Supplementary information:

α-conotoxin Vc1.1 inhibits human dorsal root ganglion neuroexcitability and mouse colonic nociception via $GABA_B$ receptors

Short title: Vc1.1 reduces visceral nociception.

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1) Supplementary Materials and Methods:

The Animal Ethics Committees of the Institute of Medical and Veterinary Science/SA Pathology, the South Australian Health and Medical Research Institute (SAHMRI) and the University of Adelaide approved experiments involving animals. Male C57BL/6J mice aged 13-17 weeks of age were used in all experiments. Mice were acquired from http://www.arc.wa.gov.au and upon arrival to the institutional animal care facility housed in standard cages, which were filled with standard bedding, in a temperature controlled environment 22°C with free access to standard laboratory chow and water. Mice were randomly assigned to treatment groups. Following TNBS administration mice were individually housed to allow for accurate clinical monitoring until the experimental day in question.

Human DRG expression studies

Thoracolumbar DRG from spinal levels T9-L1 were acquired from 4 consenting adult organ donors. Intact DRG were kept for QRT-PCR mRNA expression studies from each spinal level (T9, T10, T11, T12, L1), whilst additional DRG were dissociated to allow individual DRG neurons to be studied with single cell PCR studies or to allow whole-cell patch clamp recordings to be performed.

Patch clamp recordings on human DRG neurons

The extracellular solution contained (in mM): 145 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, adjusted to pH 7.4 with NaOH. The intracellular solution contained (in mM): 130 K-gluconate, 5 KCl, 5 NaCl, 3 Mg-ATP, 0.3 EGTA, 10 HEPES, adjusted to pH 7.3 with KOH. Signals were amplified and digitized by EPC10 amplifier (HEKA) controlled by Patchmaster software (HEKA). Recordings were performed at 32°C. To assess the rheobase, neurons were held at −70 mV and a series of increasing inward currents (500ms duration, in 20 or 50 pA steps) was applied to induce action potential firing. Recordings were made in the presence and absence of Vc1.1 (100nM) applied for 5 minutes. Alternatively, we also tested [P6O]Vc1.1, which is inactive at the $GABA_BR$, but active at the α9α10-nAChR[1]. This peptide contains a hydroxyproline (denoted by the amino acid code-O) residue in place of the proline (P) residue at position 6 of Vc1.1.

Model of chronic visceral hypersensitivity (CVH):

Colitis was induced by administration of Trinitrobenzene Sulphonic Acid (TNBS) as described previously [2-4]. Briefly, 13-week-old mice, anaesthetized with isofluorane, were administered an intracolonic enema of 0.1mL TNBS (130µL/mL of 1M solution in 30% ethanol), via a polyethylene catheter inserted 3 cm from the anus. Mice were then individually housed and observed daily for changes in body weight, physical appearance and behaviour. Previous studies using this model showed mucosal architecture, cellular infiltrate, crypt abscesses, and goblet cell depletion confirming TNBS induces significant damage by day 3-post treatment. This damage largely recovers by day 7 and is fully resolved by day 28. At the 28-day time point, the high-threshold nociceptors in these mice display significant mechanical hypersensitivity and lower mechanical activation thresholds [4]. Increased neuronal activation in the dorsal horn of the spinal cord in response to noxious colorectal distension, as well as sprouting of colonic afferent terminals within the dorsal horn has also been reported [5]. The model also induces hyperalgesia and allodynia to colorectal distension [6] and is termed Chronic Visceral Hypersensitivity (CVH) [2,3].

In vitro mouse colonic primary afferent electrophysiology and pharmacology:

Age matched healthy control or CVH mice were euthanized by CO_2 inhalation. The colon, rectum and attached mesentery were removed and afferent recordings from the splanchnic and pelvic nerves performed as described previously [4,7-9]. These sensory pathways are important as the splanchnic nerves supply the mid-to-distal colon and signal predominantly nociceptive information. In comparison, the pelvic nerves supply the colorectum, and signal a mixture of physiological and nociceptive information[4,10]. Briefly, colorectal tissue was removed and pinned flat, mucosal side up, in a specialized organ bath. The colonic compartment was superfused with a modified Krebs solution (in mM: 117.9 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.3 NaH_2PO_4, 1.2 $MgSO_4(H_2O)_7$, 2.5 $CaCl_2$, 11.1 D-glucose) and bubbled with carbogen (95% O_2, 5% CO_2) at 34°C. The splanchnic or pelvic nerves were extended into a paraffin filled recording chamber where, after fine dissection, strands were laid onto a platinum electrodes and single-unit extracellular recordings of action potentials generated.

Mechanoreceptor classification:

Receptive fields were identified by systematically stroking the mucosal surface with a still brush to activate all subtypes of mechanoreceptors. Afferents were categorized in accordance with our previously published classification system [4,7-9,11]. Once identified,

receptive fields were tested with three distinct mechanical stimuli to enable classification: static probing with calibrated von Frey hairs (2000 mg force; applied 3 times for a period of 3 sec), mucosal stroking with calibrated von Frey hairs (10mg force; applied 10 times) or circular stretch (5 g; applied for a period of 1 min). Stretch was applied using a claw attached to the tissue adjacent to the afferent receptive field and connected to a cantilever system via thread. Weights were applied to the opposite side of the cantilever system to initiate graded colonic circumferential stretch.

Serosal afferents, also termed vascular afferents, were recorded from the splanchnic pathway, have high-mechanical activation thresholds and respond to noxious distension (40mmHg), stretch (≥7g) or von Frey hair filaments (2g) but not to fine mucosal stroking (10 mg von Frey hairs; vfh) [4,8,9]. The algesic channels and receptors TRPV1 [12], TRPA1 [7,13], TRPV4 [9], $P2X_3$ [12], B1 [11], $Na_V1.8$ [14], TNFR1 [15] are highly expressed in serosal afferents. In addition, serosal afferents become mechanically hypersensitive in models of chronic visceral pain [4] and have a nociceptor phenotype [2,3, 8,16]. In the current study they are therefore referred to as 'nociceptors'. Muscular-mucosal afferents were recorded from the pelvic pathway and respond to both low-intensity circular stretch (<5 g) and to fine mucosal stroking (10mg) [4,7,9,10].

Chemicals, drugs and pharmacology

Peptides and drugs were prepared from stock solutions, diluted to appropriate final concentrations in Krebs solution, and applied via a small metal ring placed over the afferent receptive field of interest. Vc1.1, [P6O]Vc1.1 and ω-conotoxin CVID were synthesized using solid phase peptide chemistry and purified by HPLC as described previously [17]. The $GABA_B$ receptor agonist, baclofen, the $GABA_B$ receptor antagonist (2*S*)-3-[[(1*S*)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenyl-methyl)phosphinic acid hydrochloride (CGP55845), and the $Ca_V2.3$ inhibitor (SNX-482) were all purchased from Tocris Bioscience (Bristol, UK).

Once baseline splanchnic and pelvic colonic afferent responses had been established, mechanosensitivity was re-tested after application of Vc1.1 (1, 10, 100, 1000 nM). Each dose of Vc1.1 was applied to the colon mucosal surface for 10 minutes. We also tested [P6O]Vc1.1, which is inactive at the $GABA_BR$, which was also applied for 10minutes. To determine the mechanism of action of Vc1.1 the selective $GABA_BR$ antagonist (CGP55845: 5 μM), and the $Ca_V2.2$ blocker (ω-conotoxin CVID, isolated from the piscivorous cone snail *Conus catus*[18]:

1μM) or the $Ca_V2.3$ inhibitor (SNX-482, isolated from venom of the spider *Hysterocrates gigas*: 200nM) were applied alone, or in combination, for 10 minutes prior to co-incubation with Vc1.1 (1000nM).

Data analysis

Action potentials were analysed off-line using the Spike 2 wavemark function and discriminated as single units on the basis of distinguishable waveform, amplitude and duration. Data are presented as spikes/second and are expressed as mean ± SEM. In some instances data are presented as 'change from baseline'. This is calculated by determining the change in mechanosensitivity of individual afferents between the normal 'baseline' response in healthy or CVH conditions compared to the respective mechanical responses following drug or peptide addition. In the respective study this difference is then averaged across all afferents to obtain a final mean ± SEM of "change in response from baseline". *n* indicates the number of individual afferents.

Colorectal Distension (CRD):

Healthy control or CVH mice were fasted overnight with free access to water and a 5% glucose solution to ensure faecal pellets were absent for colorectal distension (CRD). Mice were then briefly anaesthetized with isoflurane anesthetic. Mice received either a single 0.2ml intracolonic enema of 0.1M phosphate buffered saline (PBS) (control mice) or Vc1.1 (1000nM in 0.1M PBS) via a polyethylene catheter inserted to a depth of 3 cm from the anus. Ten minutes after saline or Vc1.1 administration a 4cm balloon catheter was inserted into the perianal canal and secured to the tail so that the start of the balloon was 0.5cm from the anal opening. The tube was secured to the tail and the balloon catheter was attached to a sphygmomanometer pressure gauge and a 20ml syringe via a three-way stopcock. Mice were removed from the isofluorane chamber and when they regained consciousness the balloon was distended for 10 seconds to a pressure of 80mmHg, applied via the syringe. The pressure was released and the balloon deflated (0mmHg) for 5 seconds. This process was repeated five times, as per our previous studies [2,3,5]. After the fifth distension and within two minutes after the final deflation, mice were injected with an anesthetic overdose (0.2 ml/250g sodium pentobarbitone). Mice then underwent transcardial perfuse fixation. N indicates the number of animals used per study. Our previous histological studies demonstrate that this protocol does not overtly damage the colonic epithelium [2,3,5].

Tissue processing for pERK immunohistochemistry:

Healthy control or CVH mice were perfused transcardially with warm saline (0.85% physiological sterile saline) followed by ice-cold 4% paraformaldehyde (Sigma-Aldrich, MO, USA). Following perfusion, thoracolumbar (T10-L1) and lumbosacral (L6-S1) spinal cord were removed and post-fixed for 16 hours at 4^0C in 4% paraformaldehyde in 0.1M PBS. Following fixation the respective spinal cord segments were cryoprotected in 30% sucrose/PBS (Sigma-Aldrich, MO, USA) overnight at 4^0C and then placed in 50% Optimal Cutting Temperature compound (OCT; Tissue-Tek, Sakura Finetek, CA, USA) in 30% sucrose/PBS for 7 hours, before freezing in 100% OCT. Frozen sections (12µm) were cut using a cryostat and placed onto gelatin coated slides for fluorescence immunohistochemistry.

Phosphorylated MAP kinase ERK 1/2 (pERK) immunohistochemistry in the spinal cord:

Immunohistochemistry for pERK was performed in a paired fashion, with tissue from healthy control and CVH mice exposed to either intra-colonic saline or Vc1.1 at room temperature. Air-dried sections (20 min) were washed 3 times with PBS (pH7.4) and blocked for 30 min with 5% normal chicken serum (NCS)/0.2% Triton-X 100 (TX; Sigma-Aldrich, MO, USA) in PBS. Sections were then incubated with 1:200 diluted anti-phospho-p44/42 MAPK antibody (Erk1/2; Thr202/Tyr204; Cell Signalling Technology # 4370) for 18 hours. Sections were then washed 3 times with 0.2% TX/PBS and incubated for 1 hour with secondary antibody (1:200; chicken anti-rabbit AlexaFluor488, AF-488). Negative controls were prepared as above with the primary antibody omitted [2,3,5].

The average number of pERK-immunoreactive neurons within the dorsal horn of the thoracolumbar and lumbosacral spinal cord sections were compared between animals administered intra-colonic saline or Vc1.1 (1000nM) with threshold images and Image J v1.48J software. Data were analysed using Prism 5 software (GraphPad Software, San Diego, CA, USA), where appropriate, using a one-way analysis of variance (ANOVA) followed by Bonferroni *posthoc* tests. Differences between responses to noxious colorectal distension in mice administered intra-colonic saline or Vc1.1 were considered significant at a level of * $P<0.05$, ** $P<0.01$ or *** $P<0.001$.

Retrograde tracing of colonic DRG neurons

Cholera toxin subunit B conjugated to AlexaFluor 488 (CTB-488) or AlexaFluor 555

(CTB-555; Invitrogen, Carlsbad, CA) was injected at three sites sub-serosally within the wall of the distal colon of healthy control or CVH mice [3,5,7]. After 4 days, animals were humanely killed by CO_2 inhalation for subsequent DRG removal and dissociation and short-term (<2 hours) cell culture and mRNA expression studies [3,19]. Alternatively mice received an anesthetic overdose for subsequent transcardial perfusion with ice-cold fixative (4% paraformaldehyde in 0.1mol/L PBS (Sigma-Aldrich, St. Louis, MO) for immunohistochemical studies, as previously described [19].

Laser Capture microdissection (LCM) and single cell picking of mouse DRG neurons

Thoracolumbar (T10-L1) and lumbosacral (L6-S1) dorsal root ganglia (DRG) were removed from four healthy control and four CVH retrogradely traced mice 4 days after fluorescent tracer injection into the colon (CTB-AF555). DRG were digested with 4 mg/mL collagenase II (GIBCO, Life Technologies) and 4 mg/mL dispase (GIBCO) for 30 minutes at 37°C, followed by 4 mg/mL collagenase II for 10 minutes at 37°C. Neurons were mechanically dissociated into a single-cell suspension via trituration through fire-polished Pasteur pipettes. Neurons were suspended in HBSS and spot-plated onto 50-mm Zeiss duplex dishes (Carl Zeiss, Oberkochen, Germany), then maintained at 37°C in 5% CO_2 for 2 hours, allowing optimal cell adhesion. For pooling of ~200 colonic DRG neurons from healthy or CVH mice retrogradely labeled neurons were isolated using a PALM Microlaser Technologies microdissection system (Carl Zeiss) and catapulted directly into a lysis/stabilization buffer-containing carrier RNA (4ng/µL; Qiagen, Sydney, Australia) [3,7,9,19].

For single cell PCR analysis of potential co-expression of membrane receptor and ion channel targets of Vc1.1, retrogradely traced DRG neurons were identified by fluorescence on an IX70 microscope. The procedure for cell culture for neurons for single cell picking were identical to those used for laser capture microdissection, except that neurons were resuspended in DMEM with 10%FBS, 1xMEM-NEAA, 200mM GlutaMAX™, 200 units/ml penicillin, 200 µg/ml streptomycin and 100 ng/ml NGF-7S. Two hours after plating single neurons were picked using precision pulled and fire polished glass capillaries and a high fidelity micromanipulators and then stored in individual tubes. 14 TL colonic DRG neurons from 2 healthy mice were individually picked and stored for subsequent PCR analysis. Similarly, 14 TL colonic DRG neurons were individually picked and stored from 2 CVH mice. To control for contamination in the liquid of the bath a sample was taken after all traced cells were picked off each cover slip. Only clearly single cells with no other cell or debris attached to it were picked. Single cell RNA was isolated using 10µl lysis buffer with DNAse (Cells-to-Ct

kit, Ambion, Life Technologies, Australia) according to manufacturer's instructions. RT-PCR was performed using Taqman probes.

Single cell picking of human DRG neurons

Human DRG were dissociated, as per the patch clamp recordings, however, after plating single neurons were picked using precision pulled and fire polished glass capillaries and a high fidelity micromanipulators. A total of 39 human DRG neurons from 4 adult organ donors were individually picked and stored for subsequent single-cell-RT-PCR analysis. Only clearly single cells with no other cell or debris attached to it were picked. Single cell RNA was isolated the Cells-to-Ct kit (Ambion, Life Technologies, Australia) according to manufacturer's instructions. qRT-PCR was performed using human specific TaqMan probes.

Quantitative RT-PCR analysis of human DRG neurons and mouse colonic DRG neurons:

RNA was extracted from whole human (T9-L1) or mouse (T10-L1) DRG using an RNeasy Mini Kit (Qiagen, Sydney, Australia) according to the manufacturer's instructions. RNA was isolated from LCM colonic cells with a RNeasy Micro-Kit (Qiagen, Sydney, Australia). Quantitative reverse transcription-polymerase chain reaction (QRT-PCR) was performed using a Chromo4 (MJ Research, Waltham, MA) real-time instrument attached to a PTC-200 Peltier thermal cycler (MJ Research) and Opticon Monitor software (MJ Research, USA).

For mouse experiments whole DRG and pooled LCM colonic DRG experiments Qiagen QuantiTect SYBR Green RT-PCR 1-step kits were used according to the manufacturer's specifications with the following primers: $Ca_V2.2$ exon 37a primers: Forward: 5'-GCTGCGTGTTGCCGGATTCATTAT-3'; Reverse: 5'-TTCATTCGAACCAGGCGCTTGTAG-3'. $Ca_V2.2$ exon 37a+b primers: Forward: 5'-CTGAATACGACCCAGCTGCGTGTG-3'; Reverse: 5'-CCAGGCGCTTGTATGCAACTCGAG-3'. $Ca_V2.3$ primers: Forward: 5'-GCACTACATCTCTGAGCCCTATCTG-3'; Reverse: 5'-TCTCCTCCTCGCCACAGTCT-3'. $GABA_B$ R1 subunit primers: Forward: 5'-CCTGGATTCCTGTGGAAGAA-3'; Reverse: 5'-GTCAAGCCACGGTACCTGAT-3'. $GABA_B$ R2 subunit primers: Forward: 5'-ATTCTCACCGTGGGCTACAC-3'; Reverse: 5'-CACAGATCGATCAGCAGCAT. β-tubulin primers: forward: 5'-CCAAGTTCTGGGAGGTCATC-3'; reverse: 5'-TGAGAGGAGGCCTCATTGTAG-3'. (GeneWorks, Adelaide, Australia). Reaction conditions used were as follows: reverse

transcription: 50˚C (30 min); initial PCR activation: 95˚C (15 min); annealing, denaturing, and extension cycles: 94°C (15s), 55°C (30s), and 72°C (30s) repeated for 50 cycles. The size of amplified products was confirmed by gel electrophoresis. Each assay was run in at least triplicate in separate experiments. Non-template controls were performed in every PCR run. For all experiments the comparative cycle threshold method was used to quantify the abundance of target transcripts in whole ganglia and isolated neurons [7]. Quantitative data are expressed as Mean ± SD.

For PCR experiments of single human DRG neurons or single mouse colonic DRG neurons, an Ambion® Single Cell-to-CT™ Kit (Life Technologies, Sydney, Australia) was used on an Applied Biosystems® 7500 Real-Time PCR System. The following mouse TaqMan QPCR primers were used: $GABA_B$ R1 subunit: Mm00433461_m1, product size 79bp; $GABA_B$ R2 subunit: Mm01352554_m1, product size 87bp; $Ca_V2.2$: Mm01333678_m1, product size 61bp; $Ca_V2.3$: Mm00494444_m1, product size 63bp; β-actin: Mm00607939_s1, product size 115bp (Life Technologies, Sydney, Australia). The following human TaqMan QPCR primers were used: $GABA_B$ R1 subunit: Hs00559488_m1, product size 68bp; $GABA_B$ R2 subunit: Hs01554996_m1, product size 59bp; $Ca_V2.2$: Hs01053090_m1, product size 69 bp; $Ca_V2.3$: Hs00167789_m1, product size 92 bp; GAPDH: Hs02758991_g1, product size 93 bp. In both human and mouse studies the presence or absence of a target in an individual neuron was confirmed via cycle threshold analysis and GelRed™ Nucleic Acid Gel Stain gel-electrophoresis.

Immunohistochemistry of mouse colonic DRG neurons:

Two different methods were used to determine the percentage of colonic DRG neurons expressing $GABA_BR1$, $GABA_BR2$, $Ca_V2.2$ and $Ca_V2.3$ channels. The first used perfused fixed frozen sections of DRG. After transcardial perfusion of retrogradely traced healthy or CVH mice the thoracolumbar (T10–L1) DRG were removed and post-fixed for 2 hours at room temperature. After fixation, tissue for sectioning was cryoprotected in 30% sucrose/PBS (Sigma-Aldrich, St. Louis, MO, USA) overnight at 4°C and frozen in OCT compound (Tissue-Tek, Sakura Finetek, Alphen aan den Rijn, Netherlands). Serial frozen sections (10–12µm) were cut and processed for immunohistochemistry. Non-specific binding of secondary antibodies was blocked with 5% normal donkey or chicken serum diluted in 0.2% Triton-TX 200 (Sigma-Aldrich) in PBS. Tissue sections and colonic whole-mounts were incubated with primary antisera, diluted in 0.2% Triton-TX/PBS, overnight at 4°C. The specific antisera used

were $GABA_BR1$ (Abcam; ab75239, lot#GR13232-1, 1:300), $GABA_BR2$ (Abcam; ab75838, lot#GR7446-6, 1:300), $Ca_V2.2$ (Alomone; ACC-002, lot#ACC002AN3202, 1:1200), or $Ca_V2.3$ (Alomone; ACC-006, lot#ACC006AN0202, 1:1200). AF488 or AF568 conjugated donkey or chicken anti-rabbit IgG antibody were used at 1:200 for 1 hour at room temperature for visualization. Negative controls were prepared as above with the primary antibody omitted (Supp. Figure 1). Images were obtained using an epifluorescent microscope (BX51, Olympus, Hamburg, Germany) and confocal scanning microscope (SP5, Leica Microsystems, Wetzlar, Germany). Only neuronal cells with intact nuclei were included in this study and the number of labeled neurons was expressed as a percentage of neurons in the whole DRG from 6 to 8 DRG sections (30µm apart) per mouse and averaged across 4 mice. The optimal working dilution of each antibody was determined empirically. In control experiments, no labeling was observed when either primary or secondary antibodies were omitted.

The second method employed acutely dissociated neurons with identical dissociation procedures to those used for single cell picking. Dissociated neurons were plated on coverslips. The next day cells were washed three-times with PBS and fixed using 4% ice-cold paraformaldehyde for 20min. After 3 washes (5min each) cells were blocked with 10% NCS/PBS/0.1% saponin for 30min (Sigma-Aldrich, MO, USA). Cells were then incubated with $GABA_BR1$, $GABA_BR2$ $Ca_V2.2$ or $Ca_V2.3$ antibodies listed above for ~18-22 hours at 4°C. All antibodies were diluted in 0.05% saponin/5%NCS/PBS-buffer. Cover slips were washed 3-times for 5min with PBS before incubation with AlexaFluor594-conjugated chicken anti-rabbit IgG antibodies (Life Technologies, 1:800 in 1%BSA/PBS) for 1 hour at room temperature. Cover slips were washed 3-times in PBS and mounted in 6 µl Prolong Gold™ mounting media (Life Technologies). Slides were stored at 4°C and 40x images taken within 1-2 weeks using an Olympus BX51 microscope. Images were analysed using ImageJ v1.48J.

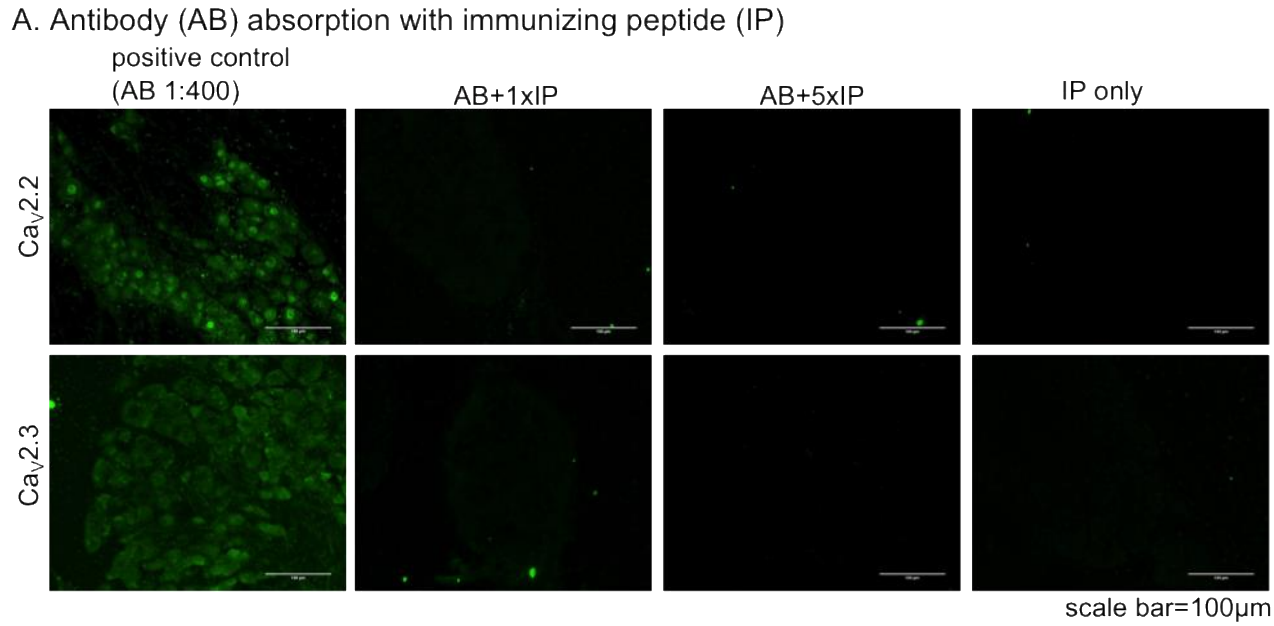
Data analysis

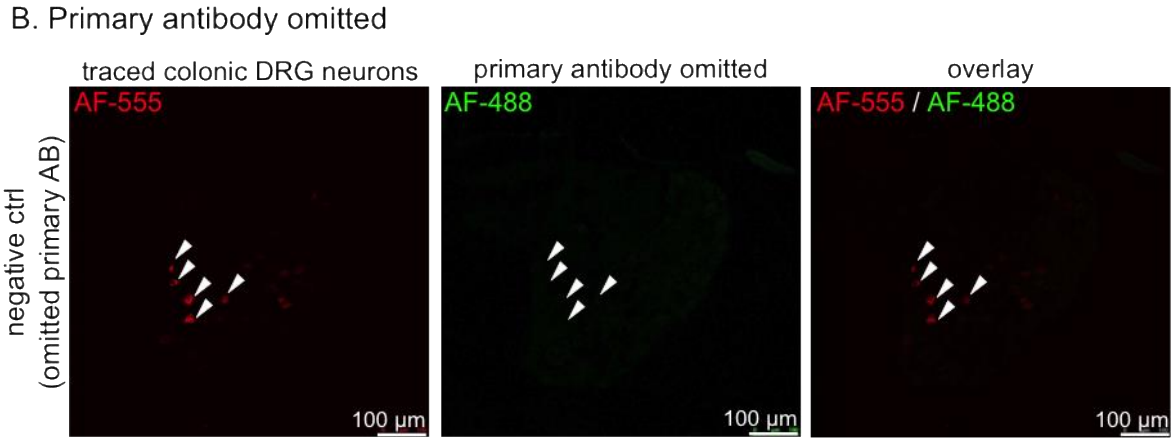
All data were analysed using Prism 5 software (GraphPad Software, San Diego, CA, USA) using either paired t-tests, or one-way or two-way analysis of variance (ANOVA) followed by appropriate *posthoc* tests. Where appropriate data were corrected for multiple comparisons. Differences between specific drug concentrations and baseline responses or expression levels between healthy and CVH mice were considered significant at * $P<0.05$, ** $P<0.01$ or *** $P<0.001$. n = the number of afferents or neurons. N = the number of animals.

<u>Supplementary Figure 1:</u> Experimental controls for immunohistochemistry using frozen DRG sections from mice.

A) Studies using $Ca_V2.2$ and $Ca_V2.3$ primary antibodies in the presence of the immunizing antigen showed a lack of immunoreactivity against $Ca_V2.2$ and $Ca_V2.3$ respectively

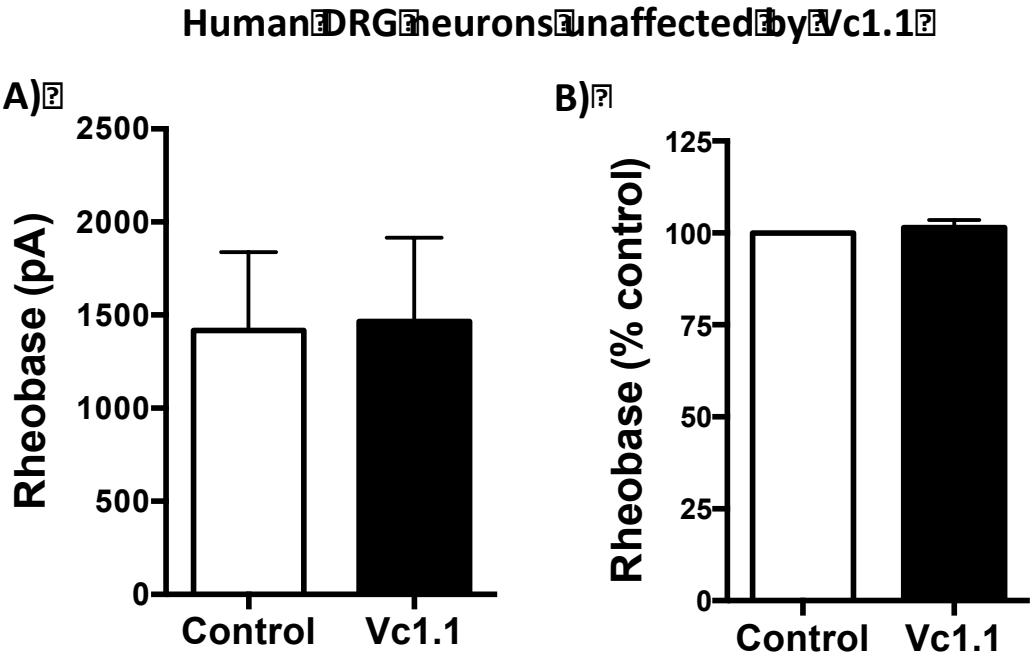
B) Primary anti-body omission studies showing a lack of immunoreactivity (AF-488), but traced colonic DRG neurons still apparent (AF-555; red neurons).





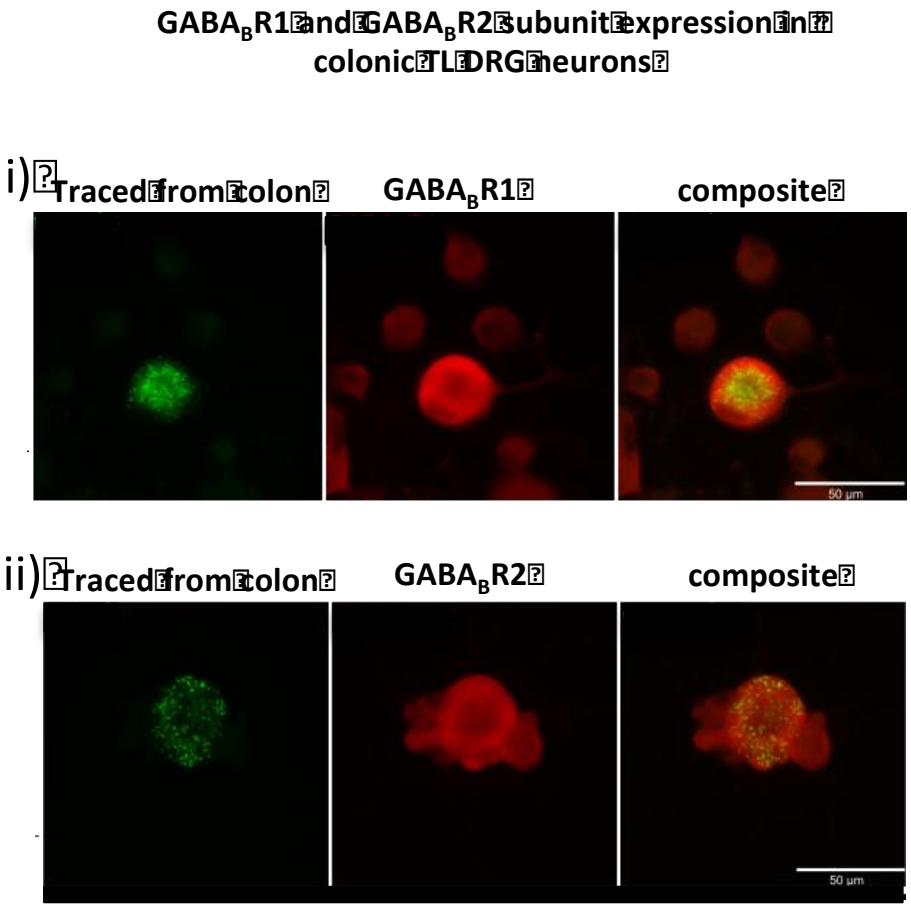
<u>Supplementary Figure 2:</u> Vc1.1 does not inhibit a sub-population of human DRG neurons.

Vc1.1 did not affect the neuronal excitability of a sub-population of human DRG neurons as defined by a lack of effect on the **A**) rheobase or **B)** percentage change in rheobase (P>0.05, n=6).



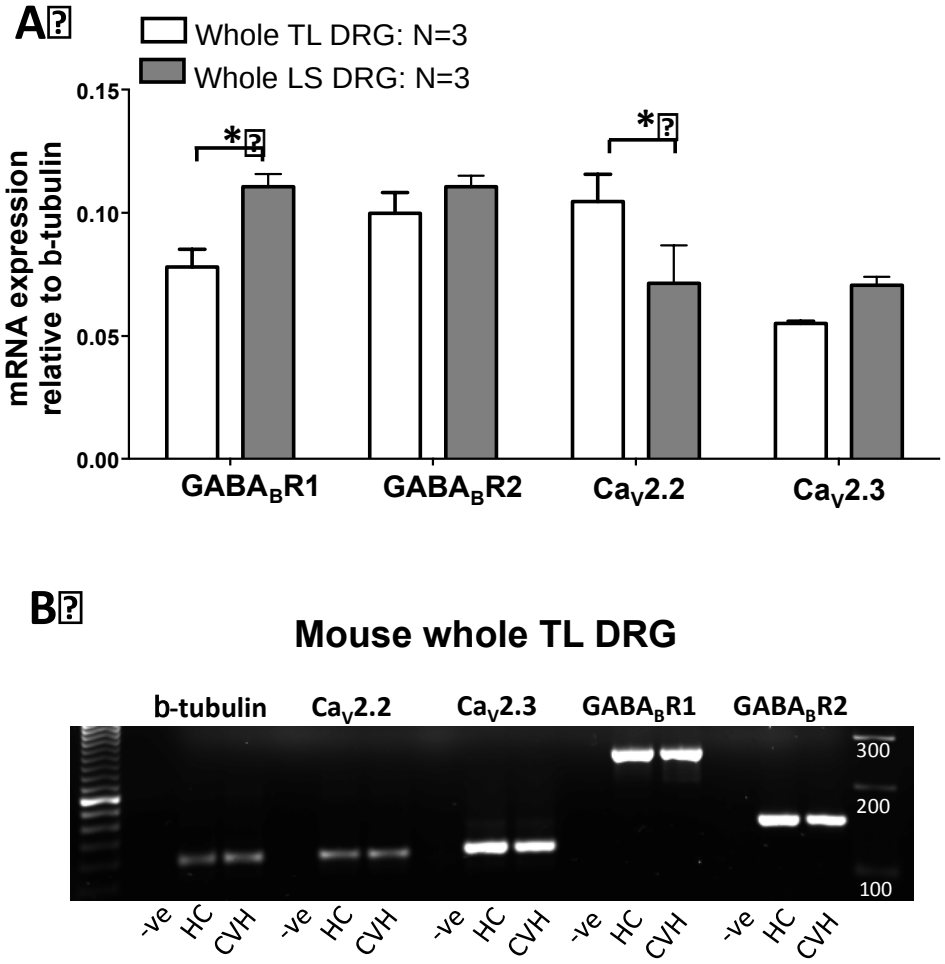
<u>Supplementary Figure 3:</u> Mouse colonic DRG neurons express $GABA_BR$.

Immunohistochemistry performed on dissociated thoracolumbar DRG from mice that had previously undergone colonic retrograde tracing with CTB-488. Examples show colonic DRG neurons expressing both $GABA_BR$ subunits: **i)** $GABA_BR1$ and **ii)** $GABA_BR2$.



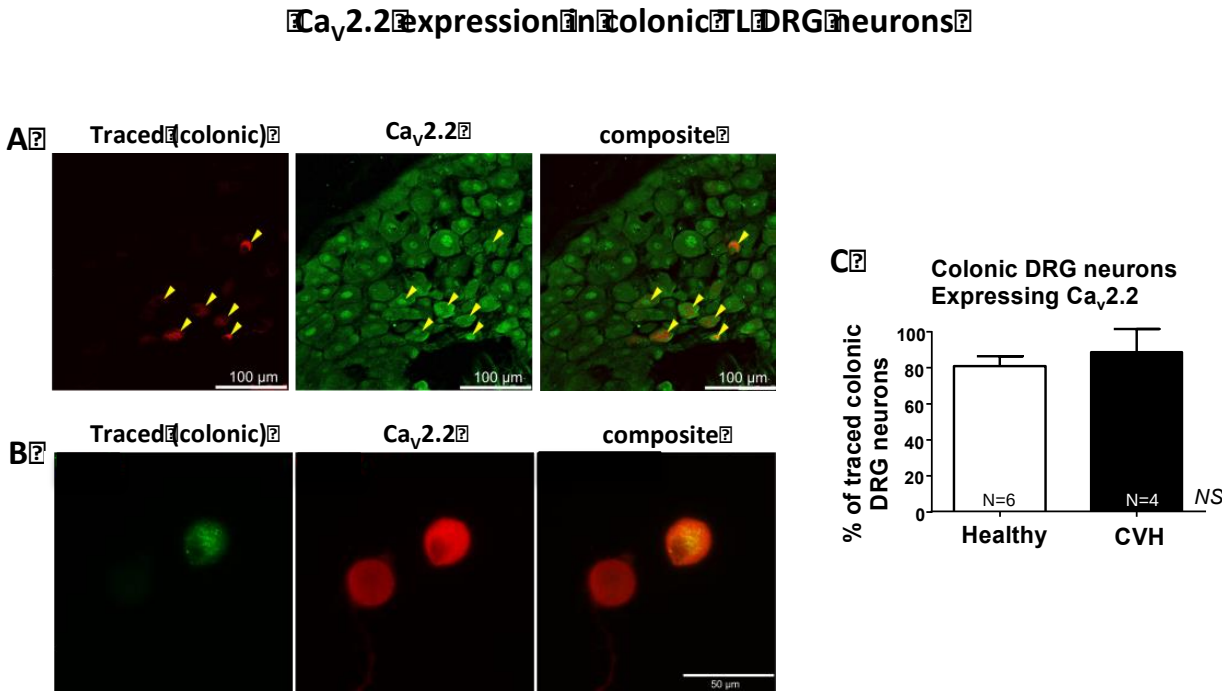
<u>Supplementary Figure 4:</u> Expression of $GABA_BR$ subunits and voltage-gated calcium channels in mouse ganglia.

A). Quantitative RT-PCR from whole thoracolumbar (TL) and lumbosacral (LS) DRG showing abundant expression of $GABA_BR1$, $GABA_BR2$, $Ca_V2.2$ and $Ca_V2.3$ mRNA. $GABA_BR1$ is more abundant in whole LS DRG (**P*<0.05), whereas $Ca_V2.2$ is more abundant in whole TL DRG (**P*<0.05, Healthy:N=3; CVH:N=3). **B)** Gel electrophoresis confirmed QPCR products from whole TL DRG from healthy (HC) and CVH mice



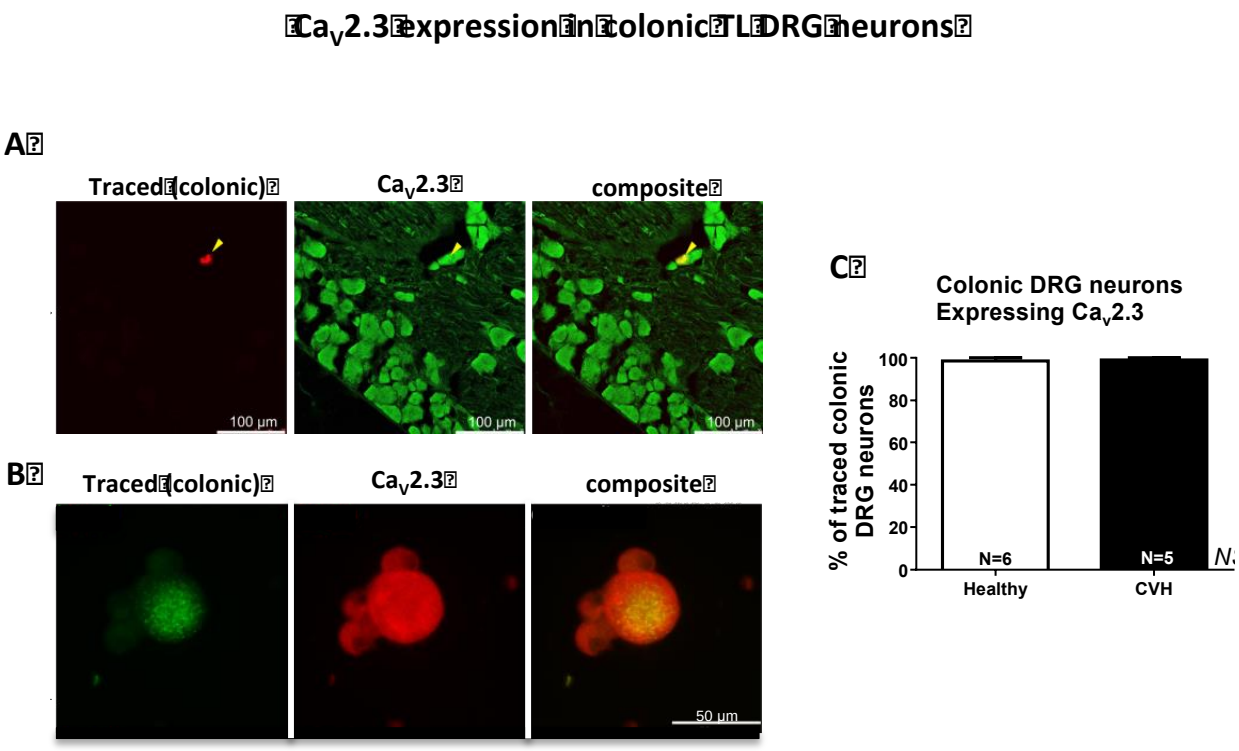
<u>Supplementary Figure 5</u>: Mouse colonic DRG neurons express the voltage-gated calcium channel $Ca_V2.2$.

Immunohistochemistry for $Ca_V2.2$ in **A)** frozen sections and **B)** acutely dissociated thoracolumbar DRG from mice that had undergone colonic retrograde tracing with CTB-488. **C).** A high percentage of colonic DRG neurons from healthy (81%) and CVH (91%) mice display $Ca_V2.2$-immunoreactivity (*NS*, not significant, Healthy, N=6 mice; CVH, N=4 mice).



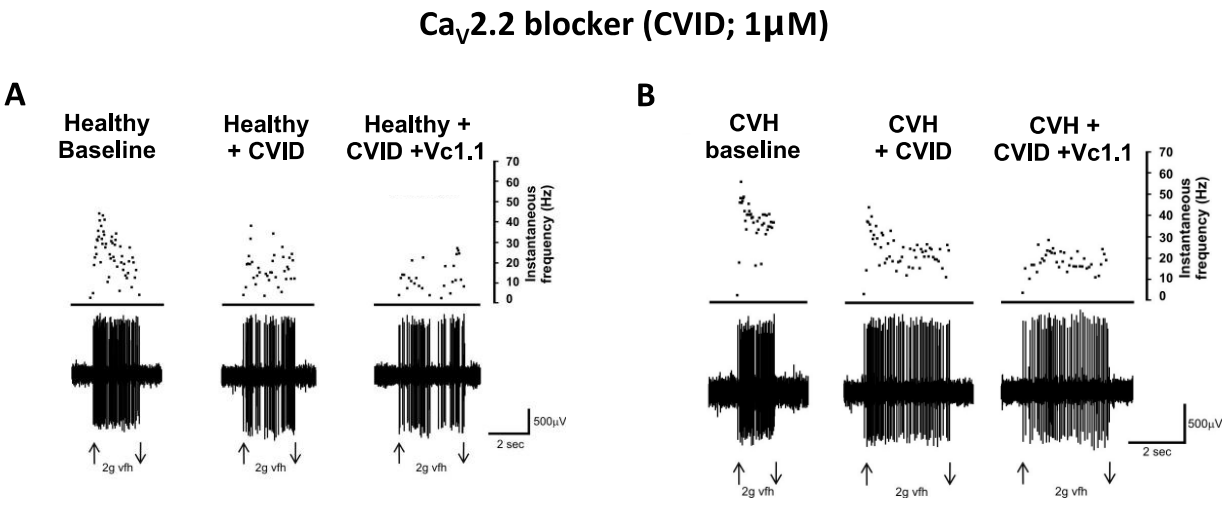
<u>Supplementary Figure 6:</u> Mouse colonic DRG neurons express the voltage-gated calcium channel $Ca_V2.3$.

Immunohistochemistry for $Ca_V2.3$ in **A)** frozen sections and **B)** acutely dissociated thoracolumbar DRG from mice that had undergone colonic retrograde tracing with CTB-488. **C).** A very high percentage of colonic DRG neurons from healthy and CVH mice display $Ca_V2.3$-immunoreactivity (*NS*, not significant, Healthy, N=6 mice; CVH, N=5 mice).



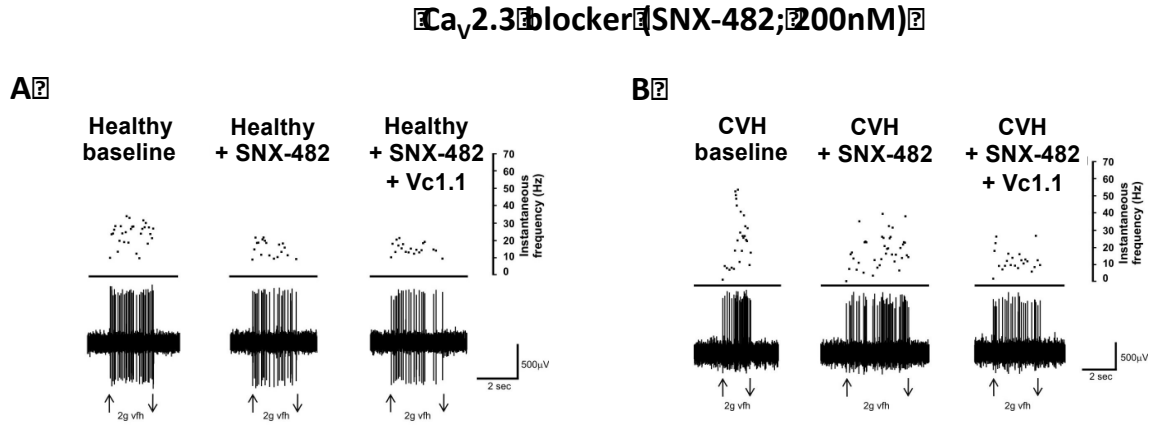
<u>Supplementary Figure 7:</u> Blocking $Ca_V2.2$ with ω-conotoxin CVID inhibits colonic nociceptors.

Examples of single-unit recordings of a **A)** healthy nociceptor and a **B)** CVH nociceptor with mechanical responses at baseline and in the presence of CVID (1 μM), and CVID (1 μM)+Vc1.1 (1000 nM). Note that Vc1.1 causes more inhibition in the presence of CVID, indicating the involvement of additional voltage-gated calcium channels in the inhibitory response.



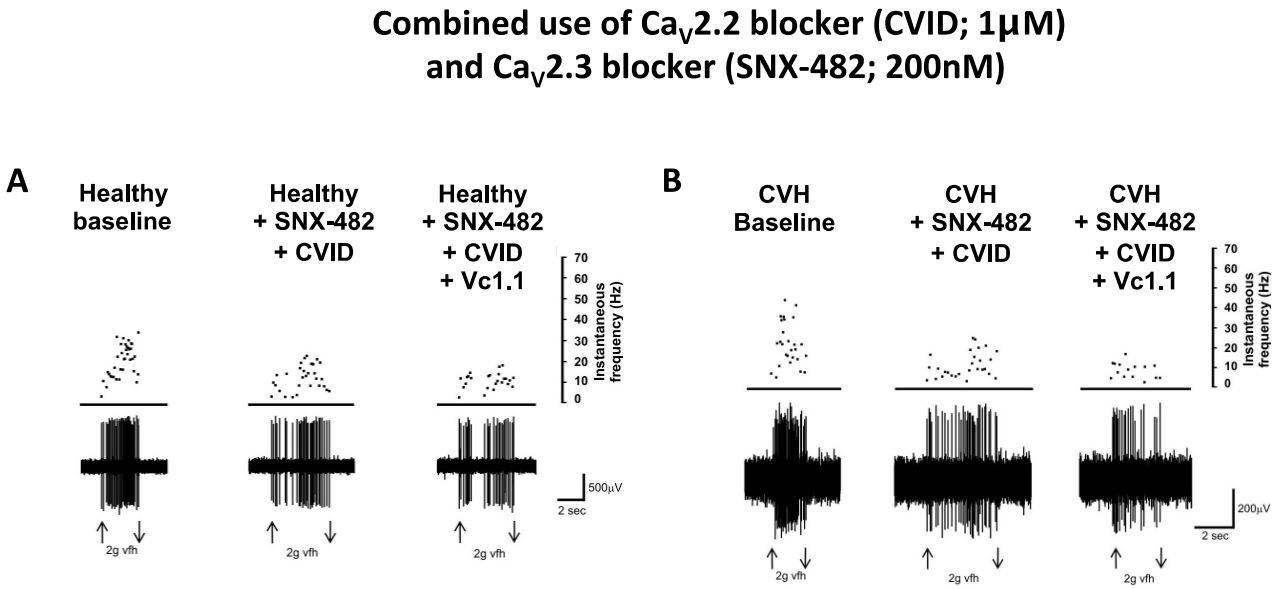
<u>Supplementary Figure 8:</u> Blocking $Ca_V2.3$ with SNX-482 causes pronounced inhibition of colonic nociceptors.

Single-unit recordings of a **A)** healthy nociceptor and a **B)** CVH nociceptor with mechanical responses at baseline and in the presence of SNX-482 (200 nM), and SNX-482 (200 nM)+Vc1.1 (1000 nM). Note that Vc1.1 causes more inhibition in the presence of SNX-482, suggesting $Ca_V2.2$ and $Ca_V2.3$ underlie the inhibitory response induced by Vc1.1.



<u>Supplementary Figure 9:</u> Blocking both $Ca_V2.2$ (CVID) and $Ca_V2.3$ (SNX-482) causes inhibition of colonic nociceptors comparable to that of Vc1.1 alone.

A) Healthy and **B**) CVH nociceptor mechanical responses at baseline and in the presence of CVID (1μM)+SNX-482 (200nM), and CVID (1μM)+SNX-482 (200nM)+Vc1.1 (1000nM).



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