Received: 13 July 2018 | Revised: 13 December 2018 | Accepted: 2 January 2019

DOI: 10.1111/bph.14608

RESEARCH PAPER

Inhibition of $Ca_v3.2$ calcium channels: A new target for colonic hypersensitivity associated with low-grade inflammation

Elodie Picard[1,2] | Frederic Antonio Carvalho[1,2] | Francina Agosti[3] | Emmanuel Bourinet[3] | Denis Ardid[1,2] | Alain Eschalier[1,2] | Laurence Daulhac[1,2] | Christophe Mallet[1,2]

[1] Université Clermont Auvergne, INSERM, CHU, NEURO-DOL Basics & Clinical Pharmacology of Pain, F-63000 Clermont-Ferrand, France

[2] ANALGESIA Institute, Université Clermont Auvergne, F-63000 Clermont-Ferrand, France

[3] CNRS, INSERM, LABEX ICST, IGF, Université Montpellier, Montpellier, France

Correspondence
Christophe Mallet, Université Clermont Auvergne, INSERM, CHU, NEURO-DOL Basics & Clinical Pharmacology of Pain, F-63000 Clermont-Ferrand, France.
Email: christophe.mallet@uca.fr

Funding information
Institut National de la Santé et de la Recherche Médicale; Centre National de la Recherche Scientifique; Université Clermont Auvergne; Université de Montpellier; I-Site CAP 20-25; Labex; Agence Nationale de la Recherche, Grant/Award Number: ANR-15-CE16-0012-01; Société Nationale Française de Gastro-Entérologie (Pierre Dunand & Marie-Thérèse Chevalier Award); FRM

Background and Purpose: Abdominal pain associated with low-grade inflammation is frequently encountered in irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) during remission. Current treatments are not very effective and new therapeutic approaches are needed. The role of $Ca_V3.2$ channels, which are important in other chronic pain contexts, was investigated in a murine model of colonic hypersensitivity (CHS) associated with low-grade inflammation.

Experimental Approach: Low doses of dextran sulfate sodium (DSS; 0.5%) were chronically administered to C57BL/6j mice in drinking water. Their inflammatory state was assessed by systemic and local measures of IL-6, myeloperoxidase, and lipocalin-2 using ELISA. Colonic sensitivity was evaluated by the visceromotor responses to colorectal distension. Functional involvement of $Ca_V3.2$ channels was assessed with different pharmacological (TTA-A2, ABT-639, and ethosuximide) and genetic tools.

Key Results: DSS induced low-grade inflammation associated with CHS in mice. Genetic or pharmacological inhibition of $Ca_V3.2$ channels reduced CHS. Cav3.2 channel deletion in primary nociceptive neurons in dorsal root ganglia ($Ca_V3.2^{Nav1.8}$ KO mice) suppressed CHS. Spinal, but not systemic, administration of ABT-639, a peripherally acting T-type channel blocker, reduced CHS. ABT-639 given intrathecally to $Ca_V3.2^{Nav1.8}$ KO mice had no effect, demonstrating involvement of $Ca_V3.2$ channels located presynaptically in afferent fibre terminals. Finally, ethosuximide, which is a T-type channel blocker used clinically, reduced CHS.

Conclusions and Implications: These results suggest that ethosuximide represents a promising drug reposition strategy and that inhibition of $Ca_V3.2$ channels is an attractive therapeutic approach for relieving CHS in IBS or IBD.

1 | INTRODUCTION

The treatment of abdominal pain is an important clinical challenge. Abdominal pain is a common symptom of two major gastrointestinal disorders, namely, inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS). IBD, which includes ulcerative colitis and Crohn's disease, is characterized by chronic inflammation in the gastrointestinal tract and affects approximately 1.4 million persons in the United States and 2.2 million Europeans (Abraham & Cho, 2009; Ananthakrishnan, 2015; Xavier & Podolsky, 2007). IBD evolves with alternating active inflammatory periods and remission phases (Podolsky, 2002). Although inflammation is thought to cause visceral

Abbreviations: CHS, colonic hypersensitivity; CRD, colorectal distension; DRG, dorsal root ganglia; DSS, dextran sulfate sodium; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; i.t., intrathecal; KI, knock-in; KO, knockout; Lcn-2, lipocalin-2; MPO, myeloperoxidase; PFA, paraformaldehyde; TBS, tris-buffered saline; TTA-A2, T-type antagonist A2; VEH, vehicle; WT, wild type

 © 2019 The British Pharmacological Society

pain during the active phase, patients continue to feel pain during quiescent periods when inflammation is reduced (low-grade inflammation; Long & Drossman, 2010; Piche et al., 2010; Stacher & Christensen, 2006). IBS is a functional gastrointestinal disorder (Chey, Kurlander, & Eswaran, 2015) characterized by the Rome IV criteria (Mearin et al., 2016). IBS affects 10–20% of the population in developed countries (Lovell & Ford, 2012) and is frequently associated with low-grade inflammation (Guilarte et al., 2007; Halpin & Ford, 2012; Piche et al., 2010). Colonic hypersensitivity (CHS) is another feature exhibited by most patients with IBD and IBS (Lembo et al., 1999). Chronic abdominal pain reduces the quality of life of both IBD (Gracie et al., 2017; Zeitz et al., 2016) and IBS (Halpin & Ford, 2012; Nellesen, Yee, Chawla, Lewis, & Carson, 2013) patients. Current therapies aim to reduce inflammation in IBD and motility disturbance in IBS. In both cases, therapy involves analgesics or antidepressant drugs. However, these therapies are not consistently effective and are associated with adverse effects (Camilleri & Boeckxstaens, 2017).

We sought to determine whether inhibition of T-type calcium channels could offer a new pharmacological strategy for reducing visceral pain and whether the channels involved were peripherally and/or centrally located. These calcium channels are major contributors to nociceptive signalling pathways (Snutch & Zamponi, 2018). This channel family comprises **$Ca_V3.1$**, **$Ca_V3.2$**, and **$Ca_V3.3$**. The $Ca_V3.2$ isoform is expressed along nociceptive pathways, including peripheral nociceptive nerve endings, the dorsal root ganglia (DRG; François et al., 2015), and presynaptic (Jacus, Uebele, Renger, & Todorovic, 2012) and postsynaptic terminals of nociceptive fibres in the spinal cord. In addition, $Ca_V3.2$ channels have emerged as a relevant target for chronic pain states, such as painful post-traumatic or diabetic neuropathy (Bourinet et al., 2005; García-Caballero et al., 2014; Obradovic et al., 2014). These channels also contribute to butyrate-induced CHS in rats (Marger et al., 2011). To further investigate the involvement of $Ca_V3.2$ channels, we used both pharmacological and genetic approaches to study their influence on CHS and the location of the channels involved.

After validation of a mouse model combining CHS and low-grade inflammation reflecting the clinical observations of IBS or IBD during the remission phase, this analysis (a) demonstrates the functional involvement of $Ca_V3.2$ channels in CHS and the major role of the channels located at the spinal afferent fibre terminals, (b) opens up new pharmacological routes based on the inhibition of these channels, and (c) argues for the prompt evaluation of an already marketed drug **ethosuximide**, which is a T-type channel blocker used clinically as an antiepileptic, as a novel pharmacological treatment for pain in IBS patients.

What is already known

- The available treatments used to relieve colonic hypersensitivity have limited efficacy or adverse side effects in most patients.

What this study adds

- It provides evidence that $Ca_V3.2$ channels, localized in C-fibres at the presynaptic level in the spinal dorsal horn, are involved in colonic hypersensitivity associated with local low-grade inflammation.

What is the clinical significance

- It suggests that $Ca_V3.2$ inhibition is an attractive therapeutic approach for relieving colonic hypersensitivity.

2 | METHODS

2.1 | Animals

All animal care and experimental procedures were approved by the local ethical committees (CEMEA Auvergne; ref 8143), performed according to European legislation (Directive 2010/63/EU) regarding the protection of animals used for scientific purposes, and complied with the recommendations of the International Association for the Study of Pain (Zimmermann, 1983). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010; Karp et al., 2015; McGrath & Lilley, 2015) and with the recommendations made by the *British Journal of Pharmacology*. All animals were housed under standard laboratory conditions (12-hr light/dark cycle, temperature 21–22°C, 55% humidity under specific pathogen-free conditions). Food and water were available ad libitum.

Eight-week-old male C57BL/6j mice purchased from Janvier Laboratories (Le Genest-Saint-Isle, France, RRID:IMSR_JAX:000664), eight-week-old C57BL/6j Cav3.2 knockout (KO) male mice originally generated by Chen et al. (2003), and their wild-type (WT) littermates were used. We also used the Cav3.2-GFP-flox knock-in (KI) mice generated by François et al. (2015), which have in the cacna1h gene, a modified exon 6 with an in-frame insertion of the ecliptic GFP sequence and flanking loxp sites to permit the GFP labelling of $Ca_V3.2$ channels and their conditional KO on Cre recombinase action. These mice enabled the design of a conditional KO specifically in small-diameter DRG neurons by crossing the KI mice with mice expressing Cre recombinase under the $Na_V1.8$ promoter (François et al., 2015; Stirling et al., 2005). These mice were designated $Cav3.2^{Nav1.8}$ KO, and their WT littermates were also used. As genetically modified animals were only available in mice, this species was used for this study.

2.2 | Low-grade inflammation associated with CHS mouse model

The mice received 0.5% dextran sulfate sodium (DSS; MW 36–50 kDa) in autoclaved drinking water ad libitum for 12 days. The control mice received only plain water, and these mice were designated water-treated animals.

2.3 | Colorectal distension

Colorectal distension (CRD) was performed using the non-invasive manometric method described by Larauche et al. (2009). A miniaturized pressure transducer catheter (model 600; Millar Instruments, Houston, TX) equipped with a custom-made balloon (1 cm wide × 2 cm long) prepared from a polyethylene plastic bag was used to create a "balloon-pressure sensor," which was calibrated at the beginning of each experiment. On the day of the experiment, the mice were accustomed to the holding device for 1 hr before the CRD. Then, the mice were briefly anaesthetized (2% isoflurane), and the balloon was inserted into the rectum such that the distal end of the balloon was 1 cm from the anal margin. Subsequently, the animals were placed in homemade restriction cages, tape-maintained on the tail, and allowed to recover for 30 min prior to CRD. The balloon, which was coupled to a pressure sensor, was connected to an electronic barostat (Distender Series II, G&J Electronics, Toronto, ON, Canada) and a preamplifier (PCU-2000 Dual Channel Pressure Control Unit, Millar Instruments) connected to the PowerLab interface. The barostat enabled the control of the balloon pressure and minimized any interference of colonic motor activity changes during balloon inflation. The signal was acquired and analysed using LabChart 7 software (ADInstruments, Paris, France, RRID:SCR_001620). The distension protocol consisted of a set of increasing distension pressures (20, 40, 60, 80, and 100 mmHg), each of which was repeated twice, which was applied for 20 s with a 4-min inter-pressure interval. The "treated signal" trace of intracolonic pressure variations was extracted from the original "raw signal" recorded by applying the method previously described by Larauche et al. (2009; Supporting Information Figure S1). Briefly, the "treated signal" trace allows for excluding the slower, tonic changes in the "raw signal" resulting from transit colonic contraction. Based on the "treated signal," the response of the animals (to each distension pressure) was calculated by subtracting the AUC of the trace during the 20 s of distension from the AUC of the trace corresponding to the 20 s preceding the distension and is expressed as the intracolonic pressure variation.

2.4 | Biological samples

Faeces were collected before and after the DSS treatment period to assay faecal lipocalin-2 (Lcn-2), which is a marker of low-grade inflammation (Chassaing et al., 2012). After the DSS treatment period and CRD, the mice were anaesthetized with an i.p. injection of a ketamine-xylazine mixture (100 and 10 mg·kg^{-1} in saline solution 0.9% NaCl) and bled from the retro-orbital venous plexus (a sample of serum was collected). Then, the mice were perfused via the ascending aorta with saline buffer, followed by 2% paraformaldehyde (PFA) in 0.1 M of Tris-buffered saline (TBS). The spleen and colon were removed before administering the PFA and their weights and lengths were measured. To evaluate the inflammatory state in the mice, a small sample (50 mg) of proximal colon was collected to assay **myeloperoxidase (MPO)** and **IL-6**.

2.5 | Enzyme-linked immunosorbent assay

All assays were performed according to the manufacturer's protocol.

2.5.1 | Faecal Lcn-2

The faecal samples were reconstituted in PBS containing 0.1% Tween 20 (100 mg·ml^{-1}) and vortexed for 20 min to form a homogenous faecal suspension. These samples were then centrifuged for 10 min at 17,115× *g* and 4°C. The clear supernatants were collected, and the Lcn-2 levels in the supernatants were measured using a DuoSet murine Lcn-2 ELISA kit (Cat. #DY1857, R&D Systems, Minneapolis, MN, Canada).

2.5.2 | Serum IL-6

After the collection of 250 μL of blood, the samples were centrifuged at 2,841× *g* for 30 min, and the supernatant corresponding to the serum was collected. IL-6 was quantified using commercially available ELISA kits (Cat. #DY406, R&D Systems).

2.5.3 | Colonic IL-6 and MPO

The colon samples were first homogenized in lysis buffer (50 mM of Tris–HCl, pH 7.4, 0.5 mM EDTA [Sigma-Aldrich, St. Louis, MO], 1% Triton X-100, 0.5 mM of phenylmethylsulfonyl fluoride [Sigma-Aldrich], 20 μM of leupeptin [Merck, Darmstadt, Germany], 100 IU·ml^{-1} of aprotinin [Sigma-Aldrich] in DMSO) and then sonicated for 10 s. The samples were centrifuged at 2,841× *g* at 4°C for 45 min. The supernatants were collected, and the total protein concentration was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). IL-6 and MPO were quantified using commercially available ELISA kits (Cat. #DY406 and #3667, respectively, R&D Systems).

2.6 | Retrograde labelling and immunofluorescence analysis

The antibody-related procedures used here comply with the recommendations made by the *British Journal of Pharmacology*. A midline laparotomy was carried out in mice anaesthetized with a ketamine/xylazine mixture (100 and 10 mg·kg^{-1} in saline solution 0.9% NaCl, respectively). Twenty microlitres of either the fluorescent retrograde neuronal tracer Fluorogold for an immunofluorescence analysis (4% in saline, hydroxystilbamidinebismethanesulfonate, Sigma-Aldrich, Germany) or dicarbocyanine dye 1.1′-dioctadecyl-3,3,3′,3-tetramethylindocarbocyanine-methane-sulfonate for an electrophysiological analysis (DiI, 50 mg·ml^{-1} in DMSO Interchim, Montluçon, France) were percutaneously injected circumferentially into the distal colon wall of the $Ca_V3.2$-GFP KI mice with a 30-gauge needle for the colonic nociceptor labelling. The overlying muscle and skin were sutured closed. After 6 days, the mice received DSS (or water) for 12 days. The retrolabelled mice were

anaesthetized on Day 12 post-DSS with a ketamine/xylazine mixture (100 and 10 mg·kg^{-1} in saline solution 0.9% NaCl, respectively) and perfused via the ascending aorta with saline buffer, followed by 2% PFA diluted in TBS. The DRGs were dissected from the perfused mice and fixed in TBS containing 2% PFA at room temperature (RT) for 60 min. Then, the tissues were washed three times for 10 min with TBS. The samples were embedded in 4% agarose (Ultrapure LMP agarose, Invitrogen, France) and sectioned at 30 µm using a vibratome (Leica VT1000S, Leica Microsystèmes, Nanterre, France). The free floating sections were blocked with TBS containing 0.05% Tween 20 (TBST), 0.2% Triton X-100, and 10% normal donkey serum at RT for 1 hr. The tissue sections were incubated with a primary antibody (1:500, chicken anti-GFP, Invitrogen; RRID: AB_2534023) diluted in blocking solution overnight at room temperature. The sections were washed four times with TBST, incubated with a secondary antibody (1:500, CF488A-conjugated donkey anti-chicken antibody, Sigma, France; RRID:AB_2721061) diluted in blocking solution at room temperature for 1 hr, washed again four times with TBST, placed on Superfrost slices, and mounted with Dako fluorescent mounting medium (Agilent Technologies, Les Ulis, France). The image acquisition was performed under a Zeiss LSM 800 microscope using the acquisition software Zen® (Carl Zeiss, Marly le Roi, France). The quantification of colonic nociceptor expressing $Ca_V3.2$ was carried out with IMARIS® software (Bitplane, Concord, MA; RRID:SCR_007370). The antibody-related procedures used comply with the recommendations made by the *British Journal of Pharmacology*.

2.7 | Electrophysiological recording

The fluorescent tracer DiI was injected into the distal colon as described above. After the DSS/water treatment, the DRG neurons were prepared as previously described (François et al., 2015). The DRG cells were treated with 2 mg·ml^{-1} of collagenase (Boehringer Mannheim, Paris, France) for 40 min at 37°C, washed with Neurobasal A/10% B27, and suspended in 2 ml of Neurobasal B27 supplemented with GlutaMax and 25 ng·ml^{-1} of nerve growth factor (NGF 7S, Thermo Fisher Scientific), 10 ng·ml^{-1} of neurotrophin 4 (Peprotech, Neuilly-Sur-Seine), and 2 ng·ml^{-1} of glial-derived neurotrophic factor (Thermo Fisher Scientific). The single-cell suspensions were obtained by three passages through three needle tips of decreasing diameter (gauges 18, 21, and 26) as previously described (Francois et al., 2013). The cells were plated in polyornithine/laminin-coated dishes and kept at 37°C until use. The whole-cell patch-clamp recordings were performed 3–16 hr after plating with an Axopatch 200B amplifier interfaced with a Digidata 1440 acquisition board and pClamp 10 software suite (Molecular devices, San Jose, CA; RRID: SCR_011323) in the voltage clamp mode. For the calcium current recordings, the extracellular solution contained the following (in mM): 2 $CaCl_2$, 10 TEACl, 2 NaCl, 1 $MgCl_2$, 130 choline Cl, 10 glucose, 5 4AP, and HEPES 10 (pH adjusted to 7.4 with TEAOH 300 mOsm). Pipettes with a resistance of 1–1.5 MΩ were filled with an internal solution containing the following (in mM): 110 CsCl, 3 $MgCl_2$, 10 EGTA, 10 HEPES, 3 Mg-ATP, and 0.6 GTP (pH adjusted to 7.4 with CsOH, 300 mOsm). There was no compensation for the series resistance. The cells were identified under an inverted microscope (IX 70 Olympus) using adapted filter sets to visualize the GFP and DiI fluorescence immediately prior to the electrophysiological recordings. The recorded images of each electrophysiologically analysed cell were obtained under the bright field, GFP, and DiI channels using a CCD camera connected to the microscope and controlled by Metamorph software (Molecular devices; RRID:SCR_002368).

2.8 | Experimental protocol used to evaluate the drug effects

The animals were randomly divided into five to 12 mice per group. The randomized treatment administrations were performed according to the method of equal blocks to simultaneously assess the effect of different treatments and avoid unverifiable and time-variable environmental influences. All experiments were performed in a quiet room by the same blinded experimenter. To ensure the methodological quality of this study, the details are reported in the Supporting Information, as recommended by Rice et al. (2008). The group size variations are due to reaching the endpoint during the DSS treatment in some rare cases and mostly to technical issues occurring during CRD that did not permit the re-use of the mice.

The acute pharmacological treatments were performed on Day 12 after the DSS administration. The colonic sensitivity was evaluated using a CRD test, and then the animals received an s.c. injection of vehicle (VEH, corresponding to the solution used to dissolve the active molecule, specific to each blockers) or various blockers of $Ca_V3.2$ channels (the T-type channel antagonist A2 [**TTA-A2**; 1 mg·kg^{-1}, 10 ml·kg^{-1}, dissolved in 0.9% NaCl saline solution, 10% DMSO, and 10% Tween80], ethosuximide [200 mg·kg^{-1}, 10 ml·kg^{-1}, dissolved in saline solution 0.9% NaCl], the peripherally acting $Ca_V3.x$ channel blocker **ABT-639** [10, 30, and 100 mg·kg^{-1}, 10 ml·kg^{-1}, CliniScience, Nanterre, France, dissolved in 10% DMSO in saline solution 0.9% NaCl]) or **morphine** (1 mg·kg^{-1}, 10 ml·kg^{-1}, dissolved in saline solution 0.9% NaCl) as a positive control. Another CRD test was performed 30 min after these injections to evaluate the drug treatment effect. ABT-639 was also administered intrathecally (i.t.) to evaluate its spinal analgesic effect (10 µg/5 µl). The mice were briefly anaesthetized by isoflurane (2.5%) during the i.t. injections. The ABT-639 doses were chosen according to the results obtained by our team in a study investigating several dose–effect relationships in another pain model (unpublished results). The second CRD tests were performed 10 min after the injection in this case due to the rapid effect of the spinal drug administration. All procedures were performed without removing the "balloon-pressure sensor" from the mice.

The repeated pharmacological treatments were performed from Days 6 Day 12 after the DSS administration. The mice received three i.p. injections·per day of ethosuximide (200 mg·kg^{-1}, 10 ml·kg^{-1}) or VEH (saline solution 0.9% NaCl, 10 ml·kg^{-1}). The effect of this drug

treatment on the colonic sensitivity was evaluated by a CRD test on Day 12 after the DSS administration.

2.9 | Data and statistical analysis

The data and statistical analysis comply with recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2015, 2018). The statistical analysis was performed using Prism 7 software, La Jolla, CA (GraphPad Prism, RRID:SCR_002798). All data are expressed as the mean ± SEM. The normality of the data distribution was tested by a KS normality test, D'Agostino and Pearson omnibus normality test, and Shapiro–Wilk normality test, and parametric and non-parametric tests were performed to analyse the normally and non-normally distributed data respectively. Non-parametric tests were used when the number of animals per experimental group was less than 10 because the results of the normality tests were not fully reliable under these conditions.

For the CRD analysis, we used a two-way ANOVA (pressure of distension and treatment), followed by a Bonferroni post hoc test for multiple comparisons. For other analyses comparing more than two groups, we used a one-way ANOVA, followed by a Bonferroni post hoc test for multiple comparisons, and when only two groups were compared, we used Mann-Whitney test. Post hoc tests were only conduced when the F value achieved the necessary level ($P < 0.05$) and there was no significant variance inhomogeneity. The data were considered statistically significant at $P < 0.05$.

2.10 | Materials

The DSS used here was supplied by MP Biomedicals formerly ICN Biomedicals, Illkirch, France; **TTA-A2** was supplied by Alomone labs, Jerusalem, Israel; ABT639 by CliniScience, Nanterre, France; ethosuximide by Interchim, Montluçon, France, and morphine by Tocris Bioscience, Bristol, UK.

2.11 | 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 (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander, Striessnig et al., 2017; Alexander, Fabbro et al., 2017).

3 | RESULTS

3.1 | DSS (0.5%) induced low-grade inflammation associated with CHS in mice

To study these gastrointestinal disorders, we first developed a CHS murine model associated with low-grade inflammation using a low dose of DSS. Compared to the control mice receiving plain water, the mice ingesting 0.5% DSS in drinking water, for 12 days, exhibited a significant increase in their responses to CRD at pressures of 80 and 100 mmHg (Figure 1a). This observation is characteristic of colonic CHS. Colon shortening and spleen enlargement are generally correlated with the extent of robust colitis (Chassaing, Aitken, Malleshappa, & Vijay-Kumar, 2014; Hendrickson, Gokhale, & Cho, 2002). In the DSS-treated animals, no significant changes in these anatomical parameters (colon or spleen weights) were observed (Figure 1b and Supporting Information Figure S2). No significant modification in serum IL-6 was found (Figure 1c, left), demonstrating the absence of severe inflammation in this model. The colonic levels of IL-6 and MPO were also not significantly changed (Figure 1c, middle and right), further highlighting the absence of local inflammation in the model. In contrast, a significant increase in the level of the low-grade inflammatory marker Lcn-2 in the faeces (Chassaing et al., 2012) was found on Day 12 in the mice receiving DSS (Figure 1d). These results confirm that long-term treatment with 0.5% DSS is a relevant model of the CHS associated with low-grade inflammation encountered in IBS or IBD during the remission phase.

3.2 | $Ca_V3.2$ calcium channels were functionally involved in CHS

To determine the role of $Ca_V3.2$ channels in CHS associated with low-grade inflammation, we genetically and pharmacologically inhibited $Ca_V3.2$ channels . The overall genetic inhibition of $Ca_V3.2$ channels in constitutive KO animals did not modify the increase in the faecal Lcn-2 level (Supporting Information Figure S3), suggesting that these channels do not contribute to low-grade inflammation. However, the DSS-induced hypersensitivity was reduced in the $Ca_V3.2$ KO mice, while this deletion had no influence under the non-pathological condition (water-treated animals; Figure 2a). This result was confirmed by pharmacological intervention. TTA-A2, which is a T-type channel blocker, had no effect on the control animals drinking plain water (Figure 2b, left) but completely suppressed DSS-induced CHS (Figure 2b, right). Similarly, an acute injection of ethosuximide significantly reduced the DSS-induced CHS (Figure 3a). Because ethosuximide is not specific for $Ca_V3.2$ channels (Gören & Onat, 2007), we compared its effects on $Ca_V3.2$ KO and littermate mice treated with DSS. Ethosuximide was as effective as morphine on CHS in the littermate mice, and in contrast to morphine, ethosuximide failed to induce any change in sensitivity in the KO mice (Figure 3b,c), demonstrating that ethosuximide-induced analgesia is dependent on $Ca_V3.2$ channels. Interestingly, three injections of ethosuximide·per day for 6 days also induced a marked decrease in CHS, assessed 24 hr after the final injection (Figure 3d).

3.3 | Deletion of $Ca_V3.2$ channels from primary nociceptive neurons in the DRG abolished the DSS-induced CHS

Next, we assessed the function of $Ca_V3.2$ channels located in primary afferent neurons in CHS. Thus, we used mice with a conditional $Ca_V3.2$ KO in primary afferent C-fibres (François et al., 2015) as

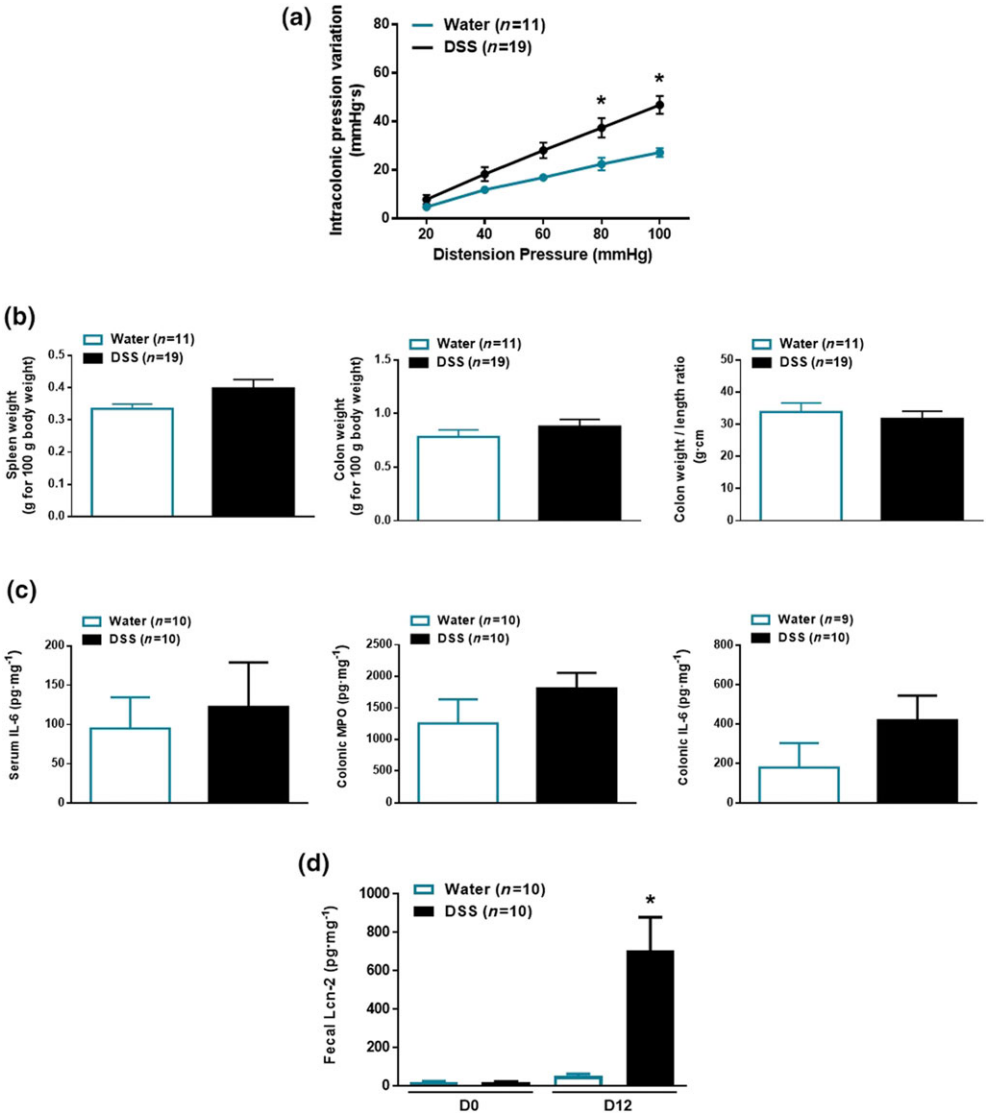

FIGURE 1 Dextran sodium sulfate (DSS; 0.5%) induced low-grade inflammation associated with colonic hypersensitivity in mice. Wild-type mice received DSS in drinking water or plain water for 12 days. (a) Intracolonic pressure variation in response to colorectal distension (CRD) to evaluate colonic sensitivity. (b) Measurement of the following anatomical parameters of biological samples collected on Day 12 (D12) after CRD: spleen weight per 100 g of body weight, colon weight per 100 g of body weight, and colon weight per length. (c) Inflammatory process as evaluated by the levels of serum IL-6, colonic IL-6, and myeloperoxidase (MPO). (d) Low-grade inflammation as evaluated by the levels of faecal lipocalin-2 (Lcn-2) on both D0 and D12. The data are presented as the mean ± SEM. *$P < 0.05$, significantly different from water; in (a), two-way ANOVA (treatment; distension pressure) followed by a Bonferroni post hoc test; in (b) and (c), one-way ANOVA followed by a Mann–Whitney test; in (d), one-way ANOVA followed by a Bonferroni post hoc test

recently described. These mice were obtained by mating the $Na_v1.8$-Cre KI strain (Stirling et al., 2005) with the Cav3.2 GFP flox line and were designated $Ca_V3.2^{Nav1.8}$ KO mice. These mice showed normal colonic sensitivity when drinking plain water (Figure 4a). However, compared with their littermates, the $Ca_V3.2^{Nav1.8}$ KO mice showed a significantly decreased response to CRD after the DSS treatment (Figure 4a). These results suggest that C-fibres containing $Ca_V3.2$ channels control the induction of CHS.

To determine whether this induction is due to the up-regulated expression of $Ca_V3.2$ channels in sensory neurons, immunohistochemistry was performed to visualize these channels in lumbar DRG sections (Figure 4b,c). To specifically study $Ca_V3.2$ channels located in DRG neurons innervating the colon, injections of the fluorescent dye Fluorogold were administered beforehand into the colon wall of the $Ca_V3.2$ -GFP-flox KI mice. The GFP immunoreactivity could be visualized in a fraction of the Fluorogold-positive neurons and indicated a tendency towards a higher prevalence at the lumbosacral level. In the water and DSS-treated animals, GFP immunoreactivity was detected in the DRG colonic neurons. However, no difference in the number of colonic sensory neurons expressing $Ca_V3.2$-GFP at the thoracic or lumbosacral levels was observed between the DSS and water-treated animals (Figure 4b,c).

As immunohistochemistry is not sufficiently specific to determine the levels of functional $Ca_V3.2$-GFP at the cell plasma membrane, we

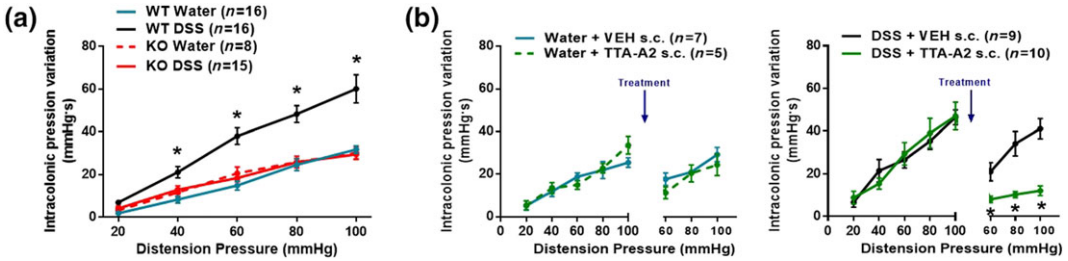

FIGURE 2 $Ca_V3.2$ channels PKC were functionally involved in colonic hypersensitivity. Groups of mice received dextran sodium sulfate (DSS) or water for 12 days. Colonic sensitivity was evaluated on Day 12 (D12) by colorectal distension (CRD). The effect of acute pharmacological treatment was assessed by first submitting the mice to a CRD set with pressures ranging from 20 to 100 mmHg; then, the pharmacological drug was administered by an s.c. injection, and after 30 min, another CRD set with pressures ranging from 60 to 100 mmHg was performed to evaluate the effect of the drug. (a) Responses of wild-type (WT) and knockout (KO) $Ca_V3.2$ mice. (b) Effect of T-type antagonist A2 (TTA-A2; 1 mg·kg^{-1}, 10 ml·kg^{-1}, s.c.) or vehicle (VEH: NaCl, 10% DMSO, 10% Tween 80, AD 10 ml·kg^{-1}, s.c.) on CRD in WT mice receiving water (left) or DSS (right). The data are presented as the mean ± SEM. In (a), *P < 0.05, significantly different from WT water; two-way ANOVA (groups; distension pressure) followed by a Bonferroni post hoc test. In (b), *P < 0.05, significant differences between the TTA-A_2 groups in the first CRD set and second CRD set; one-way ANOVA followed by a Mann–Whitney test

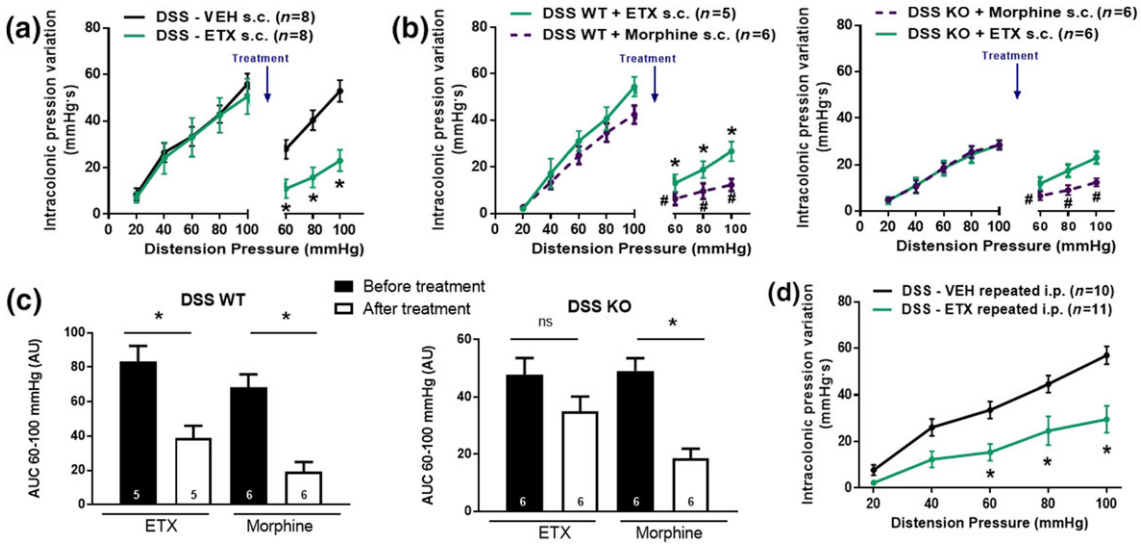

FIGURE 3 Ethosuximide treatment induced an analgesic effect that is dependent on Cav3.2 calcium channels. Groups of mice received dextran sodium sulfate (DSS) for 12 days. Colonic sensitivity was evaluated on Day 12 (D12) by colorectal distension (CRD). The effect of the acute pharmacological treatment was assessed by first submitting the mice to a CRD set with pressures ranging from 20 to 100 mmHg; then, the pharmacological drug or vehicle (VEH) was administered by an s.c. or i.t. injection, and after 30 min, another CRD set with pressures ranging from 60 to 100 mmHg was performed to evaluate the effect of the drug. The effect of the repeated ethosuximide (ETX) treatment was studied with i.p. administrations of the drugs three times a day for 6 days, and the CRD was carried out on D12 24 hr after the final injection. (a) Effect of ethosuximide (200 mg·kg^{-1}, 10 ml·kg^{-1}, s.c.) or VEH (NaCl, 10 ml·kg^{-1}, s.c.) in wild-type (WT) mice receiving DSS. (b) Responses to CRD to evaluate the effect of the acute administration of ethosuximide (200 mg·kg^{-1}, 10 ml·kg^{-1}, s.c.) or morphine (1 mg·kg^{-1}, 10 ml·kg^{-1}, s.c.) on colonic hypersensitivity in WT (left) and knockout (KO $Ca_V3.2$ right) mice receiving DSS. (c) AUC of panel (b) with pressure ranging from 80 to 100 mmHg. (d) Effect of the repeated administration of ethosuximide (200 mg·kg^{-1}, 10 ml·kg^{-1}, i.p.) or VEH (NaCl, 10 ml·kg^{-1}, i.p.) in WT mice receiving DSS. In (a, and b), *P < 0.05; significant differences between the ethosuximide groups in the first CRD set and second CRD set; #P < 0.05, significant differences between the morphine group in the first CRD set and second CRD set; Mann–Whitney test In (c), *P < 0.05, significantly different as indicated; ns: non-significant; one-Way ANOVA followed by a Mann-Whitney test. In (d), *P < 0.05, significantly different from VEH groups; two-way ANOVA (pharmacological treatment; pressure), followed by a Bonferroni post hoc test

sought to support these findings by studying the functional presence of T-type calcium current ex vivo in DiI retrolabelled colonic DRG neurons expressing $Ca_V3.2$-GFP channels to identify a potential change in the current density induced by the DSS treatment. Voltage clamp was used to record the inward calcium currents and compare the maximum current densities of both low- and high-voltage-activated channels in $Ca_V3.2$-positive colonic neurons prepared from DiI injected $Ca_V3.2$-GFP mice treated with or without DSS. All recorded DiI/GFP-positive neurons exhibited functional $Ca_V3.2$-like T-type currents, indicating that the GFP fluorescence faithfully reported the presence of

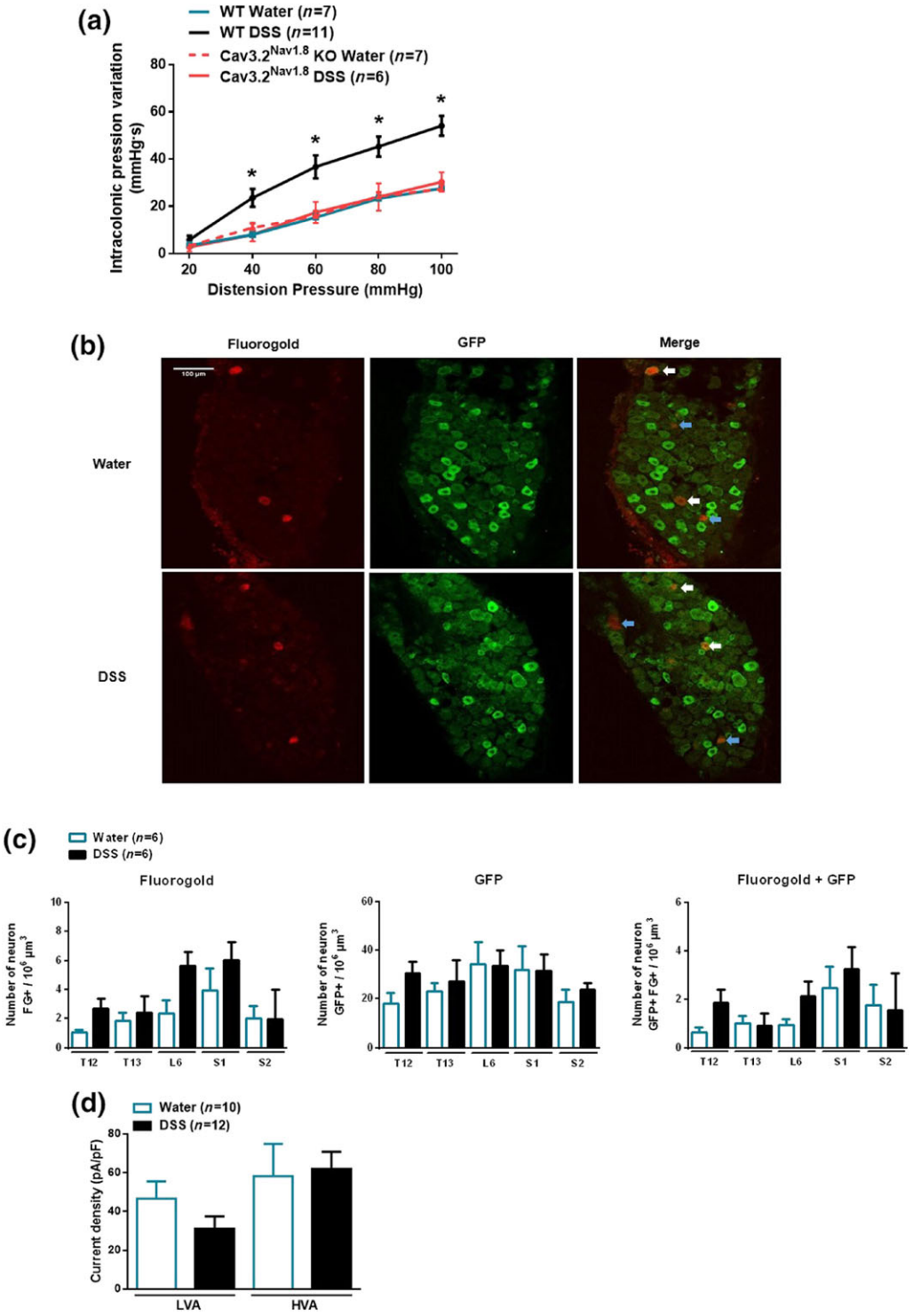

FIGURE 4 Deletion of $Ca_V3.2$ channels from primary nociceptive neurons in the dorsal root ganglion (DRG) abolished the DSS-induced colonic hypersensitivity. Wild-type and $Ca_V3.2^{Nav1.8}$ KO mice received dextran sodium sulfate (DSS) or plain water for 12 days. Colonic sensitivity was evaluated on Day 12 (D12) by colorectal distension (CRD). (a) Responses to CRD. Fluorescent tracer Fluorogold (FG, 20 µl in NaCl) was injected into the distal colon wall of $Ca_V3.2$-GFP KI mice for retrograde labelling. After 5 days, the mice received DSS or water for 12 days. *$P < 0.05$, significantly different from WT Water group in two-way ANOVA followed by a Bonferroni post hoc test. Post test? (b) Histological identification of mouse DRG colonic sensory neurons (FG positive, left), Cav3.2 channels (GFP positive, middle), and merge (right) in $Ca_V3.2$-GFP KI mice receiving water (top panel) or DSS (bottom panel). FG + GFP-positive neurons are identified by a white arrow, and FG-positive/GFP-negative neurons are identified by a blue arrow. (c) Quantitative data of the numbers of FG-positive, GFP-positive, and FG + GFP-positive neurons under the water and DSS conditions. (d) Analysis of calcium current densities of both low- and high-voltage-activated channels in $Ca_V3.2$-positive colonic neurons prepared from DiI-injected $Ca_V3.2$-GFP mice treated with DSS or water. Currents were evoked by 100-ms pulses ranging from −100 to −35 mV. The data are presented as the mean ± SEM. In (b, c and d) no significant differences between the water and DSS groups at any DRG level; one-way ANOVA

functional $Ca_V3.2$ channels within the GFP-positive neurons. However, as presented in Figure 4d, the DSS treatment did not significantly modify either the low-voltage-activated channel (i.e., T-type current) or the high-voltage-activated channel calcium current densities within these isolated DRG somata ex vivo (Figure 4d). The biophysical characteristics of the currents were also unchanged by the DSS treatment.

3.4 | Inhibition of $Ca_V3.2$ channels located on spinal terminals of C-fibres reduced CHS

The functional involvement of $Ca_V3.2$ channels in C-fibres shown by the reduction in DSS-induced CHS in the $Ca_V3.2^{Nav1.8}$ KO mice could be due to channels located in the nociceptor and/or spinal cord because, from the DRG, $Ca_V3.2$ channels can migrate to the periphery and/or presynaptic spinal levels. Therefore, the contribution of peripheral as distinct from spinal $Ca_V3.2$ channels to CHS was investigated using a pharmacological strategy. The effect of ABT-639, which is a peripherally restricted, blocker of $Ca_V3.2$ channels (Jarvis et al., 2014), was analysed after appropriate injections. Thus, systemic injections were used to block peripheral $Ca_V3.2$ channels and i.t. injections to block these channels within the spinal terminals of primary afferent neurons. We found that systemic administration of ABT-639 did not affect the responses to CRD in the DSS mice at any concentration tested (Figure 5a). In contrast, the i.t. administration of ABT-639 significantly reduced the CHS induced by DSS compared with that in the VEH control (Figure 5b), suggesting that ABT-639 has an analgesic effect only in sensory neuron spinal terminals. Although peripherally restricted by its chemical properties, when administered i.t., this drug could potentially still penetrate the spinal tissue and inhibit $Ca_V3.2$ channels at both the pre- and post-synaptic levels of afferent fibres. We therefore evaluated the effects of ABT-639 (i.t.) in $Ca_V3.2^{Nav1.8}$ KO mice deficient in presynaptic $Ca_V3.2$ channels in peripheral C-fibres. ABT-639 induced a reduction in the CHS induced by DSS in the WT littermate mice but did not modify CHS in the $Ca_V3.2^{Nav1.8}$ KO mice (Figure 6a,b). Interestingly, the systemic administration of ethosuximide also failed to induce a reduction in CHS in $Ca_V3.2^{Nav1.8}$ KO mice (Figure 6c). Together with the results obtained with ABT-639, these data suggest that this compound acts on channels at the peripheral sensory neuron endings at the spinal level, even though it crosses the blood–brain barrier. Conversely, morphine had an antihyperalgesic effect on both the littermate and $Ca_V3.2^{Nav1.8}$ KO mice , demonstrating its ability to produce analgesia in $Ca_V3.2^{Nav1.8}$ KO mice and confirming the lack of a $Ca_V3.2$ channel-dependent mechanism in the analgesic effect of morphine (Figure 5c).

Altogether, these results demonstrate the involvement of presynaptic $Ca_V3.2$ channels located at the spinal terminals of C-fibres in CHS in this model and show that CHS can be inhibited by $Ca_V3.2$ channel blockers.

4 | DISCUSSION

CHS associated with low-grade inflammation is frequently encountered in IBS and IBD patients during remission (Halpin & Ford, 2012; Lembo et al., 1999; Long & Drossman, 2010; Piche et al., 2010; Stacher & Christensen, 2006). The treatments used to relieve CHS unfortunately have limited efficacy or adverse side effects in most patients. Therefore, comprehensive studies investigating CHS

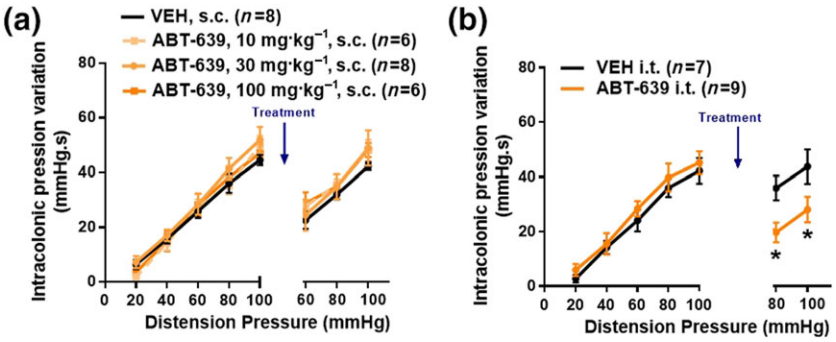

FIGURE 5 Inhibition of $Ca_V3.2$ channels located on the spinal terminals of C-fibres reduced colonic hypersensitivity. Different groups of mice received dextran sodium sulfate (DSS) for 12 days. Colonic sensitivity was evaluated on Day 12 (D12) by colorectal distension (CRD). The effect of acute pharmacological treatments was studied by first submitting the mice to a CRD set with pressure ranging from 20 to 100 mmHg; then, the drug was administered by an s.c. or i.t. injection, and after 30 or 10 min, respectively, another CRD set with pressures ranging from 60 to 100 mmHg or from 80 to 100 mmHg was performed to evaluate the effect of the drug. (a) Effect of ABT-369 (10, 30, or 100 mg·kg^{-1}, 10 ml·kg^{-1}, s.c.) or vehicle (VEH: NaCl and 10% DMSO, 10 ml·kg^{-1}, s.c.) on CRD in wild-type (WT) mice. (b) Effect of ABT-639 (10 μg/5 μl, i.t.) or vehicle (VEH: NaCl and 10% DMSO, i.t.) on CRD in WT mice. The data are presented as the mean ± SEM. *$P < 0.05$, significant differences between ABT-639 groups in the first CRD set and second CRD set; one-way ANOVA followed by a Mann-Whitney test

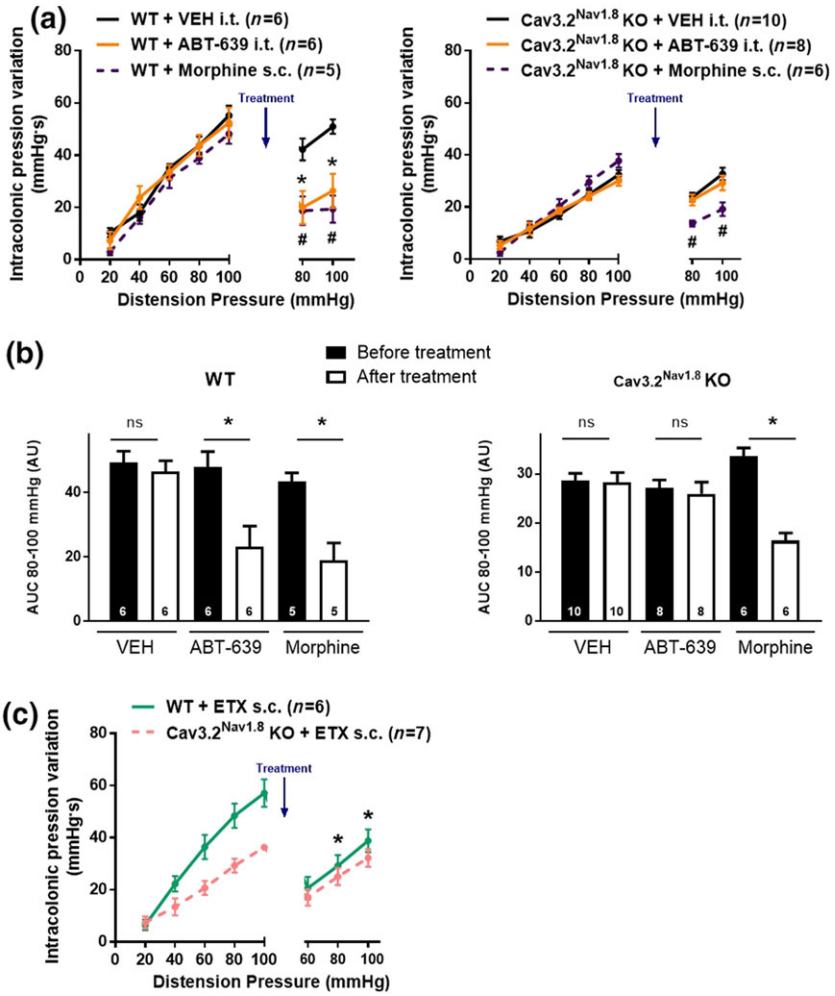

FIGURE 6 Inhibition of $Ca_V3.2$ channels located on the spinal terminals of C-fibres reduced colonic hypersensitivity. Different groups of mice received dextran sodium sulfate (DSS) for 12 days. Colonic sensitivity was evaluated on Day 12 (D12) by colorectal distension (CRD). The effect of acute pharmacological treatments was studied by first submitting the mice to a CRD set with pressure ranging from 20 to 100 mmHg; then, the drug was administered by an s.c. or i.t. injection, and after 30 or 10 min, respectively, another CRD set with pressures ranging from 60 to 100 mmHg or from 80 to 100 mmHg was performed to evaluate the effect of the drug. (a) Effect of ABT-639 (10 µg/5 µl, i.t.), VEH or morphine (1 mg·kg^{-1}, i.t.) on CRD in wild-type (WT, left) and Cav3.2$^{Nav1.8}$ knockout (KO, right) mice. (b) AUC of (a) for pressure ranging from 60 to 100 mmHg. (c) Effect of ethosuximide (ETX; 200 mg·kg^{-1}, 10 ml·kg^{-1}, s.c.) on CRD in WT and $Ca_V3.2^{Nav1.8}$ KO mice . The data are presented as the mean ± SEM. In (a), $^*P < 0.05$, significant effect of ABT-369; $^{\#}P < 0.05$, significant effect of morphine; one-way ANOVA followed by a Mann-Whitney test. In (b), $^*P < 0.05$, significantly different as indicated; one-way ANOVA followed by a Mann-Whitney test. In (c), $P < 0.05$, significant effect of ethosuximide, ns: non-significant; one-way ANOVA followed by a Mann-Whitney test

pathophysiology are required to identify novel therapeutic targets to relieve CHS. Cell excitability primarily relies on the activity of specific ion channels, and alterations in their activity, that is, channelopathies, are observed in many pathological situations, including gastrointestinal tract disorders (Beyder & Farrugia, 2016). Consistent with the importance of ion channels for visceral pain, we and other researchers have previously identified that the pronociceptive $Ca_V3.2$ T-type calcium channel isoform in visceral sensory neurons is involved in the setting of CHS (Marger et al., 2011; Matsunami, Kirishi, Okui, & Kawabata, 2011), although the sensory neuron subtypes involved has not been determined. Therefore, using a combination of a selective afferent C-fibre-specific $Ca_V3.2$ channel genetic deletion and pharmacological blockade of peripherally restricted or not-restricted T-type channel blockers, the present study highlights the involvement of C-fibre pre-synaptic $Ca_V3.2$ channels in a murine model of CHS.

To study these gastrointestinal disorders, we first developed a CHS murine model associated with low-grade inflammation using a low dose of DSS. The mice in this model presented some symptoms that are similar to those observed in IBS and IBD patients during remission. The first symptom is the presence of visceral hypersensitivity (Meleine et al., 2016; Scanzi et al., 2016), which is the most debilitating symptom in patients (Piche et al., 2010; Schirbel et al., 2010; Simrén, Törnblom, Palsson, & Whitehead, 2017). In contrast to murine models using high doses of DSS, which show strong endoscopic, macroscopic, and histological colon changes, such modifications were not observed in the present model, which is similar to endoscopy and histological colon observations in patients. However, low-grade inflammation was observed in this model, while no morphological signs of inflammation and anatomical alterations were detected, and the sensitive marker faecal Lcn-2 was increased in this model, as previously described (Chassaing et al., 2012). Such low-grade inflammation has been described in patients (Ford & Talley, 2011; Linedale & Andrews, 2017; Mumolo et al., 2018; Vivinus-Nébot et al., 2014).

To determine the role of $Ca_V3.2$ channels in CHS associated with low-grade inflammation, we genetically and pharmacologically inhibited Cav3.2 channels. Both the genetic (KO $Ca_V3.2$ mice) and pharmacological (TTA-A2) blockage strategies resulted in a suppression of CHS. These results extend the crucial role of $Ca_V3.2$ channels previously observed in other visceral pain models mostly associated with inflammation, such as colitis or cystitis (François et al., 2013; Gadotti et al., 2015; Marger et al., 2011; Matsunami et al., 2011; Matsunami et al., 2012; Terada et al., 2015; Tsubota-Matsunami, Noguchi, Okawa, Sekiguchi, & Kawabata, 2012). Moreover, these results are consistent with our previous study performed in $Ca_V3.2$

KO mice treated with a low dose of DSS (Scanzi et al., 2016). In the former study, visceral sensitivity was assessed by surgically implanted electrodes in the abdominal oblique muscle in mice that measured the visceromotor response to colorectal distention. Here, the non-invasive manometric method described by Larauche et al. (2009) was used. In the present study, we have performed colorectal distention, using a range of distention pressures from 20 to 80 mmHg, as used in many previous studies. In addition, we have also applied a 100-mmHg distension pressure as reported in previous experiments (Ness & Gebhart, 1988; Zagorodnyuk et al., 2011). Such 100- to 120-mmHg distensions are considered as acute noxious CRD stimuli (Kyloh, Nicholas, Zagorodnyuk, Brookes, & Spencer, 2011). Nevertheless, the resulting observations, with 100 mmHg, must be interpreted with caution, as acute and severe noxious stimuli are not often encountered in IBS patients. In conclusion, both methods, the visceromotor response and the non-invasive manometric method, show similar results, supporting the involvement of $Ca_V3.2$ channels in visceral hypersensitivity.

Subsequently, we explored the function of $Ca_V3.2$ channels located in primary sensitive neurons in CHS. Thus, we used mice with a conditional $Ca_V3.2$ KO in primary afferent C-fibres ($Ca_V3.2^{Nav1.8}$ KO mice) as previously described (François et al., 2015). François et al. (2015) demonstrated that $Ca_V3.2$ channels are selectively deleted from a population of C-fibres corresponding to low-threshold mechanoreceptors innervating the hairy skin that express TH, leaving their expression unchanged in A-δ fibres. Interestingly, similar to somatic DRGs, a fraction of visceral DRG neurons retrogradely traced from the colorectum or urinary bladder have also been shown to express detectable TH (Brumovsky, La, McCarthy, Hökfelt, & Gebhart, 2012), suggesting similarities in their excitability phenotype with somatic TH-positive DRGs. We showed that DSS-induced CHS is decreased in the $Ca_V3.2^{Nav1.8}$ KO mice This result demonstrated a major role for $Ca_V3.2$ channels in primary afferent C-fibres in CHS associated with low-grade inflammation.

To determine whether $Ca_V3.2$ channel involvement in CHS is due to an increased proportion of sensory neurons expressing $Ca_V3.2$ channels innervating the colon, immunohistochemistry was performed on DRG sections from GFP- $Ca_V3.2$ KI mice that have previously received fluorescent dye Fluorogold injections into the colon wall. The visualization of the GFP tag attached to the $Ca_V3.2$ channels in these KI animals compensates for the lack of a specific antibody against native $Ca_V3.2$ proteins (François et al., 2015). The detection of the $Ca_V3.2$ protein was fully consistent with the recent identification of $Ca_V3.2$ mRNA by single-cell RNAseq in several classes of colonic sensory neurons (Hockley et al., 2018). However, in the present study, we did not detect overexpression of $Ca_V3.2$ channels in the cell soma of the colonic sensory neurons following the DSS treatment.

We sought to support these findings by studying the functional presence of T-type channels in vitro in DiI retrolabelled colonic DRG neurons expressing $Ca_V3.2$-GFP channels to identify a potential change in the current density induced by the DSS treatment. No significant change in the calcium current densities in these isolated DRG somata were observed ex vivo under the water or DSS conditions. Altogether, these results show that the CHS state does not cause any change in the expression or electrophysiological properties of $Ca_V3.2$ channels at least in the ganglia. This result is similar to that reported in a recent study investigating **HCN2** ion channels in mouse models of diabetic neuropathic pain. Although these channels play a pronociceptive role in the pain context, neither their expression nor their in vitro activity is altered (Tsantoulas et al., 2017). One hypothesis is that a second downstream target dependent on $Ca_V3.2$ channels is modulated in DSS animals and involved in visceral sensibility. Therefore, the inhibition of $Ca_V3.2$ channels suppressed the action of this target. Moreover, $Ca_V3.2$ channels are expressed throughout primary sensory neurons from the receptive field to presynaptic terminals (François et al., 2015). Our results showed that this latter location was a major player in the context of visceral pain. Thus, we could not rule out the possibility that the local activation of presynaptically located $Ca_V3.2$ channels is achieved by modulating proteins, ubiquitination and deubiquitination (García-Caballero et al., 2014), and/or **PKC** involvement (Park et al., 2006). This local effect could be lost once neurons from the DRG are prepared for culture.

The functional involvement of $Ca_V3.2$ channels in C-fibres shown by the reduction in DSS-induced CHS in the $Ca_V3.2^{Nav1.8}$ KO mice can be due to channels located in nociceptors and/or the spinal cord; from the DRG, $Ca_V3.2$ channels can migrate to the periphery and/or the presynaptic spinal levels. Therefore, the contribution of peripheral, relative to spinal, $Ca_V3.2$ channels to CHS was investigated using a pharmacological strategy, that is, ABT-639, which is a $Ca_V3.2$ channel blocker that does not cross the blood–brain barrier (Jarvis et al., 2014). Administered systematically to inhibit peripheral $Ca_V3.2$ channels , ABT-639 did not affect CHS in the mice. However, the i.t. administration of ABT-639, which inhibits both spinal pre- and post-synaptic $Ca_V3.2$ channels , led to a robust reduction in CHS. The lack of an analgesic effect following the i.t. administration of ABT-639 to the $Ca_V3.2^{Nav1.8}$ KO mice confirmed the $Ca_V3.2$-dependent effect of ABT-639 and demonstrated for the first time the involvement of $Ca_V3.2$ channels located at the presynaptic level in afferent fibre terminals in the pathophysiology of CHS.

Consistent with this prospect and a translational perspective, we assessed the effect of ethosuximide, which is a low-affinity blocker of all three Ca_V3 channel isoforms and is commonly used as a treatment for absence epilepsy in children. After its acute administration, ethosuximide induced a strong analgesic effect that was maintained after prolonged treatment. Interestingly, this effect was lost in the $Ca_V3.2$ KO mice, confirming the usefulness of blocking these channels to reduce CHS. Furthermore, the lack of any effect of ethosuximide in the $Ca_V3.2^{Nav1.8}$ KO mice combined with the analgesic effect of i.t. administered ABT-639 highlight the beneficial effect on CHS through its action on spinal presynaptically located $Ca_V3.2$ channels.

In conclusion, we provide evidence using both genetic and pharmacological strategies that $Ca_V3.2$ channels are involved in CHS associated with local low-grade inflammation. Importantly, we show for the first time that $Ca_V3.2$ channels involved in CHS are localized in C-fibres at the presynaptic level in the spinal dorsal horn. These

findings highlight that $Ca_V3.2$ channels are important players in IBS and IBD during the remission phase, opening up a new possible approach to the pharmacological treatment of these patients. Thus, developing pharmacological inhibitors of $Ca_V3.2$ channels may be an attractive strategy for relieving visceral pain in such patients. Importantly, in addition to demonstrating the pathophysiological role of these channels in CHS, these results recommend the prompt inception of clinical trials of ethosuximide, which is currently in clinical use. Accordingly, our team intends to investigate the effect of ethosuximide in IBS patients (Kerckhove, Scanzi, Pereira, Ardid, & Dapoigny, 2017) for a clinical proof of concept.

ACKNOWLEDGEMENTS

This work was supported by INSERM, CNRS, Clermont Auvergne and Montpellier Universities, and ANR (ANR-15-CE16-0012-01, LABEX ICST), Pierre Dunand & Marie-Thérèse Chevalier (from the Société Nationale Française de Gastro-Entérologie), and FRM (équipe FRM 2015) grants. The authors acknowledge the support received from the Agence Nationale de la Recherche of the French government through the programme "Investissements d'Avenir" (I-Site CAP 20-25).

AUTHOR CONTRIBUTIONS

A.E. and C.M. planned the study. E.P., F.A.C., E.B., A.E., L.D., and C.M. designed the experiments. E.P., F.A.C., and E.B. performed the experiments. E.P., F.A.C., E.B., A.E., L.D., and C.M. analysed the data. E.P., C.M., and A.E. wrote the manuscript with critical input provided by F.A.C., L.D., E.B., and D.A.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

ORCID

Christophe Mallet https://orcid.org/0000-0002-0873-6763

REFERENCES

Abraham, C., & Cho, J. H. (2009). Inflammatory bowel disease. *The New England Journal of Medicine*, *361*, 2066–2078. https://doi.org/10.1056/NEJMra0804647

Alexander, S. P. H., Striessnig, J., Kelly, E., Marrion, N. V., Peters, J. A., Faccenda, E., … CGTP Collaborators (2017). The Concise Guide to PHARMACOLOGY 2017/18: Voltage-gated ion channels. *British Journal of Pharmacology*, *174*(Suppl 1), S160–S194. https://doi.org/10.1111/bph.13884

Alexander, S. P. H., Fabbro, D., Kelly, E., Marrion, N. V., Peters, J. A., Faccenda, E., … Collaborators, C. G. T. P. (2017). The Concise Guide to PHARMACOLOGY 2017/18: Enzymes. *British Journal of Pharmacology*, *174*, S272–S359. https://doi.org/10.1111/bph.13877

Ananthakrishnan, A. N. (2015). Epidemiology and risk factors for IBD. *Nature Reviews. Gastroenterology & Hepatology*, *12*, 205–217. https://doi.org/10.1038/nrgastro.2015.34

Beyder, A., & Farrugia, G. (2016). Ion channelopathies in functional GI disorders. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *311*, G581–G586. https://doi.org/10.1152/ajpgi.00237.2016

Bourinet, E., Alloui, A., Monteil, A., Barrère, C., Couette, B., Poirot, O., … Nargeot, J. (2005). Silencing of the Cav3.2 T-type calcium channel gene in sensory neurons demonstrates its major role in nociception. *The EMBO Journal*, *24*, 315–324. https://doi.org/10.1038/sj.emboj.7600515

Brumovsky, P. R., La, J.-H., McCarthy, C. J., Hökfelt, T., & Gebhart, G. F. (2012). Dorsal root ganglion neurons innervating pelvic organs in the mouse express tyrosine hydroxylase. *Neuroscience*, *223*, 77–91. https://doi.org/10.1016/j.neuroscience.2012.07.043

Camilleri, M., & Boeckxstaens, G. (2017). Dietary and pharmacological treatment of abdominal pain in IBS. *Gut*, *66*, 966–974. https://doi.org/10.1136/gutjnl-2016-313425

Chassaing, B., Aitken, J. D., Malleshappa, M., & Vijay-Kumar, M. (2014). Dextran sulfate sodium (DSS)-induced colitis in mice. *Current Protocols in Immunology*, *104*, Unit 15.25.

Chassaing, B., Srinivasan, G., Delgado, M. A., Young, A. N., Gewirtz, A. T., & Vijay-Kumar, M. (2012). Fecal lipocalin 2, a sensitive and broadly dynamic non-invasive biomarker for intestinal inflammation. *PLoS ONE*, *7*, e44328. https://doi.org/10.1371/journal.pone.0044328

Chen, C.-C., Lamping, K. G., Nuno, D. W., Barresi, R., Prouty, S. J., Lavoie, J. L., … Campbell, K. P. (2003). Abnormal coronary function in mice deficient in alpha1H T-type Ca2+ channels. *Science*, *302*, 1416–1418. https://doi.org/10.1126/science.1089268

Chey, W. D., Kurlander, J., & Eswaran, S. (2015). Irritable bowel syndrome: A clinical review. *Jama*, *313*, 949–958. https://doi.org/10.1001/jama.2015.0954

Curtis, M. J., Alexander, S., Cirino, G., Docherty, J. R., George, C. H., Giembycz, M. A., … Ahluwalia, A. (2018). Experimental design and analysis and their reporting II: Updated and simplified guidance for authors and peer reviewers. *British Journal of Pharmacology*, *175*, 987–993. https://doi.org/10.1111/bph.14153

Curtis, M. J., Bond, R. A., Spina, D., Ahluwalia, A., Alexander, S. P. A., Giembycz, M. A., … McGrath, J. C. (2015). Experimental design and analysis and their reporting: New guidance for publication in BJP. *British Journal of Pharmacology*, *172*, 3461–3471. https://doi.org/10.1111/bph.12856

Ford, A. C., & Talley, N. J. (2011). Mucosal inflammation as a potential etiological factor in irritable bowel syndrome: A systematic review. *Journal of Gastroenterology*, *46*, 421–431. https://doi.org/10.1007/s00535-011-0379-9

François, A., Kerckhove, N., Meleine, M., Alloui, A., Barrere, C., Gelot, A., … Bourinet, E. (2013). State-dependent properties of a new T-type calcium channel blocker enhance Ca(V)3.2 selectivity and support analgesic effects. *Pain*, *154*, 283–293. https://doi.org/10.1016/j.pain.2012.10.023

François, A., Schüetter, N., Laffray, S., Sanguesa, J., Pizzoccaro, A., Dubel, S., … Bourinet, E. (2015). The low-threshold calcium channel Cav3.2 determines low-threshold mechanoreceptor function. *Cell Rep*, *10*, 370–382. https://doi.org/10.1016/j.celrep.2014.12.042

Gadotti, V. M., Caballero, A. G., Berger, N. D., Gladding, C. M., Chen, L., Pfeifer, T. A., & Zamponi, G. W. (2015). Small organic molecule disruptors of Cav3.2—USP5 interactions reverse inflammatory and neuropathic pain. *Molecular Pain*, *11*, 12.

García-Caballero, A., Gadotti, V. M., Stemkowski, P., Weiss, N., Souza, I. A., Hodgkinson, V., … Zamponi, G. W. (2014). The deubiquitinating enzyme USP5 modulates neuropathic and inflammatory pain by enhancing Cav3.2 channel activity. *Neuron*, *83*, 1144–1158. https://doi.org/10.1016/j.neuron.2014.07.036

Gören, M. Z., & Onat, F. (2007). Ethosuximide: From bench to bedside. *CNS Drug Reviews*, *13*, 224–239. https://doi.org/10.1111/j.1527-3458.2007.00009.x

Gracie, D. J., Irvine, A. J., Sood, R., Mikocka-Walus, A., Hamlin, P. J., & Ford, A. C. (2017). Effect of psychological therapy on disease activity, psychological comorbidity, and quality of life in inflammatory bowel disease: A systematic review and meta-analysis. *Lancet Gastroenterol Hepatol*, *2*, 189–199. https://doi.org/10.1016/S2468-1253(16)30206-0

Guilarte, M., Santos, J., de Torres, I., Alonso, C., Vicario, M., Ramos, L., … Malagelada, J. R. (2007). Diarrhoea-predominant IBS patients show mast cell activation and hyperplasia in the jejunum. *Gut*, *56*, 203–209. https://doi.org/10.1136/gut.2006.100594

Halpin, S. J., & Ford, A. C. (2012). Prevalence of symptoms meeting criteria for irritable bowel syndrome in inflammatory bowel disease: Systematic review and meta-analysis. *The American Journal of Gastroenterology*, *107*, 1474–1482. https://doi.org/10.1038/ajg.2012.260

Harding, S. D., Sharman, J. L., Faccenda, E., Southan, C., Pawson, A. J., Ireland, S., … NC-IUPHAR (2018). The IUPHAR/BPS Guide to PHARMACOLOGY in 2018: Updates and expansion to encompass the new guide to IMMUNOPHARMACOLOGY. *Nucleic Acids Research*, *46*, D1091–D1106. https://doi.org/10.1093/nar/gkx1121

Hendrickson, B. A., Gokhale, R., & Cho, J. H. (2002). Clinical aspects and pathophysiology of inflammatory bowel disease. *Clinical Microbiology Reviews*, *15*, 79–94. https://doi.org/10.1128/CMR.15.1.79-94.2002

Hockley, J. R. F., Taylor, T. S., Callejo, G., Wilbrey, A. L., Gutteridge, A., Bach, K., … Smith, E. S. J. (2018). Single-cell RNAseq reveals seven classes of colonic sensory neuron. *Gut*, gutjnl-2017-315631. https://doi.org/10.1136/gutjnl-2017-315631

Jacus, M. O., Uebele, V. N., Renger, J. J., & Todorovic, S. M. (2012). Presynaptic Cav3.2 channels regulate excitatory neurotransmission in nociceptive dorsal horn neurons. *The Journal of Neuroscience*, *32*, 9374–9382. https://doi.org/10.1523/JNEUROSCI.0068-12.2012

Jarvis, M. F., Scott, V. E., McGaraughty, S., Chu, K. L., Xu, J., Niforatos, W., … Xia, Z. (2014). A peripherally acting, selective T-type calcium channel blocker, ABT-639, effectively reduces nociceptive and neuropathic pain in rats. *Biochemical Pharmacology*, *89*, 536–544. https://doi.org/10.1016/j.bcp.2014.03.015

Karp, N. A., Meehan, T. F., Morgan, H., Mason, J. C., Blake, A., Kurbatova, N., … Brown, S. D. M. (2015). Applying the ARRIVE guidelines to an in vivo database. *PLoS Biology*, *13*, e1002151. https://doi.org/10.1371/journal.pbio.1002151

Kerckhove, N., Scanzi, J., Pereira, B., Ardid, D., & Dapoigny, M. (2017). Assessment of the effectiveness and safety of ethosuximide in the treatment of abdominal pain related to irritable bowel syndrome—IBSET: Protocol of a randomised, parallel, controlled, double-blind and multicentre trial. *BMJ Open*, *7*, e015380. https://doi.org/10.1136/bmjopen-2016-015380

Kilkenny, C., Browne, W., Cuthill, I. C., Emerson, M., & Altman, D. G. (2010). Animal research: Reporting in vivo experiments: The ARRIVE guidelines. *British Journal of Pharmacology*, *160*, 1577–1579.

Kyloh, M., Nicholas, S., Zagorodnyuk, V. P., Brookes, S. J., & Spencer, N. J. (2011). Identification of the visceral pain pathway activated by noxious colorectal distension in mice. *Frontiers in Neuroscience*, *5*, 16.

Larauche, M., Gourcerol, G., Wang, L., Pambukchian, K., Brunnhuber, S., Adelson, D. W., … Taché, Y. (2009). Cortagine, a CRF1 agonist, induces stresslike alterations of colonic function and visceral hypersensitivity in rodents primarily through peripheral pathways. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *297*, G215–G227. https://doi.org/10.1152/ajpgi.00072.2009

Lembo, T., Naliboff, B., Munakata, J., Fullerton, S., Saba, L., Tung, S., … Mayer, E. A. (1999). Symptoms and visceral perception in patients with pain-predominant irritable bowel syndrome. *The American Journal of Gastroenterology*, *94*, 1320–1326. https://doi.org/10.1111/j.1572-0241.1999.01009.x

Linedale, E. C., & Andrews, J. M. (2017). Diagnosis and management of irritable bowel syndrome: A guide for the generalist. *The Medical Journal of Australia*, *207*, 309–315. https://doi.org/10.5694/mja17.00457

Long, M. D., & Drossman, D. A. (2010). Inflammatory bowel disease, irritable bowel syndrome, or what?: A challenge to the functional-organic dichotomy. *The American Journal of Gastroenterology*, *105*, 1796–1798. https://doi.org/10.1038/ajg.2010.162

Lovell, R. M., & Ford, A. C. (2012). Global prevalence of and risk factors for irritable bowel syndrome: A meta-analysis. *Clinical Gastroenterology and Hepatology*, *10*, 712–721.e4.

Marger, F., Gelot, A., Alloui, A., Matricon, J., Ferrer, J. F. S., Barrère, C., … Ardid, D. (2011). T-type calcium channels contribute to colonic hypersensitivity in a rat model of irritable bowel syndrome. *Proceedings of the National Academy of Sciences of the United States of America*, *108*, 11268–11273. https://doi.org/10.1073/pnas.1100869108

Matsunami, M., Kirishi, S., Okui, T., & Kawabata, A. (2011). Chelating luminal zinc mimics hydrogen sulfide-evoked colonic pain in mice: Possible involvement of T-type calcium channels. *Neuroscience*, *181*, 257–264. https://doi.org/10.1016/j.neuroscience.2011.02.044

Matsunami, M., Miki, T., Nishiura, K., Hayashi, Y., Okawa, Y., Nishikawa, H., … Kawabata, A. (2012). Involvement of the endogenous hydrogen sulfide/Ca(v) 3.2 T-type Ca2+ channel pathway in cystitis-related bladder pain in mice. *British Journal of Pharmacology*, *167*, 917–928. https://doi.org/10.1111/j.1476-5381.2012.02060.x

McGrath, J. C., & Lilley, E. (2015). Implementing guidelines on reporting research using animals (ARRIVE etc.): New requirements for publication in BJP. *British Journal of Pharmacology*, *172*, 3189–3193. https://doi.org/10.1111/bph.12955

Mearin, F., Lacy, B. E., Chang, L., Chey, W. D., Lembo, A. J., Simren, M., & Spiller, R. (2016). Bowel Disorders. *Gastroenterology*, *150*, 1393–1407. https://doi.org/10.1053/j.gastro.2016.02.031

Meleine, M., Boudieu, L., Gelot, A., Muller, E., Lashermes, A., Matricon, J., … Carvalho, F. A. (2016). Comparative effects of α2δ-1 ligands in mouse models of colonic hypersensitivity. *World Journal of Gastroenterology*, *22*, 7111–7123. https://doi.org/10.3748/wjg.v22.i31.7111

Mumolo, M. G., Bertani, L., Ceccarelli, L., Laino, G., Di Fluri, G., Albano, E., … Costa, F. (2018). From bench to bedside: Fecal calprotectin in inflammatory bowel diseases clinical setting. *World Journal of Gastroenterology*, *24*, 3681–3694. https://doi.org/10.3748/wjg.v24.i33.3681

Nellesen, D., Yee, K., Chawla, A., Lewis, B. E., & Carson, R. T. (2013). A systematic review of the economic and humanistic burden of illness in irritable bowel syndrome and chronic constipation. *Journal of Managed Care Pharmacy*, *19*, 755–764. https://doi.org/10.18553/jmcp.2013.19.9.755

Ness, T. J., & Gebhart, G. F. (1988). Colorectal distension as a noxious visceral stimulus: Physiologic and pharmacologic characterization of

pseudaffective reflexes in the rat. *Brain Research*, *450*, 153–169. https://doi.org/10.1016/0006-8993(88)91555-7

Obradovic, A. L., Hwang, S. M., Scarpa, J., Hong, S. J., Todorovic, S. M., & Jevtovic-Todorovic, V. (2014). CaV3.2 T-type calcium channels in peripheral sensory neurons are important for mibefradil-induced reversal of hyperalgesia and allodynia in rats with painful diabetic neuropathy. *PLoS ONE*, *9*, e91467.

Park, J.-Y., Kang, H.-W., Moon, H.-J., Huh, S.-U., Jeong, S.-W., Soldatov, N. M., & Lee, J. H. (2006). Activation of protein kinase C augments T-type Ca2+ channel activity without changing channel surface density. *Journal of Physiology (London)*, *577*, 513–523. https://doi.org/10.1113/jphysiol.2006.117440

Piche, T., Ducrotté, P., Sabate, J. M., Coffin, B., Zerbib, F., Dapoigny, M., … Hébuterne, X. (2010). Impact of functional bowel symptoms on quality of life and fatigue in quiescent Crohn disease and irritable bowel syndrome. *Neurogastroenterology and Motility*, *22*, 626–e174. https://doi.org/10.1111/j.1365-2982.2010.01502.x

Podolsky, D. K. (2002). Inflammatory bowel disease. *The New England Journal of Medicine*, *347*, 417–429. https://doi.org/10.1056/NEJMra020831

Rice, A. S. C., Cimino-Brown, D., Eisenach, J. C., Kontinen, V. K., Lacroix-Fralish, M. L., Machin, I., … Stöhr, T. (2008). Animal models and the prediction of efficacy in clinical trials of analgesic drugs: A critical appraisal and call for uniform reporting standards. *Pain*, *139*, 243–247. https://doi.org/10.1016/j.pain.2008.08.017

Scanzi, J., Accarie, A., Muller, E., Pereira, B., Aissouni, Y., Goutte, M., … Dapoigny, M. (2016). Colonic overexpression of the T-type calcium channel Cav 3.2 in a mouse model of visceral hypersensitivity and in irritable bowel syndrome patients. *Neurogastroenterology and Motility*, *28*, 1632–1640. https://doi.org/10.1111/nmo.12860

Schirbel, A., Reichert, A., Roll, S., Baumgart, D. C., Büning, C., Wittig, B., … Sturm, A. (2010). Impact of pain on health-related quality of life in patients with inflammatory bowel disease. *World Journal of Gastroenterology*, *16*, 3168–3177. https://doi.org/10.3748/wjg.v16.i25.3168

Simrén, M., Törnblom, H., Palsson, O. S., & Whitehead, W. E. (2017). Management of the multiple symptoms of irritable bowel syndrome. *Lancet Gastroenterol Hepatol*, *2*, 112–122. https://doi.org/10.1016/S2468-1253(16)30116-9

Snutch, T. P., & Zamponi, G. W. (2018). Recent advances in the development of T-type calcium channel blockers for pain intervention. *British Journal of Pharmacology*, *175*, 2375–2383. https://doi.org/10.1111/bph.13906

Stacher, G., & Christensen, J. (2006). Visceral hypersensitivity in irritable bowel syndrome: A summary review. *Digestive Diseases and Sciences*, *51*, 440–445. https://doi.org/10.1007/s10620-006-3152-9

Stirling, L. C., Forlani, G., Baker, M. D., Wood, J. N., Matthews, E. A., Dickenson, A. H., & Nassar, M. A. (2005). Nociceptor-specific gene deletion using heterozygous NaV1.8-Cre recombinase mice. *Pain*, *113*, 27–36. https://doi.org/10.1016/j.pain.2004.08.015

Terada, Y., Fujimura, M., Nishimura, S., Tsubota, M., Sekiguchi, F., & Kawabata, A. (2015). Roles of Cav3.2 and TRPA1 channels targeted by hydrogen sulfide in pancreatic nociceptive processing in mice with or without acute pancreatitis. *Journal of Neuroscience Research*, *93*, 361–369. https://doi.org/10.1002/jnr.23490

Tsantoulas, C., Laínez, S., Wong, S., Mehta, I., Vilar, B., & McNaughton, P. A. (2017). Hyperpolarization-activated cyclic nucleotide-gated 2 (HCN2) ion channels drive pain in mouse models of diabetic neuropathy. *Science Translational Medicine*, *9*, eaam6072.

Tsubota-Matsunami, M., Noguchi, Y., Okawa, Y., Sekiguchi, F., & Kawabata, A. (2012). Colonic hydrogen sulfide-induced visceral pain and referred hyperalgesia involve activation of both Ca(v)3.2 and TRPA1 channels in mice. *Journal of Pharmacological Sciences*, *119*, 293–296. https://doi.org/10.1254/jphs.12086SC

Vivinus-Nébot, M., Frin-Mathy, G., Bzioueche, H., Dainese, R., Bernard, G., Anty, R., … Piche, T. (2014). Functional bowel symptoms in quiescent inflammatory bowel diseases: Role of epithelial barrier disruption and low-grade inflammation. *Gut*, *63*, 744–752. https://doi.org/10.1136/gutjnl-2012-304066

Xavier, R. J., & Podolsky, D. K. (2007). Unravelling the pathogenesis of inflammatory bowel disease. *Nature*, *448*, 427–434. https://doi.org/10.1038/nature06005

Zagorodnyuk, V. P., Kyloh, M., Nicholas, S., Peiris, H., Brookes, S. J., Chen, B. N., & Spencer, N. J. (2011). Loss of visceral pain following colorectal distension in an endothelin-3 deficient mouse model of Hirschsprung's disease. *Journal of Physiology (London)*, *589*, 1691–1706. https://doi.org/10.1113/jphysiol.2010.202820

Zeitz, J., Ak, M., Müller-Mottet, S., Scharl, S., Biedermann, L., Fournier, N., … Swiss IBD Cohort Study Group (2016). Pain in IBD patients: Very frequent and frequently insufficiently taken into account. *PLoS ONE*, *11*, e0156666. https://doi.org/10.1371/journal.pone.0156666

Zimmermann, M. (1983). Ethical guidelines for investigations of experimental pain in conscious animals. *Pain*, *16*, 109–110. https://doi.org/10.1016/0304-3959(83)90201-4

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Picard E, Carvalho FA, Agosti F, et al. Inhibition of $Ca_v3.2$ calcium channels: A new target for colonic hypersensitivity associated with low-grade inflammation. *Br J Pharmacol*. 2019;176:950–963. https://doi.org/10.1111/bph.14608