Contribution of $Ca_V2.2$ and GIRK1/2 channels to membrane excitability of rodent and human dorsal root ganglion neurons

Running Title: Roles of $Ca_V2.2$ and GIRK1/2 channels in DRG neurons

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KEY POINTS SUMMARY:

• $GABA_BR1$, $Ca_V2.2$, and GIRK1 are highly expressed in the thoracolumbar dorsal root ganglia (DRGs) of both mice and humans.

• $Ca_V2.2$ inhibition and GIRK channel potentiation contribute to reduced neuronal excitability in mouse DRGs.

• The analgesic peptide, α-conotoxin Vc1.1, exerts its effects by inhibiting $Ca_V2.2$ and potentiating GIRK channels in mouse DRGs.

• Potentiation of GIRK channels does not significantly affect the excitability of colon-innervating DRGs or colonic afferents.

• These findings suggest neurons innervating different regions of the body employ distinct mechanisms to regulate neuronal excitability.

ABSTRACT

Modulation of voltage-dependent calcium and potassium channels by G protein-coupled receptors (GPCRs) plays a key role in reducing nociceptive transmission. Specifically, baclofen and the analgesic peptide α-conotoxin Vc1.1 activate $GABA_B$ receptors, resulting in the inhibition of $Ca_V2.2$ and $Ca_V2.3$ calcium channels, as well as the potentiation of GIRK1/2 potassium channels in mammalian primary afferent neurons. In this study, we examined the expression of these key ion channel targets in rodent and human dorsal root ganglion (DRG) neurons. We examined how $Ca_V2.2$ and GIRK channel antagonists, as well as a GIRK channel activator, influence the passive and active electrical properties of adult mouse DRG neurons. Additionally, we assessed the effects of α-conotoxin Vc1.1 on neuronal excitability in the presence of the selective $Ca_V2.2$ antagonist ω-conotoxin CVIE and the GIRK channel activator ML297. Furthermore, we evaluated how the GIRK channel antagonist Tertiapin-Q affects the excitability of mouse colonic DRGs and colonic afferents and explored the role of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels in regulating the membrane excitability of colonic DRGs. Our findings suggest that both $Ca_V2.2$ inhibition and GIRK channel potentiation contribute to the reduction of neuronal excitability in mouse DRGs, mediating the analgesic effects of Vc1.1 and baclofen observed *in vivo*. However, our findings indicate that GIRK channel potentiation may have a limited role in the mechanisms underlying Vc1.1 and baclofen action in colon-innervating DRGs and colonic afferents.

Keywords: dorsal root ganglia, neuronal excitability, electrophysiology, colonic afferents, analgesic peptide, baclofen

INTRODUCTION

Chronic pain is a major global health issue affecting more than 30% of the population (Cohen *et al.*, 2021). Chronic visceral pain is a particularly common form of chronic pain and is a key symptom of Irritable bowel syndrome (IBS), a chronic gastrointestinal disorder affecting 7-21% of individuals worldwide (Black & Ford, 2020; Camilleri, 2021). Although the pathophysiology of IBS is complex and not fully understood, substantial evidence indicates pain is driven by hypersensitivity of gut-innervating afferents and dysfunction along the gut-brain axis (Brierley & Linden, 2014; Grundy *et al.*, 2019). Peripheral hypersensitivity appears to be triggered by inflammatory or immune mediators, which contribute to the development of chronic visceral hypersensitivity (CVH) and neuroplastic changes within sensory pathways (Brierley & Linden, 2014; Grundy *et al.*, 2019; Van Remoortel *et al.*, 2024). These changes ultimately lead to heightened pain perception in the gastrointestinal tract (Brierley & Linden, 2014; Grundy *et al.*, 2019). Up-regulation of multiple ion channels and receptors in sensory afferents has been linked to the development of CVH in animal models (Osteen *et al.*, 2016; Castro *et al.*, 2017; Desormeaux *et al.*, 2018; Salvatierra *et al.*, 2018; Castro *et al.*, 2019; Israel *et al.*, 2019; Cardoso *et al.*, 2021; Jiang *et al.*, 2021). Consequently, targeting these ion channels and receptors may represent a promising therapeutic strategy for managing chronic visceral pain in IBS and chronic pain more broadly (Erickson *et al.*, 2018; Sadeghi *et al.*, 2018b; Grundy *et al.*, 2019; Brizuela *et al.*, 2021; Muttenthaler *et al.*, 2021).

α-Conotoxins are small, disulfide-rich peptides, typically 12−30 amino acids in length, derived from the venom of marine cone snails. These peptides are promising drug candidates for the treatment of chronic pain, and chronic visceral pain, due to their ability to selectively target a wide range of membrane receptors and ion channels (Callaghan *et al.*, 2008; Schroeder & Craik, 2012; Li *et al.*, 2021). In particular, α-conotoxin Vc1.1 has demonstrated anti-nociceptive effects *in vitro* and anti-hyperalgesic actions in various *in vivo* models of neuropathic pain (Clark *et al.*, 2010; Klimis *et al.*, 2011; Li *et al.*, 2021). Historically, the analgesic effects of Vc1.1 were attributed to its inhibitory action on neuronal nicotinic acetylcholine receptors (nAChRs) (Livett *et al.*, 2006; Frøsig-Jørgensen *et al.*, 2023). However, subsequent research has shown that its analgesic properties are primarily mediated through the activation of the G protein-coupled γ-aminobutyric acid type B receptor ($GABA_BR$) (Callaghan *et al.*, 2008; Sadeghi *et al.*, 2017; Bony *et al.*, 2022b; Frøsig-Jørgensen *et al.*, 2023). $GABA_BRs$ are broadly expressed on sensory neurons in both humans and rodents, including those innervating the gut (Castro *et al.*, 2017). Our previous studies demonstrated that Vc1.1, along with its modified and cyclised form (cVc1.1), inhibits colonic sensory afferents, with these inhibitory effects being further amplified in an animal model of IBS (Carstens *et al.*,

2016; Castro *et al.*, 2017; Castro *et al.*, 2018; Sadeghi *et al.*, 2018a). Importantly, we identified that the mechanism of action of Vc1.1 involves the activation of $GABA_B$Rs on colonic afferents, leading to downstream inhibition of voltage-gated calcium channels $Ca_V2.2$ and $Ca_V2.3$ and a reduction in the excitability of dorsal root ganglion (DRG) neurons innervating the mouse colon (Castro *et al.*, 2017; Castro *et al.*, 2018). Furthermore, we have shown that native human DRG neurons and human pluripotent stem cell (hPSC)-derived sensory neurons are inhibited by the $GABA_B$R agonists, baclofen and Vc1.1 (Castro *et al.*, 2017; St Clair-Glover *et al.*, 2025). Notably, the latter study confirmed the expression of G protein-coupled inwardly rectifying potassium (GIRK) channels in hPSC-derived sensory neurons. This is significant, as $GABA_B$R activation is known to reduce neuronal excitability through inhibition of Ca_V channels, but also but also by potentiating GIRK channels (Bony *et al.*, 2022a).

In this study, we investigated the role of GIRK channels in modulating neuronal excitability in mouse DRG neurons, including those specifically innervating the colon. We examined the contributions of $Ca_V2.2$ and GIRK channels to the passive and active electrical properties of adult mouse DRG neurons. Additionally, we explored whether Vc1.1's inhibitory effects on neuronal excitability are mediated by $GABA_B$R activation, leading to downstream modulation of $Ca_V2.2$ and GIRK1/2 channels. Finally, we assessed the role of GIRK channels in regulating the excitability of DRG neurons innervating the colon, as well as colonic afferents, to further elucidate the mechanisms by which α-conotoxins modulate colonic neuronal excitability.

MATERIALS AND METHODS

Animals

All animal procedures were conducted in accordance with the University of Wollongong Animal Ethics Committee (AEC) guidelines and regulations under protocol (AE16/10 and AE16/10r19) and SAHMRI's Animal Ethics Committee. AEC guidelines comply with the 'Australian code of practice for the care and use of animals for scientific purposes', and the ARRIVE guidelines on reporting experiments involving animals (Percie du Sert *et al.*, 2020). For University of Wollongong studies, adult male C57BL/6 mice (8-14 weeks old) were purchased from Australian Bioresources (Moss Vale, NSW, Australia) and housed in individually ventilated cages with a 12 h light/dark cycle; plastic shelter, nesting material, food pellets and water were available *ad libitum*. For studies performed at SAHMRI, male C57BL/6J mice aged 13–17 weeks were used. These mice were acquired from an in-house C57BL/6J breeding programme (strain no. 000664; originally purchased from The Jackson Laboratory, barn MP14) within SAHMRI's specific and opportunistic pathogen-free animal care facility.

Human DRG

Four L1 DRGs were collected by AnaBios (San Diego, CA, USA) from human adult organ donors (22.2 ± 2.08 years of age; male:female ratio 2:2). The samples were preserved in RNAlater (Thermo Fisher Scientific) and shipped to SAHMRI for processing (Castro *et al.*, 2017; Castro *et al.*, 2019). The AnaBios NIH compliant ethics statement is available at: https://anabios.com/ethics-statement/.

Quantitative-reverse-transcription-PCR (QRT-PCR)

Total RNA was extracted from human L1 DRG or mouse thoracolumbar (T10-L1) DRGs using PureLink RNA isolation kit (ThermoFisher Scientific). QRT-PCR was conducted with 20 ng RNA/well using the EXPRESS One-Step Superscript kit (ThermoFisher Scientific) and predesigned mouse or human-specific Taqman® probes for $GABA_BR$ subunits (*Gabbr1*, Mm00433461_m1 or Hs00559488_m1, *Gabbr2*, Mm01352554_m1 or Hs01554996_m1), $Ca_V2.2$ (*Cacna1b*, Mm01333678_m1 or Hs01053090_m1), $Ca_V2.3$ (*Cacna1e*, Mm00494444_m1 or Hs00167789_m1), GIRK1-4 (*Kcnj3*, Mm00434618_m1 or Hs04334861_s1, *Kcnj6*, Mm01215650_m1 or Hs01040524_m1, *Kcnj9*, Mm00434621_m1 or Hs05018005_m1, *Kcnj5*, Mm01175829_m1 or Hs00168476_m1), *Gapdh* (Mm99999915_g1), *Ppia* (Mm02342430_g1 or

Hs99999904_m1), and *Actb* (Mm00607939_s1 or Hs99999903_m1). mRNA was transcribed into cDNA using Supserscript IV kit (ThermoFisher Scientific). All samples were measured in duplicates. Raw data were analysed using DA2 software (ThermoFisher Scientific) and exported to Microsoft Excel to calculate relative mRNA quantities before transferring data into GraphPad for graphical representation. The comparative cycle threshold method was used to quantify the abundance of target transcripts relative to the reference genes (*Gapdh, Ppia,* and *Actb*).

Single-cell RT-PCR

A total of 53 single subserosal colon-traced dissociated TL DRG neurons were isolated from one healthy mouse (C57Bl/6J; male; 16 weeks old) and collected using a micromanipulator at 40x magnification. During the picking process, a continuous slow flow of sterile, RNA/DNAse-free PBS was applied to minimize contamination with ambient RNA. After isolating a neuron, the glass capillary containing the cell was broken into a tube containing 10 µl lysis buffer with DNAse (TaqMan® Gene Expression Cells-to-CT™ Kit; ThermoFisher Scientific). The entire sample was used for cDNA synthesis (SuperScript® VILO™ cDNA Synthesis Kit, Thermo Fisher Scientific) and diluted 1:3 to measure target expression per cell using RT-PCR for 50 cycles. For each coverslip, a bath control was collected and analysed together with samples. Expression of *Tubb3* (Mm00727586_s1) served as a positive control to confirm neuronal identity. Cells were excluded from analysis if they lacked *Tubb3* expression or tested positive for *Gfap* (Mm01253033_m1), indicating contamination with glial cells. Out of the initial 53 cells, 12 were excluded based on these criteria. The frequency of the expression of a particular target was calculated from the remaining 41 neurons.

Cell culture for patch clamp recordings of mouse DRGs

Mice were euthanised by isoflurane inhalation followed by rapid decapitation. The thoracic and lumbar DRG were exposed via laminectomy, harvested, and immediately transferred to ice cold (4°C) free of Ca^{2+}- and Mg^{2+}-free Hanks Buffered Saline Solution (HBSS). The DRGs were trimmed, removing central and peripheral nerve processes, and digested in HBSS containing collagenase type II (3 $mg.ml^{-1}$; Worthington Biomedical Corp., Lakewood, NJ, USA) and dispase (4 $mg.ml^{-1}$; GIBCO, Australia) at 37°C for 40 min. Following digestion, the ganglia were rinsed three to four times with 37°C/5% CO_2-equilibrated F-12/GlutaMAX™ (Invitrogen) supplemented with 10% heat-inactivated FBS (GIBCO™, ThermoFisher Scientific) and 1% Pen/Strep. Cells were dispersed by mechanical trituration with progressively smaller fire-polished glass Pasteur pipettes. The resulting cell suspension was filtered through a 160 µm nylon mesh (Millipore Australia Pty Ltd, North Ryde, NSW, Australia) to remove any undigested material. Dissociated DRG neurons

were plated onto 12 mm cover glass coated with poly-D-lysine (Sigma-Aldrich, Australia) and allowed to settle for ~3 hr at 37°C, supplemented with fresh media, and incubated overnight before patch clamp recordings.

Whole-cell current-clamp electrophysiology of mouse DRG neurons

Current clamp recordings of DRG neurons were carried out within 24 hr of dissociation in a extracellular (bath) solution containing (in mM): 140 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose, pH 7.4 adjusted with NaOH (~320 $mOsmol.kg^{-1}$). Fire polished borosilicate patch pipettes (World Precision Instruments) had resistances of 2-4 MΩ when filled with an internal solution containing (in mM): 130 KCl, 20 NaCl, 5 EGTA, 5 $MgCl_2$, 10 HEPES, 5 Mg-ATP, 0.2 Na_2GTP, pH 7.2 adjusted with KOH (~300 $mOsmol.kg^{-1}$). Whole-cell configuration was achieved in voltage clamp mode before transitioning to current clamp mode. Depolarizing current steps of 500 ms duration at 0.2 Hz were applied to elicit action potential firing in small to medium diameter (<30 μm) DRG neurons with resting membrane potentials (RMP) more negative than –40 mV.

Modulation of high voltage-activated N-type ($Ca_V2.2$) channels by ω-conotoxin CVIE, and GIRK channels by the antagonist Tertiapin-Q (TPQ) and the agonist ML297, was conducted via bath application of these compounds. In a series of experiments the $GABA_BR$ agonist, α-conotoxin Vc1.1, was co-applied with either CVIE or ML297. All drugs were dissolved in distilled H_2O to prepare to stock solutions at the appropriate concentration. The extracellular solutions were superfused using a peristaltic pump at a flow rate of 1 ml min^{-1} in a ~500 µl experimental chamber maintained at room temperature (21-23°C). TPQ was purchased from Abcam (Cambridge, UK), ML297 was obtained from Tocris (Bristol, UK), and ω-conotoxin CVIE and α-conotoxin Vc1.1 were synthesized as described previously (Berecki et al., 2010) and kindly provided by Profs David Craik and Richard Clark (The University of Queensland, Brisbane, Australia).

Mouse model of chronic visceral hypersensitivity (CVH)

Colitis was induced by the administration of 2,4-dinitrobenzene sulfonic acid (DNBS), as described previously (Castro *et al.*, 2022). Briefly, 13–15-week-old anaesthetized mice were administered a single intra-colonic enema of 100 µL DNBS/Ethanol solution (5 mg DNBS in 100 µL of 30% ethanol) via a polyethylene catheter to induce colonic inflammation (Castro *et al.*, 2013; Castro *et al.*, 2019). Histological examination of mucosal architecture, cellular infiltrate, crypt abscesses, and goblet cell depletion confirmed significant DNBS-induced damage by day 3 post-treatment. This damage largely resolved spontaneously by day 7 and was fully resolved by day 28

(Hughes *et al.*, 2009). High-threshold colonic nociceptors from mice at the 28-day time point displayed significant mechanical hypersensitivity and reduced mechanical activation thresholds (Hughes *et al.*, 2009; Castro *et al.*, 2013; de Araujo *et al.*, 2014; Hughes *et al.*, 2014a; Osteen *et al.*, 2016; Castro *et al.*, 2017; Castro *et al.*, 2018; Grundy *et al.*, 2018; Salvatierra *et al.*, 2018; Castro *et al.*, 2019; Castro *et al.*, 2022). This model is characterized by hyperalgesia and allodynia to colorectal distension and is therefore termed 'Chronic Visceral Hypersensitivity' (CVH) (Osteen *et al.*, 2016; Castro *et al.*, 2018; Grundy *et al.*, 2018; Salvatierra *et al.*, 2018; Castro *et al.*, 2019; Castro *et al.*, 2022; Kremsmayr *et al.*, 2024).

Retrograde tracing to identify colon-innervating DRG neurons

CTB-conjugated to AlexaFluor 488 or 555 (ThermoFisher Scientific) was injected (4 μl/injection) subserosally at three sites within the distal colon of healthy mice (single cell RT-PCR and patch clamp recordings) or CVH mice (patch clamp recordings) using a Hamilton syringe fitted with a 23-gauge needle as described previously (Harrington *et al.*, 2019).

Cell culture for patch clamp recordings and single-cell picking of colonic DRGs

Four days after retrograde tracing, mice were humanely euthanized, and thoracolumbar (TL, from T9–L1) DRG were removed and dissociated (Castro *et al.*, 2017; Castro *et al.*, 2019). DRGs were enzymatically digested first with 4 mg ml^{-1} collagenase II GIBCO, ThermoFisher Scientific) and 5.3 mg ml^{-1} dispase I (GIBCO, ThermoFisher Scientific) for 30 min at 37 ºC, followed by a second digestion with 4 mg ml^{-1} collagenase II for 10 min at 37 ºC. The dissociated neurons were resuspended in DMEM (GIBCO, ThermoFisher Scientific) supplemented with 10% fetal calf serum (Invitrogen, ThermoFisher Scientific), 2 mM L-glutamine (GIBCO, ThermoFisher Scientific), 100 μM MEM non-essential amino acids (GIBCO, ThermoFisher Scientific), 96 ng ml^{-1} NGF, and 100 mg ml^{-1} penicillin/streptomycin (GIBCO, ThermoFisher Scientific). Neurons were spot-plated onto coverslips coated with laminin (20 μg ml^{-1}; Sigma-Aldrich) and poly-D-lysine (800 μg ml^{-1}; ThermoFisher Scientific) and maintained in an incubator at 37 ºC with 5% CO_2.

Whole-cell current-clamp electrophysiology of colon-innervating DRG neurons

Dissociated DRG neurons isolated from colon-traced healthy or CVH mice were recorded on day 1 post-culture (20-30 hs after plating) (Castro *et al.*, 2018; Grundy *et al.*, 2018; Salvatierra *et al.*, 2018). The intracellular current-clamp solution contained (in mM): 135 KCl; 2 $MgCl_2$; 2 MgATP; 5 EGTA-Na; 10 HEPES-Na; adjusted to pH 7.3. The extracellular (bath) current-clamp solution contained (in mM): 140 NaCl; 4 KCl; 2 $MgCl_2$; 2 $CaCl_2$; 10 HEPES; 5 glucose; adjusted to pH 7.4. Standard-wall borosilicate glass pipettes (OD x ID x length: 1.5 mm x 0.86 mm x 7.5 cm,

Harvard) pulled and fire-polished to a resistance of 3 - 6 MΩ using a P-97 (Sutter Instruments) pipette puller were used. The protocol used was as follows, in current-clamp mode, neurons were held at -70 mV for 15 ms, hyperpolarized with a -20 pA current injection for 475 ms, then returned to -70 mV for 100 ms. Stepwise depolarizing pulses were applied in 0 pA or 25 mV increments (475 ms) from a holding potential of -70 mV, with 2 sec repetition intervals, to determine the rheobase (minimum amount of current required to elicit an action potential). Rheobase was assessed both in the presence and absence of Tertiapin-Q (100 nM) or the HCN inhibitor ZD7288 (50 µM). Neurons with a resting membrane potential more depolarized than -40 mV were excluded from recordings, as this indicated poor cell health. Recordings were amplified using an Axopatch 200A, digitized with a Digidata 1322A, sampled at 20 kHz, filtered at 5 kHz, and recorded with pCLAMP 9 software (Molecular Devices). Data was analyzed using Clampfit 10.3.2 (Molecular Devices) and Prism v9.3.1 (GraphPad).

***Ex vivo* electrophysiology**

Recordings were obtained from healthy male C57BL/6J mice. Pelvic and splanchnic afferent nerve activity was measured *ex vivo* using an isolated segment of the mouse colon ligated at either end to allow for fluid distension (Bayrer *et al.*, 2023). The tissue was mounted in a custom-built organ bath and perfused with warm, carbogenated physiological Krebs buffer (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). To suppress smooth muscle activity, the Krebs solution included the L-type calcium channel antagonist nifedipine (1 µM). The prostaglandin synthesis inhibitor indomethacin (3 µM) was also added to suppress potential inhibitory actions of endogenous prostaglandins. The pelvic and splanchnic nerve were carefully isolated and placed within a sealed glass pipette containing a microelectrode (WPI) connected to a NeuroLog headstage (NL100AK; Digitimer). Nerve activity was amplified (NL104), filtered (NL 125/126, bandpass 50–5,000 Hz, NeuroLog; Digitimer), and digitised using a CED 1401 interface (Cambridge Electronic Design, Cambridge, UK) for offline analysis with Spike2 software (Cambridge Electronic Design, Cambridge, UK). Nerve activity was measured at baseline and during ramp distension with Krebs solution or Tertiapin-Q (flow rate: 100 µl/min; pressure range: 0 to 80 mmHg) measured with a pressure transducer; NL108T2 Digitimer).

The number of action potentials exceeding a preset threshold (twice the background electrical noise) was determined per second to quantify the afferent response. Responses were compared before and after the intraluminal application of Tertiapin-Q (10 µM and 100 µM). Single-unit analysis of action potentials was conducted offline by matching individual spike waveforms

through linear interpolation using Spike2 v.10.05 software (Cambridge Electronic Design, Cambridge, UK).

RESULTS

Expression of $GABA_BR$ subunits, $Ca_V2.2$, $Ca_V2.3$, and GIRK1-4 in mouse and human thoracolumbar DRGs

Quantitative RT-PCR (QRT-PCR) analysis of whole mouse DRG samples revealed that $GABA_BR1$, $Ca_V2.2$ and GIRK1 were the predominant targets expressed in the thoracolumbar dorsal root ganglia (DRGs, T10-L1) followed by $Ca_V2.3$ and $GABA_BR2$. In contrast, GIRK2-4 exhibited very low mRNA expression levels, 51-times lower than GIRK1 for GIRK2, 106-times lower for GIRK4, and 306-times lower for GIRK3 (**Fig. 1A-B**). In mouse DRGs, the fold change in expression for each target relative to the least expressed gene was as follows: $GABA_BR1$ (1961) > $Ca_V2.2$ (1188) > GIRK1 (306) > Cav2.3 (66) > $GABA_BR2$ (34) > GIRK2 (6) > GIRK4 (3) > GIRK3 (1). A similar expression pattern was observed in human thoracolumbar DRGs (L1, **Fig. 1C-D**). However, $GABA_BR2$ expression was relatively higher in human DRGs, being four-fold lower than $GABA_BR1$, compared to mouse DRGs, where $GABA_BR2$ was 60-fold lower than $GABA_BR1$. Additionally, the expression of Cav2.3 in human DRGs was notably lower than in mice, with $Ca_V2.3$ being the least expressed target in human DRGs. Similar to mouse TL DRG, Cav2.2 was highly expressed in human L1 DRG, however, the ratio of $GABA_BR1$ to $Ca_V2.2$ was only 12 to 1 compared to the similar expression levels in mice (1.6 to 1). In human DRGs, the fold change in expression for each target relative to the least expressed gene was as follows: $GABA_BR1$ (1231) > $GABA_BR2$ (365) > GIRK1 (163) > $Ca_V2.2$ (99) > GIRK3 (13) > GIRK2 (5) > GIRK4 (3) >Ca_V 2.3 (1). Overall, these findings highlight that $Ca_V2.2$, $GABA_BR1$, and GIRK1 are the dominant isoforms in both mouse and human DRGs, suggesting their potential functional importance in thoracolumbar sensory processing.

Co-expression of $GABA_BR$ subunits, $Ca_V2.2$, $Ca_V2.3$, and GIRK channels in colon-innervating DRGs

Single-cell RT-PCR from retrogradely traced mouse colon-innervating DRGs was performed to assess the expression of $GABA_BR$, Ca_V channels, and GIRK channels specifically in colonic DRG neurons. The analysis revealed high expression levels of $Ca_V2.2$ and $GABA_BR2$, detected in 100% and 95% of colon-innervating DRG neurons, respectively (**Fig. 2A-B**). In contrast, $Ca_V2.3$ was expressed in 29 of 41 cells (71%), and $GABA_BR1$ was expressed in 26 of 41 neurons (63%, **Fig. 2A-B**). Among the 41 colonic neurons analyzed, GIRK1 was expressed in 24 neurons (58%), whereas GIRK2 was detected in only 6 neurons (15%). GIRK3 and GIRK4 expression levels were below the detection threshold and were not observed in any of the neurons analyzed (**Fig. 2A-B**). Co-expression analysis revealed that GIRK1 was highly co-expressed with $Ca_V2.2$ (100%), $Ca_V2.3$ (75%), $GABA_BR1$ (71%), and $GABA_BR2$ (96%) (**Fig. 2C**), suggesting a

strong functional link between $GABA_BR$ signaling, Ca_V channels, and GIRK channel activity in colonic sensory neurons.

Effects of the selective $Ca_V2.2$ antagonist ω-conotoxin CVIE and GIRK channel agonist ML297 on mouse DRG neuronal excitability

To investigate the roles of $Ca_V2.2$ and GIRK channels in neuronal excitability, patch clamp recordings were performed on mouse DRG neurons in the presence of the selective $Ca_V2.2$ antagonist ω-conotoxin CVIE and the GIRK channel agonist ML297 (**Fig. 3**). Application of ω-conotoxin CVIE (100 nM) reduced action potential (AP) firing frequency by approximately 50% and increased rheobase by approximately 20%, without affecting the resting membrane potential (–64.5 ± 2.9 mV, n = 6, **Fig. 3A-B**). Co-application of ω-conotoxin CVIE with α-conotoxin Vc1.1 caused membrane hyperpolarization and a further reduction in action potential (AP) firing (**Fig. 3A**). Overall, co-application of ω-conotoxin CVIE and α-conotoxin Vc1.1 significantly reduced the resting membrane potential, input resistance, and action potential firing while significantly increasing the rheobase of DRG neurons (**Fig. 3B**).

Application of the GIRK channel agonist ML297 (10 µM) produced effects similar to ω-conotoxin CVIE on resting membrane potential, rheobase, input resistance, and AP firing frequency (**Fig. 3C**). However, unlike ω-CVIE, ML297 caused significant membrane hyperpolarisation (>3.5 mV, n = 8). Previous studies have shown that $GABA_BR$-dependent GIRK channel potentiation by baclofen and Vc1.1 induces membrane hyperpolarization and reduces excitability in mouse DRG neurons (Bony *et al*., 2022a). Here, we demonstrate that co-application of ML297 with Vc1.1 (1 µM) further increased the current required to induce neuronal firing and significantly reduced firing frequency in healthy mouse DRG neurons (**Fig. 3C-D, Table 1**).

GIRK channel inhibitor Tertiapin-Q increases the excitability of adult mouse DRG neurons, and its effects are counteracted by α-conotoxin Vc1.1

The role of GIRK channels in mouse neuronal excitability was further investigated using the specific GIRK channel antagonist Tertiapin-Q (TPQ). Application of TPQ (100 nM), caused membrane depolarisation and significantly increased the excitability of mouse DRG neurons (**Fig. 4A-B, Table 1**). Specifically, this was evident by a significant increase in resting membrane potential, input resistance, and action potential firing frequency, along with a significantly lowered rheobase. Co-incubation of TPQ with Vc1.1 increased AP discharge compared to the control, suggesting that TPQ inhibited the potentiation of GIRK channel currents induced by Vc1.1. Under

normal conditions, Vc1.1 potentiation of GIRK channels would have led to a decrease in AP firing **(Fig. 5A-B)**.

GIRK channel inhibitor Tertiapin-Q does not affect the responsiveness of mouse colonic afferents

To assess the functional role of GIRK channels in colonic sensory function, we performed *ex vivo* recordings of pelvic and splanchnic nerve activity. Afferent recordings from pelvic nerves revealed that as pressure within the colon increased, action potential discharge also increased **(Fig. 6A)**. Comparisons of responses during colonic distension in the presence of vehicle (Krebs solution) versus intraluminal infusion of TPQ (10 μM or 100 μM) showed no significant differences in pelvic afferent firing or colonic compliance (**Fig. 6A-D)**. These findings indicate that TPQ does not influence the excitability of healthy mouse colonic pelvic nerve afferents responding to mechanical distension. A more detailed analysis of individual afferent responses was performed by classifying pelvic colonic afferents based on their firing characteristics at specific distension pressures (Bayrer *et al.*, 2023). The results indicated that TPQ did not alter the mechanical sensitivity of low-threshold (LT), wide dynamic range (WDR), or high-threshold (HT) pelvic afferents (**Fig. 6E**), consistent with the whole pelvic nerve responses.

Further investigations examined the effect of TPQ on splanchnic afferents (**Fig. 7**). A small but not statistically significant decrease in splanchnic nerve afferent firing was observed following intracolonic infusion of TPQ (10 μM) compared to vehicle (Krebs solution, **Fig. 7A-C**). Given that the majority of splanchnic afferents are high threshold, no sub-analysis of afferent subtypes was performed. Overall, these findings indicate that inhibiting GIRK channel currents with a GIRK channel antagonist does not affect the excitability of healthy mouse colonic pelvic or splanchnic afferents in response to mechanical distension.

GIRK channel inhibitor Tertiapin-Q does not affect the excitability of colon-innervating DRG neurons in CVH states

To further investigate the role of GIRK channels on the excitability of colonic DRG neurons, we performed whole-cell patch clamp recordings in our CVH model. These experiments were conducted on retrogradely traced colonic DRGs from CVH mice in the presence of the GIRK channel inhibitor TPQ. Unlike our findings in general DRG neurons, we observed no significant changes in the amount of injected current required to trigger an action potential before or after TPQ application, indicating no change in the rheobase (**Fig. 8A-B**). Furthermore, TPQ had no significant effect on the resting membrane potential of CVH colonic DRG neurons (**Fig. 8C**). These findings suggest that inhibiting GIRK channels with TPQ (100 nM) does not alter action potential firing in

CVH colon-innervating DRGs. This indicates that GIRK channels may not play a significant role in modulating colonic neuronal excitability in either healthy or CVH conditions.

The specific HCN channel inhibitor ZD7288 increases the excitability of healthy colonic DRG neurons

A recent study on hPSC-derived sensory neurons demonstrated that although GIRK channels regulate neuronal excitability, modulation of GIRK channels by $GABA_BRs$ was not observed, suggesting that functional GIRK1/2 channels were not coupled to $GABA_BRs$ in these neurons (St Clair-Glover *et al.*, 2025). However, this study did also show that hyperpolarisation-activated cyclic nucleotide-gated (HCN) channels play a role in regulating neuronal excitability. Specifically, when hPSC-derived sensory neurons were exposed to the HCN inhibitor ZD7288 (30 μM), both passive and active membrane properties were significantly altered (St Clair-Glover *et al.*, 2025). Given these findings, along with the lack of effect of the GIRK inhibitor Tertiapin-Q on colonic DRG neurons and colonic afferents, we investigated whether HCN channels modulate colonic DRG excitability. Therefore, we applied the HCN channel inhibitor ZD7288 and assessed the electrophysiological properties of healthy mouse colonic DRG neurons using whole-cell patch clamp recordings. Application of 50 μM ZD7288 significantly increased the excitability of colonic DRG neurons, as evidenced by a significant decrease in the amount of injected current required to elicit an action potential (**Fig. 9A-B**). However, ZD7288 (50 μM) had no significant effect on the resting membrane potential (RMP) of healthy mouse colon-innervating DRG neurons (**Fig. 9C**). Further analysis of action potential characteristics revealed no differences in the ascending or descending phases of action potentials between mouse colonic DRGs incubated with vehicle or ZD7288 (**Fig. 9D-E**).

DISCUSSION

$GABA_BRs$ are widely expressed in the somatosensory nervous system, where they play a key role in modulating pain perception and transmission (Malcangio, 2018; Benke, 2022). Targeting $GABA_BRs$ has been shown to attenuate mechanical allodynia in neuropathic pain models (Zhang *et al.*, 2022) and reduce colonic nociception, thereby alleviating visceral pain (Castro *et al.*, 2017). As G-protein-coupled receptors, $GABA_BRs$ mediate their inhibitory effects through the modulation of downstream ion channels (Alten *et al.*, 2022; Qian *et al.*, 2023). Notably, these downstream effectors are agonist-specific, with distinct channel interactions depending on the activating ligand (Shaye *et al.*, 2021). For instance, the activation of $GABA_BRs$ by the agonist baclofen inhibits three distinct calcium channels, $Ca_V2.1$, $Ca_V2.2$, and $Ca_V2.3$, whereas α-conotoxin Vc1.1 activation of $GABA_BRs$ selectively inhibits $Ca_V2.2$ and $Ca_V2.3$ channels, without affecting $Ca_V2.1$ (Berecki *et al.*, 2010). Additionally, $GABA_BR$ activation by Vc1.1 has been shown to activate G protein-gated inwardly rectifying potassium (GIRK) channels in HEK293T cells co-expressing either heteromeric human GIRK1/2 or homomeric GIRK2 subunits with $GABA_BR$ (Bony *et al.*, 2022a). This mechanism is particularly relevant given the widespread expression of GIRK channels in neurons throughout the central nervous system, including the spinal cord and sensory ganglia, where they regulate neuronal excitability and play a pivotal role in regulating numerous neurological disorders (Luscher & Slesinger, 2010; Lujan & Aguado, 2015). GIRK channels hyperpolarize neurons in response G protein-coupled receptor activation, modulating neuronal activity via GIRK-mediated self-inhibition, slow synaptic potentials, and volume transmission (Luscher & Slesinger, 2010).

Notably, GIRK channel activation via $GABA_BR$ has been shown to modulate nociceptive transmission (Ikeda *et al.*, 2000; Blednov *et al.*, 2003; Mitrovic *et al.*, 2003; Pan *et al.*, 2008). In mouse DRGs, Vc1.1-induced $GABA_BR$ activation potentiates GIRK channels, leading to membrane hyperpolarization and decreased neuronal excitability (Bony *et al.*, 2022a; Yousuf *et al.*, 2022). In the present study, we characterised the expression profiles of Ca_V and GIRK channels, as well as $GABA_BR$ subunits, in both mouse and human whole DRG. A recent study utilizing single-cell nucleus RNA sequencing data from human DRGs, reported high expression levels of Ca_V channels and $GABA_BRs$ (Jung *et al.*, 2023). These findings align with the results presented here, where we similarly identified the expression of $GABA_BRs$ and Ca_Vs and demonstrated their functional interactions. We also observed significant expression of GIRK1 channels in human DRGs, with

minimal or no expression of other GIRK channels, which is consistent with findings from the single-cell nucleus RNA sequencing data of single human DRG neurons (**Supp. Fig. 1**) (Jung *et al.*, 2023).

Additionally, we provide evidence that the inhibitory effects of α-conotoxin Vc1.1 on mouse excitability are mediated through dual mechanisms: HVA N-type calcium ($Ca_V2.2$) channel inhibition and GIRK1/2 channels potentiation. Furthermore, inhibition of GIRK1/2 channels enhanced neuronal excitability and attenuated Vc1.1's ability to suppress AP discharge. Taken together, these findings confirm that in mouse sensory DRG neurons, α-conotoxin Vc1.1 modulates neuronal excitability via $GABA_BR$ activation through both $Ca_V2.2$ channel inhibition and GIRK channel potentiation (Bony *et al.*, 2022a; Yousuf *et al.*, 2022).

Our findings provide important new insights into the downstream coupling of ion channels to $GABA_BRs$. Previous studies demonstrated that Vc1.1 enhances inwardly-rectifying K^+ currents in HEK293T cells expressing human GIRK1/2 channels and $GABA_BRs$ (Bony *et al.*, 2022a). However, more recent studies in hPSC-derived sensory neurons showed that while GIRK channels regulate neuronal excitability, modulation of GIRK channels by $GABA_BRs$ was not observed. This suggests that functional GIRK1/2 channels are not coupled to $GABA_BRs$ in these neurons (St Clair-Glover *et al.*, 2025). In the present study, we show GIRK channels are expressed in colon-innervating DRG neurons, with GIRK1 being most prevalent (58% of neurons), followed by GIRK2 (15%), while no neurons expressed GIRK3 or GIRK4. Notably, there was substantial overlap in the expression profile of $GABA_BR$, $Ca_V2.2$, and GIRK1 channels. However, our afferent recording and patch-clamp studies revealed that inhibiting GIRK channels had little effect on colonic afferent function in either healthy or CVH mice. This suggests that in mouse colonic DRGs, GIRK channels may not play a crucial role in regulating neuronal activity. These findings contrast with previous studies that reported a functional role for GIRK channels in modulating neuronal excitability in general DRG populations. This discrepancy aligns with prior research indicating that colon-innervating DRG neurons exhibit distinct functional and transcriptional profiles compared with DRG neurons that innervate other body regions (Page *et al.*, 2004; Hughes *et al.*, 2007; Hockley *et al.*, 2019). One possible explanation for this discrepancy is that $GABA_BR$-GIRK coupling may be dormant or non-functional in healthy states but becomes active under inflammatory or pathological conditions. This phenomenon has been observed with κ-opioid and oxytocin receptors, which mediate anti-nociceptive effects in colonic nociceptors only during inflammatory or post-inflammatory CVH states, but not in healthy conditions (de Araujo *et al.*, 2014; Hughes *et al.*, 2014b; Brust *et al.*, 2016; Muratspahić *et al.*, 2021; Kremsmayr *et al.*, 2024). However, our patch clamp recordings from colon thoracolumbar DRG neurons in CVH states

showed that inhibiting GIRK channels with Tertiapin-Q did not affect rheobase, further suggesting that GIRK channels do not significantly modulate neuronal function in high-threshold nociceptors from the splanchnic pathway or in LT, WDR, or HT pelvic afferents in healthy states.

The analgesic effects of α-conotoxin Vc1.1 on visceral pain are well-documented (Carstens *et al.*, 2016; Castro *et al.*, 2017; Castro *et al.*, 2018; Sadeghi *et al.*, 2018a). Vc1.1-induced activation of $GABA_BR$ has been shown to inhibit $Ca_V2.2$ and $Ca_V2.3$ channels, reducing colonic DRG neuronal output by increasing rheobase, decreasing colonic afferent excitability, reducing nociceptive signalling in the spinal dorsal horn following colonic distension, and alleviating pain responses *in vivo* (Castro et al., 2017; Castro et al., 2018; Sadeghi et al., 2018a). Notably, the anti-nociceptive effects of Vc1.1 are enhanced in hyperalgesic states, as demonstrated in a CVH model, whereby a significant upregulation of $Ca_V2.2$ exon 37a was observed (Castro *et al.*, 2017). While we did not observe a functional role for GIRK channels, our study identified a significant role for HCN channels in regulating colonic DRG neuronal excitability. HCN channels are crucial for controlling neuronal excitability and have been implicated in both somatic (Emery *et al.*, 2011) and visceral pain (Chen *et al.*, 2014). These channels are activated by membrane hyperpolarization and are permeable to both Na^+ and K^+ ions (Benarroch, 2013). Using the HCN channel blocker, ZD7288, we showed that inhibiting HCN channels altered neuronal excitability in healthy states without affecting membrane potential or action potential characteristics. This aligns with transcriptomic studies showing that a large proportion of colonic afferent DRG neurons express HCN1-4 channels (**Supp. Fig. 2)** (Hockley *et al.*, 2019). Further research is needed to determine whether $GABA_BRs$ directly couple with HCN channels in colonic DRGs and whether Vc1.1-induced activation of $GABA_BR$ modulates HCN channel activity in both healthy and disease states.

Overall, our findings confirm that in sensory mouse DRG neurons, α-conotoxin Vc1.1 regulates neuronal excitability downstream of $GABA_BR$ activation, primarily via $Ca_V2.2$ channel inhibition and GIRK channel potentiation. However, in colonic DRG neurons, while GIRK1 was the predominant GIRK channel expressed, its functional contribution to afferent responses to mechanical distension or AP discharge was minimal. This suggests that GIRK channels do not play a major functional role in colonic afferent signaling. Instead, our findings highlight a potential role for HCN channels in regulating neuronal excitability in these neurons, warranting further investigation.

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ADDITIONAL INFORMATION SECTION

Data availability statement:

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Competing interests:

Mariana Brizuela and Stuart Brierley have no competing interests in relation to the work contained within this study.

Author contributions:

SAHMRI: **M.B**: acquisition, analysis and interpretation of colonic afferent data and patch clamp data from colonic DRG neurons, drafting manuscript and figures, revisions for important intellectual content. **S.G.C**: acquisition, analysis and interpretation of QPCR from mouse and human DRG and single-cell RT-PCR from colonic DRG neurons, drafting manuscript and figures, revision for important intellectual content. **S.M.B**: conceptualization of colonic studies, interpretation of data, project administration, funding acquisition, writing, review and editing, resources, methodology, supervision. **University of Wollongong:** **A.R.B**: acquisition, analysis and interpretation of patch clamp data from adult mouse DRG neurons, drafting manuscript and figures, revisions for important intellectual content. **D.J.A**: conceptualization, interpretation of data, project administration, funding acquisition, writing, review and editing, resources, methodology, supervision.

All authors approved the final version of the manuscript; agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved; and all persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Table 1. Passive and active electrical properties of adult mouse DRG neurons in the absence (Control) and presence of either ω-conotoxin CVIE (100 nM) or ML297 (10 µM) applied alone or in combination with α-conotoxin Vc1.1 (1 µM).

	Control	ω-CVIE	ω-CVIE +Vc1.1	n	Control	ML297	ML297+Vc1.1	n
Rheobase (pA)	283.3 ± 88.2	350 ± 14.0	450 ± 122.5	6	232.5 ± 99.1	358.1 ± 123.7	408.8 ± 132.4	8
Input Resistance (MΩ)	401.7 ± 105.0	351.4 ± 107.3	298.4 ± 101.9	6	260.9 ± 42.1	237.3 ± 45.2	195.0 ± 44.5	8
RMP (mV)	-64.3 ± 2.9	-64.5 ± 2.9	-43.4 ± 23.3	6	-65.6 ± 3.4	-68.8 ± 3.6	-69.7 ± 3.5	8
AP (Hz)	8.7 ± 2.8	4.3 ± 1.7	2.7 ± 0.4	6	7.5 ± 1.9	3.0 ± 0.7	2.5 ± 0.3	8

Parameters analysed include resting membrane potential (RMP, mV), input resistance (Ri, MΩ), rheobase (pA), and action potential (AP) frequency (Hz) in response to a 500 ms depolarizing current step. Results are presented as mean ± SD, with the number of experiments indicated. Statistical significance was determined using one-way repeated measures ANOVA or the Friedman test (*$P < 0.05$).

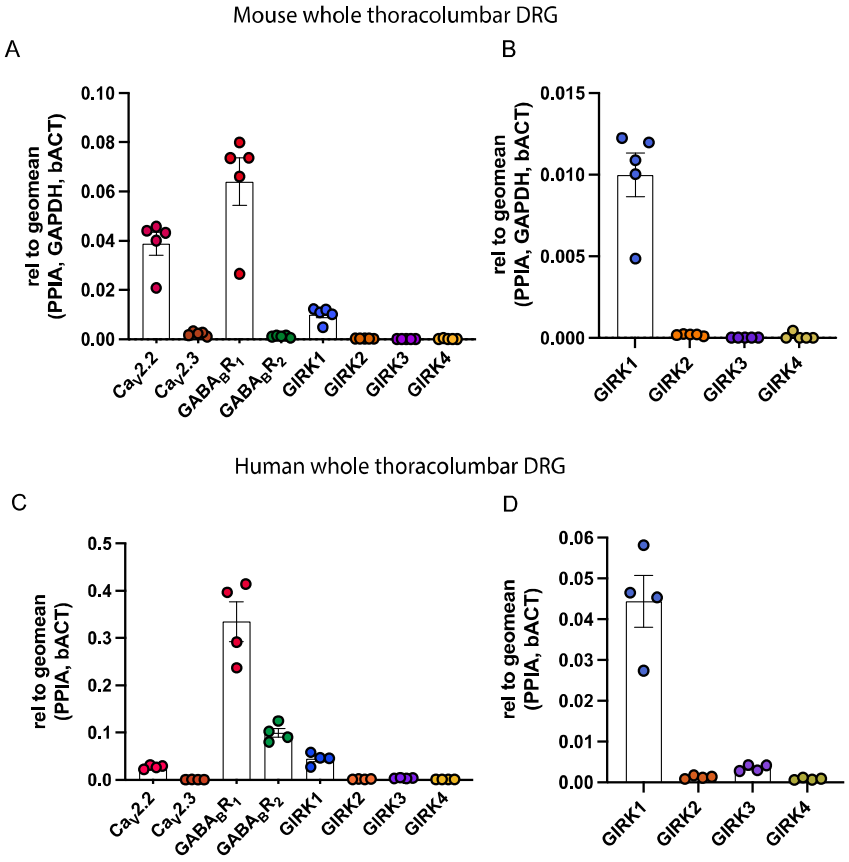


Figure 1: Expression patterns of $GABA_B$ receptors and associated ion channels in mouse and human thoracolumbar DRG (T10-L1). **(A)** Quantitative RT-PCR (QRT-PCR) analysis showing high mRNA abundance of $GABA_BR1$, $Ca_V2.2$, and GIRK1 in whole mouse thoracolumbar DRG. Data represent 5 individual mice, with each dot representing one mouse. **(B)** Inset of A, showing QRT-PCR analysis of GIRK1-4 mRNA expression in whole mouse thoracolumbar DRG (T10-L1). Results indicate low mRNA abundance for GIRK2-4. **(C)** QRT-PCR analysis of mRNA expression in whole human thoracolumbar (L1). Results show high expression of $GABA_BR1$, $GABA_BR2$, and GIRK1, while $Ca_V2.2$ is expressed at relatively lower levels compared to the abundant $GABA_BR1$ expression. **(D)** Inset of C, showing QRT-PCR analysis of GIRK1-4 in human L1 DRG, indicating low mRNA abundance of GIRK2-4. Data are from DRG samples of 4 human donors, with each dot representing an individual donor.

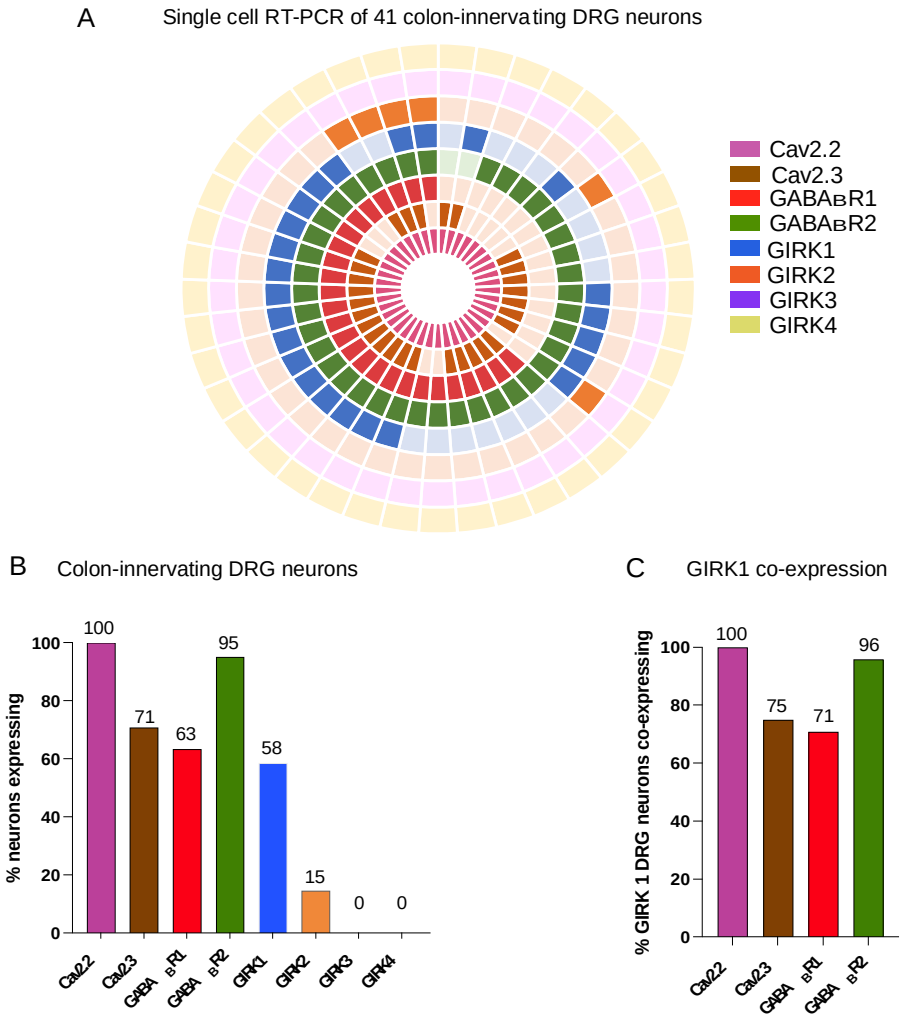


Figure 2: Expression and co-expression of ion channel and receptor genes in retrogradely traced colon-innervating mouse DRG neurons. (A) Donut plot illustrating the expression and co-expression of genes encoding $Ca_V2.2$, $Ca_V2.3$, $GABA_BR1$, $GABA_BR2$, and GIRK1-4 in 41 individual retrogradely traced colon-innervating DRG neurons (N=1 mouse). Each gene is represented by a distinct colour, with expression indicated by bold shading. GIRK4 is depicted in the outer ring, while the inner ring represents $Ca_V2.2$. Individual neurons are arranged radially, allowing for clear visualisation of gene co-expression within a single neuron from the outermost to the innermost rings. Some neurons expressed most targets, while others showed combinations of specific targets. (**B**) Single-cell RT-PCR analysis of retrogradely traced colon-innervating DRG neurons showing the percentage of colon-innervating DRG neurons expressing each transcript: $Ca_V2.2$ (100%), $Ca_V2.3$ (71%), $GABA_BR1$ (63%), $GABA_BR2$ (95%), GIRK1 (58%), GIRK2 (15%), with no detectable expression of GIRK3 or GIRK4. **(C)** Single-cell RT-PCR analysis highlighting the gene co-expression of GIRK1 with $Ca_V2.2$ (100%), $Ca_V2.3$ (75%), $GABA_BR1$ (71%), and $GABA_BR2$ (96%).

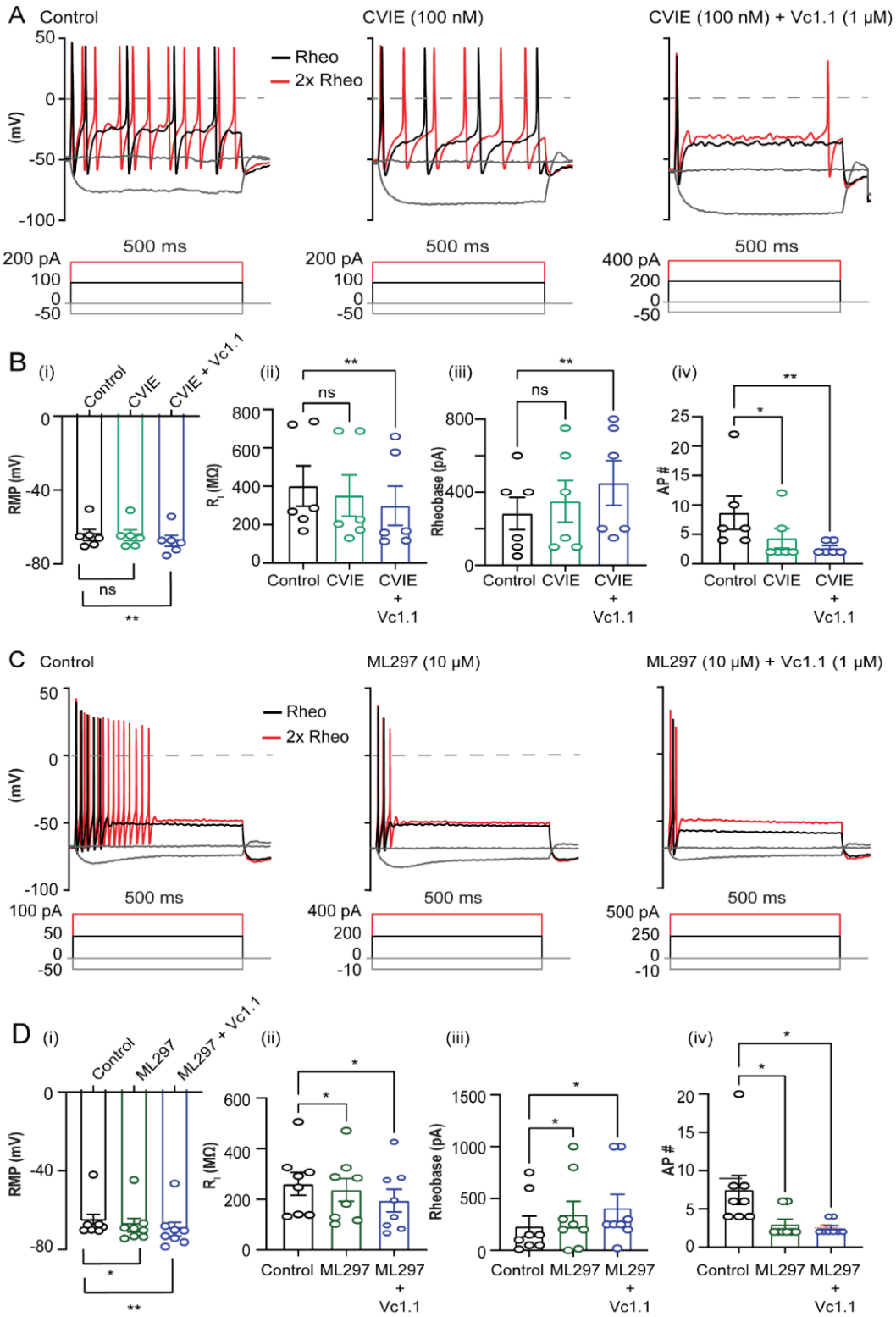


Figure 3: Effects of ω-conotoxin CVIE, ML297, and α-conotoxin Vc1.1 on the electrophysiological properties of adult mouse DRG neurons. (A) Representative voltage responses to current clamp steps recorded in a small diameter DRG neuron (23 μm) in the absence (control; **left**), and presence of CVIE (100 nM) (**middle**), and CVIE (100 nM) + Vc1.1 (1 μM)

(**right**). The dashed line indicates 0 mV. Rheobase (black) and 2x rheobase (red) are shown for both membrane potential and current. (**B**) Bar graphs summarising the effects of CVIE and CVIE + Vc1.1 on (**i**) resting membrane potential (RMP), (**ii**) input resistance (Ri), (**iii**) rheobase, and (**iv**) AP frequency (Hz) in response to 500 ms depolarizing current steps in small to medium diameter DRG neurons from adult mice. (**C**) Representative voltage responses to current clamp steps recorded in a mouse DRG neuron (24.5 μm) in the absence (control; **left**) and presence of ML297 (10 μM) (**middle**), and ML297 (10 μM) + Vc1.1 (1 μM) (**right**). The dashed line denotes 0 mV. Rheobase (black) and 2x rheobase (red) are shown for both membrane potential and current. (**D**) Bar graphs summarizing the effects ML297 and ML297 + Vc1.1 on (**i**) resting membrane potential (RMP), (**ii**) input resistance (Ri), (**iii**) rheobase, and (**iv**) AP frequency (Hz) in response to a 500 ms depolarizing current step at 2x rheobase in adult mouse DRG neurons (< 30 μm diameter). Data represent mean ± SEM, with the number of experiments indicated within each bar (n = 8). Statistical significance was determined using one-way repeated measures ANOVA or Friedman test, * $P < 0.05$.

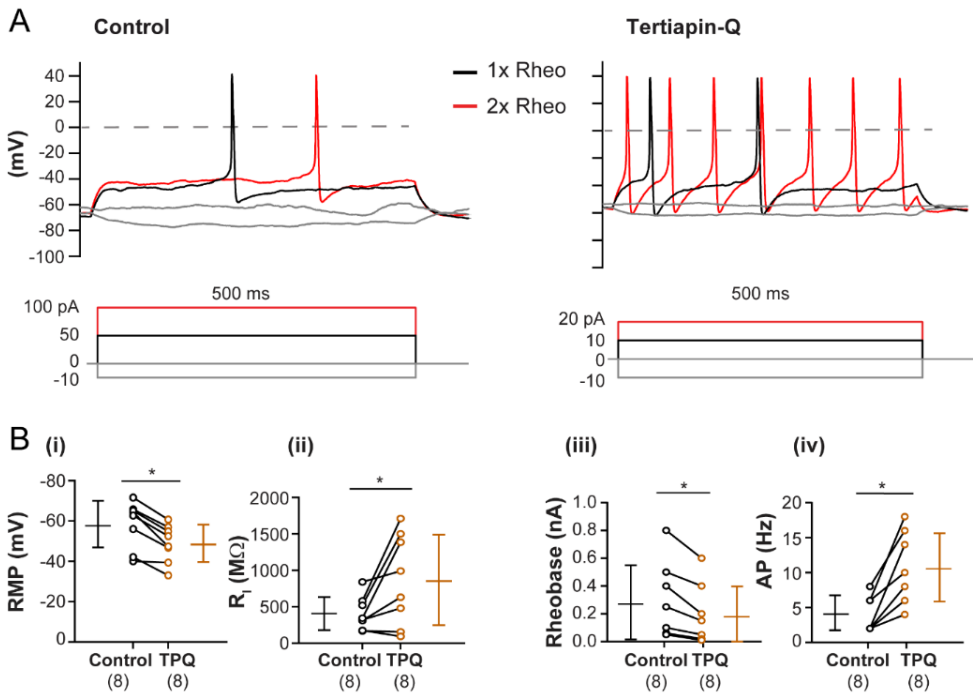


Figure 4: The GIRK channel inhibitor Tertiapin-Q (TPQ) enhances the excitability of adult mouse DRG neurons. (A) Representative voltage responses to current clamp steps recorded in a mouse DRG neuron (26 µm diameter) in the absence (control; **left**) and presence of 100 nM TPQ (**right**). The dashed line denotes 0 mV. Rheobase (black) and 2x rheobase (red) are shown for both membrane potential and current. **(B)** Paired scatter dot plots illustrating the effects TPQ (100 nM) on (**i**) resting membrane potential (RMP), (**ii**) input resistance (R_i), (**iii**) rheobase (nA), and (**iv**) AP frequency (Hz) in response to a 500 ms depolarizing current step at 2x rheobase in small to medium diameter (<30 µm) adult mouse DRG neurons. Data represent mean ± SD values, with the number of experiments indicated in parentheses. Statistical analysis was performed using a paired t-test or the Wilcoxon matched-pairs signed rank test; * $P < 0.05$.

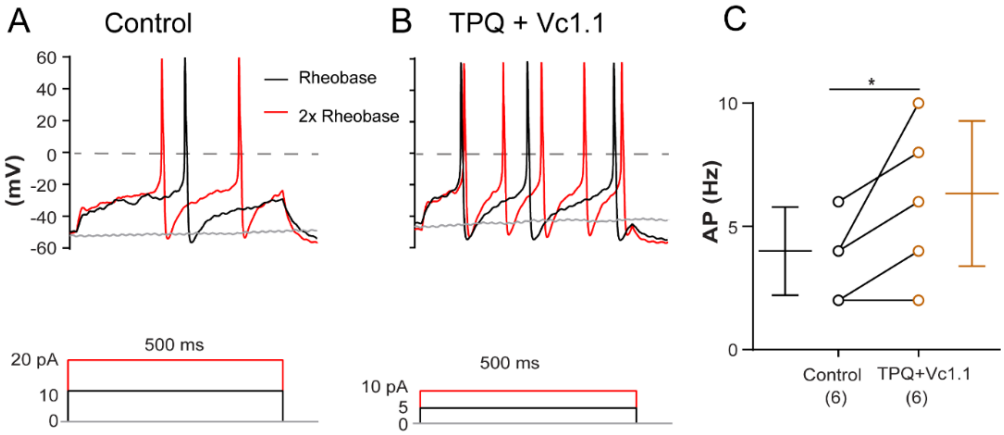


Figure 5: Lack of effect of α-conotoxin Vc1.1 on neuronal excitability in the presence of Tertiapin-Q (TPQ). (A, B) Representative voltage responses to current clamp steps recorded in a small to medium diameter mouse DRG neuron in the absence (control, A) and presence of 100 nM TPQ + 1 µM α-conotoxin Vc1.1 **(B)**. Insets show responses to 500 ms depolarizing current steps. The dashed line denotes 0 mV, while rheobase (black) and 2x rheobase (red) are indicated for both membrane potential and current. **(C)** Paired scatter dot plot showing action potential (AP) frequency (Hz) at 2x rheobase under control conditions and in the presence of TPQ (100 nM) + Vc1.1 (1 µM) in small to medium diameter (<30 µm) adult mouse DRG neurons. Data are presented as mean ± SD, with the number of experiments given in parentheses. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test, * $P < 0.05$.

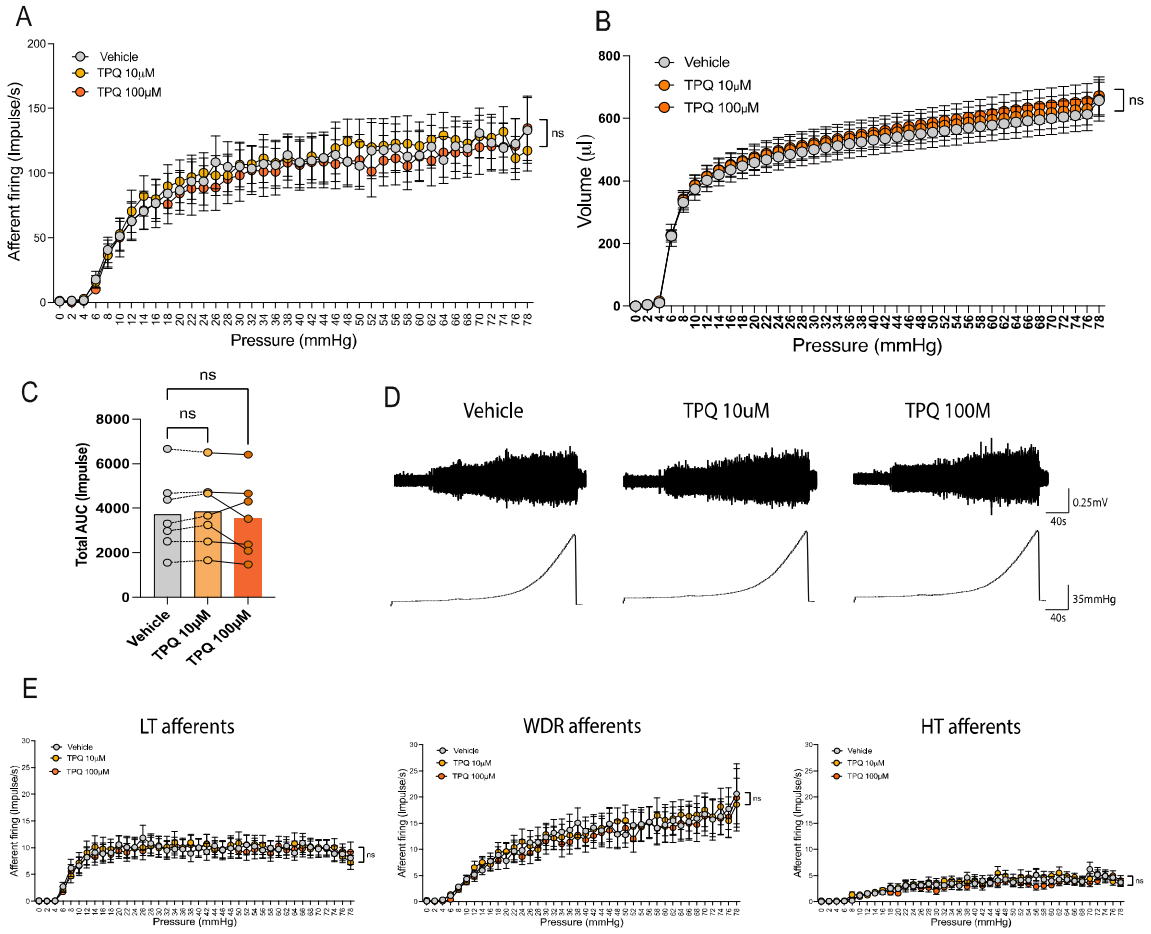


Figure 6: Lack of effect of Tertiapin-Q (TPQ) on pelvic afferent responses to colonic distension. (A) Group data showing pelvic afferent firing responses and colonic compliance **(B)** to increasing distension pressures in *ex vivo* preparations from healthy C57BL/6J mice (N = 7). Responses were recorded at baseline (vehicle-Krebs distension) and following intraluminal application of TPQ at 10 μM or 100 μM. **(C)** Comparison of the total AUC compared between vehicle and TPQ intraluminal application, showing no significant differences (ns, P > 0.05). **(D)** Representative traces of pelvic nerve afferent firing in response to colonic distension in the absence (Vehicle, left) and following TPQ application (10 and 100 μM, right). **(E)** Post-hoc single unit analysis of multiunit *ex vivo* colonic recordings. Pelvic nerve afferents were classified based on their discharge profile during ramp distension into LT (low threshold), WDR (wide dynamic range), or HT (high threshold). Total AUC for LT (n = 33), WDR (n = 25), and HT (n = 24) afferents during graded distension of the colon showed no significant effect of TPQ (10 μM or 100 μM). Data are presented as mean ± SEM. P-values are based on Two-way ANOVA (Šidak's multiple-comparisons test) or 1-way ANOVA (Friedman's multiple-comparisons test). N=7 mice.

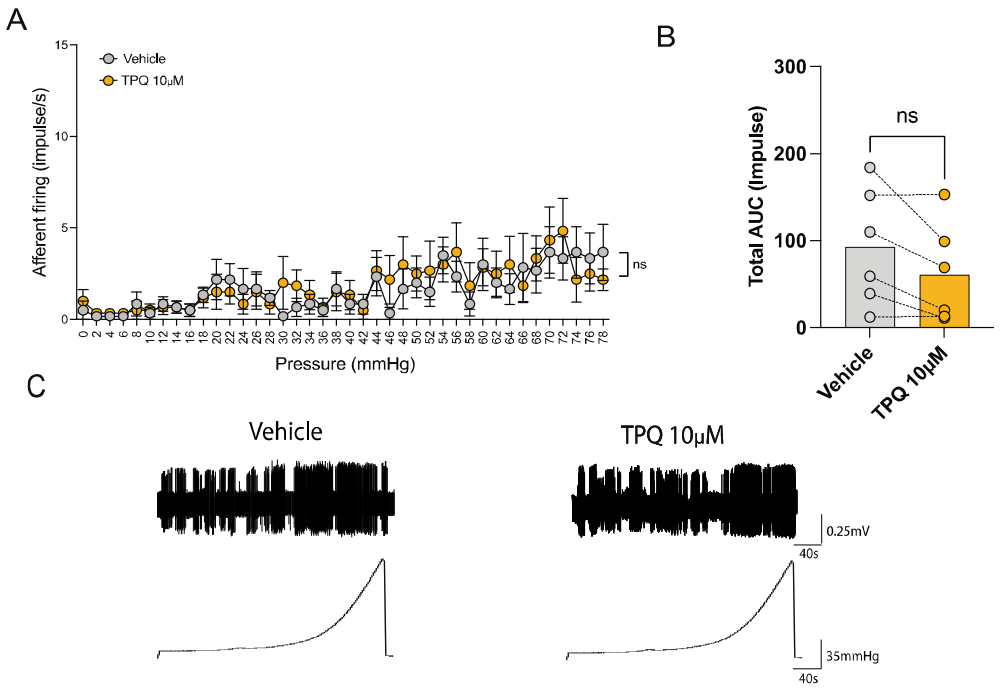


Figure 7: **Lack of effect of Tertiapin-Q on splanchnic afferent responses to colonic distension**. **(A)** Group data showing splanchnic nerve afferent firing responses to increasing colonic distension pressures in *ex vivo* preparations from healthy C57BL/6J mice (N = 6). Responses were recorded under baseline conditions (vehicle-treated Krebs distension) and following intraluminal application of TPQ (10 μM). **(B)** Comparison of the total AUC for afferent firing between vehicle-treated and TPQ-treated conditions, showing no significant differences (ns, P > 0.05). **(C)** Representative traces of splanchnic nerve afferent firing in response to colonic distension in the absence (Vehicle, left) and presence of TPQ (10 μM, right). Data are presented as mean ± SEM. Statistical analysis was performed using two-way ANOVA with Šidak's multiple-comparisons test or paired t-test.

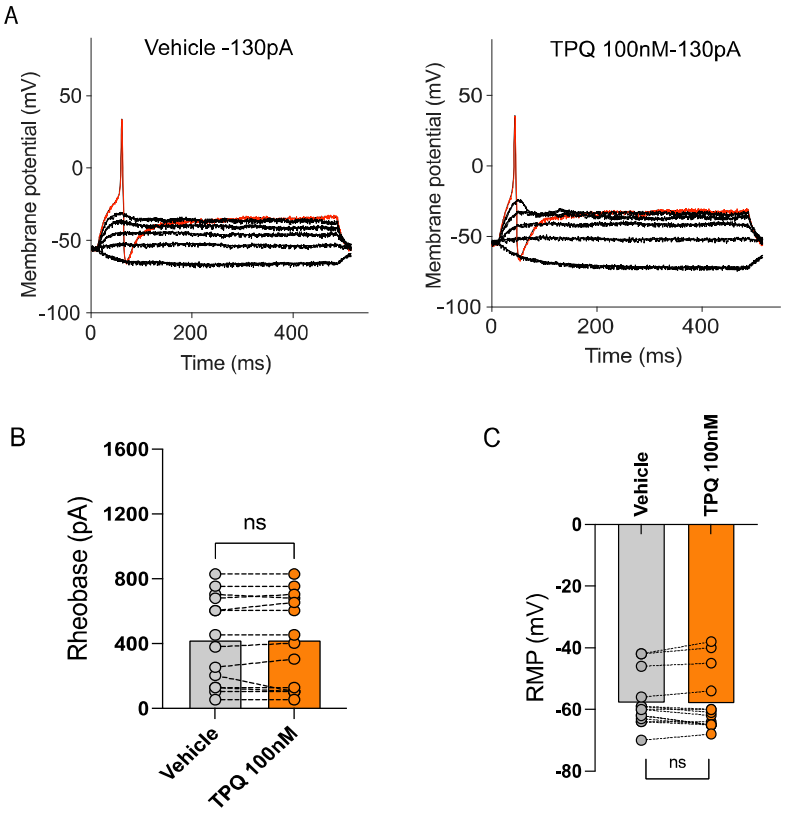


Figure 8: Lack of effect of Tertiapin-Q (TPQ) on the excitability of mouse colon-innervating DRG neurons in CVH states. (A) Representative voltage responses to current clamp steps recorded from a thoracolumbar (TL) DRG neuron in the absence (control) and presence of TPQ (100 nM). Rheobase is indicated in red (130 pA). **(B)** Bar graph showing the lack of effect of TPQ (100 nM) on rheobase or resting membrane potential (RMP) in colon-innervating DRG neurons. Data are presented as mean ± SEM. *P* values are based on paired t-tests. n = 14 neurons, N = 3 mice.

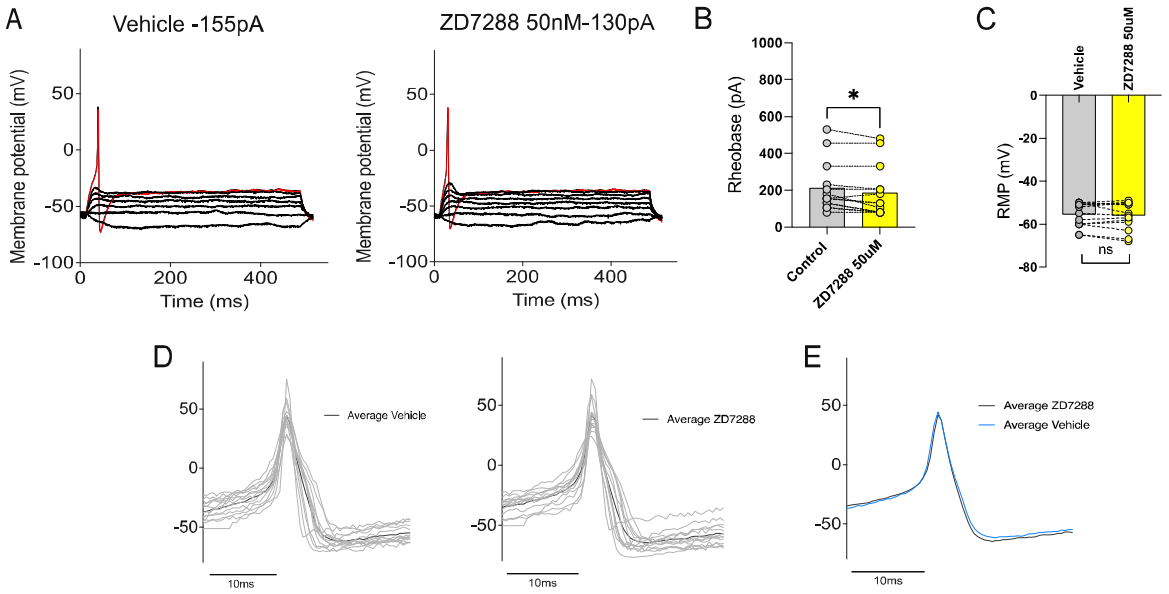
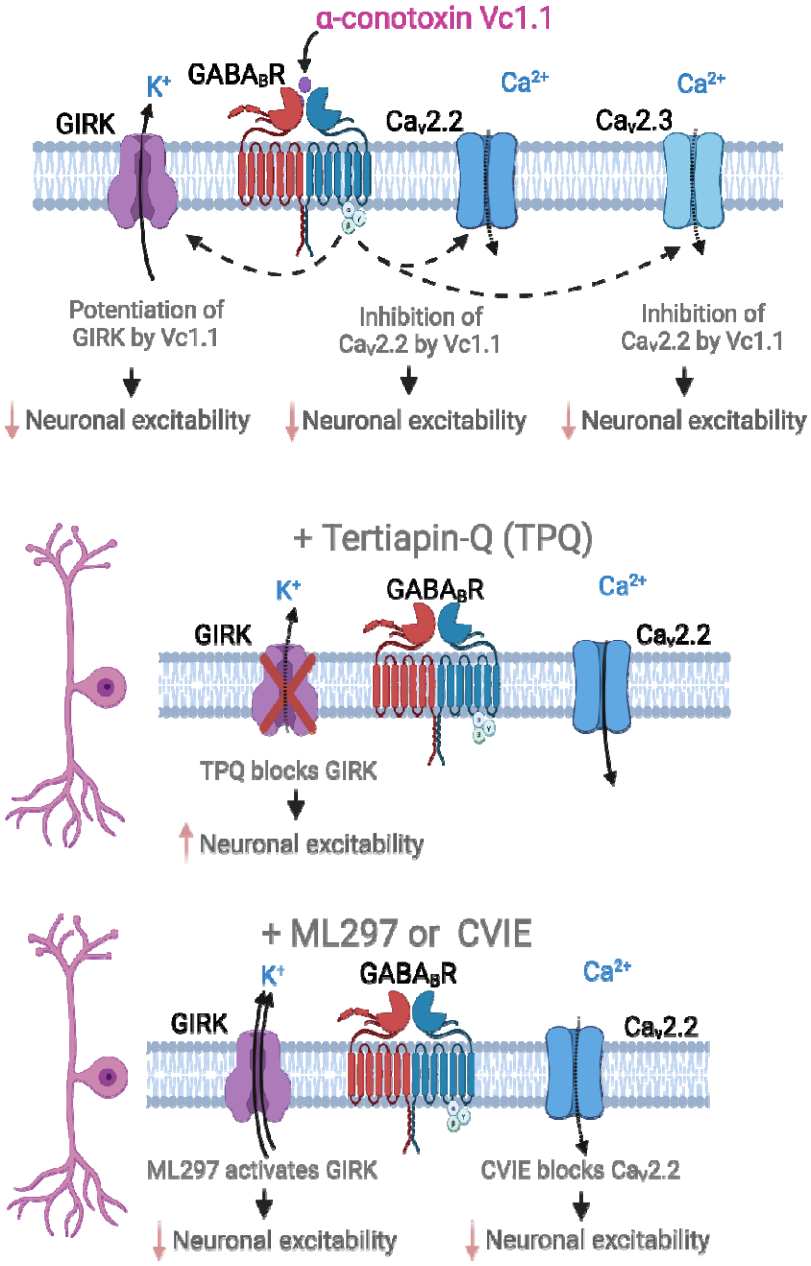


Figure 9: The HCN channel blocker ZD7288 enhances the excitability of mouse colon-innervating DRG neurons.

(A) Representative voltage responses to current clamp steps recorded in a colon-innervating DRG neuron in the absence (control, vehicle) and presence of ZD7288 (50 µM). Rheobase is indicated in red. **(B, C)** Bar graph showing the effects of ZD7288 on rheobase and resting membrane potential (RMP) in colon-innervating DRG neurons. Data are presented as mean ± SEM. P values were determined using a paired t-test. **(D)** Representative action potential (AP) traces recorded under each condition (grey), with the average waveform overlaid in black and aligned to peak depolarisation. **(E)** Overlayed average AP waveforms show no significant differences between vehicle (blue) and ZD7288 (black). n = 14 neurons, N = 3 mice.

FIGURE:



Whole-cell patch-clamp recordings from mouse sensory neurons demonstrated that α-conotoxin Vc1.1 significantly reduces neuronal activity by inhibiting $Ca_V2.2$ and $Ca_V2.3$ and potentiating GIRK channels in 'general' mouse dorsal root ganglion (DRG) neurons, highlighting its dual role in modulating sensory neuron excitability.

Inhibition of GIRK channels with the antagonist Tertiapin-Q (TPQ) increased neuronal excitability; however, this effect was not observed in DRG neurons that innervate the mouse colon.

Inhibition of high voltage-activated calcium channels ($Ca_V2.2$) by the $Ca_V2.2$ antagonist CVIE and activation of the G protein-coupled inwardly rectifying potassium (GIRK) channels by the agonist ML297 both contribute to a reduction in neuronal excitability in 'general' mouse DRG neurons. Figure created with Biorender