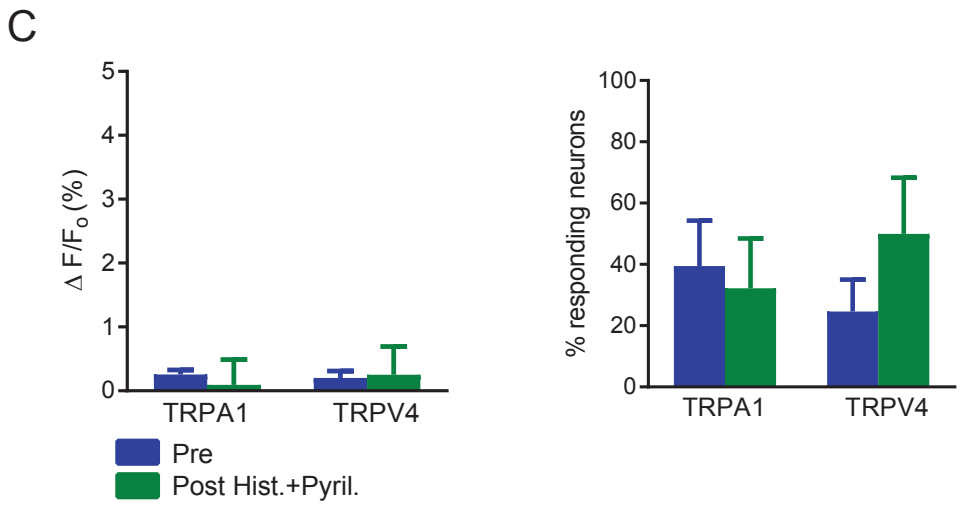

A
Fluo 4
1
2
HuCD
1
2
B
High K+
ΔF/F0 (%)
15
10
5
1
10 20 30 40 50
Seconds
C
HS
IBS
0.01 Δ F/Fo
5 sec
10 nM CA
10 nM CA
Δ F/Fo (%)
% responding neurons
**
***
***
***
10 nM
1 μM
10 nM
1 μM
D
HS
IBS
0.01 Δ F/Fo
5 sec
0.1 nM GSK
0.1 nM GSK
Δ F/Fo (%)
% responding neurons
**
***
***
***
0.1 nM
1 nM
0.1 nM
1 nM

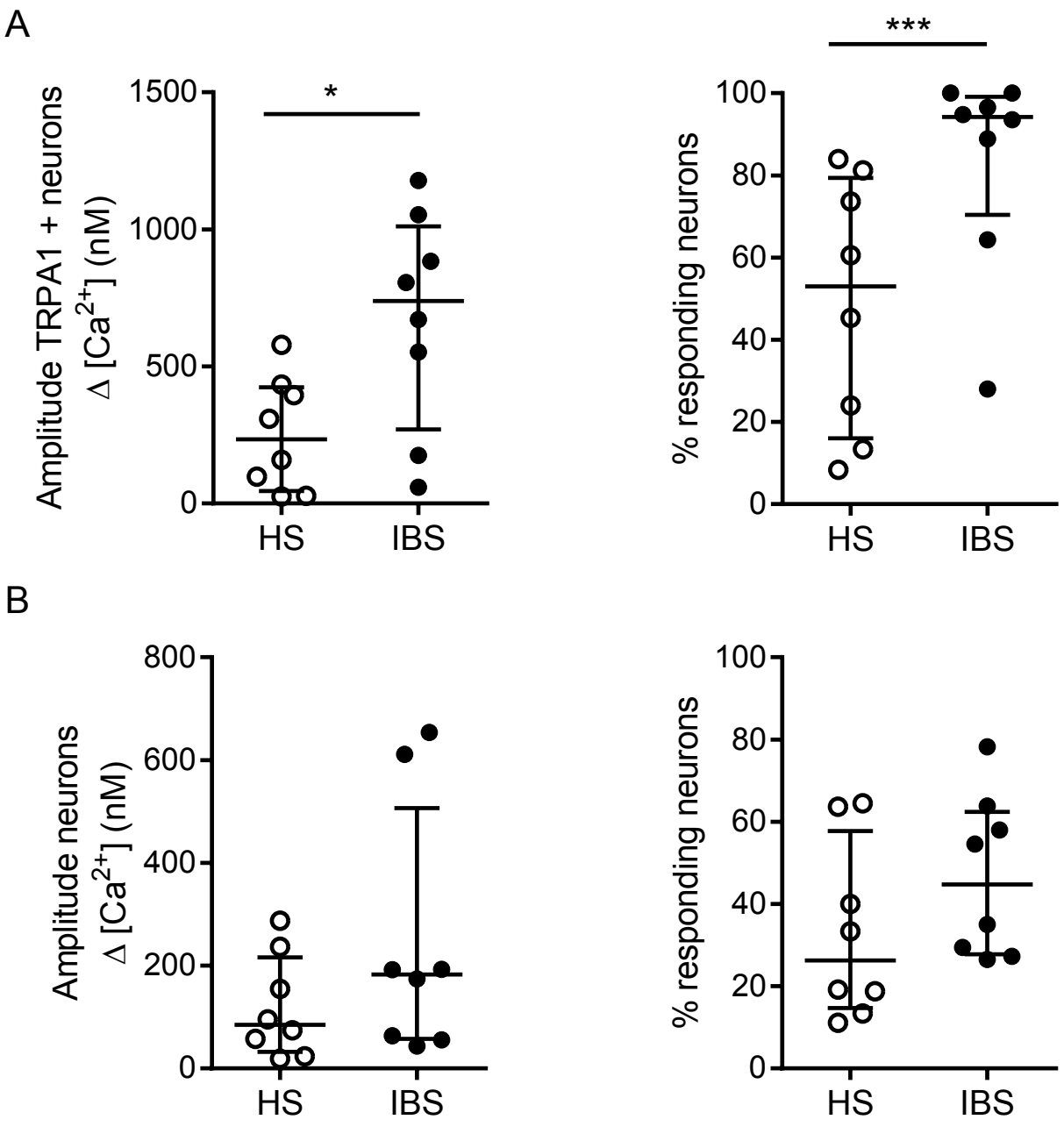

10^-3
relative TRPA1 expression
4
2
HS
IBS

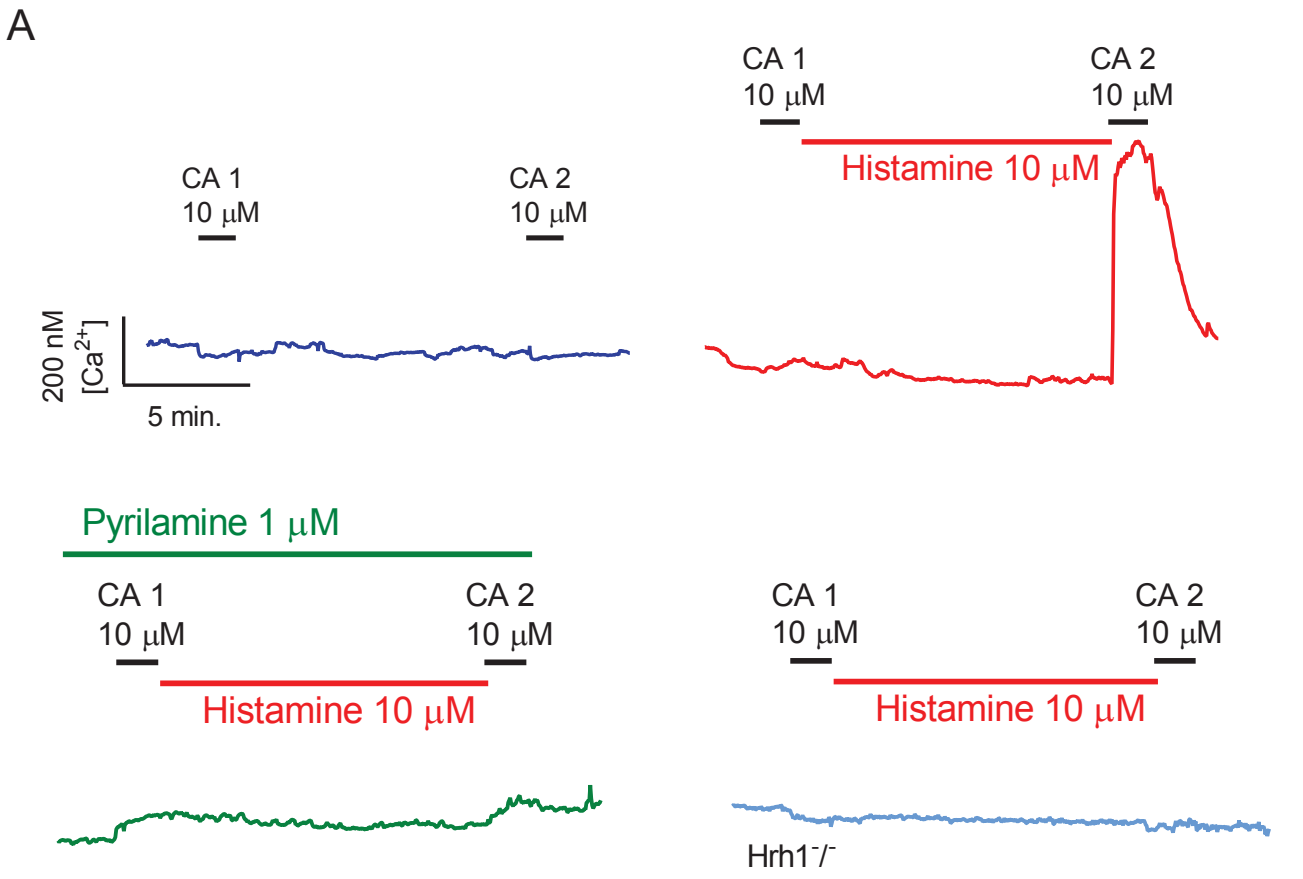

10^-5
relative TRPV4 expression
6
4
2
HS
IBS

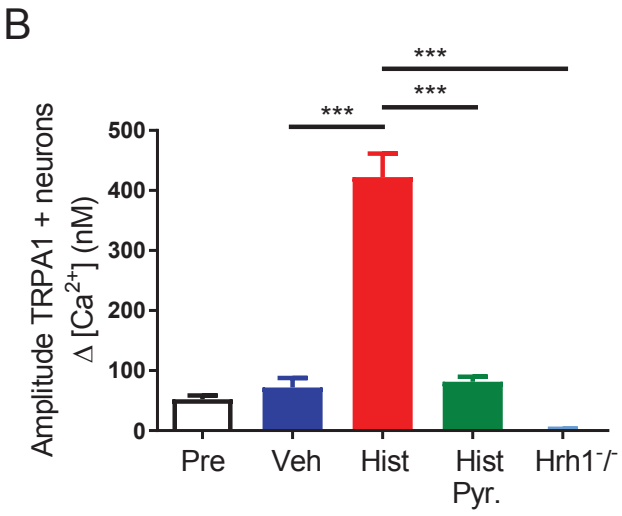

A
TRPA1
0.01 Δ F/Fo
5 sec
10 nM CA
Histamine
10 nM CA
Histamine
10 nM CA
+ Pyrilamine
TRPV4
0.01 Δ F/Fo
5 sec
1 nM GSK
Histamine
1 nM GSK
Histamine
1 nM GSK
+ Pyrilamine

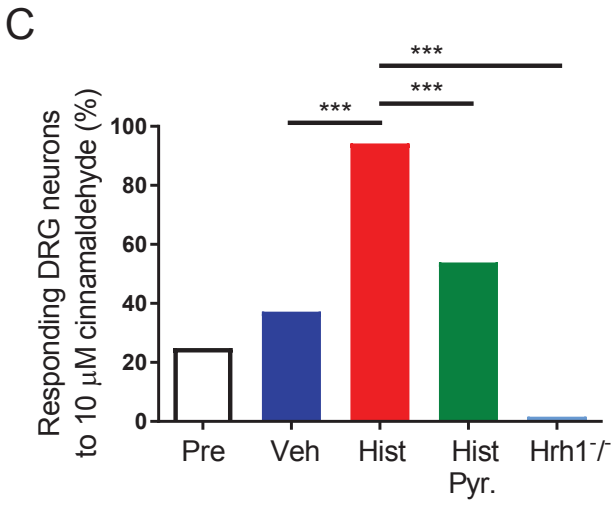

B
Δ F/Fo (%)
TRPA1
TRPV4
Pre
Post Hist.
% responding neurons
TRPA1
TRPV4

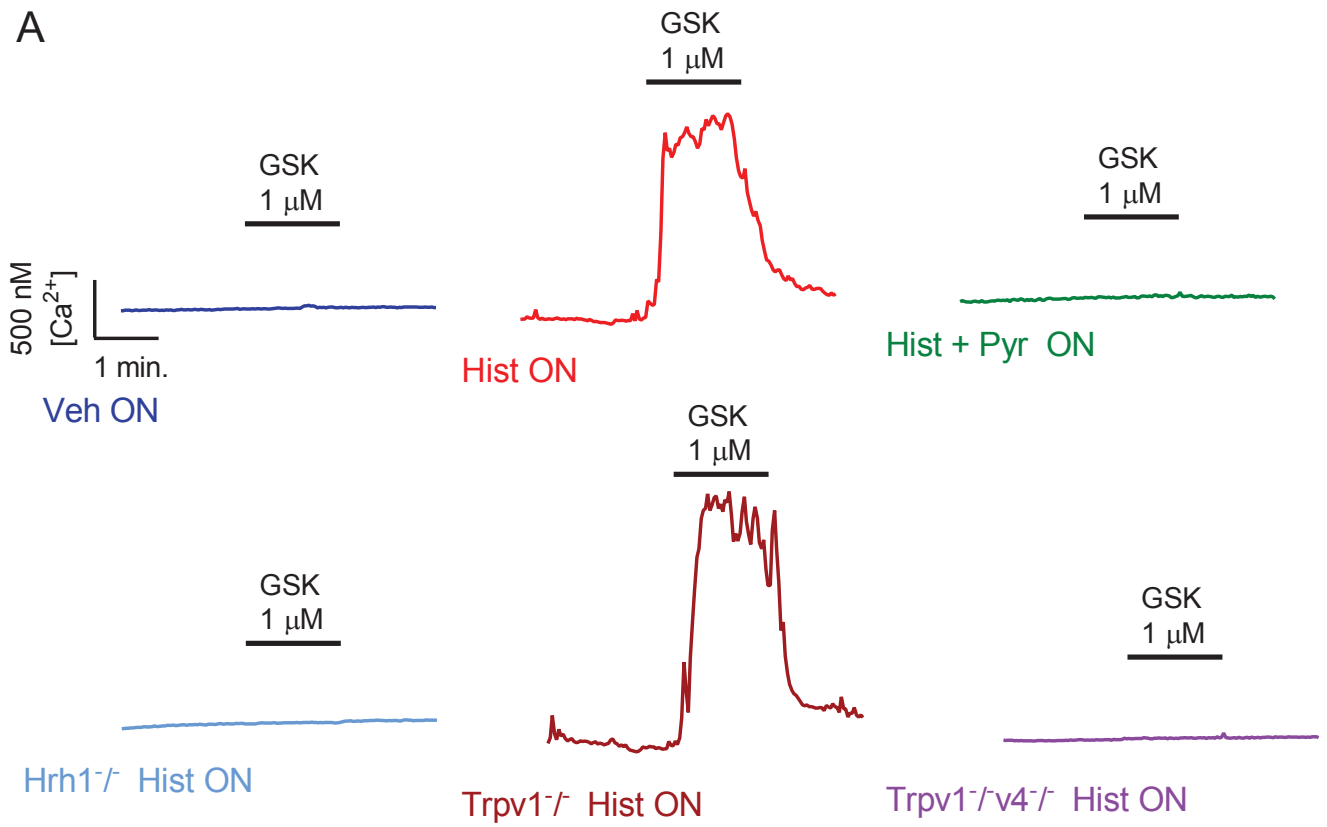

C
Δ F/Fo (%)
TRPA1
TRPV4
Pre
Post Hist.+Pyril.
% responding neurons
TRPA1
TRPV4

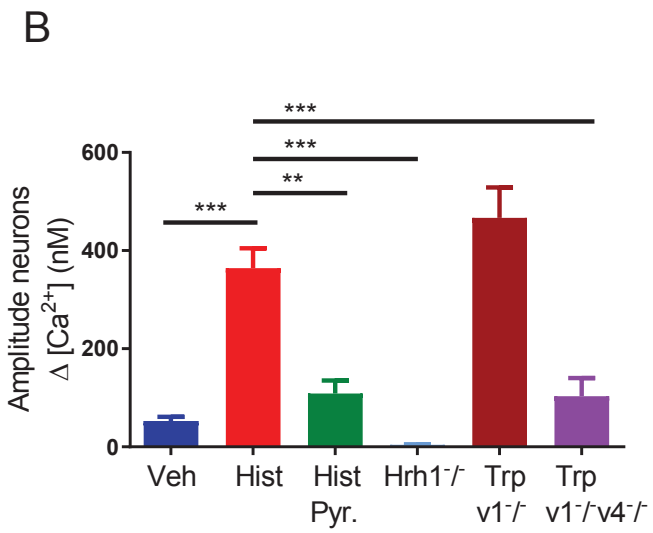

A
Amplitude TRPA1 + neurons
Δ [Ca2+] (nM)
1500
1000
500
0
*
HS
IBS
% responding neurons
100
80
60
40
20
0
***
HS
IBS
B
Amplitude neurons
Δ [Ca2+] (nM)
800
600
400
200
0
HS
IBS
% responding neurons
100
80
60
40
20
0
HS
IBS

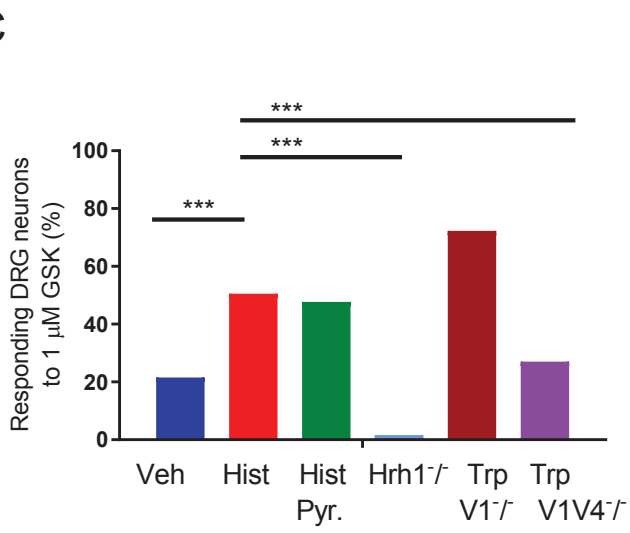

A
CA 1
10 μM
CA 2
10 μM
200 nM
[Ca2+]
5 min.
CA 1
10 μM
CA 2
10 μM
Histamine 10 μM
Pyrilamine 1 μM
CA 1
10 μM
CA 2
10 μM
Histamine 10 μM
CA 1
10 μM
CA 2
10 μM
Histamine 10 μM
Hrh1-/-

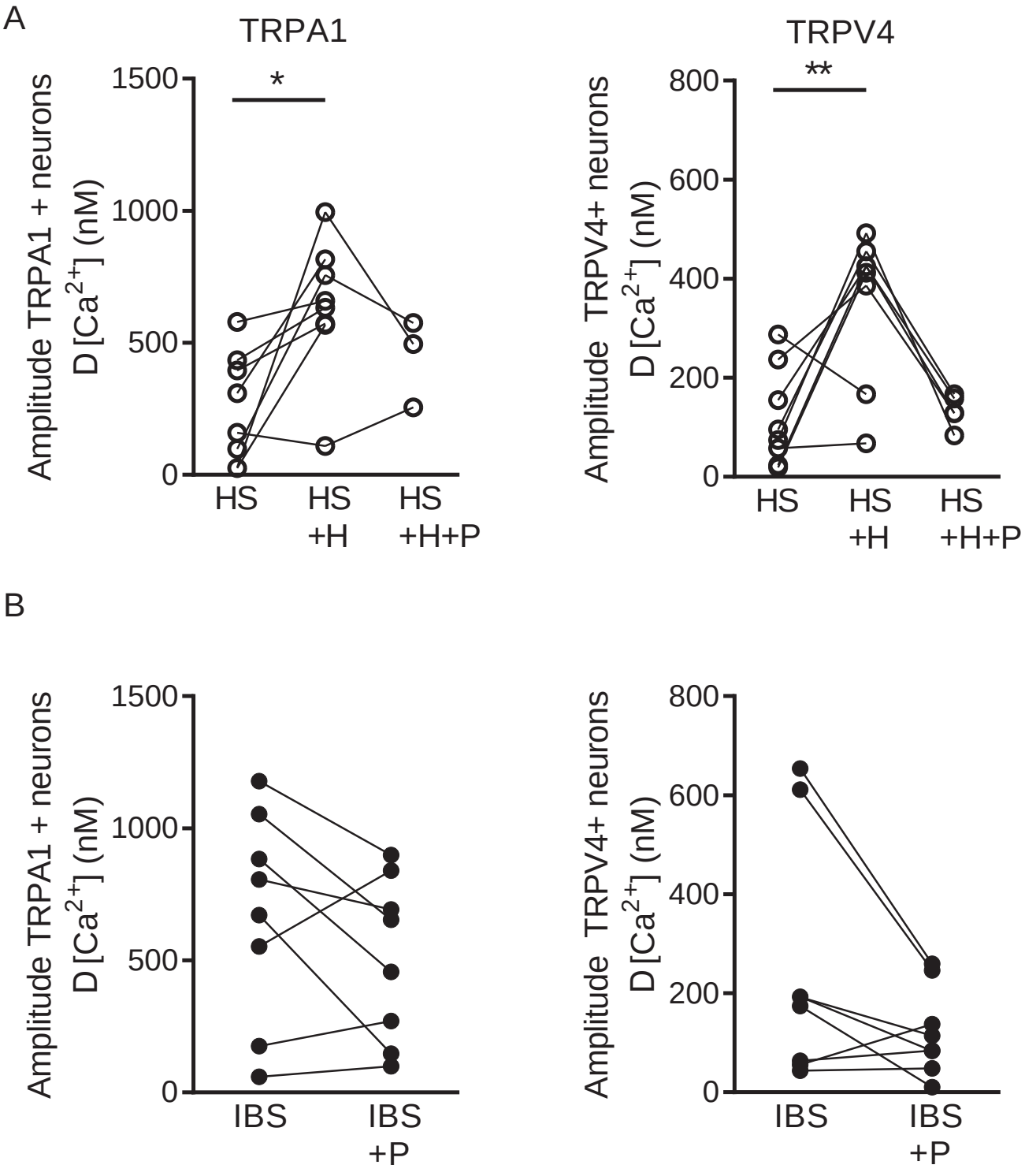

B
Amplitude TRPA1 + neurons
Δ [Ca2+] (nM)
500
400
300
200
100
0
***
***
***
Pre
Veh
Hist
Hist
Pyr.
Hrh1-/-


C
Responding DRG neurons
to 10 μM cinnamaldehyde (%)
100
80
60
40
20
0
***
***
***
Pre
Veh
Hist
Hist
Pyr.
Hrh1-/-

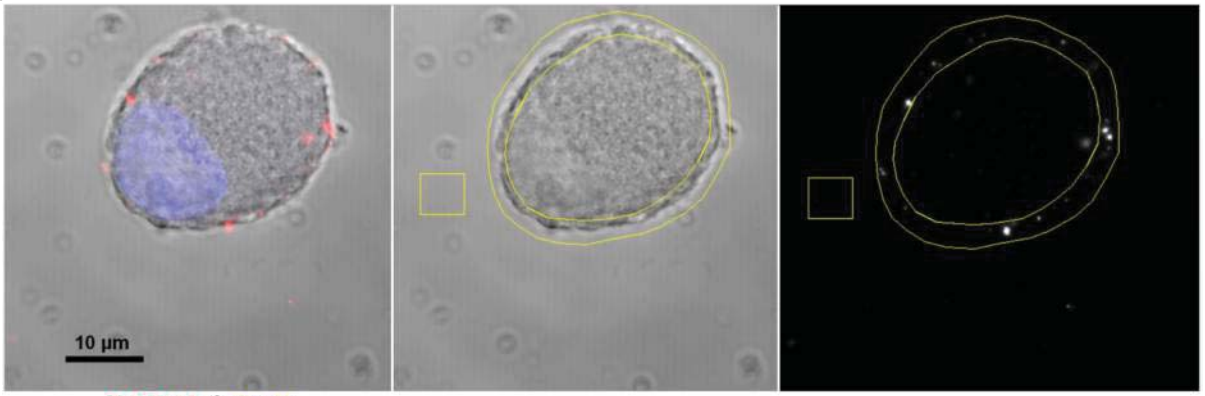

A
GSK
1 μM
GSK
1 μM
GSK
1 μM
500 nM
[Ca2+]
1 min.
Veh ON
Hist ON
Hist + Pyr ON
GSK
1 μM
GSK
1 μM
GSK
1 μM
Hrh1-/- Hist ON
Trpv1-/- Hist ON
Trpv1-/-v4-/- Hist ON


B
***
***
**
***
600
400
200
0
Amplitude neurons
Δ [Ca2+] (nM)
Veh
Hist
Hist Pyr.
Hrh1-/-
Trp v1-/-
Trp v1-/-v4-/-

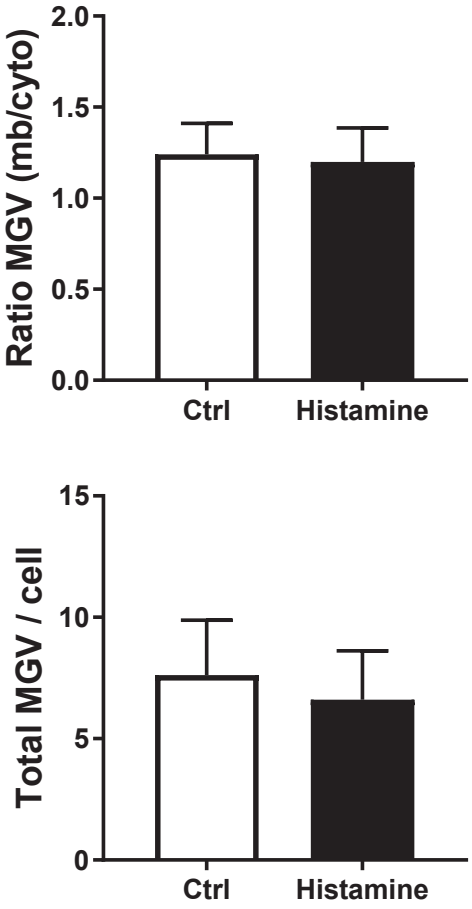

C
***
***
***
100
80
60
40
20
0
Responding DRG neurons
to 1 μM GSK (%)
Veh
Hist
Hist Pyr.
Hrh1-/-
Trp V1-/-
Trp V1V4-/-


A
TRPA1
TRPV4
Amplitude TRPA1 + neurons
D [Ca2+] (nM)
Amplitude TRPV4+ neurons
D [Ca2+] (nM)
*
**
1500
1000
500
0
800
600
400
200
HS
HS +H
HS +H+P
B
IBS
IBS +P

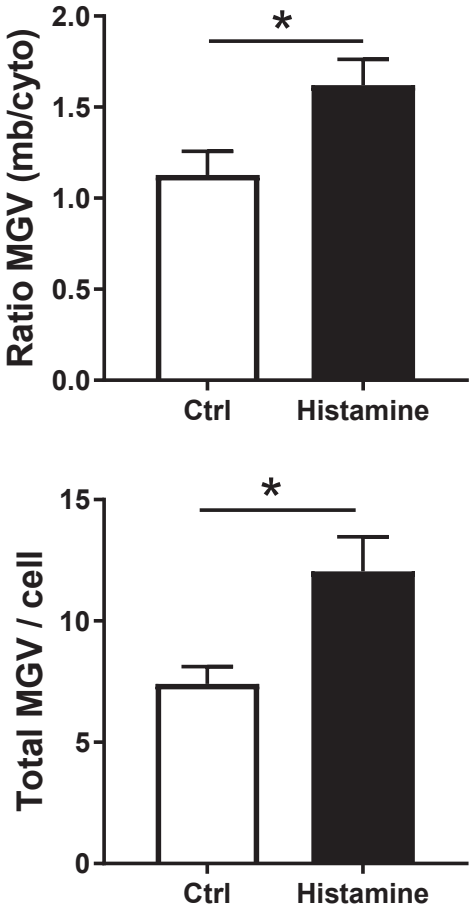
A


10 µm
TRPV4 / DAPI

B


TRPA1
Ratio MGV (mb/cyto)
2.0
1.5
1.0
0.5
0.0
Ctrl
Histamine
Total MGV / cell
15
10
5
0
Ctrl
Histamine

C


TRPV4
Ratio MGV (mb/cyto)
*
2.0
1.5
1.0
0.5
0.0
Ctrl
Histamine
Total MGV / cell
*
15
10
5
0
Ctrl
Histamine