Supplementary information

Chronic linaclotide treatment reduces colitis-induced neuroplasticity and reverses persistent bladder dysfunction

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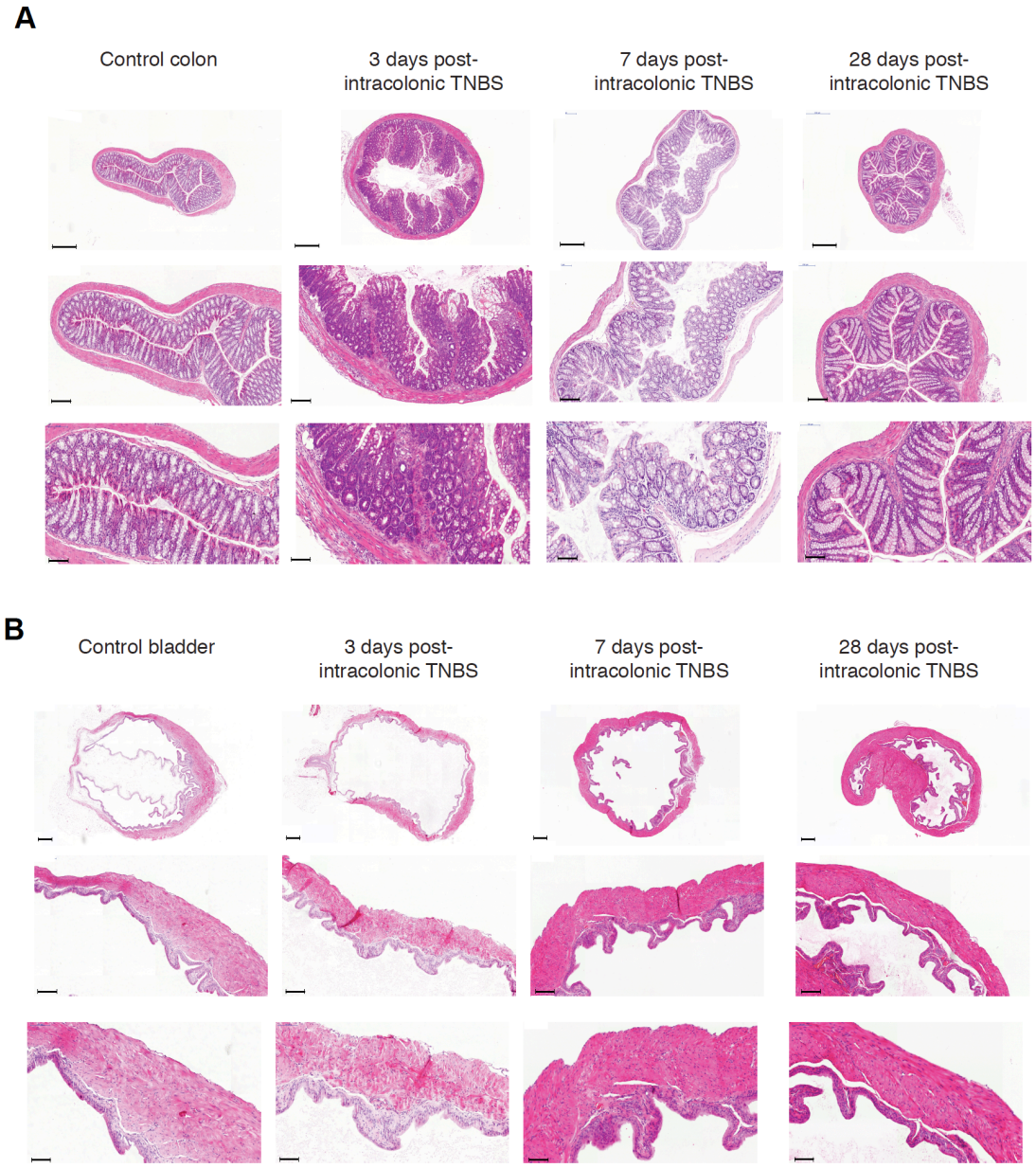
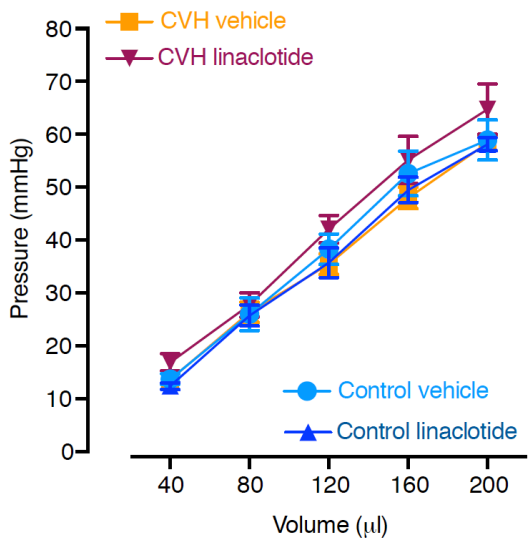
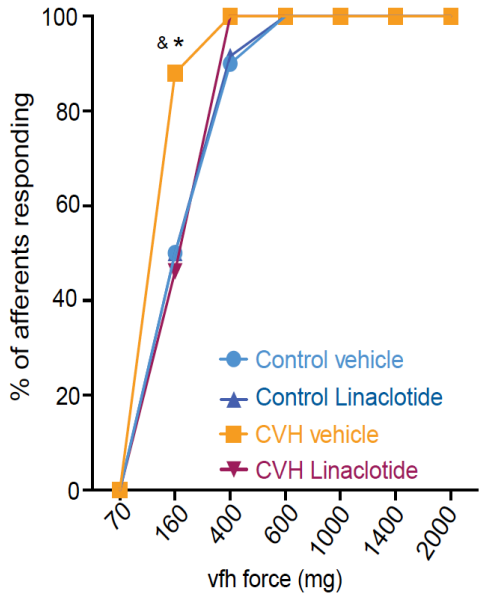

A
Control colon
3 days post-intracolonic TNBS
7 days post-intracolonic TNBS
28 days post-intracolonic TNBS
B
Control bladder
3 days post-intracolonic TNBS
7 days post-intracolonic TNBS
28 days post-intracolonic TNBS
C
**
*
Colon
Bladder
MPO (U/mg protein)
350
300
250
200
150
100
50
0
Control
3 days
7 days
28 days
post-intracolonic TNBS administration

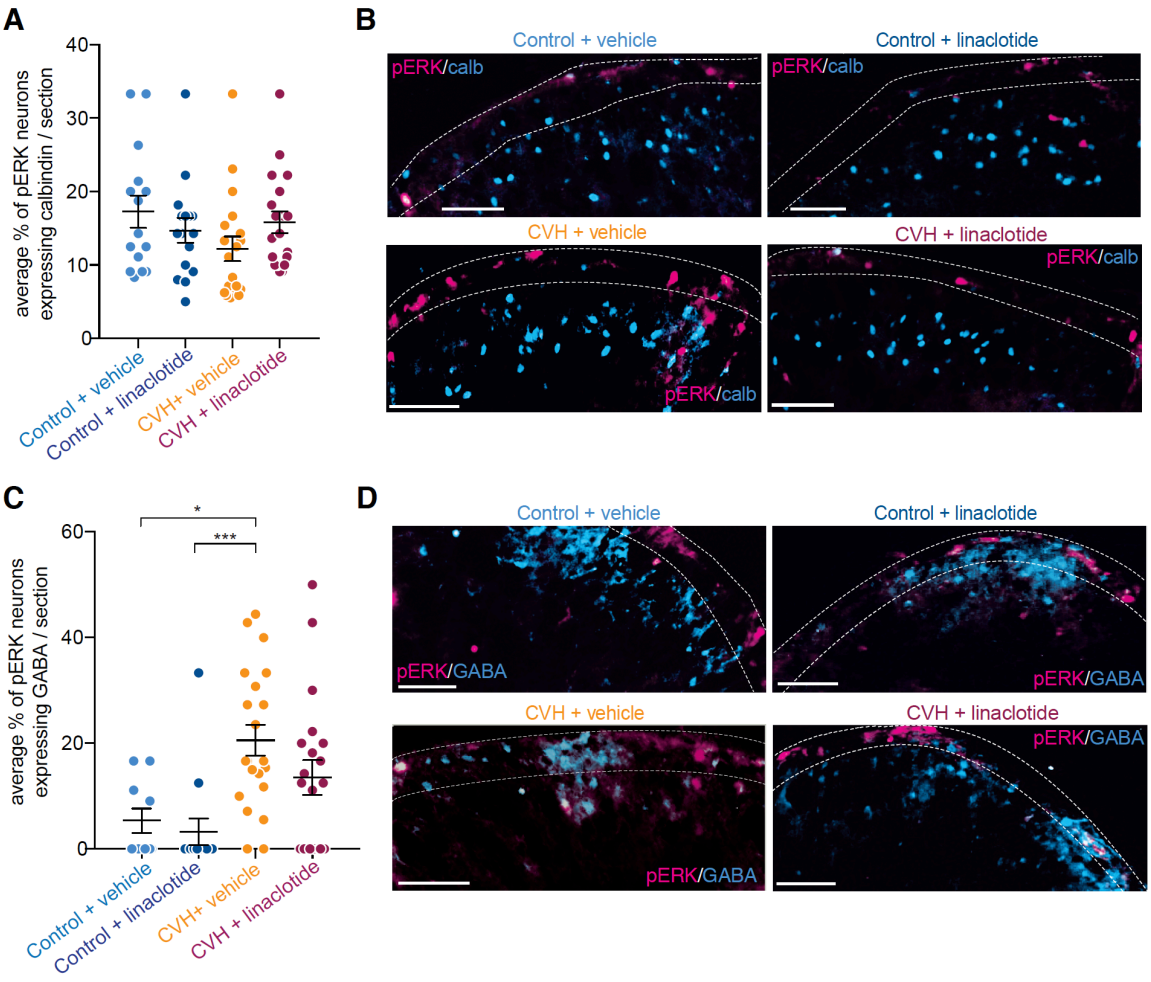
Supplementary Figure 1: Intra-colonic administration of 2,4,6-trinitrobenzenesulphonic acid (TNBS) causes inflammation to the colon but not the bladder **(A)** Representative histological sections at increasing magnification of haematoxylin and eosin (H&E) staining in colon samples from control mice, and mice at 3 days, 7 days and 28 days post-intracolonic administration of TNBS. Panels show significant damage to the colon at day 3 post-intracolonic TNBS administration with crypt segmentation, oedema, and local inflammation. Scale bars for the top row of panels = 500 μm; middle row of panels = 200 μm; lower row of panels = 25 μm. **(B)** Representative histological sections at increasing magnification of H&E staining in bladder samples from control mice, and mice at 3 days, 7 days and 28 days post-intracolonic administration of TNBS. Panels show no signs of inflammation and no change in the bladder wall at day 3, 7 or 28 post-intracolonic TNBS administration. Scale bars; top row of panel = 500 μm; middle row of panels = 200 μm; lower row of panels = 25 μm. **(C)** Myeloperoxidase (MPO) activity is significantly enhanced in the colon at day 3 post-TNBS (***P*<0.01), and returns to control levels by 28 days post-intracolonic TNBS administration (**P*<0.05, Control: N=4 mice, 3 days post-TNBS: N=5 mice, 7 days post-TNBS: N=3 mice, 28 days post-TNBS: N=3 mice). In contrast, intracolonic administration of TNBS has no effect on bladder MPO activity at any time point tested. Data represent mean ± SEM. P values are based on analysis by a two-way ANOVA with Tukey's multiple comparison tests **(C)**.



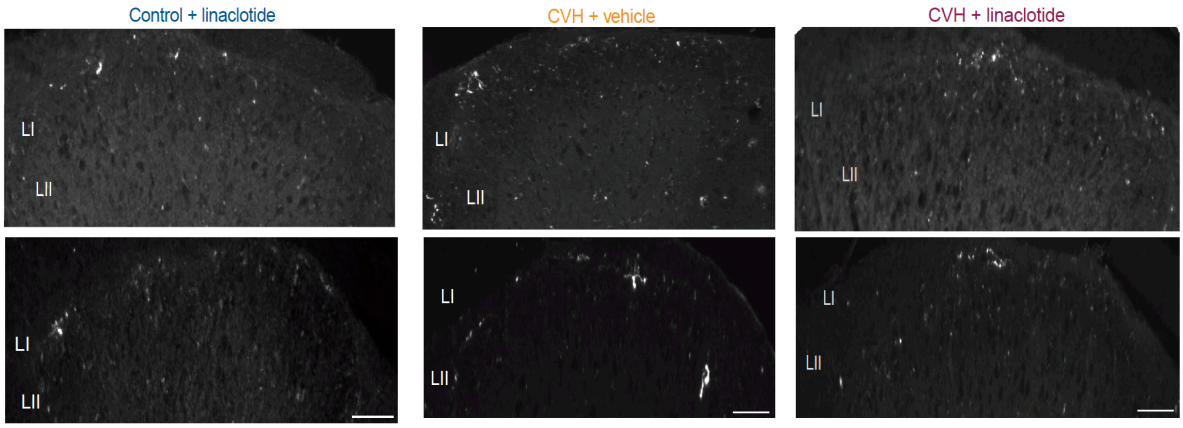
Supplementary Figure 2: In vivo colonic compliance to balloon distension is unaltered in CVH mice and in treatment groups. Group data collected from in vivo visceromotor response (VMR) experiments to colorectal balloon distension (CRD), showing that colonic compliance is unaltered in CVH mice treated with vehicle (N=10 mice), or CVH mice administered linaclotide (3µg/kg) for 14 days (N=9 mice) compared with control mice treated with vehicle (N=7 mice) and control mice administered linaclotide (3µg/kg) for 14 days (N=7 mice). Data represent mean ± SEM and were analyzed by two-way ANOVA.



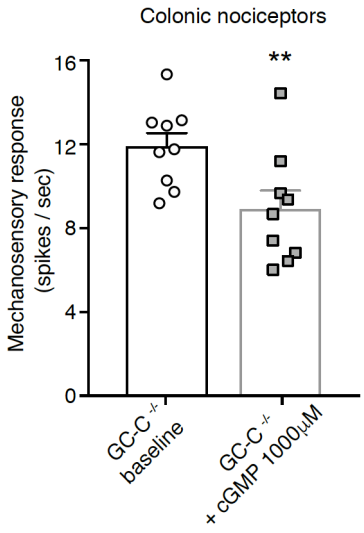
Supplementary Figure 3: Mechanical activation thresholds of colonic nociceptors are decreased in CVH and normalised with linaclotide treatment. Group data from ex vivo colonic afferent recordings showing the mechanical activation thresholds of high-threshold nociceptors to von frey hair (vfh) stimulation. Data are from CVH mice treated with vehicle (n=9 afferents), CVH mice administered linaclotide (3μg/kg) for 14 days (n=13 afferents) compared with control mice treated with vehicle (n=12 afferents) and control mice administered linaclotide (3μg/kg) for 14 days (n=12 afferents). Significantly more nociceptors from CVH vehicle treated mice responded at lower stimulation vfh intensities compared with control vehicle treated mice (*P<0.05). CVH mice administered linaclotide displayed normalised activation thresholds compared with CVH vehicle treated animals ($^{\&}$ P<0.05). Data represent the percentage of afferents responding to vfh stimulation at increasing stimulation intensities. P values are based on χ^2 analysis.



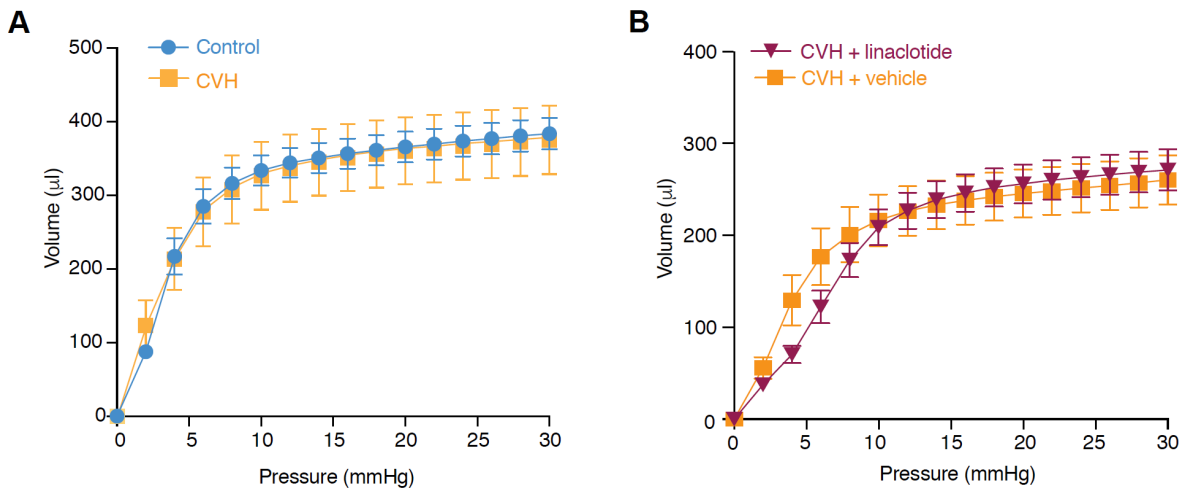
Supplementary Figure 4: The neurochemistry of dorsal horn neurons activated by noxious colorectal distension is altered in CVH mice. (A). Quantitative data showing the average percentage of pERK-immunoreactive (pERK-IR) neurons per section that co-express calbindin in the dorsal horn (lamina I (LI) and II (LII)). No significant differences were observed between groups (P>0.05, N=3 mice per group). **(B)** Histological sections of pERK-IR within the spinal cord of control and CVH mice chronically administered vehicle or linaclotide. pERK-IR neurons are shown in magenta, with calbindin-immunoreactive neurons shown in blue. Co-labelled neurons appear white. Scale bars:100μm. **(C)** Quantitative data showing the average percentage of pERK-IR neurons per section co-expressing GABA in the dorsal horn (lamina I (LI) and II (LII)). Significantly more pERK-IR neurons in CVH mice administered vehicle co-express GABA than control mice administered vehicle (*P<0.05) or linaclotide (***P<0.001, N=3 mice per group). **(D)** Histological sections of pERK-IR within the spinal cord of control and CVH mice chronically administered vehicle or linaclotide. pERK-IR neurons are shown in magenta, with GABA-immunoreactive neurons shown in blue. Co-labelled neurons appear white. Scale bars:100μm. Data represent mean ± SEM. P-values are based on a one-way ANOVA with Tukey's multiple comparison tests **(A,C)**.



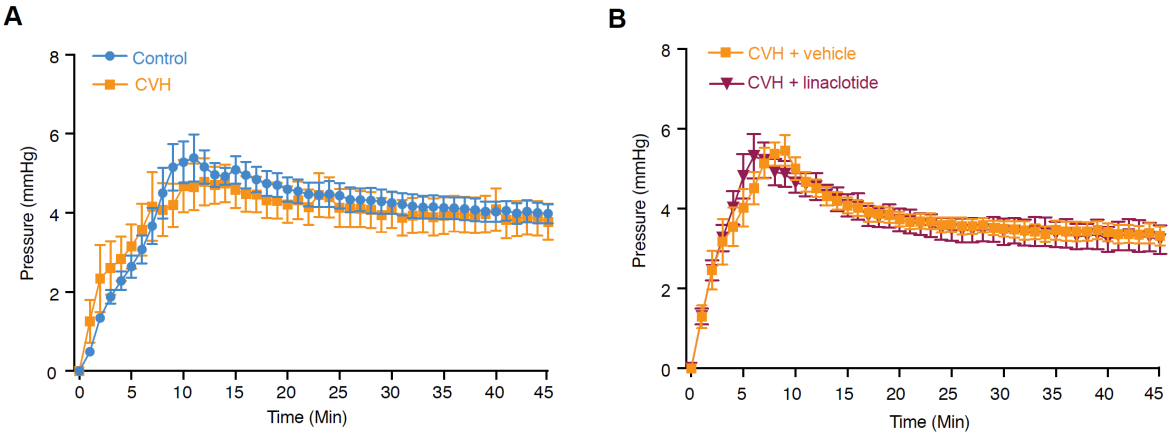
Supplementary Figure 5: Sprouting of colonic afferent central terminals in CVH mice is reversed with linaclotide treatment. Fluorescent images of the data presented in Figure 3C, showing sections of dorsal horn with tracer accumulation within the central terminals of colon-innervating afferents from control mice chronically administered linaclotide, or CVH mice administered vehicle, or linaclotide. Scale bars:100μm.



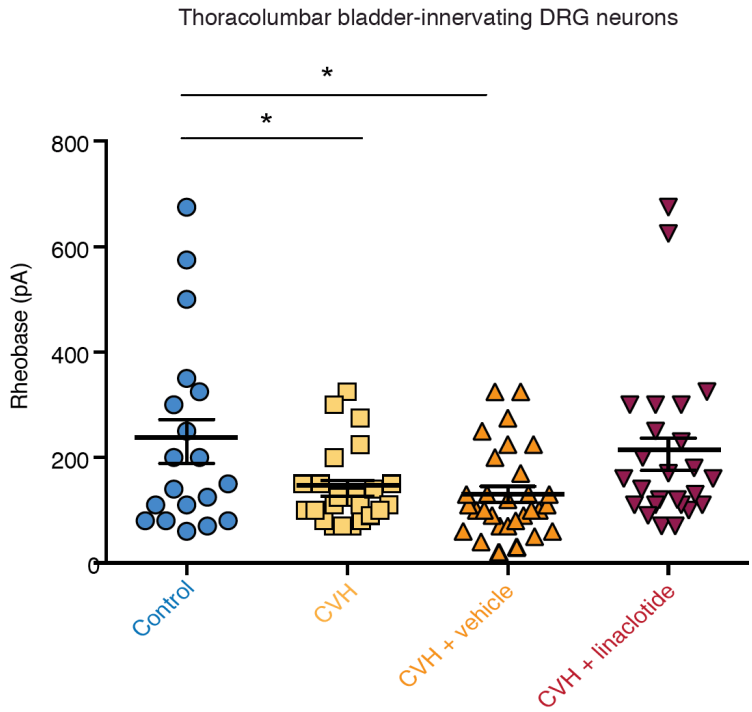
Supplementary Figure 6: cGMP inhibits colonic nociceptors from guanylate cyclase-C (GC-C) null mutant ($^{-/-}$) mice. Group data from ex vivo colonic afferent recordings showing that exogenously applied cGMP (1000 μM) inhibits colonic nociceptors from GC-C (*gucy2c*) null mutant $^{(-/-)}$ mice (**P<0.01; n=9 afferents, from N=3 mice). Data represent mean ± SEM. P values are based on analysis by a paired t-test.



Supplementary Figure 7: Bladder compliance to distension is unaltered in CVH mice and in vehicle and linaclotide treatment groups **(A)** Group data from ex vivo bladder afferent recordings showing that bladder compliance is unchanged in CVH mice (N=11 mice) relative to control mice (N=10 mice). **(B)** Group data from ex vivo bladder afferent recordings showing that bladder compliance is unchanged in CVH mice administered linaclotide (3µg/kg; N=13 mice) for 14 days compared to CVH mice administered vehicle (N=6 mice) for 14 days. Data represent mean ± SEM and were analysed by two-way ANOVA **(A, B).**



Supplementary Figure 8: Bladder contractility is unaltered in CVH mice and in vehicle and linaclotide treatment groups. **(A, B)** Bladder contractility from control (N=10) and CVH mice (N=11) and vehicle-treated (N=6) or linaclotide-treated CVH (N=13) mice. In both cases data show that bladders were partially filled (~7mmHg) then the infusion was stopped but the outflow tap remained closed, keeping a set volume within a moderately distended bladder. Bladders accommodated over time with no significant difference found between healthy and CVH states, nor between CVH mice treated with vehicle or linaclotide. Data represent mean ± SEM, and were analyzed by two-way ANOVA **(A, B)**.



Supplementary Figure 9: Thoracolumbar bladder-innervating dorsal root ganglion (DRG) neurons are hypersensitive in CVH states, which is normalised with linaclotide treatment. Whole-cell current-clamp recordings of retrogradely traced bladder-innervating thoracolumbar (TL) DRG neurons. Group data show reduced rheobase of TL DRG neurons from CVH mice (*P<0.05, n=23 neurons) and CVH mice treated with vehicle for 14 days (*P<0.05, n=30 neurons) versus neurons from control mice (n=19 neurons). Bladder-innervating DRG neurons from CVH mice treated daily with oral administration of linaclotide for 14 days (n=25 neurons) showed a normalised rheobase. Data represent mean ± SEM, P values are based on analysis by one-way ANOVA with Bonferroni post-hoc test.

SUPPLEMENTARY METHODS

Animals

The South Australian Health and Medical Research Institute (SAHMRI), Flinders University, The University of Adelaide and the University of Tübingen Animal Ethics Committees approved experiments involving animals, which conformed to regulatory standards and the ARRIVE guidelines. Male C57BL/6J mice aged 13-17 weeks were used in all experiments using adult mice. Mice were acquired from an in-house C57BL/6J breeding programme (JAX strain #000664; originally purchased from The Jackson Laboratory; breeding barn MP14; Bar Harbor, ME, USA) within SAHMRI's specific and opportunistic pathogen-free animal care facility. Mice were group housed (five mice per cage) within individual ventilated cages (IVC), filled with chip coarse dust-free aspen bedding (Cat#–ASPJMAEBCA; PuraBed, Niederglatt, Switzerland). Cages were stored on IVC racks within a temperature-controlled environment of 22°C and a 12 h light/12 h dark cycle. Mice had free access to LabDiet® JL Rat and Mouse/Auto6F chow (Cat#5K52, St. Louis, MO; USA) and autoclaved reverse osmosis water. Mice had an average weight of ~29 g on the experimental day.

Experimental study design

Mice were randomly assigned to study sub-groups and the order of treatment was randomised. Mice were assigned to either 1) control or 2) 2,4,6-trinitrobenzenesulfonic acid (TNBS) treatment groups. Following TNBS administration, mice were individually housed within individual ventilated cages (IVC) to allow for accurate clinical monitoring until the experimental day. Control or CVH (28 days post-intracolonic TNBS administration) mice were then randomly assigned to one of three groups: those that received i) no gavage, or ii) once daily 100μL oral gavage of linaclotide (3μg/kg/day; provided by Ironwood Pharmaceuticals, Cambridge, MA, USA) for 14 days, or iii) vehicle (water) once daily 100μL oral gavage for 14 days. For groups ii) and iii) daily oral administration of linaclotide or vehicle began from 14 days post-TNBS administration (or time/day matched for control animals) until the day of experimentation, at 28 days post-TNBS administration.

Mouse model of chronic visceral hypersensitivity (CVH)

Colitis was induced by administration of TNBS as described previously(1-4). Briefly, after an overnight fast with free access to a 5% glucose solution, 13-week-old mice anaesthetised via isoflurane inhalation (isoflurane 2-4% in oxygen) were administered a single intracolonic enema of 0.1ml (130μL/ml in 35% ethanol) TNBS (P2297, Sigma-Aldrich, Sydney, Australia) via a polyethylene catheter (1,2,5).

In vivo visceromotor responses (VMR) to colorectal distension (CRD)

Using abdominal electromyography (EMG) we assessed visceral sensitivity in vivo in fully awake animals

(6-8). VMR was assessed in four cohorts: **1)** control mice administered a once-daily oral gavage of vehicle (water) for 14 days **2)** control mice administered a once-daily oral gavage of linaclotide (3µg/kg/day) for 14 days, **3)** CVH mice administered a once-daily oral gavage of vehicle (water) for 14 days, or **4)** CVH mice administered a once-daily oral gavage of linaclotide (3µg/kg/day) for 14 days. Four days prior to the VMR and under isoflurane anaesthesia, two Teflon-coated stainless-steel wires (Advent Research Materials Ltd, Oxford, UK) were sutured into the right abdominal muscle and tunnelled subcutaneously to be exteriorised at the base of the neck. On the day of VMR, mice were briefly anaesthetised using isoflurane and a lubricated balloon (2.5cm length) was inserted into the colorectum up to 0.25cm past the anal verge and connected to a barostat (Isobar 3, G&J Electronics, Willowdale, Canada) for graded and pressure-controlled balloon distension. Distensions were applied at 20-40-60-80 mmHg (each of 20s duration) applied at a 4min-intervals. Following the final distension mice were humanely killed by cervical dislocation. EMG electrodes were relayed to a data acquisition system and the signal was recorded (NL100AK headstage), amplified (NL104), filtered (NL 125/126, Neurolog, Digitimer Ltd, bandpass 50–5000 Hz) and digitised (CED 1401, Cambridge Electronic Design, Cambridge, UK) to a PC for off-line analysis using Spike2. The analogue EMG signal was rectified and integrated with the area under the curve (AUC) during the distension (20s) corrected for the baseline activity (AUC pre-distension,20s). Colonic compliance was assessed by applying graded volumes (40-200µL, 20s duration) to the balloon in the colorectum, while recording the corresponding colorectal pressure (6,7).

Ex vivo single fibre colonic nociceptor recordings

Ex vivo colonic nociceptor recordings were from four cohorts described in the VMR study section**.** On the day of experimentation, mice were humanely killed by CO_2 inhalation and the colon and rectum (5–6 cm) and attached splanchnic nerves were removed and afferent recordings from splanchnic nerves performed as described previously(5,9). We recorded from serosal afferents, also termed vascular afferents from the splanchnic pathway as described previously (9,10). These colonic afferents have high mechanical activation thresholds and respond to noxious distension (40mmHg), stretch (≥7g) or von frey hair (vfh) filaments (2g) but not to fine mucosal stroking (10mg vfh) (3,4,10). They are therefore referred to as colonic 'nociceptors'. Baseline mechanosensitivity was determined in response to application of 70mg, 160mg, 400mg, 600mg, 1000mg, 1400mg and 2000mg vfh probes to the afferent receptive field for 3 seconds. This process was repeated 3–4 times, separated each time by 10 seconds. The threshold for afferent activation was also determined. Some experiments also utilised mice lacking GC-C (*gucy2c*$^{-/-}$ in house breeding colony at SAHMRI, Adelaide, Australia), which were generated as described previously (11).

Visualisation of pERK-activated neurons within the dorsal horn of the spinal cord following noxious colorectal distension (CRD).

Mice studied in these experiments were from the four cohorts described in the VMR section above. Mice were briefly anaesthetised with isoflurane and a 2.5cm balloon catheter was inserted into the perianal canal and secured to the tail so that the start of the balloon sat 0.25cm from the anal opening(1-3). Upon regaining consciousness, the balloon was distended for 10 seconds to a pressure of 80mmHg and the balloon deflated for 5 seconds. This process was repeated 5 times(12). After the fifth distension, mice were given an anaesthetic overdose (Lethabarb, intraperitoneal) and underwent transcardial perfusion with heparinised saline and warm 0.1M phosphate buffer (Sigma-Aldrich, MO, USA) followed by ice-cold 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (Sigma-Aldrich). Following transcardial perfusion, thoracolumbar (T10-L1) spinal cord was removed, and post-fixed for 16 hours at 4°C in 4% PFA, then cryoprotected in 30% sucrose/0.1M phosphate buffer (Sigma-Aldrich) overnight at 4°C and then placed in 50% Optimal Cutting Temperature compound (OCT; Tissue-Tek, Sakura Finetek, CA, USA) in 30% sucrose/phosphate buffer solution for 7 hours, before block freezing in 100% OCT. Frozen sections (12µm) were cut using a cryostat and placed onto gelatin-coated slides. Immunohistochemistry for pERK was performed in a paired fashion, with tissue from healthy and CVH mice exposed to linaclotide or saline run simultaneously. Sections were incubated with 5% normal chicken serum/0.2% Triton-X 200 (Sigma-Aldrich) in PBS (0.2% TX-PBS) for 30 minutes at room temperature to block non-specific binding of secondary antibodies. Sections were then incubated for 18 hours at room temperature with monoclonal anti-sera rabbit anti-phospho-p44/42 MAPK (Erk1/2),(Thr202/Tyr204) (D13.14.4E) (pERK; 1:200; #4370 Cell Signalling Technology, MA, USA; RIID: AB_2315112) diluted in 0.2 % TX-PBS alone or combined with either mouse anti-Calbindin D28K (clone CB955; 1:800; #C8666 Sigma; RIID AB_2313712) or mouse anti-GABA (clone GB-69;1:1000;#A0310,Sigma;RIID AB_476667). Sections were washed 3x with 0.2% TX-PBS before being incubated for 1 hour at room temperature with secondary antibody chicken anti-rabbit IgG (H+L) AlexaFluor488 (1:200; #A-21441, Molecular Probes, ThermoFisher Scientific, MA,USA) and goat anti-mouse (IgG1) AlexaFluor594 (1:200; #A-21125). Sections were then washed 3x with 0.2% TX-PBS before mounting in ProLong Gold anti-fade (#P36930, ThermoFisher Scientific). Negative controls were prepared as above with the primary antibody omitted (12). For quantification, dorsal horn neurons with intact nuclei and immunoreactivity above that of negative controls, where the primary antibody was omitted, were counted. The number of pERK-immunoreactive neurons for each mouse was obtained from 10 histological sections (50µm apart) from each spinal segment (T10-T12, T13-L1), visualised by standard microscopy with 10x magnification. The percentage of pERK-immunoreactive neurons co-labelled with either calbindin or GABA were obtained from 5 sections per spinal segment per mouse.

Retrograde tracing to label the central terminals of colonic afferents

A small aseptic abdominal incision was made in anaesthetised (isoflurane 2-4% in oxygen) mice and

0.5% Cholera Toxin subunit B conjugated to AlexaFluor®555 (CTB-555;#C22843, ThermoFisher Scientific) injected (3μl/injection site) at three sites into the wall of the colorectum. Injections were made using a Hamilton syringe (5μl,65RN-7633-01, Hamilton, NV, USA) attached to a 23-gauge needle (RN custom point 4). The abdominal incision was sutured closed and mice allowed to recover and monitored for seven days prior to undergoing transcardial perfuse fixation, dissection and cryosectioning of spinal cord (as described above) in order to visualise the CTB labelled central projections of colonic afferents within the thoracolumbar dorsal horn of the spinal cord. Quantification of CTB-555 terminal density and area covered in the dorsal horn was performed as previously described (12). Briefly, images collected at 10x magnification at 5 second exposure of the dorsal horn area from the central canal to laminae I. Using Image J FIJI (NIH, MD, USA) analysis module, all images were scaled to 5.62 pixels/μm and the CTB-555 immunoreactive regions were selected using manually set thresholds of 75/255–85/255 for each image. The area covered by the regions with immunoreactivity within threshold was then measured using Image J based on the calibrated image scale, with the area covered by CTB-555 expressed as μm^2. Density (IntDen) was also obtained using the same parameters, giving the sum values of pixel intensity in the selected area equivalent to the product of area and mean gray value (ImageJ). These measurements were made from 6-10 sections of spinal cord per spinal segment per mouse and averaged across mice for each experimental group.

Retrograde tracing to identify colon-innervating or bladder-innervating DRG neurons and cell culture for patch clamp recordings.

CTB-conjugated to AlexaFluor 488 (CTB-488; #C22841, ThermoFisher Scientific) was injected at 1) three sites sub-serosally within the wall of the distal colon(1,3,12) or 2) three sites into the bladder wall (4μl/injection) using a Hamilton syringe attached to a 23-gauge needle. After 4 days, mice were humanely killed for subsequent T10-L1 DRG removal and dissociation. DRGs were digested with 4mg ml^{-1} collagenase II (#17101015, GIBCO, ThermoFisher Scientific) and 4mg ml^{-1} dispase II (#17105041,GIBCO,ThermoFisher Scientific) for 30min at 37°C, followed by 4mg ml^{-1} collagenase II for 10min at 37°C. Neurons were resuspended in DMEM (# 11995065, GIBCO, ThermoFisher Scientific) containing 10% fetal calf serum (Invitrogen, ThermoFisher Scientific, MA, USA), 2mM L-glutamine (#25030081, GIBCO, ThermoFisher Scientific), 100μM MEM non-essential amino acids (#11140076, GIBCO, ThermoFisher Scientific) and 100mg ml^{-1} penicillin/streptomycin (#15070063, GIBCO, ThermoFisher Scientific). Neurons were spot-plated on coverslips coated with laminin (20 μg ml^{-1}; #L2020, Sigma-Aldrich) and poly-D-lysine (800μg ml^{-1}; ThermoFisher Scientific) and maintained in an incubator at 37°C in 5% CO_2.

Patch clamp recordings of colon-innervating or bladder-innervating DRG neurons. Whole-cell patch-clamp recordings were obtained from fluorescently labelled thoracolumbar colon-

innervating DRG neurons 24-48hr after plating, using fire-polished glass electrodes with a resistance of 2–5MΩ. The membrane potential was held at −65mV. In current clamp mode, a series of depolarising pulses (10pA current step, 500ms duration) were applied from the holding potential and the amount of current required to elicit an action potential (rheobase) determined in normal external bath solution and following either the addition of cGMP (1μM,10μM,100μM) or linaclotide (1000nM), which were applied with a gravity-driven multi-barrel perfusion system(7). Rheobase was determined at baseline or following a 2min continuous perfusion with either cGMP (1μM,10μM,100μM) or linaclotide (1000nM) dissolved in external bath solution. Intracellular solutions: (mM): 135:KCl; 2:$MgCl_2$; 2:MgATP; 5:EGTA-Na; 10:HEPES-Na; adjusted to pH 7.4. Extracellular bath solutions: (mM): 140:NaCl; 4:KCl; 2:$MgCl_2$; 2:$CaCl_2$; 10: HEPES-Na; 5:glucose; adjusted to pH 7.4 (Sigma-Aldrich). A neuron inhibited by cGMP or linaclotide was defined as exhibiting a ≥10% change in rheobase from baseline(1,4,7). Whole-cell recordings were also made from fluorescently labelled bladder-innervating DRG neurons 36–48hr after plating, using criteria and solutions described above.

FRET-based cGMP imaging in DRG neurons

DRG were isolated from R26-CAG-mcGi500(L1) embryos that express a membrane-targeted version of the FRET-based cGMP sensor cGi500(13), termed mcGi500, under control of the ubiquitous CAG promoter. mcGi500 was derived from the original cGi500 sensor by attaching the N-terminal myristoylation site of the MARCKS protein. The R26-CAG-mcGi500(L1) mouse line was generated as described (14). C57BL/6 female mice were mated with R26-CAG-mcGi500(L1) heterozygous male mice. Plug-positive females were separated 0.5 dpc. At day 12.5 dpc, pregnant females were euthanised with CO_2 and R26-CAG-mcGi500(L1) transgenic embryos, identified by their YFP fluorescence, were used for further preparation. Spinal cord with attached DRG was dissected from E12.5 transgenic embryos. For dissociated DRG cell culture, 10-20 DRG were collected, trypsinised and suspended in DRG medium containing DMEM/F12 (ThermoFisher Scientific) supplemented with 10% horse serum (Life Technologies, ThermoFisher Scientific), 0.3mg/mL L-glutamine (Life Technologies, ThermoFisher Scientific), 1% penicillin/streptomycin (Invitrogen,ThermoFisher Scientific), 8mg/mL glucose (CarlRoth, Karlsruhe, Germany), and 100ng/mL nerve growth factor (Alomone Labs, Jerusalem, Israel). For DRG explant culture, the trypsinisation step was omitted, DRG were cut into halve and directly transferred to a culture dish containing DRG medium. Cells and explants were plated on poly-D-lysine/laminin-coated coverslips and grown at 37°C and 6% CO_2 in a humidified incubator. FRET/cGMP imaging was performed 24 hours after plating using an imaging setup as described previously (14,15). Briefly, the imaging setup was based on an inverted Axiovert 200 microscope (Carl Zeiss Microscopy GmbH,Jena,Germany) equipped with a Plan NeoFluar40×/1.30 oil objective, a light source with excitation filter switching device (Oligochrome, TILL Photonics GmbH, Graefelfing, Germany), a DualView beam splitter with 516nm dichroic mirror and emission filters for CFP (480/30nm) and YFP (535/40nm) (Photometrics, AZ, USA) and a charge-coupled device camera (Retiga 2000R, QImaging, BC,

Canada). The cells were superfused at room temperature at a flow rate of 1mL/min with imaging buffer (mM: 140:NaCl; 5:KCl; 1.2:$MgSO_4$; 2.5:$CaCl_2$; 5:glucose; 5:HEPES; pH:7.4) without drugs or with CNP (Tocris, Bristol,UK), uroguanylin (Peptide Institute Inc.,Osaka,Japan) or cGMP (Biolog Life Science, Bremen, Germany). Permeabilisation of cells was performed using 100μg/mL β-escin (Sigma-Aldrich,MO,USA). FRET data were analysed using ImageJ (NIH), and OriginPro (OriginLab Corp., MA, USA) software. F_{480} and F_{535} traces were background-corrected before calculating the F_{480}/F_{535} ratio R. $\Delta F_{480}/F_{480}$, $\Delta F_{535}/F_{535}$ and $\Delta R/R$ traces were obtained by normalisation to the baseline recorded for 2-3 min at the beginning of each experiment (14,15). For $\Delta R/R$ peak area calculation, the OriginPro peak area analyser was used.

Human DRG neuron patch-clamp recordings

Thoracolumbar DRG (T9-L1) were acquired from human adult organ donors during the removal of the vital organs for transplantation as described previously(1). The harvested DRG were immediately processed and cultured for down-stream patch clamp studies. In some studies, an inflammatory soup consisting of histamine (10μM: H7250), PGE-II (10μM: P5640), serotonin (10μM: H9523), bradykinin (10μM: B3259, Sigma-Aldrich,USA) was added to the culture medium for 2 hours before patch-clamp recordings. Whole-cell patch-clamp recordings were performed in current-clamp mode in response to depolarising current pulses (100pA increment, 6-12 steps, 800ms duration). Signals were amplified and digitised by EPC10 amplifier (HEKA Instruments, MA, USA) controlled by Patchmaster software (HEKA). Determining the rheobase in control buffer was followed by a 2 min continuous perfusion with either cGMP (1μM) dissolved in external bath solution, after which a second recording was made to determine rheobase in the presence of cGMP. This process was repeated with 10μM and 100μM cGMP with rheobase re-determined at each concentration. Recordings were performed at 32°C. Extracellular solutions (mM): 145:NaCl; 3:KCl; 2:$CaCl_2$; 1:$MgCl_2$; 10:HEPES; 10:glucose; adjusted to pH 7.4 with NaOH, and 300-320 mOsm. Intracellular solutions (mM): 130:K-gluconate; 5:KCl; 6.6:NaCl; 1:$MgCl_2$; 0.3:EGTA; 10:HEPES; 10:Glucose; adjusted to pH 7.3 with KOH, and 290-310 mOsm.

Bladder voiding pattern analysis

The 4 cohorts of mice described in the VMR section were individually housed in cages lined with filter paper for 3 hours with free access to food/water as described previously(16). Filter paper was collected immediately prior to intra-colonic TNBS administration and at days 7,14,21 and 28 post-TNBS administration or the same time point for controls. Filter paper was imaged using a Bio-Rad ultraviolet trans-illuminator and digitised into binary images using ImageJ software. The number and size of urine spots was determined using pre-set thresholds within ImageJ software, and for the purposes of this study, the number of small (500-1000 pixels), medium (1001-100,000 pixels) and large (>100,000 pixels) spots were quantified.

Ex vivo bladder afferent recordings

Whole nerve recordings were conducted using an ex vivo model previously described(16,17). On the day of experimentation, control or CVH mice administered linaclotide (3µg/kg/day) or vehicle once daily for 14 days were humanely killed by CO_2 inhalation. The whole pelvic region was dissected and placed in a recording chamber (30ml), continually perfused with gassed (95% O_2,5% 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^{o}C. The urethra and dome were cannulated to enable recording of intravesical pressure and efficient evacuation of fluid. This also allowed for subsequent studies determining the responsiveness of bladder afferents to intravesically applied linaclotide (1000 nM) or serosally applied αβMe-ATP (30 µM), carbachol (1 µM) or capsaicin (30 µM) (compounds from Sigma-Aldrich). The pelvic and hypogastric nerves were dissected into fine multiunit branches, and afferent activity was recorded by a neurolog headstage (NL100, Digitimer Ltd, UK), amplified, filtered (NL215, band pass 300-4000 Hz) and captured by a computer via a power 1401 interface and Spike 2 software (version 7, CED, UK).

Ex vivo bladder contractions

Chemosensitivity responses of bladder afferents were performed on partially filled bladders with stable intravesical pressures. Once afferent nerve output to graded distension was stable, bladders were partially filled (≈7mmHg) with the outflow tap closed. Infusion was stopped but the outflow tap remained closed, keeping a set volume within a moderately distended bladder. The preparation was left in this state for 45 minutes until bladder pressure and baseline nerve activity were once again stable (18). Carbachol (1 µM), αβMe-ATP (30 µM), and capsaicin (10 µM) were added sequentially by bolus dose (1 ml) with a washout period of 30 minutes between each compound addition. Bladder contraction was measured as a change in intravesical pressure (mmHg) and afferent nerve activity as the number of spikes crossing a pre-set threshold per second above baseline.

In-situ hybridisation mRNA detection of guanylate cyclase-C

In situ detection of mouse guanylyl cyclase-C (*gucy2c*) transcripts was performed in formalin-fixed paraffin-embedded bladder and colon sections from GC-C$^{+/+}$ and GC-C$^{-/-}$ mice (RNAscope® 2.5 LS Reagent Kit–BROWN,#322100,Advanced Cell Diagnostics, CA, USA) according to the manufacturer's instructions. Briefly, 5 µm bladder or colon sections were de-paraffinised and pretreated with protease digestion followed by hybridisation for 2hrs with the target probe for GC-C (*gucy2c*; #436598, RNAscope® LS 2.5 Probe-Mm-Gucy2c, Advanced Cell Diagnostics). An HRP-based signal amplification system was hybridized to the target probe before colour development with 3,3′-diaminobenzidine

tetrahydrochloride. The dihydrodipicolinate reductase (*DapB)* gene (#312038, Advanced Cell Diagnostics) was used as a negative control.

Q-RT-PCR for GC-C in bladder and colonic epithelial cells

Bladders and colons were excised from mice, cut longitudinally, pinned urothelial/epithelial side up on a sylgard-coated petri dish and washed 3x in fresh MEM media containing 1% PSF/0.7% HEPES. Bladders/colons were incubated with 2.5mg/ml sispase in MEM (containing PSF and HEPES) for 2hrs at room temperature. The urothelial/epithelial layers were gently scraped with a blunt scalpel and cells immediately placed into 0.25% trypsin-EDTA solution and then incubated at 37°C for 10 minutes. The cell suspension was then spun at 1500rpm for 5 minutes at 4°C, the media aspirated and the pellet was resuspended in K-SFM media containing 1% PSF. RNA was isolated using the PureLink RNA Micro kit (#12183-016, Invitrogen, ThermoFisher Scientific) according to manufacturer's instructions. All samples were DNAse treated (# 12185-010, Life Technologies, ThermoFisher Scientific). A NanoDrop spectrometer determined Quantity and purity. Q-RT-PCR was performed using EXPRESS One-Step Superscript® qRT-PCR Kit (#11781-01K, Life Technologies, ThermoFisher Scientific) with commercially available hydrolysis TaqMan® probes (GCC:Mm01267705_m1; TrpV4:Mm00499025_m1; Hprt:Mm00446968_m1; Life Technologies, ThermoFisher Scientific) and DEPC-treated water (#AM9916, AMBION, ThermoFisher Scientific). Exon-spanning primers were used for all assays. Samples were run in duplicates. RT-PCR curves were analysed using 7500 Software v.2.06 from Life Technologies. Quantification cycles (Cq) were exported to Excel and relative abundance was estimated using delta Cq method.

Haematoxylin and eosin

Whole bladder and distal colon were removed from mice immediately after euthanasia in 4 different sets of mice: i) control; or those following TNBS treatment at ii) 3 days, iii) 7 days, or iii) 28 days post-TNBS. Tissue was immersed in cold 4% PFA, processed, embedded in paraffin, and sectioned at 10 µm thickness. Sections were then stained with haematoxylin and eosin, imaged with a nanozoomer™ and assessed using panoramic viewer.

Myeloperoxidase (MPO)

Bladder and descending colon were cut longitudinally, rinsed in ice-cold PBS, and snap frozen in liquid nitrogen. Tissue was defrosted on ice, weighed, and put in 1.5ml HTAB buffer (0.5% Hexadecyltrimethylammonium bromide, pH6): 2.5g HTAB (H5882, Sigma, Australia) added to 500ml of 50mM PBS, pH6). The tissue was then homogenised with a cooled TissueLyser™ (Qiagen, Hilden, Germany) and sonicated for 1min. 20µl of tissue homogenate was added to 200µl of an o-dianisidine dihydrochloride (o-dianisidine) solution containing H_2O_2. Absorption data was recorded on a VERSAmax

plate reader (Molecular Devices) at 450nm wavelength for 4 min with 30 sec interval-reading. All samples were run in triplicates and a blank was included.

Mouse dual retrograde tracing to simultaneously identify colon- and bladder-innervating DRG neurons or their central terminals

CTB-555 was injected (3μl/injection site) at three sites (midline and two lateral) into the wall of the colorectum as described above. This was followed by CTB-488 injections into three sites into the bladder wall (4μl/injection) using a separate Hamilton syringe attached to a 23-gauge needle. Mice were then allowed to recover, housed individually and monitored for i) four days, in order to visualise CTB-labelled afferent neurons in the DRG; or ii) seven days, in order to visualise the CTB-labelled central projections of colonic- or bladder-innervating DRG neurons within the dorsal horn of the lumbosacral spinal cord. DRG were processed using the CLARITY method, which removes lipids, rendering tissue transparent whilst preserving ultrastructure, in order to visualise CTB-labelled neurons in intact whole DRG. Four days after retrograde tracing surgery (CTB-555 for colon, CTB-488 for bladder) mice were given an Lethabarb overdose, (intraperitoneal administration) and subsequently transcardially perfuse-fixed as previously described above (12), however, ice cold 0.1M phosphate buffer was used prior to 16% PFA (in 0.1 M phosphate buffer) as per the protocol described in(19). DRG (L1 and L6) were removed to identify CTB-labelled colonic or bladder afferent neurons and placed in 16% PFA-hydrogel solution (4% acrymalyde, 0.25% VA-044 in PBS, Sigma-Aldrich). Samples were then kept at 4°C for a minimum of 1 day. Residual oxygen was then removed from samples, as oxygen inhibits hydrogel polymerisation, using a standard vacuum pump and desiccation chamber attached to a nitrogen gas supply (19). Samples were degassed in their tubes for 15 minutes before transferred to a 37°C water bath for 5 hours until hydrogel solution had uniformly polymerised. Tissue was removed from the hydrogel and underwent passive clearing in 8% SDS/200mM boric acid solution (Sigma-Aldrich), gently shaking at 37°C in a water bath. Buffer was changed every three days until samples were transparent at which point they were washed with PBS-triton X-100 (0.2%) solution for 7 days at 37°C prior to placing individual DRG in 18-well glass slides. Wells with DRG were filled with RapiClear 1.47 refractive index solution (# RC147001, SunJin Lab, Taiwan) and coverslipped 1 hour prior to microscopy.

Identifying the CTB-labelled spinal central projections of colonic or bladder afferents within the dorsal horn was as per described in the colonic central terminals section above. Fluorescence was visualised with a confocal laser scanning microscope (Leica TCS SP8X, Wetzlar, Germany). Images (1024×1024 pixels) were obtained with 20X oil immersion lenses (spinal cord slice) and 10X dry lens (DRG). Differential visualisation of fluorophores was achieved using 495 nm-excitation and 503/538 nm-emission detection settings for AF-488, 552nm-excitation and 558-606 nm-emission detection settings for AF-555. Sequential scanning was used to collect separate emissions from AF-488 and AF-555 labels using four-line averaging. Spinal cord slices were optically sectioned (5μm) and projected images were

reconstructed (45-50μm). DRG were optically sectioned (10μm) and projected images reconstructed for each DRG (230-390μm). Images were processed using Leica LAS Lite and Image J software.

Statistics

Data are expressed as mean ± SEM or the % of neurons/afferents. Figures were prepared in GraphPad Prism 7 Software (San Diego, CA, USA). N equals the number of animals, whilst n equals the number of neurons/afferents. Differences were considered significant at a level of *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. VMR to CRD data were statistically analysed by generalised estimating equations followed by LSD post-hoc test using SPSS 23.0 (IMB, USA). All other data were analysed using GraphPad Prism 7 (San Diego,CA,USA). These data were analysed using either 1) one-way ANOVA. If normally distributed repeated measures one-way ANOVA, with Post hoc analysis conducted by making all possible comparisons among the treatment groups with the Tukey's tests. If data were non-parametric, they were analyzed by Kruskal-Wallis one-way ANOVA, with Dunn's test was used to assess any post hoc differences by comparisons among all groups. 2) If normally distributed repeated measures two-way ANOVA with post-hoc Bonferroni or Tukey's or tests, and 3) paired or unpaired 2-tailed t-tests or 4) χ^2 analysis. The specific tests used to analyse each data set is indicated within the individual figure legends.

Study approval

The Animal Ethics Committees of the South Australian Health and Medical Research Institute (SAHMRI), Flinders University, The University of Adelaide and the University of Tübingen approved experiments involving animals. All experiments conformed to the relevant regulatory standards and the ARRIVE guidelines. All human tissues used for the study were obtained by legal consent from organ donors in the US. ANABIOS Corporation's procurement network and includes only US based Organ Procurement Organizations and Hospitals. Policies for donor screening and consent are the ones established by the United Network for Organ Sharing (UNOS). Organizations supplying human tissues to ANABIOS follow the standards and procedures established by the US Centres for Disease Control (CDC) and are inspected biannually by the Department of Health and Human Services (DHHS). Tissue distribution is governed by internal IRB procedures and compliance with HIPAA regulations regarding patient privacy. All transfers of donor organs to ANABIOS are fully traceable and periodically reviewed by US Federal authorities.

Author contributions

A.D, J.C, J.M, C.B.K, I.S-S and S.M.B designed, performed and analysed the VMR to CRD studies. J.C and S.M.B designed, performed and analysed the colonic afferent recordings. A.M.H and S.M.B designed,

performed and analysed the pERK and retrograde tracing studies. S.P, R.F, C.B.K and I.S-S designed, performed and analysed the FRET cGMP experiments. L.G, G.Y.R and S.M.B designed, performed and analysed the patch clamp experiments on mouse colon- and bladder-innervating DRG neurons. L.G and S.M.B designed, performed and analysed the bladder afferent recordings and bladder voiding patterns analysis. P.M, A.G, I.S-S, and S.M.B designed, performed and analysed the patch clamp recordings on human DRG neurons. P.G and G.H designed, performed and analysed the *in-situ* hybridisation studies. SG.C and S.M.B designed, performed and analysed the Q-RT-PCR and MPO experiments. A.M.H and SG.C performed DRG CLARITY experiments. L.G and SG.C performed the histology experiments. All authors contributed to the discussion and interpretation of the results. S.M.B wrote the manuscript with contributions and suggestions from all authors.

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