Cyclic analogues of α-conotoxin Vc1.1 inhibit colonic nociceptors and provide analgesia in a mouse model of chronic abdominal pain

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Abstract

**Background and Purpose:** Patients with irritable bowel syndrome suffer from chronic visceral pain (CVP) and limited analgesic therapeutic options are currently available. We have shown that α-conotoxin Vc1.1 induced activation of GABA$_B$ receptors on the peripheral endings of colonic afferents and reduced nociceptive signalling from the viscera. However, the analgesic efficacy of more stable, cyclized versions of Vc1.1 on CVP remains to be determined.

**Experimental Approach:** Using ex vivo colonic afferent preparations from mice, we determined the inhibitory actions of cyclized Vc1.1 (cVc1.1) and two cVc1.1 analogues on mouse colonic nociceptors in healthy and chronic visceral hypersensitivity (CVH) states. Using whole-cell patch clamp recordings, we also assessed the inhibitory actions of these peptides on the neuronal excitability of colonic innervating dorsal root ganglion neurons. In vivo, the analgesic efficacy of these analogues was assessed by determining the visceromotor response to colorectal distension in healthy and CVH mice.

**Key Results:** cVc1.1 and the cVc1.1 analogues, [C2H,C8F]cVc1.1 and [N9W] cVc1.1, all caused concentration-dependent inhibition of colonic nociceptors from healthy mice. Inhibition by these peptides was greater than those evoked by linear Vc1.1 and was substantially greater in colonic nociceptors from CVH mice. cVc1.1 also reduced excitability of colonic dorsal root ganglion neurons, with greater effect in CVH neurons. CVH mice treated with cVc1.1 intra-colonically displayed reduced pain responses to noxious colorectal distension compared with vehicle-treated CVH mice.

**Conclusions and Implications:** Cyclic versions of Vc1.1 evoked significant anti-nociceptive actions in CVH states, suggesting that they could be novel candidates for treatment of CVP.

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Running title: Cyclized Vc1.1 reduces visceral nociception

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Key Words:
Chronic visceral hypersensitivity, G protein-coupled receptor, analgesia, α-conopeptides, voltage-gated calcium channels, GABA_B receptor
Abbreviations:
- $\text{Ca}_\text{V}2.1$: P/Q-type voltage-gated calcium channel
- $\text{Ca}_\text{V}2.2$: N-type voltage-gated calcium channel
- $\text{Ca}_\text{V}2.3$: R-type voltage-gated calcium channel
- CTB-488: Cholera toxin subunit B conjugated to AlexaFluor 488
- cVc1.1: cyclized Vc1.1
- CVH: chronic visceral hypersensitivity
- CVP: chronic visceral pain
- DRG: dorsal root ganglion
- EMG: electromyographic
- GABA$_\text{R}$: γ-aminobutyric acid receptor B
- IBS: Irritable Bowel Syndrome
- TL: Thoracolumbar
- Vc1.1: ‘linear’ Vc1.1
- VGCCs: voltage-gated calcium channels
- Vfh: von Frey hair
- VMR: Visceromotor response
Abstract:

Background and purpose: Patients with Irritable Bowel Syndrome suffer from chronic visceral pain (CVP); however, limited analgesic therapeutic options are currently available. We have shown that α-conotoxin Vc1.1-induced activation of GABA\(_B\) receptors on the peripheral endings of colonic afferents reduces nociceptive signalling from the viscera. However, the analgesic efficacy of more stable, cyclized versions of Vc1.1 on CVP remains to be determined.

Experimental approach: Using ex vivo colonic afferent preparations, we determined the inhibitory actions of cyclized Vc1.1 (cVc1.1) and two cVc1.1 analogues on mouse colonic nociceptors in healthy and chronic visceral hypersensitivity (CVH) states. Using whole-cell patch clamp recordings, we also assessed the inhibitory actions of these peptides on the neuronal excitability of colonic innervating dorsal root ganglion (DRG) neurons. The in vivo analgesic efficacy of these analogues was assessed by determining the visceromotor response to colorectal distension in healthy and CVH mice.

Key results: cVc1.1 and the cVc1.1 analogues [C2H,C8F]cVc1.1, and [N9W]cVc1.1 all caused concentration-dependent inhibition of colonic nociceptors from healthy mice. The inhibitory actions of these peptides were larger than those evoked by linear Vc1.1, and were substantially greater in colonic nociceptors from CVH mice. cVc1.1 also reduced the excitability of colonic DRG neurons, with greater effect in CVH neurons. CVH mice intra-colonically administered cVc1.1 displayed significantly reduced pain responses to noxious colorectal distension compared with vehicle-treated CVH mice.

Conclusions and implications: We show that cyclic versions of Vc1.1 evoke significant anti-nociceptive actions in CVH states, suggesting they are novel candidates for the treatment for CVP.
Introduction:

Irritable bowel syndrome (IBS) is a prevalent, chronic gastrointestinal disorder that negatively impacts the quality of life for ~11% of the global population (Chey et al., 2015; Enck et al., 2016). IBS is characterized by abdominal pain, discomfort, bloating and altered bowel habits (Chey et al., 2015; Enck et al., 2016). Although the pathophysiology of IBS is not completely understood, it is becoming clear that changes to peripheral cellular and sensory mechanisms play key roles in the associated pain (Bellono et al.; Brierley & Linden, 2014; Enck et al., 2016). In particular, chronic visceral hypersensitivity (CVH) of colonic afferents is implicated in the development and maintenance of chronic visceral pain (CVP) in IBS patients (Brierley & Linden, 2014). Characteristic features of CVH include; nociceptor hypersensitivity, increased signalling of noxious colorectal distension (CRD) within the spinal cord, and allodynia and hyperalgesia to colorectal distension (Castro et al., 2017; Castro et al., 2013; de Araujo et al., 2014). Recent evidence suggests sensory afferents display up-regulation of various membrane receptors and ion channels in animal models of CVH (Brierley, 2016), making them targets for analgesic treatment.

The α-conotoxin family of venom-derived peptides from marine cone snails, provide a rich source of novel disulfide-bonded peptides that target a wide variety of membrane receptors and ion channels (Adams & Berecki, 2013; Lewis et al., 2000; Schroeder & Craik, 2012; Vetter & Lewis, 2012). In particular, α-conotoxin Vc1.1, a ‘linear’ 16-amino acid synthetic version of a peptide derived from Conus victoriae, has anti-nociceptive actions in vitro and anti-hyperalgesic actions in numerous in vivo models of neuropathic pain (Clark et al., 2010; Klimis et al., 2011; Satkunanathan et al., 2005). We recently showed that Vc1.1 inhibits sensory afferent pathways within the splanchnic and pelvic innervation of the colon and that these inhibitory actions of Vc1.1 are enhanced in an animal model of CVH (Castro et al., 2017). Notably, we demonstrated that this inhibitory effect occurs via Vc1.1 activation of the γ-aminobutyric acid receptor B (GABA\textsubscript{B}R), which is expressed by colonic afferents, and the subsequent down-stream inhibition of the voltage-gated calcium channels (VGCCs) Ca\textsubscript{V}2.2 (N-type) and Ca\textsubscript{V}2.3 (R-type) (Castro et al., 2017). We also demonstrated that Vc1.1 reduces the excitability of human dorsal root ganglion (DRG) neurons via a GABA\textsubscript{B}R-mediated mechanism (Castro et al., 2017). These studies confirmed recent recombinant cell line studies demonstrating the human GABA\textsubscript{B}R is the primary, and high affinity target for Vc1.1 (Callaghan...
& Adams, 2010; Clark et al., 2010), rather than the originally proposed target, α9α10 nAChR subtype (Vincler et al., 2006; Vincler & McIntosh, 2007; McIntosh et al., 2009). Furthermore, these studies show that GABA<sub>B</sub>R activation by Vc1.1 causes down-stream inhibition of the VGCCs Cav2.2 (N-type) and Cav2.3 (R-type), but not Cav2.1 (P/Q-type) (Adams et al., 2012; Berecki et al., 2014).

These findings are promising for the development of Vc1.1 as a therapeutic treatment for CVP, particularly as Vc1.1 (also called ACV1) has been used in Phase I and Phase IIA clinical trials for the treatment of neuropathic pain (ASX, 2006a; ASX, 2006b; ASX, 2007). In these studies, ACV1 was reported to be safe and well tolerated, with a clean safety and side-effect profile. Despite such promise, peptides as therapeutics can be limited by their susceptibility to degradation within the body. However, the use of synthetic cyclization, whereby the N and C termini are joined by a linker sequence of residues, can stabilize the structure of the peptide and reduce the susceptibility to proteolysis, thus potentially enhancing therapeutic potential (Clark et al., 2010). We have previously shown that cyclization of Vc1.1 improves its stability and is orally active (Clark et al., 2010). Intriguingly, this cyclized Vc1.1 (called cVc1.1) has two additional serendipitous benefits, which are likely a result of its extra rigidity. First, it is a more potent inhibitor of the GABA<sub>B</sub>R-modulated Cav2.2 channel, the proposed target for analgesia, than the native ‘linear’ Vc1.1. Second, it is also more selective for the inhibition of voltage-gated calcium channel currents over the α9α10 nAChR subtype when compared to ‘linear’ Vc1.1 (Clark et al., 2010). Therefore, it has increased potency for GABA<sub>B</sub>R-mediated inhibition of VGCCs and a reduced activity at the α9α10 nAChR compared to linear Vc1.1 (Clark et al., 2010; Yu et al., 2015). Accordingly, cVc1.1 has been used as an orally active peptide, which displays analgesic activity in rat models of neuropathic pain (Clark et al., 2010; Yu et al., 2015). Therefore, in the present study we assessed the anti-nociceptive activity of cVc1.1 and two modified cyclic analogues of Vc1.1 in colonic afferents and colonic DRG neurons from healthy and CVH mice. One of the cyclic analogues of Vc1.1, [C2H,C8F]cVc1.1, was designed to be a simplified version of cVc1.1 in terms of its production, whereas the second, [N9W]cVc1.1, was designed based on increased potency at the α9α10 nAChR. We also compared the most potent cVc1.1 analogue to Vc1.1 using an in vivo model assessing visceral sensitivity in both healthy and CVH mice.
Methods:

Animals:

The Animal Ethics Committees of the South Australian Health and Medical Research Institute (SAHMRI), The University of Adelaide and Flinders University approved experiments involving animals. All experiments performed conformed to the relevant regulatory standards and the ARRIVE guidelines (McGrath & Lilley, 2015). A total of 116 male C57BL/6J mice aged 13-17 weeks of age were used in the reported experiments. We have previously demonstrated that mice provide an appropriate model for investigating chronic visceral hypersensitivity relevant to clinical conditions such as IBS (Brierley & Linden, 2014; Castro et al., 2013). Therefore, we chose mice as the species for the current study. Mice were acquired from an in-house C57BL/6J breeding program (from strain # 000664 originally purchased from Jackson laboratory) within SAHMRI’s specific and opportunistic pathogen free animal care facility. Mice were group housed (5 mice per cage) within individual ventilated cages (IVC), which were filled with aspen wood chip bedding (CA PURA CHIP ASPEN COARSE (Cat# ASPJMAEB; EcoPure, UK). These cages were stored on IVC racks in specific housing rooms within a temperature controlled environment of 22°C and a 12 hr light/ 12 hr dark cycle. Mice were fed at libitum with Jackson lab diet: 5K52 JL RAT & MOUSE/AUTO 6F DIET (Cat# ASSPECIAL) and reverse osmosis purified water. IVC cages contained Jackson lab bedding: CA PURA CHIP ASPEN COARSE (Cat# ASPJMAEB). Mice had an average weight of ~ 29 g on the experimental day.

Mice were randomly assigned to healthy control or Trinitrobenzene Sulphonic Acid (TNBS) treatment groups. Following TNBS administration, mice were individually housed in individual ventilated cages to allow for accurate clinical monitoring until the experimental day in question. Mice were randomly assigned to study sub-groups, whilst the order of treatment was also randomised. Where possible, investigators were blinded to either the drugs being administered or the treatment group during analysis.
Model of chronic visceral hypersensitivity (CVH):

Colitis was induced by administration of TNBS as described previously (Castro et al., 2017; Castro et al., 2013; de Araujo et al., 2014; Hughes et al., 2009b; Hughes et al., 2014; Osteen et al., 2016). Briefly, 13-week-old mice, anaesthetized with isofluorane, were administered an intracolonic enema of 0.1mL TNBS (135 μL/mL of 1M solution in 35% ethanol), via a polyethylene catheter inserted 3 cm from the anus. Mice were then individually housed and monitored up to 3 times daily for clinical assessment for changes in body weight, physical appearance and behaviour. Our previous studies using this model show mucosal architecture, cellular infiltrate, crypt abscesses, and goblet cell depletion confirming TNBS induces significant damage of the colonic mucosa by day 3-post treatment. This damage largely spontaneously 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 (Hughes et al., 2009b). Mice from this model also display 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 (Harrington et al., 2012). This model also induces hyperalgesia and allodynia to colorectal distension (Adam et al., 2006), and is therefore termed ‘Chronic Visceral Hypersensitivity’ (CVH) (Castro et al., 2017; Castro et al., 2013; de Araujo et al., 2014; Hughes et al., 2009b; Hughes et al., 2014; Osteen et al., 2016).

Ex-vivo single fibre colonic splanchnic afferent recording preparation:

Mice were humanely killed, by CO$_2$ inhalation at days 0 (healthy), and 28 (CVH) after TNBS administration. The colon and rectum (5–6 cm) and attached splanchnic nerves were removed and afferent recordings from splanchnic nerves were performed as described previously (Brierley et al., 2004; Brierley et al., 2005b; Hughes et al., 2009b). Briefly, colons were removed, dissected open 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$_2$PO$_4$, 1.2 MgSO$_4$ (H$_2$O)$_7$, 2.5 CaCl$_2$, 11.1 D-glucose), bubbled with carbogen (95% O$_2$, 5% CO$_2$) at a temperature of 34°C. All solutions contained the L-type calcium channel antagonist nifedipine (1 μM) to suppress smooth muscle activity and the prostaglandin synthesis inhibitor indomethacin (3 μM) to block endogenous prostaglandin production. The nerve bundle was extended into a paraffin-filled recording compartment in
which finely dissected strands were laid onto a mirror, and single fibres placed on the platinum recording electrode. Action potentials, generated by mechanical stimuli to the colon’s receptive field, were recorded by a differential amplifier, filtered and sampled (20 kHz) using a 1401 interface (Cambridge Electronic Design, Cambridge, UK) and stored on a PC for off-line analysis.

**Colonic afferent classification and selection:**
Receptive fields were identified by systematically stroking the mucosal surface of the colon with a stiff brush to activate all subtypes of mechanoreceptors. Categorization of afferents properties was in accordance with our previously published classification system (Brierley et al., 2004; Brierley et al., 2005b). Once identified, receptive fields were tested with three distinct mechanical stimuli to enable classification: static probing with calibrated von Frey hairs (vfh) (2 g force; applied 3 times for a period of 3 sec), mucosal stroking with calibrated vfh (10 mg force; applied 10 times) or circular stretch (5 g; applied for a period of 1 min). We tested the effect of cVc1.1 and the cVc1.1 analogues [C2H,C8F]cVc1.1, and [N9W]cVc1.1 on serosal afferents, also termed vascular afferents (Brookes et al., 2013), recorded from the splanchnic pathway. These colonic afferents have high-mechanical activation thresholds and respond to noxious distension (40 mmHg), stretch (≥7 g) or vfh filaments (2 g) but not to fine mucosal stroking (10 mg vfh) (Brierley et al., 2004; de Araujo et al., 2014; Hughes et al., 2013; Osteen et al., 2016). The algesic ion channels and receptors, NaV1.1 (Osteen et al., 2016), TRPV1 (Brierley et al., 2005b), TRPA1 (Brierley et al., 2011; Hughes et al., 2009a), TRPV4 (Brierley et al., 2008), P2X3 (Brierley et al., 2005b), bradykinin B1 (Brierley et al., 2005a), NaV1.8 (Beyak, 2010), and TNFR1 (Hughes et al., 2013) are highly expressed in these afferents. In addition, serosal afferents become mechanically hypersensitive in models of chronic visceral pain (Hughes et al., 2009b) and have a nociceptor phenotype (Brierley et al., 2004; Carstens et al., 2016; Castro et al., 2017; Castro et al., 2013; de Araujo et al., 2014; Osteen et al., 2016). In the present study, they are therefore referred to as colonic ‘nociceptors’.

**Peptide application to colonic afferents:**
The peptides cVc1.1, [C2H,C8F]cVc1.1, and [N9W]cVc1.1 were prepared from stock solutions, diluted to appropriate final concentrations (1, 10, 100 or 1000 nM) in Krebs solution. These concentrations of peptide were applied sequentially to the same individual.
afferent receptive field. There was no washout duration *per se* with the next drug added within ~30 seconds of completing mechanical testing following the previous drug addition. This process involved determining baseline splanchnic colonic nociceptor mechanosensitivity in response to application of 3x 3 sec 2g vfh probes to the afferent receptive field. A small chamber was then applied to the mucosal surface of the colon, which surrounded the afferent receptive field. Residual Krebs solution within the chamber was aspirated and 1 nM of the respective peptide applied for 5 minutes. Mechanical sensitivity was then re-tested in response to application of 3x 3 sec 2g vfh probes to the afferent receptive field. This process was then repeated for 10, 100 and 1000 nM of the respective peptide and mechanical sensitivity re-tested after each concentration (Carstens et al., 2016; Castro et al., 2017; Castro et al., 2013; de Araujo et al., 2014; Osteen et al., 2016)

**Statistical analysis of afferent recording data:**

Action potentials were analyzed off-line using the Spike 2 (version 5.21) software (Cambridge Electronic Design, Cambridge, UK) and discriminated as single units based on distinguishable waveforms, amplitudes and durations. Data are expressed as mean ± SEM. *n* = the number of afferents recorded. *N* = the number of animals used for those specific experiments. 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 peptide addition. This difference is then averaged across all afferents with a cohort to obtain a final mean ± SEM of “change in response from baseline”. Data were statistically compared using Prism 7 software (GraphPad Software, San Diego, CA, USA), and where appropriate, were analyzed using a one or two-way analysis of variance (ANOVA) with Bonferroni post hoc tests. Differences were considered significant and at a level of *P* < 0.05, and as per the British Journal of Pharmacology guidelines, only reported as *P* < 0.05, with no presentation of multiple levels of significance.

**Retrograde tracing to identify colonic thoracolumbar (TL) DRG neurons:**

Cholera toxin subunit B conjugated to AlexaFluor 488 (CTB-488); Invitrogen, Carlsbad, CA) was injected at three sites sub-serosally within the wall of the distal colon of healthy control or CVH mice (Brierley et al., 2009; Castro et al., 2017; de Araujo et al., 2014;
Harrington et al., 2012). After 4 days, animals were humanely killed by CO$_2$ inhalation for subsequent TL (T10-L1) DRG removal and dissociation.

**Cell culture of colonic DRG neurons:**

DRGs (thoracic 9 to lumbar 1) were digested with 4 mg ml$^{-1}$ collagenase II (GIBCO, Invitrogen) and 4 mg ml$^{-1}$ dispase (GIBCO) for 30 min at 37°C, followed by 4 mg ml$^{-1}$ collagenase II for 10 min at 37°C. A single-cell suspension was achieved via trituration of DRG’s through fire-polished Pasteur pipettes of descending diameter. Neurons were resuspended in DMEM (GIBCO) containing 10% FCS (Invitrogen), 2 mM L-glutamine (GIBCO), 100 μM MEM non-essential amino acids (GIBCO) and 100 mg ml$^{-1}$ penicillin/streptomycin (Invitrogen). Neurons were spot-plated on clean 13 mm coverslips cut in half and coated with laminin (20 μg ml$^{-1}$) and poly-D-lysine (800 μg ml$^{-1}$) and maintained in an incubator at 37°C in 5% CO$_2$.

**Patch clamp recordings of colonic DRG neurons:**

Whole-cell patch clamp recordings were made from fluorescently labelled colonic thoracolumbar DRG neurons (from N=5 mice/group) 24 - 48h after plating, using fire-polished glass electrodes with a resistance of 2–5 MΩ. Inclusion criteria for cells included: 1) being retrogradely traced, 2) having small diameter (maximum soma diameter <20 μm), 3) having a resting membrane potential more negative than $-40$ mV, 4) a series resistance of $<10$ MΩ, and 5) a capacitance of $\leq$30 pF. Resting membrane potential was $-49.19 \pm 1.0$ mV for healthy colonic innervating DRG neurons and $-47.64 \pm 1.3$ mV for CVH colonic innervating DRG neurons. For all colonic DRG neurons, the membrane potential was held at $-70$ mV. In current clamp mode, a series of depolarizing pulses (10 pA current step, 500 ms 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 Vc1.1 (10 nM) or cVc1.1 (10 nM). Control solutions and peptides were applied with a gravity driven multi-barrel perfusion system positioned within 1 mm of the neuron under investigation, as used previously (Brierley et al., 2011). This involved an initial baseline rheobase recording being made during continuous perfusion of normal external bath solution. This was followed by a 2 min continuous perfusion with either Vc1.1 (10 nM) or cVc1.1 (10 nM) diluted in external bath solution, after which a second recording was made to
determine rheobase in the presence of the individual peptides. Pipettes were filled with intracellular solutions contained (in mM): 135 KCl; 2 MgCl\(_2\); 2 MgATP; 5 EGTA-Na; 10 HEPES-Na; adjusted to pH 7.4. Extracellular bath solutions contained (in mM): 140 NaCl; 4 KCl; 2 MgCl\(_2\); 2 CaCl\(_2\); 10 HEPES-Na; 5 glucose; adjusted to pH 7.4.

**Statistical analysis of patch clamp data:**

All data were analyzed using Prism 7 software (GraphPad Software, San Diego, CA, USA) using paired t-tests, or one-way ANOVAs, to compare before and after effects of Vc1.1 or cVc1.1 on rheobase. Data are expressed as mean ± SEM. A neuron inhibited by Vc1.1 or cVc1.1 was defined as exhibiting a ≥10% change in rheobase from baseline control, as described previously (Castro et al., 2017; Osteen et al., 2016). An un-paired t-test was used to determine differences in the rheobase of colonic DRG neurons from healthy and CVH mice. Differences between specific drug and baseline responses were considered significant at a level of * P < 0.05. n = the number of colonic innervating DRG neurons recorded. N = the number of animals used for those specific experiments.

**Visceromotor responses (VMR) to colorectal distension:**

Noxious distension of the colorectum triggers the VMR, a nociceptive brainstem reflex consisting of the contraction of the abdominal muscles (Ness & Gebhart, 1988). Using abdominal electromyography (EMG), this technique allows assessment of visceral sensitivity in vivo in fully awake animals (Christianson & Gebhart, 2007; Deiteren et al., 2014). Under isoflurane anaesthesia, the bare endings of two Teflon-coated stainless steel wires (Advent Research Materials Ltd, Oxford, UK) were sutured into the right abdominal muscle and tunneled subcutaneously to be exteriorized at the base of the neck for future access. At the end of the surgery, mice received prophylactic antibiotic (Baytril®; 5mg/kg s.c.) and analgesic (buprenorphine; 0.4 mg/10 kg s.c.), were housed individually and allowed to recover for at least three days before assessment of VMR. On the day of VMR assessment, mice were briefly anaesthetized using isoflurane and received a 100 µl enema of 1 µM Vc1.1, or 1 µM cVc1.1 or vehicle (sterile H\(_2\)O). A lubricated balloon (2.5 cm length) was gently introduced through the anus and inserted into the colorectum up to 0.25 cm past the anal verge. The balloon catheter was secured to the base of the tail and connected to a barostat (Isobar 3, G&J Electronics, Willowdale, Canada) for graded and pressure-controlled balloon distension.
Mice were allowed to recover from anaesthesia in a restrainer with dorsal access for 15 minutes prior to initiation of the distension sequence. Distensions were applied at 20-40-60-80 mmHg (20 s duration) at a 4 min-interval so that the last distension was performed 30 min after intra-colonic treatment. Following the final distension, mice were humanely killed by cervical dislocation. The 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 digitized (CED 1401, Cambridge Electronic Design, Cambridge, UK) to a PC for off-line analysis using Spike2 (Cambridge Electronic Design). The analog EMG signal was rectified and integrated. To quantify the magnitude of the VMR at each distension pressure, the area under the curve (AUC) during the distension (20 s) was corrected for the baseline activity (AUC pre-distension, 20 s).

**Colonic compliance:**

Colonic compliance was assessed by applying graded volumes (40-200 µL, 20 s duration) to the balloon in the colorectum of fully awake mice, while recording the corresponding colorectal pressure as described previously (Deiteren et al., 2014).

**Statistical analysis of VMR data:**

Data are present as mean ± SEM, where N represents the number of animals. Data were statistically analyzed by generalized estimating equations followed by LSD post hoc test when appropriate using SPSS 23.0. Analysis and figures were prepared in GraphPad Prism 7 Software, San Diego, CA, USA).

**Design of cVc1.1 and cVc1.1 analogue variants:**

Vc1.1 was synthesized via solid-phase peptide chemistry as described previously (Clark et al., 2006). Cyclic Vc1.1 (cVc1.1) was engineered by joining the N- and C-termini of the peptide without affecting the three-dimensional structure or biological activity (Clark et al 2010). cVc1.1 was designed because a major obstacle generally impeding the use of bioactive peptides as drugs is their susceptibility to enzymatic degradation and lack of oral bioavailability. The cyclic variant proved to be stable and orally active in the rat chronic constriction injury model of neuropathic pain (Clark et al 2010).
Two mutants of the cyclic peptide were also studied (Figure 1). [N9W]cVc1.1 was designed based on the increased potency at α9α10 nAChR of ‘linear’ Vc1.1 resulting from the substitution N9W (Yu et al., 2013). This variant was instrumental in determining that Vc1.1 preferentially blocks the α10(+)/α9(-) orthosteric binding site (Yu et al., 2013). [N9W]cVc1.1 is 30-fold more active than the parent peptide at the human α9α10 nAChR, with an IC₅₀ of 33 nM compared with the rat α9α10 nAChR of 975 nM. A one-disulfide variant of cVc1.1, namely [C2H,C8F]cVc1.1, was also designed to reduce the possibility of disulfide bond shuffling, which can limit the bioavailability of disulfide-rich peptides (Yu et al., 2015). The disulfide bond between the first and third Cys was replaced by two hydrophobic residues, which we showed by NMR spectroscopy analysis to form a small hydrophobic core to the peptide. The cost of this simplifying mutation is that the inhibitory activity of [C2H,C8F]cVc1.1 falls by three-fold compared to the native peptide at the human α9α10 (IC₅₀ of 13 μM) and at voltage-gated calcium channels via GABA₆ activation (IC₅₀ of 900 pM) (Yu et al., 2015).

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/2018 (Alexander et al., 2015a; Alexander et al., 2015b; Alexander et al., 2015c).

Results:

Cyclized Vc1.1 (cVc1.1) inhibits colonic nociceptor mechanosensitivity

We have previously shown that Vc1.1 inhibits colonic nociceptors from both healthy and CVH mice. However, we observed that Vc1.1 evoked a larger inhibitory effect in colonic afferents from a mouse model of CVH (Castro et al., 2017). Given that cVc1.1 has improved stability, increased potency and efficacy for GABA₆R-mediated inhibition of VGCC currents than Vc1.1 (Clark et al., 2010; Yu et al., 2015), we hypothesized that cVc1.1 should evoke greater inhibition of colonic nociceptors. To test this hypothesis, we performed ex vivo single fibre afferent recordings of colonic nociceptors from both healthy and CVH mice (Castro et al., 2017; Castro et al., 2013; de Araujo et al., 2014; Osteen et al., 2016). We assessed colonic nociceptor
mechanosensitivity before and after increasing doses of cVc1.1 (1 nM, 10 nM, 100 nM, 1 μM) and observed that cVc1.1 concentration-dependently inhibited colonic nociceptor mechanosensitivity from both healthy (IC₅₀: 12.2 nM, Figure 2A) and CVH (IC₅₀: 3.5 nM, Figure 2B) mice. Notably, at each of the cVc1.1 concentrations tested, CVH colonic nociceptors displayed lower mechanosensory responses than in healthy nociceptors, with greatest inhibition observed at a concentration of 1 μM (Figure 2C, 2D, 2E). A direct comparison of the inhibitory actions evoked by ‘linear’ Vc1.1 and cyclic cVc1.1 demonstrated, at each concentration tested, that cVc1.1 caused greater inhibition of colonic nociceptors than Vc1.1 in colonic nociceptors from both healthy (IC₅₀’s Vc1.1: 23.4 nM vs. cVc1.1: 12.2 nM, Figure 2F) and CVH (IC₅₀’s Vc1.1: 10.2 nM vs. cVc1.1: 3.5 nM, Figure 2G) mice. Overall, these data show that cVc1.1 causes potent inhibition of colonic nociceptors, particularly those from CVH mice.

**cVc1.1 inhibits colonic DRG neurons with greater efficacy in CVH mice.**

We have previously shown that Vc1.1 reduces the excitability of a sub-population of human DRG neurons (Castro et al., 2017). We therefore wanted to determine if both Vc1.1 and cVc1.1 inhibited colonic DRG neurons from healthy mice and determine if their inhibitory actions were enhanced in CVH mice. To assess the excitability of colonic DRG neurons, we performed whole-cell patch clamp recordings in current clamp mode. Accordingly, we determined the effects Vc1.1 and cVc1.1 had on the rheobase, the amount of injected current required to fire an action potential, in colonic DRG neurons from healthy and CVH mice. ‘Linear’ Vc1.1 (10 nM) had a modest, but significant effect on reducing the neuronal excitability of large population of colonic DRG neurons from healthy mice (8.5 ± 1.8 % inhibition relative to baseline, Figure 3Ai, Aii, Aiii). Furthermore, cVc1.1 (10 nM) also caused inhibition of a subpopulation of colon-innervating DRG neurons from healthy mice (22.8 ± 5.1 % inhibition relative to baseline Figure 3Bi, Bii, Biii), an effect which was greater than that observed with Vc1.1 (Figure 3Aiii).

Colonic DRG neurons from CVH mice displayed pronounced hyper-excitability compared with their healthy control counterparts, as reflected in their rheobase (Healthy: 222.8 ± 20.29 pA; n=32 neurons, vs. CVH: 145.8 ± 8.49 pA; n=33 neurons, *P<0.05, unpaired t-test; Figure 3A, 3B, 3C, 3D). Vc1.1 caused significant inhibition of a subpopulation of colon-
innervating DRG neurons from CVH (10.5 ± 1.6% inhibition relative to CVH baseline, Figure 3Ci, 3Cii, 3Ciii). Furthermore, cVc1.1 also caused significant inhibition of neuroexcitability in a large sub-population of CVH colonic DRG neurons (26.7 ± 6.8% inhibition relative to CVH baseline, Figure 3Dii, 3Diii, 3Diii). These data indicated that cVc1.1 has increased efficacy in reducing neuronal excitability compared to Vc1.1 at the same concentration in CVH mice. Overall, these findings are consistent with our ex vivo afferent recording studies, which demonstrate that cVc1.1 causes greater inhibition of colonic nociceptors from CVH mice.

Effects of cVc1.1 analogues on colonic nociceptor mechanosensitivity.

Given that cVc1.1 has potent inhibitory effects on colonic nociceptors, we investigated if several analogues of cVc1.1, which have modifications to improve synthesis and production, also inhibit colonic nociceptors. The cVc1.1 analogue, [C2H,C8F]cVc1.1 has a similar three-dimensional structure and activity to Vc1.1. However, since it has only one possible disulfide isomer, the cost of peptide synthesis and purification is reduced compared to the parent peptide (Yu et al., 2015). Application of [C2H,C8F]cVc1.1 caused inhibition of healthy colonic nociceptors (IC₅₀: 8.6 nM, Figure 4A), and inhibition in CVH colonic nociceptors (IC₅₀: 7.0 nM, Figure 4B, 4C, 4D, 4E). The other cyclic mutant, [N9W]cVc1.1 also inhibited colonic nociceptors from both healthy (IC₅₀: 6.3 nM, Figure 5A) and CVH mice (IC₅₀: 23.1 nM, Figure 5B, 5C, 5D, 5E).

A direct comparison of anti-nociceptive efficacy of the analogues shows that ‘linear’ Vc1.1 caused the least amount of inhibition in both healthy (Figure 6A) and CVH (Figure 6B) states. Conversely, cVc1.1 evoked the greatest degree of inhibition in healthy and CVH colonic nociceptors (Figure 6A, 6B).

Intra-colonic administration of Vc1.1 or cVc1.1 inhibits in vivo colonic pain responses in mice with CVH.

To translate our ex vivo and in vitro findings to an in vivo model, we recorded the VMR, a nociceptive brainstem reflex consisting of the contraction of the abdominal muscles, in response to CRD (Ness & Gebhart, 1988). By using abdominal EMG, we were able to assess the effect of intra-colonically administered Vc1.1 or cVc1.1 on visceral sensitivity in vivo in fully awake healthy or CVH mice (Christianson & Gebhart, 2007; Deiteren et al., 2014) (Carstens et al., 2016). We chose Vc1.1 and cVc1.1 as they had the least and highest anti-
Nociceptive actions in our colonic nociceptor recordings, respectively. Intra-colonic administration of linear Vc1.1 (1 μM) had no significant effect on the VMR of healthy mice compared with the VMRs of vehicle treated mice (Figure 7A). In contrast, linear Vc1.1 significantly reduced the VMR of CVH mice to CRD, particularly at noxious distension pressures of 40 mmHg and 60 mmHg (Figure 7B). Intra-colonic administration of cVc1.1 did not affect VMRs in healthy mice (Figure 7C, 7E). However, cVc1.1 did significantly reduce enhanced VMRs to CRD in CVH mice at noxious distension pressures of 40, 60 and 80 mmHg (Figure 7D, 7E). Notably, colonic compliance was not affected by intra-colonic administration of cVc1.1 in either healthy or CVH mice (Figure 7F), suggesting that the anti-nociceptive actions of cVc1.1 occur via inhibition of colonic nociceptors, rather than relaxation of the colonic smooth muscle.

Discussion:

This study provides evidence that cyclized analogues of the α-conotoxin Vc1.1 inhibit colonic nociception and that these inhibitory effects are enhanced in a mouse model of CVH. Notably, the cyclized Vc1.1 analogues, cVc1.1 and [C2H,C8F]cVc1.1, evoked greater anti-nociceptive effects than Vc1.1, the ‘linear’ native version of the peptide. We also demonstrate that peripheral administration of the cVc1.1 inhibits visceral pain in a mouse model of CVH. These findings highlight the potential therapeutic value of cyclized versions of Vc1.1 in the treatment of CVP.

Comparing the anti-nociceptive and analgesic effects of Vc1.1 relative to cVc1.1.

We recently showed that ‘linear’ Vc1.1 inhibits mouse colonic nociceptors and low-threshold distension-sensitive colonic afferents (Castro et al., 2017). Although Vc1.1 can act upon both the GABABR and α9α10 nAChR with IC50’s in the nM range (Adams et al., 2012; Mohammadi & Christie, 2015), we have demonstrated that ‘linear’ Vc1.1 evokes inhibition of colonic afferents via activation of the GABABR, as the inhibitory effects of Vc1.1 on colonic afferents were blocked by a selective GABABR antagonist. Conversely, the inhibitory actions of Vc1.1 can be mimicked by the GABABR agonist baclofen, and recapitulated by using inhibitors of the VGCCs CaV2.2 and CaV2.3, which are known downstream targets of Vc1.1-
induced GABA<sub>B</sub>R activation (Berecki et al., 2014; Castro et al., 2017). Importantly for translation to humans, ‘linear’ Vc1.1 also reduces human DRG neuroexcitability, via a GABA<sub>B</sub>R-mediated mechanism. Correspondingly, both mouse colonic DRG neurons and human DRG neurons co-express the GABA<sub>B</sub>R subunits R1 and R2, plus Ca<sub>V</sub>2.2 and/or Ca<sub>V</sub>2.3, which combined are the required molecular components for Vc1.1-induced inhibition (Castro et al., 2017). This anti-nociceptive action of Vc1.1 also translates in vivo, as intra-colonic administration of Vc1.1 to mice inhibits the signalling of noxious information from the colon into the spinal cord (Castro et al., 2017).

In the present study, we took these observations further and showed that both ‘linear’ Vc1.1 and cyclized cVc1.1 cause a greater reduction in the excitability of colonic DRG neurons from mice with CVH. Moreover, both Vc1.1 and cVc1.1 administration reduced colonic pain in CVH mice. One possible explanation for the increased efficacy of both Vc1.1 and cVc1.1 during CVH is our observation that colonic DRG neurons from CVH mice display up-regulation of Ca<sub>V</sub>2.2 exon-37a (Castro et al., 2017), which is a known nociceptive variant of Ca<sub>V</sub>2.2 (Altier et al., 2007). In this scenario, the increase in Ca<sub>V</sub>2.2 exon-37a expression may contribute, in part, to the hyper-excitability of colonic DRG neurons from CVH mice. Correspondingly, we have observed that the selective Ca<sub>V</sub>2.2 inhibitor, α-conotoxin CVID, causes greater inhibition of colonic nociceptors from CVH mice, relative to healthy mice (Castro et al., 2017). Consequently, Vc1.1 or cVc1.1 activation of GABA<sub>B</sub>R would result in a greater net inhibition of VGCC currents in CVH neurons relative to healthy colonic DRG neurons.

What is becoming apparent from our work and those of others is that the downstream effector channels of GABA<sub>B</sub>R appear to be linked to the agonist, the manner in which GABA<sub>B</sub>R is activated and the ion channels expressed by the cell in question. Recent studies have shown that baclofen-induced activation of GABA<sub>B</sub>R results in the downstream blockade of Ca<sub>V</sub>2.1, Ca<sub>V</sub>2.2 and Ca<sub>V</sub>2.3. However, Vc1.1 induced activation of GABA<sub>B</sub>R results in the downstream inhibition of Ca<sub>V</sub>2.2 and Ca<sub>V</sub>2.3, but not Ca<sub>V</sub>2.1 (Berecki et al., 2014). Furthermore, whilst baclofen-induced activation of GABA<sub>B</sub>R may couple to GIRK (Kir3) channels in some cell types (Takeda et al., 2015), previous studies have shown that neither Vc1.1, nor a related α-conopeptide Rg1A, target GIRK channels via GABA<sub>B</sub>R (Huynh et al.,
These findings suggest that activation of GABA<sub>B</sub>R by Vc1.1 and cVc1.1 results in a more biased, less promiscuous set of downstream inhibitory targets than baclofen. The reason for these actions are not clear at present, but may involve GPCR biased signaling and recruitment of selective second messenger systems.

The increased efficacy of cVc1.1 relative to ‘linear’ Vc1.1 is probably a result of the improved stability of cVc1.1, and increased affinity of the peptide for the GABA<sub>B</sub>R, which results in greater inhibition of VGCC currents compared with ‘linear’ Vc1.1 (Clark et al., 2010; Yu et al., 2015). This interpretation is consistent with studies using Vc1.1 and cVc1.1 in rat models of neuropathic pain. Although Vc1.1 has clear anti-nociceptive actions in vitro and anti-hyperalgesic actions in vivo (Clark et al., 2010; Klimis et al., 2011; Satkunanathan et al., 2005), it is not orally active. By contrast, cVc1.1 is orally active and displays significant analgesic activity in rat chronic constriction injury models of neuropathic pain (Clark et al., 2010; Yu et al., 2015). Accordingly, our collective findings indicate that cVc1.1 has greater analgesic properties for both visceral pain and neuropathic pain than linear Vc1.1.

**Cyclic analogues of cVc1.1.**

Although cVc1.1 has clear anti-nociceptive and analgesic properties, potentially it can be improved further. One approach was to focus on the disulfide bond network. Because there are four cysteine residues in the peptide primary sequence of cVc1.1, it can form three disulfide bond isomers, with only one of them being active (Yu et al., 2015). The formation of multiple isomers can complicate synthesis procedures and significantly increase the cost of peptide production, especially on the scale of therapeutic production. However, recent studies have shown for other disulfide-rich conotoxins that only certain disulfide bonds are crucial for activity and stability of the peptide (Barnham et al., 1998; Carrega et al., 2005; Flinn et al., 1999; Khoo et al., 2009; Pennington et al., 1999; Yu et al., 2015). Therefore, we used in silico modelling to design disulphide-deleted variants and found that removing one disulfide bond of cVc1.1 to produce [C2H,C8F]cVc1.1, leads to a well-folded peptide (Yu et al., 2015). This peptide has a similar three-dimensional structure and activity to Vc1.1, but has a larger hydrophobic core than cVc1.1 and, potentially, additional surface salt bridge interactions. The advantage of [C2H,C8F]cVc1.1 is that it has only one possible disulfide isomer, reducing the cost of peptide synthesis and purification compared to the parent peptide (Yu et al., 2015). Specifically, cVc1.1 folds into two isomers in a 72:28 ratio (Clark et
al., 2010), whereas [C2H,C8F]cVc1.1 forms only one isomer, therefore gaining an immediate improvement of 28% in folding yield (Yu et al., 2015). Here, we found that [C2H,C8F]cVc1.1 inhibited the mechanosensitivity of colonic nociceptors from healthy mice, and that this inhibitory effect was greater in CVH colonic nociceptors. In both cases, the inhibitory effect of [C2H,C8F]cVc1.1 was greater than that observed with Vc1.1, but less than that of cVc1.1. These observations are consistent with their respective IC$_{50}$ values determined for inhibition of rat DRG neuron Ca$_V$2.2 channels, and human Ca$_V$2.3 channels (Berecki et al., 2014; Callaghan et al., 2008; Clark et al., 2010; Yu et al., 2015).

Another mutant of the cyclic peptide, [N9W]cVc1.1, designed in an earlier study to preferentially target human over rat nAChRs, but also act via GABA$_B$R, inhibited colonic nociceptors from both healthy and CVH mice. However, [N9W]cVc1.1 evoked less inhibition than cVc1.1, which has a greater efficacy on GABA$_B$R, and a reduced affinity at nAChRs. Taken together, these data again suggest that Vc1.1 inhibits colonic nociception via a GABA$_R$-mediated mechanism, rather than a nAChR-mechanism, as we have demonstrated previously (Castro et al., 2017).

The lack of in vivo analgesic effect observed in healthy mice with intra-colonic administration of either ‘linear’ Vc1.1 or cVc1.1 contrasts with our previous findings with a truncated form of Vc1.1, an 8-amino acid peptide called Vc1.1(1-8). In these earlier studies, we found that the same intra-colonic delivery of this shorter peptide caused significant reductions in colonic pain response in healthy animals (Carstens et al., 2016). The reasons for these differences may relate to the size of the peptide and the time it takes to reach efficacious concentrations at the afferent ending of the nociceptor within the colonic wall. In CVH mice, we did observe analgesic actions of both linear Vc1.1 and cVc1.1 following intra-colonic administration. This key difference between the healthy and CVH states may relate to increased permeability of the colonic epithelium in this post-inflammatory CVH model. Such increases in mucosal permeability have also been reported in patients with IBS (Brierley & Linden, 2014; Enck et al., 2016). Notably, the inhibitory effect of Vc1.1 at higher distension pressures (80 mmHg) in CVH mice was more variable than that observed with cVc1.1. As these recordings were performed at the furthest time point after administration, this
variability may relate to reduced stability of Vc1.1 relative to cVc1.1, which increases the rationale for designing and developing cyclized analogues of Vc1.1.

In conclusion, our findings demonstrate an anti-nociceptive action for cVc1.1 and cVc1.1 analogues in colonic nociceptors. This anti-nociceptive action is greater in a model of CVH than that observed in healthy mice. The use of cyclized Vc1.1 analogues increases both the anti-nociceptive and analgesic effects relative to ‘linear’ Vc1.1. Because altered visceral sensory function is a hallmark of IBS, cVc1.1 represents a potential novel therapy to reduce nociceptive stimuli from the colon and rectum to the CNS. These findings highlight the potential therapeutic value of cyclized Vc1.1 analogues in the treatment of CVP.

**Author contributions:**
S.M.B, D.J.A and D.J.C conceived the study. S.M.B, D.J.A, J.C, L.G, A.D, A.M.H, T.O.D, J.M, SG-C, G.Y.R, and J.M designed, conducted and analyzed experiments. D.J.C, Q.K. and R.Y. synthesized Vc1.1, cVc1.1 and the associated cVc1.1 analogues and assisted with critical revision of the manuscript for important intellectual content. S.M.B wrote the paper and all authors contributed to revising the manuscript.

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**Competing Financial Interests and Conflict of Interests:**
The authors have no competing interests.
References:


McGrath JC, & Lilley E (2015). Implementing guidelines on reporting research using animals (ARRIVE etc.): new requirements for publication in BJP. Brit J Pharmacol. 172 3189–3193


Yu R, Kompella SN, Adams DJ, Craik DJ, & Kaas Q (2013). Determination of the \(\alpha\)-conotoxin Vc1.1 binding site on the \(\alpha_9\alpha_{10}\) nicotinic acetylcholine receptor. J Med Chem. 56: 3557-3567.

cVc1.1 is an engineered peptide in which a cyclizing linker (blue) was added to confer stability and oral activity to the analgesic peptide Vc1.1. This peptide comprises two disulfide bonds, which are shown in red. The conserved positions of the cVc1.1 variants are shown using lighter colour fonts to highlight the substituted positions.
Figure 2: Cyclized Vc1.1 (cVc1.1) inhibits colonic nociceptors from healthy and chronic visceral hypersensitivity (CVH) mice.

(A) Healthy colonic nociceptor mechanosensitivity is significantly reduced following increasing concentrations of cVc1.1, applied to the mucosal epithelium, for 5 minutes at each concentration. cVc1.1 at 100 nM and 1 μM significantly reduced colonic nociceptor mechanosensitivity compared with baseline responses (* P<0.05 one-way ANOVA, n=8 afferents from N=5 healthy mice, * P<0.05 Bonferroni-posthoc).
(B) In a model of CVH, colonic nociceptors display increased mechanosensitivity at baseline, but are potently and concentration-dependently inhibited by cVc1.1. Concentrations of cVc1.1 at 10 nM, 100 nM, and 1 μM significantly reduced the mechanical response of colonic nociceptors compared with baseline responses (* P<0.05 one-way ANOVA, n=10 afferents from N=5 CVH mice, * P<0.05 Bonferroni-posthoc).

(C) Change in mechanosensitivity induced by cVc1.1 in healthy and CVH nociceptors compared to their respective baseline responses. cVc1.1 caused more inhibition, at every concentration assessed, in CVH nociceptors compared with healthy nociceptors (* P<0.05 two-way ANOVA, n=8 afferents from N=5 healthy mice, n=10 CVH from N=5 CVH mice).

(D) Original recordings of a colonic nociceptor from a healthy control mouse, showing action potential firing in response to a 2 g von Frey hair (vfh) probe at baseline and in the presence of cVc1.1 (1 μM).

(E) Original recordings of a colonic nociceptor from a CVH mouse, showing action potential firing in response to a 2 g vfh probe at baseline and reduced action potential firing in the presence of cVc1.1 (1 μM).

(F) Comparative inhibition of healthy colonic nociceptors by cVc1.1 vs. Vc1.1 expressed as change in nociceptor mechanosensitivity respect to baseline response. Overall, cVc1.1 (n=8 afferents, N=5 healthy mice) caused greater inhibition of healthy colonic nociceptors relative to Vc1.1 (n=10 afferents, N=5 healthy mice). (* P<0.05 two-way ANOVA, Bonferroni-posthoc: * P<0.05: 10 nM, * P<0.05: 100 nM, * P<0.05: 1 μM). Vc1.1 data are re-analyzed from (Castro et al., 2017). Data for cVc1.1’s effects on healthy nociceptors are also shown in Panel C.

(G) Comparative inhibition of CVH colonic nociceptors by cVc1.1 vs. Vc1.1 expressed as percentage inhibition from baseline response. Overall, cVc1.1 (n=10 afferents, N=5 mice) caused greater inhibition of CVH colonic nociceptors relative to Vc1.1 (n=10 afferents, N=5 mice). (* P<0.05 two-way ANOVA, Bonferroni-posthoc: * P<0.05: 10 nM, * P<0.05: 1 μM). Vc1.1 data are re-analyzed from (Castro et al., 2017). Data for cVc1.1’s effects on CVH nociceptors are also shown in Panel C.
Figure 3: α-Conotoxins Vc1.1 and cVc1.1 inhibit excitability in mouse colon-innervating dorsal root ganglion (DRG) neurons from healthy and CVH mice.

(A) (i) Vc1.1 (10 nM) caused a modest, but significant inhibition of a subpopulation (8 of 13) colonic DRG neurons from healthy mice, as determined by an increase in rheobase. (* P<0.05, n=8 neurons from N=5 healthy mice, paired t-test). (ii) Vc1.1 (10 nM) did not effect a small subpopulation of colon-innervating DRG neurons (5 of 13 neurons tested). (iii) Current
clamp recordings of colon-innervating DRG neurons from healthy mice in the absence and presence of Vc1.1 (10 nM). Representative whole-cell current clamp recording of a retrogradely traced colon-innervating DRG neuron in response to 500 ms 10pA step current injection at rheobase.

(B) (i) cVc1.1 (10 nM) significantly increases the rheobase of a subpopulation of healthy colonic DRG neurons indicating that cVc1.1 also inhibits neuroexcitability (* P<0.05, n=10 neurons of 19 tested from N=5 healthy mice, paired t-test). (ii) cVc1.1 (10 nM) did not effect a subpopulation of colon-innervating DRG neurons (9 of 19 neurons tested) from healthy mice. (iii) Current clamp recordings of colon-innervating DRG neurons from healthy mice in the presence and absence of cVc1.1 (10 nM).

(C) (i) In colon-innervating DRG neurons from CVH mice, Vc1.1 caused a significant increase in rheobase, indicative of a reduction in neuroexcitability (* P<0.05, n=9 of 15 neurons tested from N=5 CVH mice, paired t-test). (ii) Vc1.1 (10 nM) did not effect a small subpopulation of colon-innervating DRG neurons (6 of 15 neurons tested) from CVH mice. (iii) Current clamp recordings of colon-innervating DRG neurons from CVH mice in the absence and presence of Vc1.1 (10 nM).

D) (i) cVc1.1 caused a significant increase in the rheobase of a subpopulation of CVH colonic DRG neurons (15 of 18 neurons tested from N=5 CVH mice), indicative of a reduction in neuroexcitability (* P<0.05, paired t-test). (ii) cVc1.1 (10 nM) did not effect a subpopulation of colon-innervating DRG neurons (3 of 18 neurons tested) from CVH mice. (iii) Current clamp recordings of colon-innervating DRG neurons from CVH mice in the presence and absence of cVc1.1 (10 nM).
Figure 4: cVc1.1 analogue, [C2H,C8F]cVc1.1, inhibits the mechanosensitivity of colonic nociceptors from healthy and CVH mice.

(A) The mechanosensitivity of colonic nociceptors from healthy mice is reduced following increasing concentrations of [C2H,C8F]cVc1.1 applied to the mucosal epithelium, for 5 minutes at each concentration. Significant reductions in healthy colonic nociceptor mechanosensitivity relative to baseline responses were observed at 10 nM, 100 nM and 1 μM (* P<0.05, n=8 afferents from N=5 healthy mice, one-way ANOVA, Bonferroni-posthoc).

(B) [C2H,C8F]cVc1.1 potently and concentration-dependently inhibited CVH nociceptors at 10 nM, 100 nM and 1 μM (* P<0.05, n=9 afferents from N=5 CVH mice, one-way ANOVA, Bonferroni-posthoc).

(C) [C2H,C8F]cVc1.1 caused significantly more inhibition of CVH colonic nociceptors at 100 nM (* P<0.05), and 1 μM (* P<0.05) compared with responses in healthy nociceptors (n=8 afferents from N=5 healthy mice, n=9 afferents from N=5 CVH mice, two-way ANOVA, Bonferroni posthoc).

(D) Original recordings of a healthy colonic nociceptor, showing action potential firing in response to a 2 g vfh probe at baseline and in the presence of [C2H,C8F]cVc1.1 (1 μM).

(E) Original recordings of a CVH colonic nociceptor showing action potential firing in response to a 2 g vfh probe at baseline (control) and in the presence of [C2H,C8F]cVc1.1 (1 μM).
Figure 5: [N9W]cVc1.1 inhibits the mechanosensitivity of colonic nociceptors from healthy and CVH mice.

(A) Healthy colonic nociceptor mechanosensitivity is significantly reduced following application of [N9W]cVc1.1 at concentrations of 10 nM, 100 nM and 1 μM (* P<0.05, n=7 afferents from N=5 healthy mice, one-way ANOVA, Bonferroni posthoc).

(B) [N9W]cVc1.1 inhibited CVH colonic nociceptors at concentrations of 100 nM (* P<0.05) and 1 μM (* P<0.05). (n=7 afferents from N=5 CVH mice, one-way ANOVA, Bonferroni posthoc). 

(C) [N9W]cVc1.1 caused significantly more inhibition at 1 μM (*P <0.05) in CVH colonic nociceptors compared with healthy colonic nociceptors (n=7 afferents from N=5 healthy mice, n=7 afferents from N=5 CVH mice, two-way ANOVA, Bonferroni posthoc).

(D) Healthy colonic nociceptor recording, showing action potential firing in response to a 2 g vfh probe at baseline and in the presence of [N9W]cVc1.1 (1 μM).

(E) Recording of a CVH colonic nociceptor showing action potential firing in response to a 2 g vfh probe at baseline and in the presence of [N9W]cVc1.1 (1 μM).
Figure 6: Direct comparison of the inhibitory effects of the various Vc1.1 analogues on colonic nociceptors from healthy and CVH mice.

(A) Overall cVc1.1 evoked the largest inhibitory effect on healthy colonic nociceptors, with Vc1.1 evoking the least amount of inhibition. The cVc1.1 analogues evoked inhibition between that of Vc1.1 and cVc1.1. Analogues inhibited colonic nociceptors from healthy mice in the following rank order cVc1.1 > [N9W]cVc1.1 > [C2H,C8F]cVc1.1 > Vc1.1. *P<0.05: 10 nM Vc1.1 vs. 10 nM cVc1.1; * P<0.05: 100 nM Vc1.1 vs. 100 nM cVc1.1; * P<0.05: 1000 nM Vc1.1 vs. 1000 nM cVc1.1. Vc1.1: n=10 afferents from N=5 mice; cVc1.1: n=8 afferents from N=5 healthy mice; [C2H,C8F]cVc1.1: n=8 afferents from N=5 healthy mice, [N9W]cVc1.1: n=7 afferents from N=5 healthy mice. Analysis represents a total of 33 afferents from N=20 healthy mice.

(B) Colonic nociceptors from CVH mice displayed the greatest degree of inhibition in the presence of cVc1.1, with Vc1.1 causing the least amount of inhibition. Analogues inhibited colonic nociceptors from CVH mice in the following rank order cVc1.1 > [N9W]cVc1.1 > [C2H,C8F]cVc1.1 > Vc1.1. *P<0.05: 10 nM Vc1.1 vs 10 nM cVc1.1; *P<0.05: 1000 nM Vc1.1 vs 1000 nM cVc1.1. Vc1.1: n=10 afferents from N=5 CVH mice; cVc1.1: n=10 afferents from N=5 CVH mice; [C2H,C8F]cVc1.1: n=9 afferents from N=5 CVH mice, [N9W]cVc1.1: n=7 afferents from N=5 CVH mice. Analysis represents a total of 36 afferents from N=20 CVH mice.
Figure 7: Effects of Vc1.1 and cVc1.1 on pseudo-related pain responses to colorectal distension in healthy and CVH mice

(A) In healthy mice visceromotor responses (VMRs) to colorectal distension (CRD) were not significantly changed by intra-colonic administration of linear Vc1.1 (1 μM) relative to vehicle administration (P>0.05); Vehicle: N=10 mice; Vc1.1: N=6 mice. Data expressed as area under the curve of the corresponding EMG signal.
(B) In CVH mice, intra-colonic administration of linear Vc1.1 (1 μM) significantly inhibited the VMR to CRD, particularly at distension pressures of 40 mmHg and 60 mmHg (* P<0.05; Vehicle: N=10 mice, Vc1.1: N=7 mice).

(C) Intra-colonic administration of cVc1.1 (1 μM) did not affect VMRs to CRD in healthy mice relative to vehicle treated mice (P>0.05); Vehicle: N=8 mice, cVc1.1: N=7 mice).

(D) In CVH mice, intra-colonic administration of cVc1.1 significantly reduced VMRs to colorectal distension, particularly at distension pressures of 40 mmHg (* P<0.05), 60 mmHg (* P<0.05) and 80 mmHg (* P<0.05). (Vehicle: N=10 mice, cVc1.1: N=8 mice).

(E) Representative tracing of the raw abdominal electromyographic (EMG) signals assessing the VMR to 60 mmHg (20 s duration, indicated by horizontal bar) of CRD in healthy control (HC) mice after (i) vehicle or (ii) cVc1.1 and in mice with CVH after (iii) vehicle or (iv) cVc1.1. Length of recording shown 45 seconds.

(F) Colonic compliance in both healthy and CVH mice were not altered by intra-colonic cVc1.1 (1 μM) administration relative to intra-colonic vehicle administration (P>0.05).