Histamine induces peripheral and central hypersensitivity to bladder distension via the histamine H_1 receptor and TRPV1

* [1,2,3] Luke Grundy, * [1,2,3] Ashlee Caldwell, [1,2,3] Sonia Garcia-Caraballo, [1,2,3] Andelain Erickson, [1,2,3] Gudrun Schober, [1,2,3] Joel Castro, [1,2,3] Andrea M. Harrington, [1,2,3] Stuart M. Brierley

[1] Visceral Pain Research Group, Centre for Neuroscience, College of Medicine and Public Health, Flinders University, Bedford Park, South Australia, 5042, AUSTRALIA.

[2] Hopwood Centre for Neurobiology, Lifelong Health Theme, South Australian Health and Medical Research Institute (SAHMRI), North Terrace, Adelaide, South Australia 5000, AUSTRALIA,

[3] Centre for Nutrition and Gastrointestinal Diseases, Discipline of Medicine, Faculty of Health and Medical Science, University of Adelaide, North Terrace, Adelaide, South Australia 5000, AUSTRALIA.

* These authors made equal contributions

Corresponding Author:

Prof. Stuart M. Brierley, Ph.D. Visceral Pain Research Group, Level 7, South Australian Health and Medical Research Institute (SAHMRI), North Terrace, Adelaide, SA 5000, AUSTRALIA.

Email: stuart.brierley@flinders.edu.au

Competing financial interests and conflict of interests:

The authors have nothing to declare

Abstract

Interstitial Cystitis/Bladder Pain Syndrome (IC/BPS) is a common chronic pelvic disorder with sensory symptoms of urinary urgency, frequency, and pain, indicating a key role for hypersensitivity of bladder-innervating sensory neurons. The inflammatory mast cell mediator histamine has long been implicated in IC/BPS, yet the direct interactions between histamine and bladder afferents remain unclear. Here we show, using a mouse *ex vivo* bladder afferent preparation, that intravesical histamine enhanced the mechanosensitivity of sub-populations of afferents to bladder distension. Histamine also recruited 'silent afferents', which were previously unresponsive to bladder distension. Furthermore, *in vivo* intravesical histamine enhanced activation of dorsal horn neurons within the lumbosacral spinal cord, indicating increased afferent signaling into the central nervous system. qRT-PCR revealed significant expression of histamine receptor subtypes (*Hrh1-Hrh3*) in mouse lumbosacral dorsal root ganglia (DRG), bladder detrusor smooth muscle, mucosa, and isolated urothelial cells. In DRG, *Hrh1* was the most abundantly expressed. Acute histamine exposure evoked calcium influx in select populations of DRG neurons but did not elicit calcium transients in isolated primary urothelial cells. Histamine-induced mechanical hypersensitivity *ex vivo* was abolished in the presence of the histamine H_1 receptor (H_1R) antagonist pyrilamine and was not present in preparations from mice lacking the TRPV1 receptor. Together, these results indicate that histamine enhances the sensitivity of bladder afferents to distension via interactions with H_1R and TRPV1. This hypersensitivity translates to increased sensory input and activation in the spinal cord, which may underlie the symptoms of bladder hypersensitivity and pain experienced in IC/BPS.

Introduction

Interstitial Cystitis/Bladder Pain Syndrome (IC/BPS) is a common, chronic pelvic disorder that negatively impacts approximately 5% of the Western population (67, 68). IC/BPS patients experience hypersensitivity to bladder distension, inducing symptoms of urinary urgency, frequency, and bladder allodynia (pain perception at lower distension volumes) compared to healthy subjects (7, 12, 36). Although the etiology underlying IC/BPS remains unresolved, given the sensory nature of the symptoms (33), changes in the sensitivity of the bladder-innervating sensory afferent nerves responsible for regulating micturition likely play a key role.

Bladder afferents are located throughout the bladder wall, innervating the detrusor smooth muscle and mucosa (80). As the bladder fills, mechanosensitive afferents transduce bladder stretch into sensory signals that are relayed into the spinal cord, where they feed into autonomic circuits responsible for maintaining continence (15, 23). Conscious discrimination of bladder fullness and the urge to urinate occurs when afferent signaling reaches the 'micturition threshold', triggering a combination of reflexive and cognitive events that ultimately result in the conscious decision to urinate (15, 18, 23). In addition to responding to mechanical stimuli or distension, the peripheral terminals of bladder afferents express an array of pro- and anti-nociceptive ion channels and receptors capable of detecting mediators released in response to bladder damage and inflammation (33, 82). Inflammatory mediators have consistently been shown to sensitize sensory afferents innervating the bladder, such that bladder afferents become hypersensitive to distension (16, 19, 33, 61, 82). Befittingly, inflammation of the bladder is implicated in the pathogenesis of IC/BPS (16, 25, 27, 33, 61).

Mast cells play an important role in the pathophysiology of a variety of inflammatory diseases. Accordingly, they are located in mucosal and epithelial tissues throughout the body, including the bladder (76, 86, 91). Upon activation, mast cells release a number of potent inflammatory mediators, including but not limited to cytokines, proteases, and histamine (84, 87). Importantly, mast cells are located in close proximity to nerve endings, and histamine has been shown to activate sensory afferents innervating both the skin and gastrointestinal tract (47, 51, 55). Clinical and pre-clinical studies have shown that mast cells may be a crucial component in the pathogenesis and symptomology of chronic bladder pain. Mast cell numbers are increased in bladder tissue from IC/BPS patients compared to healthy subjects (55, 76, 86, 91), and mast cell mediators, particularly tryptase, histamine, and methylhistamine, are also significantly upregulated in bladder tissue and urine of IC/BPS patients compared to controls (8, 20). Induction of IC/BPS like symptoms in mast cell deficient mice, including inflammation and pain, is significantly attenuated in two separate pre-clinical models of cystitis (6, 74), while a recent study using an experimental autoimmune cystitis model demonstrated that the accumulation of mast cells in the bladder is required for IC/BPS-associated allodynia (2). Additionally,

histamine has been implicated in bladder neuroinflammation, as it is known to act as both a neuromodulator and a pro-inflammatory agent (72).

Although a number of studies have explored the action of neuropeptides on histamine release from mast cells (58, 78) and the role of histamine on detrusor smooth muscle and pain responses (72, 75, 81), a complete understanding of the direct interactions between histamine and bladder-innervating sensory afferent neurons remains lacking. In this study, we addressed this issue by investigating the effects of histamine on bladder afferent mechanosensitivity using *ex vivo* recordings, retrogradely traced bladder-innervating dorsal root ganglia (DRG) neurons, and spinal cord signaling *in vivo*. We also characterized the expression profiles of histamine receptor subtypes *Hrh1-Hrh4* in bladder muscle, mucosa, isolated urothelial cells, lumbosacral DRG, and individual retrogradely traced bladder-innervating DRG neurons. These data provide the first comprehensive analysis of bladder afferent sensitivity to histamine and provide evidence for a mechanism whereby increased histamine release from mast cells during IC/BPS contributes to bladder afferent sensitization and the sensory symptoms of urgency, frequency, and pelvic pain.

Methods

Ethics and animals

In this study, we used 12- to 18-week-old male and female C57BL/6J mice via an in-house breeding colony at the South Australian Health and Medical Research Institute (SAHMRI). Some experiments utilized a TRPV1 knockout (*Trpv1*$^{-/-}$) mouse strain with a C57BL/6J genetic background, also bred in-house at SAHMRI. All experiments were approved by and performed in accordance with the SAHMRI Animal Ethics Committee approvals (SAM195 and SAM190). Mice at SAHMRI were originally acquired from an in-house C57BL/6J breeding program (JAX strain #000664; originally purchased from The Jackson Laboratory; breeding barn MP14; Bar Harbor, ME) and then bred within the specific and opportunistic pathogen-free animal care facility at SAHMRI. Mice were group housed (5 mice per cage) within individual ventilated cages filled with chip coarse dust-free aspen bedding (Cat#–ASPJMAEBCA; PuraBed, Niederglatt, Switzerland). Cages were stored on individual ventilated cage racks within a temperature-controlled environment of 22°C and a 12-hour light/12-hour dark cycle. Mice had free access to LabDiet JL Rat and Mouse/Auto6F chow (Cat #5K52, St. Louis, MO) and autoclaved reverse osmosis water. Sample sizes of each experiment are detailed in the corresponding figure legends.

Ex vivo bladder afferent nerve recording

Nerve recording was performed using a previously described *ex vivo* model (31-34). Mice were humanely killed via CO_2 inhalation, and the entire lower abdomen was removed and submerged in a modified organ bath under continual perfusion with gassed (95% O_2 and 5% CO_2) Krebs-bicarbonate solution (composition in mmol/L: 118.4 NaCl, 24.9 $NaHCO_3$, 1.9 $CaCl_2$, 1.2 $MgSO_4$, 4.7 KCl, 1.2 KH_2PO_4, 11.7 glucose) at 35°C. The bladder, urethra, and ureters were exposed by removing excess tissue. Ureters were tied with 4-0 perma-hand silk (Ethicon, #LA53G). The bladder was catheterized (PE 50 tubing) through the urethra and connected to a syringe pump (NE-1000) to allow a controlled fill rate of 30 µL/min with saline (NaCl, 0.9%). A second catheter was inserted through the dome of the bladder, secured with silk, and connected to a pressure transducer (NL108T2; Digitimer) to enable intravesical pressure recording during graded distension. Pelvic nerves, isolated from all other nerve fibers between the pelvic ganglia and the spinal cord, were dissected into fine multiunit branches and a single branch was placed within a sealed glass pipette containing a microelectrode (WPI) attached to a Neurolog headstage (NL100AK; Digitimer). Nerve activity was amplified (NL104), filtered (NL 125/126, band pass 50–5,000 Hz, Neurolog; Digitimer), and digitized (CED 1401; Cambridge Electronic Design, Cambridge, UK) to a PC for offline analysis using Spike2 software (Cambridge Electronic Design, Cambridge, UK). The number of action potentials crossing a pre-set threshold at twice the background electrical noise, was determined per second, to quantify the afferent response. Single-unit analysis was performed

offline by matching individual spike waveforms through linear interpolation using Spike2 version 5.18 software. A single unit was deemed to be responsive to histamine if a greater than 20% increase in neuronal excitability was detected.

Afferent recording experimental protocols

At the start of each afferent recording experiment, control bladder distensions were performed with intravesical infusion of saline (NaCl, 0.9%) at a rate of 30 µL/min to a maximum pressure of 30 mmHg at 10 min intervals to assess the viability of the preparation and reproducibility of the intravesical pressure and neuronal responses to distension. The volume in the bladder was extrapolated from the known fill rate (30 µL/min) and the time taken (s) to reach maximum pressure (30 mmHg). Compliance was determined by plotting intravesical pressure against the calculated volume. We did not observe spontaneous phasic contractions during bladder distension as has been reported by others(37). After a stable baseline was maintained, the saline in the infusion pump was replaced by histamine (300 µM; Sigma, #H7125-1G), with or without the histamine 1 receptor antagonist pyrilamine (100 µM, Sigma, P5514-5G). The single nerve bundle isolated and inserted into the glass electrode during the dissection process was contained within the recording electrode for the entire experiment, allowing multiunit afferent nerve recordings to be performed and comparisons between afferent firing rates to be compared in the same nerve fibers during intra-bladder incubation with saline and histamine (300 µM). Spontaneous baseline firing between bladders distensions was also recorded before and after intravesical histamine administration. Data were obtained immediately before and 5-15 minutes after histamine was introduced into the bladder.

In vivo spinal dorsal horn activation

In vivo bladder infusion

In vivo bladder infusion in anaesthetized mice was performed as previously described (32). 12-week old female mice were anaesthetized (isoflurane 2% - 4% in oxygen) and a catheter (PE 50 tubing) was inserted into the bladder via the urethra. Correct catheter placement was determined by inserting gently until meeting resistance (bladder dome) and receding slightly (2-3 mm) to avoid damaging the bladder wall. Urine was removed using a suction syringe. Subsequently, a new catheter (PE 50 tubing) was inserted, primed with either vehicle (saline; N = 5 mice) or histamine (100 µM; N = 5 mice), and 100 µL of solution was then infused gently, to fill but not fully distend the bladder, and allowed to incubate for 5 minutes. Following removal of the compound, mice were administered an anesthetic overdose (intraperitoneal injection, 0.125 mL/250 g Lethabarb®; Virbac Australia) followed by transcardial perfuse fixation.

Transcardial perfuse fixation and tissue dissection

After anesthetic overdose, the thoracic cavity was opened and 0.5 mL heparinized saline (50 IU in 5 mL; Pfizer) was injected into the left ventricle followed by insertion of a 22-gauge needle attached to tubing and a peristaltic perfusion pump. The right atrium was cut open allowing for drainage of perfusate. Warm phosphate buffer (0.1 M; 21.7 mM NaH_2PO_4 [Chem Supply, #SA061-500G], 81 mM Na_2HPO_4 [VWR Chemicals, #102494C], pH 7.2) was then perfused, followed by ice-cold 4% paraformaldehyde (PFA; Sigma-Aldrich, #158127) in 0.1 M phosphate buffer. After complete PFA perfusion, the lumbosacral spinal cord (determined by the level of dorsal root ganglia (DRG) root insertion points; the lowest rib was used as an anatomical marker of DRG level T13) was removed and postfixed in 4% PFA in 0.1 M phosphate buffer at 4°C for 18 to 20 hours. After post-fixation, spinal cords were cryoprotected in 30% sucrose/phosphate buffer (Sigma-Aldrich, #S9378) for two days at 4°C, followed by 48-hour incubation at 4°C in 50% optimal cutting temperature (OCT; VWR, #C361603E)/30% sucrose/phosphate buffer solution before freezing in 100% OCT using liquid nitrogen-cooled 2-methylbutane Sigma-Aldrich, #M32631). Frozen sections of the spinal cord were cryosectioned (10 µm thick) and placed onto gelatin-coated slides for immunofluorescence labelling processing. Sections were performed serially and distributed over 6 slides, which were used for immunofluorescence localization of phosphorylated-MAP-kinase ERK 1/2 (pERK). The number of pERK-immunoreactive neurons were counted in L6-S1 spinal cord sections, with spinal segments determined *ex vivo* by the shape of the dorsal horn (54).

Immunohistochemistry of phosphorylated-MAP-kinase ERK 1/2 within the spinal cord

The dorsal horn neurons activated by bladder infusion were identified by labelling for neuronal activation marker, phosphorylated-MAP-kinase ERK 1/2 (pERK), using a method described previously (9, 32-34, 38). The details for the primary and secondary antisera can be found in Table 1. pERK activation was determined rather than c-Fos as ERK phosphorylation is far more rapid (minutes compared to hours) (24). As the nature of the specific peripheral stimuli applied is short duration, the rapid onset of pERK provides a better correlate. Further, spinal activation to a range of peripheral stimuli is increasingly being identified via quantification of pERK activation (9, 32, 34, 39). After air drying for 1 hour, sections were washed with 0.2% Triton X-100 (Sigma-Aldrich, #T8787) in 0.1-M phosphate-buffered saline (PBS-T) to remove excess OCT. Non-specific binding of secondary antibody was blocked with 5% normal chicken serum diluted in 0.2% PBS-T. Tissue sections were incubated with pERK primary antisera diluted in PBS-T overnight (18 hours) at room temperature. Sections were then washed in PBS-T and incubated for 1 hour at room temperature with secondary antibody conjugated to AlexaFluor 594. Following secondary antibody incubation, sections were washed in PBS-T before

mounting in ProLong Diamond Antifade (Life Technologies, #P-36962) and coverslipping. Negative controls were prepared as above with the primary antibody omitted. Slides were allowed to set for 24 hours before visualization.

Microscopy

Fluorescence was visualized with an epifluorescence microscope (Olympus BX51, Tokyo, Japan) using 10× objective and 3-second exposure, with 590 nm excitation and 610/630 nm emission settings. Images were analyzed using ImageJ software (NIH). Other than making moderate adjustments for contrast and brightness, the images were not manipulated in any way.

Spinal cord pERK neuronal counts and analysis

Neuronal counts were analyzed from previously saved digital photomicrographs, with only neurons with intact nuclei counted. The number of pERK-immunoreactive (pERK-IR) neurons in a quadrant of the L6-S1 dorsal horn was obtained from minimum 6 sections/animal viewed at 10x magnification. The mean number of pERK-IR neurons (± SEM) in the superficial dorsal horn (SDH; LI-II), the dorsal grey commissure (DGC), and the sacral parasympathetic nuclei (SPN) in sacral spinal segments was compared between vehicle- and histamine-treated mice. These regions are known to have roles in nociceptive signaling and autonomic reflexes (33, 88).

Retrograde tracing

A small aseptic abdominal incision was made in anaesthetized (2% – 4% isoflurane in oxygen) mice. Cholera Toxin subunit B conjugated to AlexaFluor 488 (CTB-488, 0.5% diluted in 0.1 M phosphate buffered saline [PBS] pH 7.4; ThermoFisher Scientific) was injected at three sites into the bladder wall (3 µL/injection) using a 5µL Hamilton syringe attached to a 30-gauge needle (32, 33). The needle was inserted subserosally, parallel with the bladder muscle, to ensure that CTB was not injected into the bladder lumen. The abdominal incision was sutured closed and analgesic (Buprenorphine (Temvet); 0.1 mg/kg; Troy Laboratories Pty Ltd, APVMA #67612) and antibiotic (Amoxicillin; 50 mg/kg; Amoxil, AUSTR11137) given subcutaneously as mice regained consciousness. Mice were then housed individually and allowed to recover. After 4 days, mice were humanely euthanized for subsequent lumbosacral (LS; L5-S1) dorsal root ganglia (DRG) removal and the neurons isolated and cultured to visualize CTB-labelled bladder innervating neurons amongst the DRG neurons.

Cell culture of bladder-innervating DRG neurons

Four days after bladder retrograde tracing, mice were humanely euthanized via CO_2 inhalation and lumbosacral (LS; L5-S1) dorsal root ganglia (DRG) were removed. DRG were digested in Hanks'

balanced salt solution (HBSS; pH 7.4; Life Technologies, #14170161) containing 2.5 mg/mL collagenase II (GIBCO, ThermoFisher Scientific, #17101015) and 4.5 mg/mL dispase (GIBCO, ThermoFisher Scientific, #17105041) at 37°C for 30 min. The collagenase-dispase solution was aspirated and replaced with HBSS containing collagenase (4.5 mg/mL) only for 10 min at 37°C. Following subsequent washes in HBSS, DRG were mechanically disrupted and cells dissociated in 600 µL complete DMEM (Dulbecco's Modified Eagle Media [DMEM; GIBCO, ThermoFisher Scientific, #11995065]; 10% Fetal Calf Serum [Invitrogen, ThermoFisher Scientific, MA, USA]; 2 mM L-glutamine [GIBCO, ThermoFisher Scientific, #25030081], 100 µM MEM non-essential amino acids [GIBCO, ThermoFisher Scientific, #11140076], 100 mg/mL penicillin/streptomycin [GIBCO, ThermoFisher Scientific, #15070063], and 96 µg/L nerve growth factor-7S [Sigma, N0513-0.1MG]) via trituration through fire-polished Pasteur pipettes of descending diameter, and centrifuged for 1 min at 50 g (32, 34, 38, 64). Neurons were resuspended in 360 µL complete DMEM and spot-plated (30 µL) onto 13 mm coverslips coated with laminin (20 µg/mL; Sigma-Aldrich, #L2020) and poly-D-lysine (800 µg/mL; ThermoFisher Scientific). Coverslips were incubated at 37°C in 5% CO_2 for 2-3 hours to allow neurons to adhere before flooding with 1.7 mL complete DMEM. Cultured neurons were maintained in an incubator at 37°C in 5% CO_2 for 18-48 hours for calcium imaging or for 2 hours for cell picking for single-cell RT-PCR.

Isolation of mouse urothelial cells

Primary urothelial cells were isolated and cultured from mice as performed previously (11, 28, 31). Following euthanasia by CO_2 inhalation, bladders were removed and dissected in sterile PBS with a single cut from the urethral opening along one side to the dome, pinned and stretched out urothelial side up, and incubated with DMEM containing 2.5 mg/mL dispase, 10% Fetal Calf Serum, and 1 µM/mL HEPES (Sigma, #H3375, pH 7.0) for 3 hours at room temperature. Cells were collected by gentle scraping of the urothelium with a blunt scalpel and dissociated in 0.025% trypsin EDTA (GIBCO, ThermoFisher Scientific, #25200072) at 37°C in 5% CO_2 for 10 min with gentle intermittent trituration. The cell suspension was added to DMEM containing 10% Fetal Calf Serum to deactivate the trypsin before centrifugation (15 min, 1500 rpm, 4°C). Cells were then resuspended in keratinocyte serum-free media (KSFM; Invitrogen, #17005042) before being plated onto collagen I-coated coverslips (diluted 1:20 in H2O; Sigma; #A10483-01). Coverslips were left for 4 hours in an incubator at 37°C in 5% CO_2 before flooding with KSFM (2 mL/well). Cells were maintained at 37°C in 5% CO_2 for 24-48 hours and used for calcium imaging.

Cytokeratin 7 immunohistochemistry

Immunohistochemical labelling for transitional epithelium was performed on isolated urothelial cells using monoclonal antibody cytokeratin 7 (CK7). The details of the primary and secondary

antibodies used are in Table 2. Coverslips were washed with 0.1 M phosphate-buffered saline (PBS) three times and fixed with ice-cold 4% PFA at 4°C for 45 minutes. Coverslips were washed with saponin 0.05% (Sigma-Aldrich; #47036) + 2% FBS in 0.1 M PBS (SF-PBS) to remove excess PFA and permeabilize cell membranes. Nonspecific binding of secondary antibodies was blocked with 3% bovine serum albumin (Sigma-Aldrich; #A9418) diluted in 0.05% SF-PBS for 1 hour. Coverslips were incubated with primary antisera diluted in SF-PBS overnight (28 hours) at 4°C. Sections were then washed 3x in PBS and incubated in the dark for 2 hours at room temperature with secondary antibody conjugated to AlexaFluor 488. Cells were then washed in SF-PBS before mounting in Prolong Gold Antifade with DAPI and coverslipping. Negative controls were prepared as above with the primary antibody omitted. Slides were allowed to set for 24 hours before visualization.

Microscopy

Fluorescence was visualized with a confocal laser scanning microscope (Leica TCS SP8X; Leica Microsystems, Wetzlar, Germany). Images (1024 x 1024 pixels) were obtained using a x63 lens (software zoom x1.3) and sequential scanning (4- to 5-line average). Separation of fluorophores was achieved using white line laser tuned to 495-nm excitation and 505- to 534-nm emission detection settings for AF-488 and 405-nm excitation and 425- to 475-nm emission detection settings for DAPI. Confocal settings were optimized to reduce background staining by adjusting the white light laser intensity, emission window (as described above), and amplifier gain [726.7 offset: x0.07 (AF488); 10 offset: x0.1 (DAPI)]. These settings were saved and used for all imaging.

Calcium imaging of cultured urothelial cells and DRG neurons

Cultured DRG neurons and urothelial cells (18-48 hours) were loaded with 2 µM Fura-2-acetoxymethyl ester (Fura-2; Invitrogen, ThermoFisher Scientific, #F1221) in 0.01% pluronic F-127 (Invitrogen, ThermoFisher Scientific, #P3000MP) for 30 min at 37°C and washed with HEPES buffer (10 mM HEPES sodium salt [4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt; Sigma, #H7006-100G], 140 mM NaCl [Chem Supply, #SA046-3KG], 4 mM KCl [Chem Supply, #PA054-500G], 5 mM D-glucose anhydrous [Chem Supply, #GA018-500G], 2 mM $CaCl_2$ [Scharlau, #CA01951000], and 2 mM $MgCl_2$ [Sigma, #M8266-100G], pH 7.40) for 10 min before imaging at room temperature (23°C). Fura-2 was excited at 340 and 380 nm, and emissions were measured at 510 nm using a Nikon TE300 Eclipse microscope equipped with a Sutter DG-4/OF wavelength switcher, an Omega XF04 filter set for Fura-2, a Photonic Science ISIS-3 intensified CCD camera, and Universal Interface Card MetaFluor software. Retrogradely traced bladder DRG neurons were identified by the presence of the CTB-488 tracer, visible under excitation at 480 nm. 480 nm excitation was used as an initial bladder neuron identifier only; images were not obtained at 480 nm throughout the experiment to prevent unnecessary

photobleaching. Fluorescence images were obtained every 5 s leading up to and immediately following drug application, and every 20 s for washout period, using a 20x objective. Data were recorded and further analyzed using MetaFluor software. After an initial baseline reading to ensure cell fluorescence was stable, indicating healthy cells, DRG neurons and urothelial cells were stimulated with histamine (100 µM; Sigma, #H7125-1G), and changes in intracellular calcium (Ca^{2+}_i) were monitored in real time. Ionomycin (2 µM) and KCl (40 mM) were applied as positive controls in urothelial and neuronal experiments, respectively. Ca^{2+}_i is expressed as the ratio between the fluorescence signals at 340- and 380-nm (Fura-2 (340/380)). Baseline Ca^{2+}_i was determined by averaging 8-12 fluorescence ratio readings immediately prior to stimulation. Peak response was calculated by subtracting the relevant baseline score from the maximum fluorescence ratio reading following drug application (Δ340/380). Baseline fluorescence was normalized to a ratio of 1 for example traces.

mRNA expression analysis in DRG, cultured urothelial cells and bladder mucosal and detrusor layers

Tissue collection

16- to 18-week-old mice were humanely euthanized via CO_2 inhalation and the bladder and lumbosacral (L5-S1) dorsal root ganglia (DRG) removed. DRG were frozen in dry ice in pairs by spinal level (L5, L6, S1) and stored at -80˚C for RNA extraction and quantitative reverse-transcription polymerase chain reaction (RT-qPCR). The bladder was opened, stretched out and pinned flat, urothelial side up. For tissue RT-qPCR, the mucosal and detrusor layers were gently peeled apart, frozen in dry ice and stored at -80˚C for RNA extraction and RT-qPCR. For urothelial cell RT-qPCR, urothelial cells were isolated as for cell culture above. Following resuspension of cells in keratinocyte serum-free media (KSFM; Invitrogen, #17005042), cell count and viability were determined using the Countess Automated Cell Counter (Invitrogen, ThermoFisher Scientific). Cells were pelleted, frozen in dry ice and stored at -80˚C for RNA extraction and RT-qPCR.

RNA extraction

RNA was extracted using the PureLink RNA Micro kit (Invitrogen, Victoria, Australia, #12183-016; DRG pairs and isolated urothelial cells) or the PureLink RNA Mini kit (Invitrogen, #12183018A; bladder mucosa and detrusor) with DNAse treatment (Life Technologies, #12185-010) according to the manufacturer's instructions. A NanoDrop Lite spectrophotometer (Thermofisher Scientific) determined RNA quantity and purity. RNA was reverse transcribed to cDNA using SuperScript VILO Master Mix (Invitrogen, #11755250) as per the manufacturer's instructions. cDNA was stored at -20°C for qPCR.

Quantitative reverse-transcription polymerase chain reaction (QRT-PCR)

QRT-PCR was performed using Taqman Gene Expression Master Mix (Applied Biosystems, Victoria, Australia, #4369016) with commercially available hydrolysis probes (TaqMan; Life Technologies, see Table 3 for details) and RNAse-free water (AMBION, Victoria, Australia, #AM9916). For each reaction, 10 µL of qPCR MasterMix, 1 µL of TaqMan primer assay, 4 µL of water, and 5 µL of cDNA (1:2 dilution in RNA-free H_2O) from each sample was tested in duplicate for each target. *Actb* (β-actin) was used as an endogenous control for DRG pairs. *Gapdh* and *Hprt* were used as endogenous controls for mucosal and detrusor tissue layers and isolated urothelial cells. Assays were run for 45 cycles on a 7500 Fast Real-Time PCR System (Applied Biosystems) machine, using 7500 Fast software, v2.0.6. mRNA quantities are expressed as $2^{-\Delta Ct}$ relative to reference gene *Actb* (DRG pairs) or *Gapdh* x *Hprt* (geometric mean; mucosa, detrusor and urothelial cells). Using Prism 8 software (GraphPad, San Diego, CA), data were analyzed by 1-way analysis of variance (ANOVA) with Tukey's multiple comparison test.

Single-cell RT-PCR of individual bladder-innervating dorsal root ganglia neurons

Under continuous perfusion of sterile and RNA-/DNase-free PBS, retrogradely traced single bladder dorsal root ganglia (DRG) neurons were identified using a fluorescence microscope and collected into the end of a fine glass capillary using a micromanipulator (32). The glass capillary containing the cell was then broken into a sterile Eppendorf tube containing 10 µL of lysis buffer with DNAse (TaqMan Gene Expression Cells-to-CT Kit; Invitrogen, #4399002). For each coverslip of cells, a bath control was also taken and analyzed together with cells. After lysis and termination of DNAse treatment, samples were immediately frozen on dry ice and stored at -80°C until cDNA synthesis was performed. RNA was reverse transcribed to cDNA using SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme (Invitrogen, #11766500) as per the manufacturer's instructions. cDNA was stored at -20°C for real time PCR. Tubulin-3 expression served as a neuronal marker and positive control. Expression was considered positive if a complete curve was observed before 50 cycles.

Data Analysis and Statistics

Data are presented as mean ± SEM or the % of neurons or afferents. N indicates the number of animals, whilst n indicates the number of independent neurons or afferents. Statistical significance was considered as $P<0.05$ and reported at a level of * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$. In some cases, # is used to indicate significance of at least $P < 0.05$ for multiple comparisons. Data were analyzed using Prism 8 (GraphPad, San Diego, CA, USA), using one- or two-way analysis of variance

(ANOVA) with Tukey's or Sidak's post hoc analyses dependent on data distribution, or student's t-tests, for parametric data. Non-parametric data were analyzed using Kruskal-Wallis test with Dunn's multiple comparisons, Mann-Whitney analysis, or Wilcoxon matched-pairs signed rank test. Data were tested for Gaussian distribution in order to determine the correct statistical tests. The specific tests used to analyze each data set is indicated within the individual figure legends.

Results

Histamine enhances bladder afferent sensitivity to bladder distension

In order to determine whether the presence of histamine within the bladder influences the activity of afferent sensory neurons innervating the bladder wall, we performed multiunit recordings of bladder-innervating pelvic afferents in response to graded bladder distension using an *ex vivo* preparation. Bladders were distended first with saline as a control, followed by histamine. As the pressure within the bladder increased with distension, there was a corresponding increase in afferent activity (**Figure 1A** and **1B**). We did not observe any significant effects on mechanosensitivity with 10-30μM histamine concentrations (Supp. Figure 1, URL: https://figshare.com/s/beaf7c9c8bf91b1d9ac4, DOI: https://doi.org/10.6084/m9.figshare.10116524). In contrast, 300μM histamine significantly enhanced the afferent firing rate at distension pressures of and above 12 mmHg compared to the saline control distensions (**Figure 1A** and **1C**). The pressure-to-volume relationship in the bladder during distension was also analyzed to ensure that muscle compliance and contractility was not altered during the protocol, thus affecting afferent firing. No difference in muscle compliance was observed between saline and histamine protocols (**Figure 1D**). These data suggest that the significant changes observed in bladder afferent mechanosensitivity with histamine instillation were due to a direct effect on afferents, and not secondary to changes in the ability of the bladder to accommodate an increase in volume.

To further characterize the enhanced mechanosensitivity observed *ex vivo* in response to histamine, the single afferent units that comprise the multiunit response were analyzed (**Figure 2**). Overall, three distinct populations of afferent responses were observed. One, 45% of bladder afferents responded to histamine with an increase in mechanosensitivity (**Figure 2A, 2E(i)** and **2F**), reflecting the overall outcome observed in whole nerve recordings. Two, 50% of mechanosensitive bladder afferents were unresponsive to histamine, showing no difference in firing rates upon histamine application (**Figure 2B, 2E(ii)** and **2F**). Three, the remaining 5% of bladder afferents were shown to be a distinct functional subclass, identified as 'silent afferents' such that they were non-responsive during bladder distension with saline, but accrued mechanosensitivity in the presence of histamine (**Figure 2C** and **2F**). All responding afferents showed a significant increase (≥20% above baseline) in afferent firing in response to bladder distension with histamine (**Figure 2E(i)**). These results demonstrate that histamine

is able to sensitize a subset of mechanosensitive afferents in the bladder and recruit a population of 'silent afferents' to become mechanosensitive, ultimately resulting in an enhanced mechanosensory afferent response to bladder distension.

Bladder-infused histamine activates dorsal horn neurons in the lumbosacral spinal cord

Following the identification of increased sensitivity in the bladder afferents *ex vivo*, we aimed to determine whether this hypersensitivity is relayed centrally, at the level of the spinal cord. We performed *in vivo* bladder infusion of histamine or saline (control) and analyzed the number of dorsal horn neurons with pERK-immunoreactivity (pERK-IR) as an indicator of spinal cord neuronal activation (Figure 3). Following saline infusion, pERK-IR was observed in sections of lumbosacral spinal cord in the dorsal grey commissure (DGC), the superficial dorsal horn (SDH), and the sacral parasympathetic nucleus in sacral spinal segments (SPN; **Figure 3A** and **3B**). Infusion of histamine into the bladder resulted in significantly greater numbers of pERK-IR neurons relative to saline infusion (**Figure 3A** and **3B**) in the DGC (Figure 3A(ii)) and the SDH (Figure 3A(iii)), with no significant difference in the SPN (Figure3A(iv)). Catheter insertion alone did not evoke pERK-immunoreactivity (Figure 3B(ii)). These results indicate that the histamine-evoked hypersensitivity of bladder afferent peripheral endings is relayed into the spinal cord and enhances central signaling.

Histamine receptor subtypes Hrh1, Hrh2 and Hrh3 are expressed in lumbosacral dorsal root ganglia (L5-S1), with Hrh1 identified in ~24% of bladder innervating afferent neurons

To begin to explore the potential mechanism of action of histamine on bladder afferents, we used QRT-PCR to investigate the mRNA expression of the four histamine receptor subtypes, *Hrh1-Hrh4*, relative to the housekeeper *Actb* (β-actin) in the lumbosacral (LS; L5-S1) DRG. mRNA for *Hrh1-Hrh3*, but not *Hrh4*, which was found in spleen (positive control tissue, Supp. Fig 2 URL:https://figshare.com/s/150eb77ed470f6533c58, DOI: https://doi.org/10.6084/m9.figshare.10247108), was expressed within the LS DRG (**Figure 4A** and **4B**), with small differences observed between the DRG pairs across L5, L6 and S1 (Figure 4A). As *Hrh1* was the highest expressed receptor within LS DRG and is known to play a significant role in irritant response mechanisms, we performed single cell RT-PCR to determine *Hrh1* mRNA expression in individual bladder innervating LS DRG neurons. We found that 23.6% of bladder-innervating afferent neurons expressed mRNA for *Hrh1* (Figure 4C). In comparison, mRNA expression of transient receptor potential vanilloid 1 (*Trpv1*), which has been implicated in histamine activity in other visceral organs, was

identified in 84.4% of bladder-innervating DRG neurons in an independent subset of bladder neurons (Figure 4C).

Histamine evokes direct activation in a small subpopulation of bladder DRG neurons

Having identified that histamine has a significant effect on the afferent sensitivity of the bladder both peripherally and at the level of the spinal cord, we aimed to determine whether this effect was as a result of direct neuronal activation. Retrogradely traced bladder afferent cell bodies from LS DRG were dissected, dissociated and cultured for live cell calcium imaging to measure intracellular calcium transients in response to short (1 minute) incubations in histamine. Direct application of histamine evoked calcium transients in a population of LS DRG neurons, including a small population of bladder-innervating neurons (2 out of 63; **Figure 5A and 5B**). While these numbers do not equate to the percentage of cells expressing histamine receptors, this data demonstrates that histamine can activate bladder neurons directly.

The direct action of histamine on bladder afferents was also examined in the *ex vivo* bladder afferent preparation by measuring direct firing, in response to histamine between bladder distensions. Interestingly, whilst histamine evoked marked increases in afferent firing to bladder distension (Figure 1), in the same ex vivo preparations we did not observe action potential firing in response to histamine compared to saline application (**Figure 5C and 5D**). Taken together, these results indicate that the observed increase in peripheral and central afferent signaling in response to histamine *ex vivo* and *in vivo* are not simply a result of direct, independent activation of the neurons.

Histamine does not evoke calcium transients in isolated primary urothelial cells

As the effects observed during our *ex vivo* and *in vivo* experiments resulted from histamine infused into the bladder lumen and the urothelium has been shown to exert a sensory role within the bladder by responding to and releasing a number of neuromodulators, we sought to determine whether urothelial cells play a role in mediating histamine-induced sensitivity in the bladder. To do this, we assessed the expression and function of histamine receptors in isolated primary urothelial cells via QRT-PCR and live cell calcium imaging (Figure 6). We confirmed that the isolation process was generating pure cultures of urothelial cells through positive immunolabelling of CK7 in isolated urothelial cells (Figure 6A). Similar to what we observed in the LS DRG, primary urothelial cells demonstrated expression of *Hrh1-Hrh3* mRNA, with *Hrh4* mRNA expression levels below reliable detection limits (Figure 6B). Interestingly, relative expression of *Hrh3* was significantly higher than that of *Hrh1* and *Hrh2* in primary urothelial cells. Relative expression was also determined in the whole

mucosa (including urothelium and underlying interstitial tissue with the detrusor smooth muscle removed) and the whole detrusor smooth muscle separately (**Figure 6C**). Expression of each of the histamine receptor subtypes relative to housekeepers *Gapdh* and *Hprt* was higher in the mucosa than the isolated urothelial cells but of a similar ratio, with *Hrh3* the highest expressed receptor. Expression in the detrusor muscle was highest for *Hrh2* and *Hrh1*, with significantly lower expression of *Hrh3*. As with isolated urothelial cells, *Hrh4* mRNA was below the detectable limits for both bladder mucosa and detrusor (**Figure 6C**).

Given the relative abundance of histamine receptors in urothelial cells, we used live-cell calcium imaging to determine whether isolated urothelial cells are directly activated in the presence of histamine, as a potential mechanism contributing to the enhancement of bladder mechanosensitivity we observed *ex vivo* and *in vivo*. Histamine application was unable to evoke calcium transients in any urothelial cells across multiple independent experiments (**Figure 6D** and **6E).** The administration of 2 µM ionomycin following wash-out of histamine as a control for urothelial cellular responsiveness demonstrated that the cultured cells were healthy and able to respond with increases in intracellular calcium. These results suggest that while histamine receptors are present in the bladder and specifically in urothelial cells, histamine is unable to directly trigger calcium influx in an isolated urothelial culture.

Histamine H_1 receptor (H_1R) mediates histamine-evoked enhanced mechanosensitivity to bladder distension *ex vivo*

Given that *Hrh1* was the most abundantly expressed histamine receptor subtype in LS DRG, and the significant proportion of bladder-innervating afferent neurons expressing *Hrh1* mRNA, we sought to determine what role this receptor subtype plays in mediating histamine activity in the bladder. Application of pyrilamine, a H_1R antagonist, blocked histamine-evoked mechanical hypersensitivity in an *ex vivo* bladder afferent preparation (**Figure 7A** and **7B**). Furthermore, pyrilamine had no effect on baseline mechanosensitivity, indicating H_1R is not an essential component of bladder mechanosensitivity under normal conditions. Pyrilamine also had no effect on bladder muscle compliance (**Figure 7C**), indicating that, as with the histamine-induced mechanosensitivity without pyrilamine, the inhibition of this response is at the afferent level rather than a secondary effect from altered bladder capacity. These results suggest that the enhanced mechanosensitivity observed in response to histamine is mediated by H_1R.

TRPV1 expression is required for histamine-evoked hypersensitivity to bladder distension *ex vivo*

TRPV1 has previously been implicated in H_1R-mediated histamine responses in other organs (79, 90). As our results show a high proportion of bladder-innervating afferent neurons expressing *Trpv1* mRNA and a critical role for H_1R in the histamine-induced mechanosensitization *ex vivo*, we aimed to determine whether the effect of histamine was lost in preparations from *Trpv1*$^{-/-}$ mice (**Figure 8**). Supporting previous studies, when we compared control (saline) bladder distensions between *Trpv1*$^{-/-}$ and wildtype mice in the *ex vivo* bladder preparation we found a significant reduction in mechanosensitivity in the *Trpv1*$^{-/-}$ mice (**Supp. Figure 3**, **URL:**https://figshare.com/s/7a767726ba71bdf13c70, **DOI**: https://doi.org/10.6084/m9.figshare.9845549). Importantly, while the overall magnitude of the response was blunted compared to the wildtype mice, the afferent response to distension in the *Trpv1*$^{-/-}$ mice demonstrated a graded increase in firing in response to increased intraluminal pressure in the bladder, of an equivalent pattern to the wildtype preparation. In the *Trpv1*$^{-/-}$ mice, histamine failed to elicit a change in overall bladder afferent mechanosensitivity compared to saline control responses (**Figure 8A**). Consistent with healthy control mice, no changes in muscle compliance were observed (**Figure 8B**). Interestingly, analysis of the single units in the *Trpv1*$^{-/-}$ preparations revealed that while the overall histamine response was abolished, a small proportion (20%) of single units maintained histamine sensitivity (**Figure 8D** and **8E**). These histamine-sensitive afferents demonstrated a significant increase in mechanosensitivity following histamine administration, as with histamine-sensitive afferents in the wildtype mice. We also did not observe the recruitment of silent afferents in the presence of histamine in the *Trpv1*$^{-/-}$ experiments. Furthermore, histamine failed to evoke calcium transients in dissociated bladder-innervating LS DRG from *Trpv1*$^{-/-}$ mice (**Figure 8F**), whilst the percentage of non-traced LS DRG neurons responding to histamine in *Trpv1*$^{-/-}$ mice (4.1%) was reduced compared to wild type mice (9.4%; see **Figure 5A** and **8F**). Taken together, these results suggest that TRPV1 is important in mediating histamine-induced mechanosensitivity to bladder distension, however a component of the histamine response is also TRPV1-independent.

Discussion

The chronic sensory symptoms of urgency, frequency and pelvic pain experienced by IC/BPS patients implicate altered bladder afferent activity as a key component in the pathophysiology of this disorder. Despite this, the specific mechanisms underlying a switch from normosensitivity (healthy state) to hypersensitivity in IC/BPS are still unclear, and thus effective treatments are currently lacking (33). Previous studies have indicated that a relationship exists between the release of histamine from mast cells and the sensitization of bladder afferent neurons in IC/BPS that contributes to the symptoms of hypersensitivity, including pelvic pain (2, 6, 58, 72, 74, 75, 78, 81). In this study, for the first time, we show that histamine is able to directly sensitize mechanosensitive bladder afferent neurons via mechanisms regulated by the histamine H_1 receptor (H_1R) and transient receptor potential vanilloid 1 (TRPV1).

Our results demonstrate that a population of primary afferent neurons innervating the bladder exhibit enhanced mechanosensitivity in the presence of histamine. Furthermore, we identified a sub-population of 'silent afferents' that became mechanosensitive only in the presence of histamine. These neurons represent a distinct functional subclass previously reported to be responsible for signaling 'high-threshold' or 'nociceptive' stimuli, responding only to noxious levels of distension, that can be sensitized by chemical mediators (57, 69, 93). Together, these data suggest that if an increase in histamine occurs in the bladder wall, it has the ability to sensitize bladder afferents to mechanical distension, such that they fire more frequently in response to a range of bladder distension pressures, potentially leading to exaggerated sensation. Our data is supported by previous studies showing a role for histamine in the activation and sensitization of sensory afferents innervating both the skin and gastrointestinal tract (44, 47, 51, 55, 77), as well as intracranial nociceptors (92). Lower concentrations of histamine had no effect on bladder mechanosensitivity ex-vivo. The primary role of the urothelium is to provide a barrier to the toxic solutes in the urine, however, we and others utilizing these techniques have shown that by using relatively high concentrations of experimental compound we are able to overcome this barrier to target the underlying mechanosensory afferent nerves (32, 71). Whilst this provides the limitation that the exact concentrations at the afferent terminals is unknown, and prevents meaningful washout of the agonist in question, it provides significant value in proof of concept studies such as this. It is also important to consider that the concentrations of histamine that bladder nerve terminals are exposed to in health and disease are unknown, but likely exceed those found in the urine. Mast cells are the primary source of histamine (84, 87), and a number of previous studies have identified increased activated mast cell counts in IC/BPS patients (55, 76, 85, 86, 91) as well as increased urinary levels of histamine in these patients (8, 20). Mast cells have been identified in extremely close proximity to nerve endings (1, 42, 55, 59, 83), allowing direct mast cell nerve interactions that likely

involve high localized concentrations of histamine and which may promote the underlying hypersensitivity associated with IC/BPS that contributes to urinary frequency and urgency.

Bladder-innervating primary afferents project into the lumbosacral spinal dorsal horn, where subpopulations of neurons within the DGC, SDH, and the SPN have been previously identified within the bladder sensory pathways (4, 5, 16, 23, 33, 60, 66). Such pathways are known to project up to the brain as well as form local connections to spinal reflex arcs, feeding into networks which are important for both micturition and nociception (23, 26, 33, 88). Using pERK-IR to examine dorsal horn neuronal activation we confirmed that the exaggerated peripheral afferent signal stimulated by histamine evokes enhanced neuronal activation within these dorsal horn spinal cord regions (23, 60, 66, 88). We have previously demonstrated that during graded bladder distension from physiological to noxious bladder distension pressures, similar regions of the spinal cord are activated, with nociceptive signaling identified by an increase in the number of pERK-IR neurons within those same regions, rather than activation of a distinct nociceptive pathway (33). Similarly, the presence of histamine in the bladder *in vivo* increases activation in these same regions, rather than activating distinct irritant pathways.

Having established a direct involvement for histamine in mediating both peripheral and central sensitivity to bladder distension, we sought to understand the specific mechanism of this interaction. We show that histamine sensitizes bladder afferents via a H_1R-dependent mechanism, as histamine-evoked hypersensitivity to distension was completely abolished in the presence of the H_1R antagonist pyrilamine. This is an interesting finding as afferents innervating the colon are also activated by histamine via a H_1R-dependent mechanism (90). Similarly, histamine also activates cutaneous DRG neurons, although this effect appears more promiscuous, by acting on H_1R, H_3R and H_4R (53, 72). The down-stream mechanisms of H_1R have also been identified in these populations of DRG neurons. Histamine sensitizes TRPV1 channels via H_1R in irritable bowel syndrome (IBS), a chronic visceral sensitivity disorder (90). In cutaneous DRG neurons, TRPV1 also mediates H_1R-dependent histamine-sensitization (79, 90), with coupling between histamine receptors and TRPV1 occurring via activation of phospholipase C and phospholipase A2 (65).

Our QRT-PCR data show *Hrh1* is the most abundant of the histamine receptors in LS DRG, and we confirmed *Hrh1* mRNA expression in ~24% of bladder-innervating LS DRG neurons with single cell RT-PCR. We also identified that TRPV1 is an essential integrator of histamine-induced mechanosensitivity in the bladder. No overall increase in mechanosensitivity was observed in the presence of histamine in *Trpv1*$^{-/-}$ mice and nor were calcium transients to histamine observed in dissociated *Trpv1*$^{-/-}$ mouse bladder DRG neurons. While the overall effect of histamine on mechanosensitivity is absent in *Trpv1*$^{-/-}$ mice, a sub-population of bladder afferents are still histamine

sensitive, albeit a reduced percentage compared to wild-type mice. As with many GPCRs, the activation of histamine receptors results in several intracellular processes (35, 52, 65); evidently a subset of histamine-sensitive bladder afferents respond in a TRPV1-independent manner. Along these lines, histamine also sensitizes colonic neurons via TRPV1(90) and a related TRP channel, TRPV4 (10). In the current study, we found that ~84% of bladder-innervating LS DRG neurons express TRPV1. This is consistent with a number of previous studies from both rat and mouse bladder innervating DRG showing between 48-90% express TRPV1 or show functional responses to the TRPV1 agonist capsaicin (14, 22, 50). Although TRPV1 is generally considered a marker of polymodal nociceptors or C-fibers, we and others have found *Trpv1*$^{-/-}$ mice exhibit alterations in afferent firing at physiological distension pressures (13), building on a wealth of research suggesting traditional nomenclature and characterizations of 'C-fibers' as nociceptors should not be relied upon as a proxy for functional characterization of sensory neurons in the bladder (17, 22, 70).

The question remains as to whether the histamine-dependent sensitivity of bladder afferents also involved non-neuronal mechanisms. This is important as the urothelium is known to express various ion channels and receptors that enable it to mediate sensory signaling by responding to chemical and mechanical stimuli (3, 56, 89). Given the demonstrated increase of histamine and metabolites of histamine in the urine of IC/BPS patients (8, 20), we explored the potential role of urothelial cells in mediating histamine-enhanced bladder afferent mechanosensitivity. Here we confirmed the expression of histamine receptors *Hrh1-Hrh3* in all layers of the mouse bladder but did not detect *Hrh4*. These findings differ from previously reported results in mice but are comparable to results from human tissue (62, 63, 74). *Hrh4* is predominantly expressed in granulocytes and we validated this probe against mRNA isolated from the spleen. Importantly, our mRNA expression data demonstrated expression of *Hrh1, Hrh2* and *Hrh3* for the first time in isolated urothelial cells, highlighting the potential for urothelial cells in mediating histamine-induced modulation of mechanosensitivity. Notably, in mucosa and primary urothelial cells *Hrh3* displayed the highest abundance of all histamine receptors, which differs to our data in DRG, where *Hrh1* is most abundant. Furthermore, in detrusor muscle mRNA *Hrh2* and *Hrh1* were significantly more abundant than *Hrh3*, demonstrating clear differences with expression profiles in DRG, detrusor muscle, mucosa and primary urothelial cells. Despite the relative expression of histamine receptor subtypes in isolated urothelial cell culture, application of histamine did not induce calcium influx in these cells. Our primary mouse urothelial cell culture was confirmed to be of urothelial origin, but the proportion of umbrella, intermediate, and basal cells remains undetermined. Previous studies have indicated that whilst this type of primary culture isolates a heterogeneous urothelial cell population (48), the majority of these cells are of basal and intermediate origin and exhibit functional responses to a range of ion channel and

GPCR agonists (21, 29, 30, 45, 48, 49). Whilst this does not preclude an effect of histamine on bladder urothelial cells, as we saw no responses to histamine in any of the cells from this study, our data indicates that intracellular calcium responses are not directly initiated by short duration exposure to histamine. Further dissection of this mechanism following the development of techniques to isolate individual populations of urothelial cells or in-vitro imaging of the mucosa would provide valuable insight into these interactions. Two considerations remain unanswered here. The first, that *Hrh3* as the highest expressed receptor in urothelial cells may be contributing in its capacity to function as an inhibitory receptor in healthy states. Second, while short term application allows us to explore the direct activity of histamine on cells via receptors, IC/BPS is a chronic condition, with diagnoses often occurring several months or even years after symptom onset (46). As such, the expression of histamine receptors in the urothelial cells may be important in the long-term potentiation of symptoms, if not acute activation.

The findings of the current study raise an interesting question as to role of histaminergic mechanisms within the bladder. We can draw parallels from other systems, whereby histamine evokes somatic itch via activation or sensitization of peripheral afferents innervating the skin, (40, 41, 43, 53, 72, 73, 77). As the physiological response to cutaneous itch is scratching to remove harmful irritants, the corresponding physiological response in the bladder would be achieved via micturition-induced irritant expulsion. This would involve histamine sensitizing bladder afferents to mechanical stimuli and triggering increases in the frequency of micturition, and thus experiencing sensations of urgency at lower distension volumes via an increase in mechanosensitivity may be a fundamental protective mechanism.

In conclusion, this study has demonstrated for the first time that histamine in the urinary bladder causes enhanced mechanosensitivity to bladder distension as well as recruitment of silent afferents, which translates to increased neuronal activation in the spinal cord dorsal horn. As such, in conditions such as IC/BPS, where mast cells and urinary histamine concentrations are increased, we propose that histamine contributes to the sensitization of mechanosensitive bladder afferent pathways resulting in symptoms of urgency, frequency and suprapubic pain at physiological distension pressures.

References

1. Bauer O and Razin E. Mast Cell-Nerve Interactions. *Physiology* 15: 213-218, 2000.
2. Bicer F, Altuntas CZ, Izgi K, Ozer A, Kavran M, Tuohy VK, and Daneshgari F. Chronic pelvic allodynia is mediated by CCL2 through mast cells in an experimental autoimmune cystitis model. *American journal of physiology Renal physiology* 308: F103-113, 2015.
3. Birder L and Andersson KE. Urothelial signaling. *Physiol Rev* 93: 653-680, 2013.
4. Birder LA and de Groat WC. Induction of c-fos expression in spinal neurons by nociceptive and nonnociceptive stimulation of LUT. *Am J Physiol* 265: R326-333, 1993.
5. Birder LA, Roppolo JR, Erickson VL, and de Groat WC. Increased c-fos expression in spinal lumbosacral projection neurons and preganglionic neurons after irritation of the lower urinary tract in the rat. *Brain Res* 834: 55-65, 1999.
6. Bjorling DE, Jerde TJ, Zine MJ, Busser BW, Saban MR, and Saban R. Mast cells mediate the severity of experimental cystitis in mice. *J Urol* 162: 231-236, 1999.
7. Bogart LM, Berry SH, and Clemens JQ. Symptoms of interstitial cystitis, painful bladder syndrome and similar diseases in women: a systematic review. *J Urol* 177: 450-456, 2007.
8. Boucher W, el-Mansoury M, Pang X, Sant GR, and Theoharides TC. Elevated mast cell tryptase in the urine of patients with interstitial cystitis. *Br J Urol* 76: 94-100, 1995.
9. Castro J, Harrington AM, Garcia-Caraballo S, Maddern J, Grundy L, Zhang J, Page G, Miller PE, Craik DJ, Adams DJ, and Brierley SM. alpha-Conotoxin Vc1.1 inhibits human dorsal root ganglion neuroexcitability and mouse colonic nociception via GABAB receptors. *Gut* 66: 1083-1094, 2017.
10. Cenac N, Altier C, Motta JP, d'Aldebert E, Galeano S, Zamponi GW, and Vergnolle N. Potentiation of TRPV4 signalling by histamine and serotonin: an important mechanism for visceral hypersensitivity. *Gut* 59: 481-488, 2010.
11. Chess-Williams R, Sellers DJ, Brierley SM, Grundy D, and Grundy L. Purinergic receptor mediated calcium signalling in urothelial cells. *Scientific Reports* 9: 16101, 2019.
12. Clemens JQ, Mullins C, Ackerman AL, Bavendam T, van Bokhoven A, Ellingson BM, Harte SE, Kutch JJ, Lai HH, Martucci KT, Moldwin R, Naliboff BD, Pontari MA, Sutcliffe S, and Landis JR. Urologic chronic pelvic pain syndrome: insights from the MAPP Research Network. *Nat Rev Urol* 16: 187-200, 2019.
13. Daly D, Rong W, Chess-Williams R, Chapple C, and Grundy D. Bladder afferent sensitivity in wild-type and TRPV1 knockout mice. *J Physiol* 583: 663-674, 2007.
14. Dang K, Bielefeldt K, and Gebhart GF. Cyclophosphamide-induced cystitis reduces ASIC channel but enhances TRPV1 receptor function in rat bladder sensory neurons. *Journal of neurophysiology* 110: 408-417, 2013.
15. de Groat WC, Griffiths D, and Yoshimura N. Neural control of the lower urinary tract. *Compr Physiol* 5: 327-396, 2015.
16. de Groat WC and Yoshimura N. Afferent nerve regulation of bladder function in health and disease. *Handb Exp Pharmacol*: 91-138, 2009.

17. de Groat WC and Yoshimura N. Afferent nerve regulation of bladder function in health and disease. *Handb Exp Pharmacol*: 91-138, 2009.
18. de Groat WC and Yoshimura N. Anatomy and physiology of the lower urinary tract. *Handb Clin Neurol* 130: 61-108, 2015.
19. DeBerry JJ, Schwartz ES, and Davis BM. TRPA1 mediates bladder hyperalgesia in a mouse model of cystitis. *Pain* 155: 1280-1287, 2014.
20. el-Mansoury M, Boucher W, Sant GR, and Theoharides TC. Increased urine histamine and methylhistamine in interstitial cystitis. *J Urol* 152: 350-353, 1994.
21. Everaerts W, Vriens J, Owsianik G, Appendino G, Voets T, De Ridder D, and Nilius B. Functional characterization of transient receptor potential channels in mouse urothelial cells. *American journal of physiology Renal physiology* 298: F692-F701, 2010.
22. Forrest S, Osborne P, and Keast J. Characterization of bladder sensory neurons in the context of myelination, receptors for pain modulators, and acute responses to bladder inflammation. *Frontiers in Neuroscience* 7, 2013.
23. Fowler CJ, Griffiths D, and de Groat WC. The neural control of micturition. *Nat Rev Neurosci* 9: 453-466, 2008.
24. Gao YJ and Ji RR. c-Fos and pERK, which is a better marker for neuronal activation and central sensitization after noxious stimulation and tissue injury? *Open Pain J* 2: 11-17, 2009.
25. Grover S, Srivastava A, Lee R, Tewari AK, and Te AE. Role of inflammation in bladder function and interstitial cystitis. *Ther Adv Urol* 3: 19-33, 2011.
26. Grundy L and Brierley SM. Cross-organ sensitization between the colon and bladder: to pee or not to pee? *Am J Physiol Gastrointest Liver Physiol* 314: G301-G308, 2018.
27. Grundy L, Caldwell A, and Brierley SM. Mechanisms Underlying Overactive Bladder and Interstitial Cystitis/Painful Bladder Syndrome. *Front Neurosci* 12: 931, 2018.
28. Grundy L, Chess-Williams R, Brierley SM, Mills K, Moore KH, Mansfield K, Rose'Meyer R, Sellers D, and Grundy D. NKA enhances bladder-afferent mechanosensitivity via urothelial and detrusor activation. *American journal of physiology Renal physiology* 315: F1174-F1185, 2018.
29. Grundy L, Chess-Williams R, Brierley SM, Mills K, Moore KH, Mansfield K, Rose'Meyer R, Sellers D, and Grundy D. NKA enhances bladder-afferent mechanosensitivity via urothelial and detrusor activation. *American Journal of Physiology-Renal Physiology* 315: F1174-F1185, 2018.
30. Grundy L, Daly DM, Chapple C, Grundy D, and Chess-Williams R. TRPV1 enhances the afferent response to P2X receptor activation in the mouse urinary bladder. *Scientific Reports* 8: 197, 2018.
31. Grundy L, Daly DM, Chapple C, Grundy D, and Chess-Williams R. TRPV1 enhances the afferent response to P2X receptor activation in the mouse urinary bladder. *Sci Rep* 8: 197, 2018.

32. Grundy L, Erickson A, Caldwell A, Garcia-Caraballo S, Rychkov G, Harrington A, and Brierley SM. Tetrodotoxin-sensitive voltage-gated sodium channels regulate bladder afferent responses to distension. *Pain* 159: 2573-2584, 2018.
33. Grundy L, Harrington AM, Caldwell A, Castro J, Staikopoulos V, Zagorodnyuk VP, Brookes SJH, Spencer NJ, and Brierley SM. Translating peripheral bladder afferent mechanosensitivity to neuronal activation within the lumbosacral spinal cord of mice. *Pain*, 2018.
34. Grundy L, Harrington AM, Castro J, Garcia-Caraballo S, Deiteren A, Maddern J, Rychkov GY, Ge P, Peters S, Feil R, Miller P, Ghetti A, Hannig G, Kurtz CB, Silos-Santiago I, and Brierley SM. Chronic linaclotide treatment reduces colitis-induced neuroplasticity and reverses persistent bladder dysfunction. *JCI Insight* 3, 2018.
35. Haaksma EE, Leurs R, and Timmerman H. Histamine receptors: subclasses and specific ligands. *Pharmacol Ther* 47: 73-104, 1990.
36. Hanno P and Dmochowski R. Status of international consensus on interstitial cystitis/bladder pain syndrome/painful bladder syndrome: 2008 snapshot. *Neurourol Urodyn* 28: 274-286, 2009.
37. Heppner TJ, Tykocki NR, Hill-Eubanks D, and Nelson MT. Transient contractions of urinary bladder smooth muscle are drivers of afferent nerve activity during filling. *The Journal of General Physiology* 147: 323-335, 2016.
38. Inserra MC, Israel MR, Caldwell A, Castro J, Deuis JR, Harrington AM, Keramidas A, Garcia-Caraballo S, Maddern J, Erickson A, Grundy L, Rychkov GY, Zimmermann K, Lewis RJ, Brierley SM, and Vetter I. Multiple sodium channel isoforms mediate the pathological effects of Pacific ciguatoxin-1. *Sci Rep* 7: 42810, 2017.
39. Jiang GY, Dai MH, Huang K, Chai GD, Chen JY, Chen L, Lang B, Wang QX, St Clair D, McCaig C, Ding YQ, and Zhang L. Neurochemical characterization of pERK-expressing spinal neurons in histamine-induced itch. *Sci Rep* 5: 12787, 2015.
40. Jutel M, Akdis M, and Akdis CA. Histamine, histamine receptors and their role in immune pathology. *Clin Exp Allergy* 39: 1786-1800, 2009.
41. Kajihara Y, Murakami M, Imagawa T, Otsuguro K, Ito S, and Ohta T. Histamine potentiates acid-induced responses mediating transient receptor potential V1 in mouse primary sensory neurons. *Neuroscience* 166: 292-304, 2010.
42. Keith IM, Jin J, and Saban R. Nerve-mast cell interaction in normal guinea pig urinary bladder. *Journal of Comparative Neurology* 363: 28-36, 1995.
43. Kim BM, Lee SH, Shim WS, and Oh U. Histamine-induced Ca(2+) influx via the PLA(2)/lipoxygenase/TRPV1 pathway in rat sensory neurons. *Neurosci Lett* 361: 159-162, 2004.
44. Koda H and Mizumura K. Sensitization to mechanical stimulation by inflammatory mediators and by mild burn in canine visceral nociceptors in vitro. *J Neurophysiol* 87: 2043-2051, 2002.
45. Konthapakdee N, Grundy L, O'Donnell T, Garcia-Caraballo S, Brierley SM, Grundy D, and Daly DM. Serotonin exerts a direct modulatory role on bladder afferent firing in mice. *J Physiol* 0.

46. Koziol JA, Clark DC, Gittes RF, and Tan EM. The natural history of interstitial cystitis: a survey of 374 patients. *J Urol* 149: 465-469, 1993.
47. Kreis ME, Haupt W, Kirkup AJ, and Grundy D. Histamine sensitivity of mesenteric afferent nerves in the rat jejunum. *Am J Physiol* 275: G675-680, 1998.
48. Kullmann FA, Artim D, Beckel J, Barrick S, de Groat WC, and Birder LA. Heterogeneity of muscarinic receptor-mediated Ca2+ responses in cultured urothelial cells from rat. *American journal of physiology Renal physiology* 294: F971-F981, 2008.
49. Kullmann FA, Shah MA, Birder LA, and Groat WCd. Functional TRP and ASIC-like channels in cultured urothelial cells from the rat. *American Journal of Physiology-Renal Physiology* 296: F892-F901, 2009.
50. La JH, Schwartz ES, and Gebhart GF. Differences in the expression of transient receptor potential channel V1, transient receptor potential channel A1 and mechanosensitive two pore-domain K+ channels between the lumbar splanchnic and pelvic nerve innervations of mouse urinary bladder and colon. *Neuroscience* 186: 179-187, 2011.
51. LaMotte RH, Dong X, and Ringkamp M. Sensory neurons and circuits mediating itch. *Nat Rev Neurosci* 15: 19-31, 2014.
52. Latorraca NR, Venkatakrishnan AJ, and Dror RO. GPCR Dynamics: Structures in Motion. *Chem Rev* 117: 139-155, 2017.
53. Lee JS, Han JS, Lee K, Bang J, and Lee H. The peripheral and central mechanisms underlying itch. *BMB Rep* 49: 474-487, 2016.
54. Lein ES, Hawrylycz MJ, Ao N, Ayres M, Bensinger A, Bernard A, Boe AF, Boguski MS, Brockway KS, Byrnes EJ, Chen L, Chen L, Chen TM, Chin MC, Chong J, Crook BE, Czaplinska A, Dang CN, Datta S, Dee NR, Desaki AL, Desta T, Diep E, Dolbeare TA, Donelan MJ, Dong HW, Dougherty JG, Duncan BJ, Ebbert AJ, Eichele G, Estin LK, Faber C, Facer BA, Fields R, Fischer SR, Fliss TP, Frensley C, Gates SN, Glattfelder KJ, Halverson KR, Hart MR, Hohmann JG, Howell MP, Jeung DP, Johnson RA, Karr PT, Kawal R, Kidney JM, Knapik RH, Kuan CL, Lake JH, Laramee AR, Larsen KD, Lau C, Lemon TA, Liang AJ, Liu Y, Luong LT, Michaels J, Morgan JJ, Morgan RJ, Mortrud MT, Mosqueda NF, Ng LL, Ng R, Orta GJ, Overly CC, Pak TH, Parry SE, Pathak SD, Pearson OC, Puchalski RB, Riley ZL, Rockett HR, Rowland SA, Royall JJ, Ruiz MJ, Sarno NR, Schaffnit K, Shapovalova NV, Sivisay T, Slaughterbeck CR, Smith SC, Smith KA, Smith BI, Sodt AJ, Stewart NN, Stumpf KR, Sunkin SM, Sutram M, Tam A, Teemer CD, Thaller C, Thompson CL, Varnam LR, Visel A, Whitlock RM, Wohnoutka PE, Wolkey CK, Wong VY, et al. Genome-wide atlas of gene expression in the adult mouse brain. *Nature* 445: 168-176, 2007.
55. Letourneau R, Pang X, Sant GR, and Theoharides TC. Intragranular activation of bladder mast cells and their association with nerve processes in interstitial cystitis. *Br J Urol* 77: 41-54, 1996.
56. Merrill L, Gonzalez EJ, Girard BM, and Vizzard MA. Receptors, channels, and signalling in the urothelial sensory system in the bladder. *Nat Rev Urol*, 2016.
57. Michaelis M, Habler HJ, and Jaenig W. Silent afferents: a separate class of primary afferents? *Clin Exp Pharmacol Physiol* 23: 99-105, 1996.

58. Minogiannis P, El-Mansoury M, Betances JA, Sant GR, and Theoharides TC. Hydroxyzine inhibits neurogenic bladder mast cell activation. *Int J Immunopharmacol* 20: 553-563, 1998.
59. Mittal A, Sagi V, Gupta M, and Gupta K. Mast Cell Neural Interactions in Health and Disease. *Frontiers in Cellular Neuroscience* 13, 2019.
60. Nadelhaft I, Vera PL, Card JP, and Miselis RR. Central nervous system neurons labelled following the injection of pseudorabies virus into the rat urinary bladder. *Neurosci Lett* 143: 271-274, 1992.
61. Nazif O, Teichman JM, and Gebhart GF. Neural upregulation in interstitial cystitis. *Urology* 69: 24-33, 2007.
62. Neuhaus J, Schulte-Baukloh H, Stolzenburg JU, Speroni di Fenizio P, Horn LC, Ruffert H, Hartenstein S, Burger M, Schulze M, and Schwalenberg T. Individual receptor profiling as a novel tool to support diagnosis of bladder pain syndrome/interstitial cystitis (BPS/IC). *World J Urol* 30: 693-700, 2012.
63. Neuhaus J, Weimann A, Stolzenburg JU, Dawood W, Schwalenberg T, and Dorschner W. Histamine receptors in human detrusor smooth muscle cells: physiological properties and immunohistochemical representation of subtypes. *World J Urol* 24: 202-209, 2006.
64. Osteen JD, Herzig V, Gilchrist J, Emrick JJ, Zhang C, Wang X, Castro J, Garcia-Caraballo S, Grundy L, Rychkov GY, Weyer AD, Dekan Z, Undheim EA, Alewood P, Stucky CL, Brierley SM, Basbaum AI, Bosmans F, King GF, and Julius D. Selective spider toxins reveal a role for the Nav1.1 channel in mechanical pain. *Nature* 534: 494-499, 2016.
65. Panula P, Chazot PL, Cowart M, Gutzmer R, Leurs R, Liu WL, Stark H, Thurmond RL, and Haas HL. International Union of Basic and Clinical Pharmacology. XCVIII. Histamine Receptors. *Pharmacol Rev* 67: 601-655, 2015.
66. Park SK, Devi AP, Bae JY, Cho YS, Ko HG, Kim DY, and Bae YC. Synaptic connectivity of urinary bladder afferents in the rat superficial dorsal horn and spinal parasympathetic nucleus. *J Comp Neurol*, 2019.
67. Parsons JK, Kurth K, and Sant GR. Epidemiologic issues in interstitial cystitis. *Urology* 69: 5-8, 2007.
68. Patnaik SS, Lagana AS, Vitale SG, Buttice S, Noventa M, Gizzo S, Valenti G, Rapisarda AMC, La Rosa VL, Magno C, Triolo O, and Dandolu V. Etiology, pathophysiology and biomarkers of interstitial cystitis/painful bladder syndrome. *Arch Gynecol Obstet* 295: 1341-1359, 2017.
69. Prato V, Taberner FJ, Hockley JRF, Callejo G, Arcourt A, Tazir B, Hammer L, Schad P, Heppenstall PA, Smith ES, and Lechner SG. Functional and Molecular Characterization of Mechanoinsensitive "Silent" Nociceptors. *Cell Rep* 21: 3102-3115, 2017.
70. Robinson DR and Gebhart GF. Inside information: the unique features of visceral sensation. *Mol Interv* 8: 242-253, 2008.
71. Rong W, Spyer KM, and Burnstock G. Activation and sensitisation of low and high threshold afferent fibres mediated by P2X receptors in the mouse urinary bladder. *J Physiol* 541: 591-600, 2002.

72. Rosa AC and Fantozzi R. The role of histamine in neurogenic inflammation. *Br J Pharmacol* 170: 38-45, 2013.
73. Rossbach K, Nassenstein C, Gschwandtner M, Schnell D, Sander K, Seifert R, Stark H, Kietzmann M, and Baumer W. Histamine H1, H3 and H4 receptors are involved in pruritus. *Neuroscience* 190: 89-102, 2011.
74. Rudick CN, Bryce PJ, Guichelaar LA, Berry RE, and Klumpp DJ. Mast cell-derived histamine mediates cystitis pain. *PLoS One* 3: e2096, 2008.
75. Rudick CN, Schaeffer AJ, and Klumpp DJ. Pharmacologic attenuation of pelvic pain in a murine model of interstitial cystitis. *BMC Urol* 9: 16, 2009.
76. Sant GR, Kempuraj D, Marchand JE, and Theoharides TC. The mast cell in interstitial cystitis: role in pathophysiology and pathogenesis. *Urology* 69: 34-40, 2007.
77. Schmelz M. Neurophysiology and itch pathways. *Handb Exp Pharmacol* 226: 39-55, 2015.
78. Shanahan F, Denburg JA, Fox J, Bienenstock J, and Befus D. Mast cell heterogeneity: effects of neuroenteric peptides on histamine release. *J Immunol* 135: 1331-1337, 1985.
79. Shim WS, Tak MH, Lee MH, Kim M, Kim M, Koo JY, Lee CH, Kim M, and Oh U. TRPV1 mediates histamine-induced itching via the activation of phospholipase A2 and 12-lipoxygenase. *J Neurosci* 27: 2331-2337, 2007.
80. Spencer NJ, Greenheigh S, Kyloh M, Hibberd TJ, Sharma H, Grundy L, Brierley SM, Harrington AM, Beckett EA, Brookes SJ, and Zagorodnyuk VP. Identifying unique subtypes of spinal afferent nerve endings within the urinary bladder of mice. *J Comp Neurol* 526: 707-720, 2018.
81. Stromberga Z, Chess-Williams R, and Moro C. Histamine modulation of urinary bladder urothelium, lamina propria and detrusor contractile activity via H1 and H2 receptors. *Sci Rep* 9: 3899, 2019.
82. Sun B, Li Q, Dong L, and Rong W. Ion channel and receptor mechanisms of bladder afferent nerve sensitivity. *Auton Neurosci* 153: 26-32, 2010.
83. Theoharides TC. The mast cell: a neuroimmunoendocrine master player. *Int J Tissue React* 18: 1-21, 1996.
84. Theoharides TC, Alysandratos KD, Angelidou A, Delivanis DA, Sismanopoulos N, Zhang B, Asadi S, Vasiadi M, Weng Z, Miniati A, and Kalogeromitros D. Mast cells and inflammation. *Biochim Biophys Acta* 1822: 21-33, 2012.
85. Theoharides TC, Kempuraj D, and Sant GR. Mast cell involvement in interstitial cystitis: a review of human and experimental evidence. *Urology* 57: 47-55, 2001.
86. Theoharides TC, Sant GR, el-Mansoury M, Letourneau R, Ucci AA, Jr., and Meares EM, Jr. Activation of bladder mast cells in interstitial cystitis: a light and electron microscopic study. *J Urol* 153: 629-636, 1995.
87. Theoharides TC, Valent P, and Akin C. Mast Cells, Mastocytosis, and Related Disorders. *N Engl J Med* 373: 163-172, 2015.
88. Todd AJ. Neuronal circuitry for pain processing in the dorsal horn. *Nat Rev Neurosci* 11: 823-836, 2010.

89. Winder M, Tobin G, Zupancic D, and Romih R. Signalling molecules in the urothelium. *BioMed research international* 2014: 297295, 2014.
90. 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, Ghesquiere B, Cirillo C, Kortekaas I, Carmeliet P, Peetermans WE, Vermeire S, Rutgeerts P, Augustijns P, Hellings PW, Belmans A, Vanner S, Bulmer DC, Talavera K, Vanden Berghe P, Liston A, and Boeckxstaens GE. Histamine Receptor H1-Mediated Sensitization of TRPV1 Mediates Visceral Hypersensitivity and Symptoms in Patients With Irritable Bowel Syndrome. *Gastroenterology* 150: 875-887.e879, 2016.
91. Yamada T, Murayama T, Mita H, and Akiyama K. Subtypes of bladder mast cells in interstitial cystitis. *Int J Urol* 7: 292-297, 2000.
92. Zhang XC, Strassman AM, Burstein R, and Levy D. Sensitization and activation of intracranial meningeal nociceptors by mast cell mediators. *J Pharmacol Exp Ther* 322: 806-812, 2007.
93. Zhang Y, Li S, Yecies T, Morgan T, Cai H, Pace N, Shen B, Wang J, Roppolo JR, de Groat WC, and Tai C. Sympathetic afferents in the hypogastric nerve facilitate nociceptive bladder activity in cats. *American journal of physiology Renal physiology* 316: F703-F711, 2019.

Tables

Table 1: Spinal cord immunohistochemistry primary and secondary antisera

	Species raised in	RRID/AF conjugate	Manufacturer, Catalogue No.	Dilution
Primary antisera: Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP(tm) Rabbit mAb #4370	Rabbit	AB_2315112	Genesearch, #4370L	1:200
Secondary Antisera: anti-Rabbit IgG	Chicken	AlexaFluor 594	Life Technologies, #A-21442	1:200

Table 2: Urothelial immunolabelling primary and secondary antisera details

	Species raised in	RRID/AF conjugate	Manufacturer, Catalogue No.	dilution
Primary antisera: Cytokeratin 7 Monoclonal Antibody (OV-TL 12/30)	Mouse	AB_10989596	ThermoFisher, # MA5-11986	1:50
Secondary Antisera: anti-Mouse IgG1	Goat	AlexaFluor 488	ThermoFisher, # A-21121	1:1000

Table 3 - Primers used for RT-qPCR receptor expression assays

Gene Alias	Gene Target	Assay ID
β-actin (reference gene)	*Actb*	Mm00607939_s1
HPRT (reference gene)	*Hprt*	Mm00446968_m1
Gapdh (reference gene)	*Gapdh*	Mm99999915_g1
Tubulin-3 (neuronal marker)	*Tubb3*	Mm00727586_s1
Histamine Receptor subtype 1	*Hrh1*	Mm00434002_s1
Histamine Receptor subtype 2	*Hrh2*	Mm00434009_s1
Histamine Receptor subtype 3	*Hrh3*	Mm00446706_m1
Histamine Receptor subtype 4	*Hrh4*	Mm00467634_m1
Transient receptor potential vanilloid 1	*Trpv1*	Mm01246300_m1

Figure Legends

Figure 1: Histamine enhances bladder afferent mechanosensitivity to graded bladder distension *ex vivo*

Graded distension of the bladder in an *ex vivo* nerve recording preparation was performed with saline followed by 300 µM histamine. (A) Administration of 300 µM histamine resulted in an increased afferent firing rate (impulses per second; imp/s) compared to saline at distension pressures at and above 18 mmHg (N = 10; *** P < 0.001). (B) An example experiment showing an increase in raw afferent nerve activity in the presence of 300 µM histamine compared to saline in response to increased intraluminal pressure. (C) The maximum afferent firing rate (imp/s) of bladder afferents per mouse was significantly increased (** P < 0.01; Δ = 43.7 ± 10.05 imp/s) in the presence of 300 µM histamine. (D) No change in muscle compliance (pressure-to-volume relationship) was observed between saline and histamine protocols, suggesting that the observed effect is neuronal and not muscular. Data are represented as mean ± SEM (A, D) or maximum imp/s (C). P values above are based on two-way ANOVA (A, D) with subsequent Sidak's post hoc test significance at individual data points indicated on (A), or paired t-test (C).

Figure 2: Single unit analysis of *ex vivo* bladder afferent mechanosensitivity in the presence of histamine

Bladder afferent nerve recordings following histamine administration in the *ex vivo* bladder afferent preparation can be separated into three distinct populations. **(A)** Responding afferents show a significant increase in afferent firing (impulses per second; imp/s) in the presence of 300 µM histamine compared to saline (n = 36 out of 81; **** P < 0.0001). **(B)** Non-responding afferents show no change in mechanosensitivity between saline and histamine protocols (n = 41 out of 81; P > 0.05). **(C)** 'Silent afferents' are mechanically insensitive in saline control distension but become sensitive to distension in the presence of 300 µM histamine (n = 4 out of 81; **** P < 0.0001). **(D)** An example experiment showing graded increase in intraluminal pressure up to 30 mmHg, resulting in raw afferent nerve activity as above **(A(ii), B(ii), C(ii)). (E)** The peak afferent firing rate of individual bladder afferent units **(i)** responding to 300 µM histamine (n = 40, **** P < 0.0001; Δ = 6.70 ± 0.58 imp/s), or **(ii)** not responding to 300 µM histamine (n = 41, ns P > 0.05; Δ = -0.17 ± 0.27 imp/s). **(F)** Pie chart demonstrating the percentage of afferent units from each population (45% responding afferents, 50% non-responding afferents, 5% 'silent afferents'). Data are represented as mean ± SEM **(A-C)**, maximum imp/s **(E)**, or percentage of afferents **(F)**. P values above are based on two-way ANOVA **(A - C)** with subsequent Sidak's post hoc test significance at individual data points indicated on **(A)** and **(C)** (# represents significance of at least P < 0.05), or Wilcoxon matched-pairs signed rank test **(E)**.

Figure 3: Histamine enhances bladder afferent signaling in the dorsal horn of the spinal cord *in vivo*

pERK immunoreactivity (pERK-IR) was measured to determine central signaling in the lumbosacral (LS; L5-S1) spinal cord in response to peripheral administration of saline or 100 µM histamine into the bladder. **(A)** pERK-IR was significantly increased in the LS dorsal horn **(i)** following 100 µM histamine administration (N = 5) compared to saline administration (N = 5; **** $P < 0.0001$). pERK-IR was observed in the dorsal grey commissure (DGC) **(ii)**, superficial dorsal horn (SDH) **(iii)**, and the sacral parasympathetic nucleus (SPN) **(iv)**, with significantly higher pERK-IR in the presence of 100 µM histamine compared to saline in the DGC (**** $P < 0.0001$) and the SDH (**** $P < 0.0001$), while no difference was observed in the SPN in sacral segments (ns, $P > 0.05$). **(B)** Representative pERK-labelled LS spinal cord images at each spinal level, as determined by the shape of the dorsal horn as demonstrated in **(i)** dorsal horn maps, from mice (ii) that received only catheter insertion, or were treated with **(iii)** saline and **(iv)** 100 µM histamine. White arrows indicate pERK-IR neurons. CC = central canal. Data are represented as the number of pERK-IR neurons per section of LS spinal cord, with a minimum of 6 sections per mouse. P values above are based on Mann Whitney tests **(A(i) - Aiii)**, or an unpaired t-test **(Aiv)**. Scale bar represents distance of 100µm **(B).** L6 and S1 dorsal horn atlas images **(Bi)** adapted from the Allen Spinal Cord Atlas available at **http://mousespinal.brain-map.org** (54). Image credit: Allen Institute. 2008 Allen Institute for Brain Science.

Figure 4: Neuronal mRNA expression of histamine receptor subtypes

(A) QRT-PCR was used to determine how mRNA expression of histamine receptor subtypes *Hrh1, Hrh2, Hrh3, Hrh4* relative to *Actb* differs between lumbosacral (LS; L5, L6, S1) dorsal root ganglia (DRG; N = 5). Each dot represents paired DRG from each level. *Hrh1* mRNA expression was significantly more abundant than *Hrh2* (*** P < 0.001) and *Hrh3* (**** P < 0.0001) at L5 and L6 (* P < 0.05, and **** P < 0.0001 respectively). *Hrh2* and *Hrh3* were also significantly differently expressed at L6 (** P < 0.01), however no differences between *Hrh1, Hrh2 and Hrh3* were observed in S1. *Hrh4* mRNA expression was not detected in all LS DRG (below detectable limit; bdl). (B) Global expression of histamine receptor subtypes *Hrh1, Hrh2, Hrh3* and *Hrh4* relative to *Actb* in pooled lumbosacral (LS; L5, L6 and S1) dorsal root ganglia (DRG; N = 5). Each sample contained one DRG pair (at L5, L6 or S1) and was run in duplicate for each target and mean target Ct relative to mean *Actb* Ct determined ($2^{-\Delta Ct}$). *Hrh1* was significantly more abundant than *Hrh2* (*** P < 0.001) and *Hrh3* (**** P < 0.0001). *Hrh4* mRNA expression was not detected (below detectable limit; bdl). (C) The percentage of retrogradely labelled bladder-innervating DRG neurons expressing *Hrh1* and *Trpv1* was determined using single cell RT-PCR. Overall, 23.6% of bladder-innervating DRG neurons were positive for *Hrh1* mRNA expression (13 out of 55 neurons). 84.4% of an independent subset of retrogradely labelled bladder-innervating DRG neurons were positive for *Trpv1* mRNA expression (65 out of 77 neurons). Data are represented as mean ± SEM ($2^{-\Delta Ct}$) on a linear scale (A-B), or percentage of neurons (C). P values above are based on one-way ANOVA with Tukey's multiple comparison test, comparing *Hrh1, Hrh2* and *Hrh3* (A-B).

Figure 5: Direct effect of histamine on bladder afferents *in vitro* and *ex vivo*

Direct responses of bladder afferent neurons to histamine were determined using live cell calcium imaging *in vitro* and afferent nerve recordings without bladder distension in the *ex vivo* model. (A) A small population of isolated and cultured primary bladder afferent neurons from lumbosacral (LS) dorsal root ganglia (DRG) responded to 100 µM histamine (3%; 2 out of 63 neurons), compared to 9.4% (44 out of 466 neurons) of untraced LS DRG neurons (*** $P < 0.001$; $N = 10$). (B) Example calcium imaging experiment. Traced neurons are shown in green (1 out of 4 responding), untraced neurons are shown in grey (10 out of 28 responding). Fura-2 fluorescence ratio (340/380) indicates relative intracellular calcium, baseline fluorescence normalized to ratio of 1. 40 mM KCl was used as a positive control for neuronal responsivity. (C) Afferent firing (impulses per 180 seconds (imp/180s) in response to instillation of 300 µM histamine (5-15 minutes) in the bladder is not significantly different to baseline control (saline) in the *ex vivo* model without distension (ns; $P > 0.05$). (D) An example experiment showing very low levels of multiunit raw afferent nerve activity in the presence of saline (control) or 300 µM histamine before bladder distension (afferent firing in direct response to the compound) measured in 5 impulses per second (5imp/s). Data are represented as mean percentage of neurons per experiment ± SEM (total 27 experiments) (A) or mean ± SEM (C). P values above are based on a Mann Whitney test (A) or a Wilcoxon matched-pairs signed rank test (C).

Figure 6: Histamine does not evoke calcium transients in isolated primary urothelial cells

(A) Cytokeratin 7 (CK7) staining (green) was used to confirm cell type. Cell nuclei were identified by DAPI staining (blue). **(B)** Histamine receptor subtypes *Hrh1, Hrh2* and *Hrh3* were all expressed in isolated urothelial cells (N = 3), while *Hrh4* expression was below the detectable limit (bdl). Expression of target receptors is relative to the geomean of *Gapdh* and *Hprt*. All receptors were measured in duplicates. *Hrh3* showed significantly higher relative expression compared to Hrh1 (**** P < 0.0001) and *Hrh2* (**** P < 0.0001). **(C)** mRNA expression profiles of histamine receptor subtypes *Hrh1, Hrh2, Hrh3, Hrh4* relative to genes *Gapdh* and *Hprt* (*Gapdh***Hprt*) differ between mucosal and detrusor smooth muscle layers of the bladder (N = 4). Each sample was run in duplicate for each target and mean target Ct relative to geomean of mean housekeeper Ct values was determined ($2^{-\Delta Ct}$). *Hrh3* expression was significantly higher than *Hrh1* (**** P < 0.0001) and *Hrh2* (****P < 0.0001) in the mucosa, corresponding to the expression profiles observed in isolated urothelial cells in **(B)**. Conversely, *Hrh1* (** P < 0.01) and *Hrh2* (*** P < 0.001) were both significantly more abundant than *Hrh3* in the detrusor smooth muscle. Between the tissue types, *Hrh2* expression was significantly higher in the detrusor smooth muscle than in the mucosa (** P < 0.01, represented as '+'), while *Hrh3* expression was significantly higher in the mucosa than in the detrusor layer (*** P < 0.001, represented as '^'). *Hrh4* mRNA expression was not detected (below detectable limit; bdl). **(D)** An example calcium imaging experiment demonstrating that application of 100 µM histamine did not evoke calcium transients in Fura-2 loaded urothelial cells. Application of 2 µM ionomycin was used as a positive control for calcium influx (represented as an increase in Fura-2 340/380 fluorescence ratio) in calcium imaging experiments. **(E)** Example images of urothelial cells from a calcium imaging experiment **(i)** prior to experiment imaged via light filter, and imaged as 340/380 fluorescence ratio to determine changes in intracellular calcium **(ii)** at the start of the experiment, **(iii)** following addition of 100 µM histamine, and **(iv)** following addition of 2 µM ionomycin. An increase in 340/380 fluorescence ratio and hence intracellular calcium is depicted by color change on a heat scale from blue (baseline intracellular calcium) to yellow (high intracellular calcium) or red (very high intracellular calcium). Data are represented as mean ± SEM ($2^{-\Delta Ct}$) on a linear scale. P values above are based on one-way ANOVA with Tukey's multiple comparison test, comparing *Hrh1, Hrh2* and *Hrh3*.

Figure 7: Histamine H_1 receptor (H_1R) antagonist pyrilamine blocks histamine-evoked increase in bladder mechanosensitivity

Graded distension of the bladder in an *ex vivo* nerve recording preparation was performed with saline and 300 µM histamine in the presence of Hrh1 antagonist pyrilamine (100 µM). (A) No changes were observed in afferent firing rate (impulses per second; imp/s) following administration of 100 µM of pyrilamine alone or 100 µM pyrilamine with 300 µM histamine compared to saline at any distension pressure (0 – 30 mmHg; N = 6; ns $P > 0.05$). (B) An example experiment showing raw afferent nerve activity in the presence of (i) saline, 100 µM pyrilamine, and 100 µM pyrilamine with 300 µM histamine in succession, increasing in response to (ii) graded increase in intraluminal pressure. (C) No change in muscle compliance (pressure-to-volume relationship) was observed between saline, pyrilamine, or pyrilamine and histamine protocols, suggesting afferent responses were not affected by changes in musculature. Data are represented as mean ± SEM. P values above are based on two-way ANOVA with subsequent Sidak's post hoc test significance at individual data points (ns, $P > 0.05$).

Figure 8: Histamine-evoked increase in bladder afferent mechanosensitivity *ex vivo* is severely reduced in *Trpv1*$^{-/-}$ mice

Graded distension of the bladder in a *Trpv1*$^{-/-}$ *ex vivo* nerve recording preparation was performed with saline followed by 300 µM histamine. **(A)** No change in overall afferent firing rate (impulses per second; imp/s) was observed following administration of 300 µM histamine compared to saline at any distension pressure (0 – 30 mmHg; N = 5; P > 0.05). **(B)** No change in muscle compliance (pressure-to-volume relationship) was observed between saline and histamine protocols, suggesting afferent responses were not affected by changes in musculature. **(C)** An example experiment showing **(i)** an equivalent increase in raw afferent nerve activity in the presence of 300 µM histamine compared to saline in response to **(ii)** increased intraluminal pressure. **(D)** Single unit analysis showed that the majority of *Trpv1*$^{-/-}$ afferents (n = 32 out of 40; ns P > 0.05; Δ = -0.33 ± 0.34 imp/s) were histamine insensitive **(ii)**, with no change in overall afferent firing rate (imp/s) between saline and 300 µM histamine protocols, while a small proportion of *Trpv1*$^{-/-}$ single afferent units **(i)** showed a significant increase in overall afferent firing rate (imp/s) between saline and 300 µM histamine protocols (n = 8 out of 40 afferents; *** P < 0.001; Δ = 5.125 ± 3.60 imp/s). **(E)** Pie chart depicting the proportion of histamine responsive (20%) and histamine insensitive (80%) single unit afferents. **(F)** Isolated bladder-innervating lumbosacral (LS; L5-S1) dorsal root ganglia (DRG) neurons from *Trpv1*$^{-/-}$ mice (N = 6) were unresponsive to 100 µM histamine (0 out of 36 neurons). A small percentage of untraced LS DRG neurons from *Trpv1*$^{-/-}$ mice responded to 100 µM histamine (10 out of 242 neurons, 4.1%). Data are represented as mean ± SEM **(A, B, F)**, maximum imp/s **(D)**, or mean percentage of neurons per experiment ± SEM (total 21 experiments) **(E)**. P values above are based on two-way ANOVA **(A, B)** with subsequent Sidak's post hoc test significance at individual data points (ns, P > 0.05), or Wilcoxon matched-pairs signed rank test or paired t-test **(D)**.