Olorinab (APD371), a peripherally acting, highly selective, full agonist of the cannabinoid receptor 2, reduces colitis-induced acute and chronic visceral hypersensitivity in rodents

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Abstract
Abdominal pain is a key symptom of inflammatory bowel disease and irritable bowel syndrome, for which there are inadequate therapeutic options. We tested whether olorinab—a highly selective, full agonist of the cannabinoid receptor 2 (CB2)—reduced visceral hypersensitivity in models of colitis and chronic visceral hypersensitivity (CVH). In rodents, colitis was induced by intrarectal administration of nitrobenzene sulfonic acid derivatives. Control or colitis animals were administered vehicle or olorinab (3 or 30 mg/kg) twice daily by oral gavage for 5 days, starting 1 day before colitis induction. Chronic visceral hypersensitivity mice were administered olorinab (1, 3, 10, or 30 mg/kg) twice daily by oral gavage for 5 days, starting 24 days after colitis induction. Visceral mechanosensitivity was assessed in vivo by quantifying visceromotor responses (VMRs) to colorectal distension. Ex vivo afferent recordings determined colonic nociceptor firing evoked by mechanical stimuli. Colitis and CVH animals displayed significantly elevated VMRs to colorectal distension and colonic nociceptor hypersensitivity. Olorinab treatment significantly reduced VMRs to control levels in colitis and CVH animals. In addition, olorinab reduced nociceptor hypersensitivity in colitis and CVH states in a concentration- and CB2-dependent manner. By contrast, olorinab did not alter VMRs nor nociceptor responsiveness in control animals. Cannabinoid receptor 2 mRNA was detected in colonic tissue, particularly within epithelial cells, and dorsal root ganglia, with no significant differences between healthy, colitis, and CVH states. These results demonstrate that olorinab reduces visceral hypersensitivity through CB2 agonism in animal models, suggesting that olorinab may provide a novel therapy for inflammatory bowel disease—and irreversible bowel syndrome—associated abdominal pain.

Keywords: Nociceptors, Visceral hypersensitivity, Colon, Inflammatory bowel disease, Irritable bowel syndrome, Abdominal pain, Cannabinoid receptor 2, Cannabinoid receptor agonists, Visceral pain, Olorinab, Colitis, Colonic nociception, Visceral afferents

1. Introduction
Inflammatory bowel diseases (IBDs), including Crohn disease and ulcerative colitis, are chronic relapsing gastrointestinal disorders with increasing prevalence worldwide. Approximately 70% of patients with IBD experience abdominal pain throughout their disease, and ~50% experience chronic pain for ≥5 years, profoundly impacting their quality of life. Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder characterized by recurrent abdominal pain and altered bowel habits (eg, constipation, diarrhea, or both). More than 90% of patients experience abdominal pain at least weekly; of all IBS symptoms, abdominal pain most severely disrupts quality of life and is associated with illness severity. Pain management is imperative in the care of patients with IBD or IBS, underscoring the need for therapies that specifically target abdominal pain.

Activation of colon-innervating spinal sensory afferents by mechanical and chemical stimuli are key processes contributing to abdominal pain. Inflammatory mediators released during colitis can directly activate and sensitize colon-innervating afferents, resulting in enhanced responsiveness to chemical and mechanical stimuli, known as visceral hypersensitivity. These changes in colonic afferent function result in enhanced nociceptive signals sent to the spinal cord. Therefore, a key therapeutic strategy for abdominal pain associated with IBD and IBS is preventing or reducingafferent sensitization.

Cannabinoids are an attractive treatment for abdominal pain in IBD and IBS. The crystal structures, activation, and signaling mechanisms of the 2 known cannabinoid receptors, cannabinoid...
receptor 1 (CB₁) and 2 (CB₂), were recently identified. Cannabinoid receptor 1 is widely distributed and highly expressed in the brain, where it mediates the psychoactive effects of cannabis. By contrast, CB₂ is mainly expressed in immune cells and peripheral tissues, including the colonic mucosa and enteric nervous system (ENS). Increased CB₂ expression has been demonstrated in preclinical models of gastrointestinal inflammation and gut tissue from humans with IBD or IBS. Cannabinoid receptor agonists showed activity in preclinical models of visceral and neuropathic pain, and endocannabinoid-like dietary supplements alleviated abdominal pain and discomfort in a phase 2b study in patients with IBS. However, clinical development of these candidates has been limited, possibly because of psychoactive side effects from a lack of selectivity for CB₂ vs CB₁ or loss of activity from partial CB₂ agonism.

Olorinab (APD371) is an oral, peripherally acting visceral analgesic and highly selective full agonist of CB₂. Its >1000-fold functional selectivity for CB₂ over CB₁ and low brain penetration minimize the risk of psychoactive effects. Olorinab activated endogenous CB₂ in primary rat splenocytes, human HL-60 cells, and primary human B cells and demonstrated antinoceptive efficacy in preclinical models of chronic pain. In a phase 2a study in patients with quiescent to mildly active Crohn disease, olorinab was well tolerated and improved abdominal pain. Based on these findings, we assessed the activity of olorinab in reducing visceral hypersensitivity in rodent models of IBD and IBS.

2. Methods

2.1. Animal studies

All experiments were performed in accordance with the guidelines of the Animal Ethics Committees of the South Australian Health and Medical Research Institute and Flinders University (ethics project SAM276). Male C57BL/6J mice and Sprague-Dawley rats were used in all experiments. Mice and rats were sourced from the specific and opportunistic pathogen-free facility at the South Australian Health and Medical Research Institute Animal Bioresources. The facility is accredited by the Office of the Gene Technology Regulator and as a physical containment level 2 facility (license number, cert 3767). The facility also has approval to breed and rederive lines. Mice were originally purchased from The Jackson Laboratory (strain code, 400; Wilmington, MA). Rats were originally purchased from Charles River Laboratories (strain code, 400; Bar Harbor, ME). Rats were housed individually and monitored daily for changes in body weight, physical appearance, and behavior. Chronic visceral hypersensitivity induction was performed as previously described. Briefly, 10- to 11-week-old male C57BL/6J mice were fasted overnight with access to 5% glucose solution before administration of an intracolonic enema of DNBS (6.5 mg in 30% ethanol; total volume of 0.1 mL) under isoflurane anesthesia. After DNBS administration, animals were housed individually and monitored daily for changes in bowel weight, physical appearance, and behavior. Chronic visceral hypersensitivity was assessed 28 days after DNBS administration. Male littermates were used as healthy control animals.

2.1.3. Olorinab treatment

Olorinab doses were selected based on the pharmacokinetic profile of the compound in rats and efficacious doses in other preclinical models of pain. Accordingly, our initial in vivo visceromotor response (VMR) studies were performed in rats. Healthy control or colitis rats were orally administered either vehicle control (0.5% methylcellulose) or olorinab (3 or 30 mg/kg) twice daily (BID) by oral gavage for 5 days starting 1 day before DNBS or TNBS administration (Fig. 1A). Based on our initial findings in rats, we expanded the study to CVH mice and used 4 doses of olorinab. For the CVH studies, mice were orally administered either vehicle control (0.5% methylcellulose) or olorinab (1, 3, 10, or 30 mg/kg) BID by oral gavage for 5 days starting at 24 days after DNBS administration (Fig. 1B).

2.2. In vivo visceral pain assessment: visceromotor response to colorectal distension

The VMR is a nociceptive brainstem reflex consisting of the contraction of the abdominal muscles in response to noxious distension of the colorectum. We measured the VMRs to CRD as an objective measure of visceral sensitivity in fully conscious animals. These studies were performed in healthy, colitis, and CVH animals.

2.2.1. Surgical implantation of electrodes

The VMR was assessed by electromyography (EMG) to quantify abdominal muscle contractions in response to non-noxious and noxious CRD as previously published. We performed these studies in both rats (healthy and colitis) and mice (healthy and CVH). Briefly, 3 days before VMR assessment, rats and mice were anesthetized with isoflurane, and a 1 cm incision was made just superior to the right inguinal ligament, exposing the external oblique abdominal muscle. Two polytetrafluoroethylene-coated stainless-steel wires (Advent Research Materials Ltd, Witney, Bar Harbor, ME). Rats were originally purchased from Charles River Laboratories (strain code, 400; Bar Harbor, ME). Rats were housed individually and monitored daily for changes in body weight, physical appearance, and behavior. Chronic visceral hypersensitivity induction was performed as previously described. Briefly, 10- to 11-week-old male C57BL/6J mice were fasted overnight with access to 5% glucose solution before administration of an intracolonic enema of DNBS (6.5 mg in 30% ethanol; total volume of 0.1 mL) under isoflurane anesthesia. After DNBS administration, animals were housed individually and monitored daily for changes in bowel weight, physical appearance, and behavior. Chronic visceral hypersensitivity was assessed 28 days after DNBS administration. Male littermates were used as healthy control animals.

2.1. Animal models of colitis (inflammatory bowel disease–like model)

Colitis was induced by administration of trinitrobenzene sulfonic acid (TNBS; 1 M solution, Cat# 92822; Sigma-Aldrich, St. Louis, MO) or 2,4-dinitrobenzene sulfonic acid (DNBS; Cat# 556971; Sigma-Aldrich) as described previously. Briefly, 13-week-old male C57BL/6J mice and 6- to 7-week-old male Sprague-Dawley rats were fasted overnight with access to 5% glucose solution. After the fasting period, isoflurane-anaesthetized animals were administered an intracolonic enema of DNBS (6.5 mg in 30% ethanol; total volume of 0.1 mL) for mice and TNBS (12 mg in 35% ethanol; total volume of 0.3 mL) for rats. Animals were then individually housed with unlimited access to soaked food and 5% glucose solution and were subsequently observed daily for changes in body weight, physical appearance, and behavior.

2.1.2. Mouse model of chronic visceral hypersensitivity (irritable bowel syndrome–like model)

Chronic visceral hypersensitivity (CVH) induction was performed as described. Briefly, 10- to 11-week-old male C57BL/6J mice were fasted overnight with access to 5% glucose solution before administration of an intracolonic enema of DNBS (6.5 mg in 30% ethanol; total volume of 0.1 mL) under isoflurane anesthesia. After DNBS administration, animals were housed individually and monitored daily for changes in body weight, physical appearance, and behavior. Chronic visceral hypersensitivity was assessed 28 days after DNBS administration. Male littermates were used as healthy control animals.

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United Kingdom) were sutured into the muscle approximately 5 mm apart. The electrodes were tunneled subcutaneously and exteriorized at the base of the neck for future access. All rats and mice received prophylactic antibiotics (Baytril [enrofloxacin], Bayer AG; 5 mg/kg subcutaneously) and pain relief (buprenorphine 0.05 mg/kg subcutaneously). After surgery, animals were single housed to protect the EMG electrodes.

2.2.2. Assessing visceromotor response to colorectal distension

On the day of VMR assessment, rats and mice were briefly sedated with isoflurane and received a 500 μL saline enema to remove any fecal pellets in the distal colon. A lubricated, latex balloon (4.0 cm in length for rat studies, 2.5 cm in length for mouse studies) was gently passed through the anus and inserted up to 0.5 cm proximal to the anal verge. Once in position, the balloon catheter was secured to the base of the tail and connected to a barostat (ISOBAR-3, G&J Electronics Inc, Toronto, Canada) for pressure-controlled rapid inflation. Animals were then transferred to a restrainer with dorsal access, and the EMG electrodes were relayed to a data acquisition system. Rats and mice were allowed to regain consciousness for at least 10 minutes (mean, 29 minutes; range, 13-37 minutes) before the distension sequence was initiated. Distensions were applied by the barostat in a pressure-controlled fashion, ranging from the non-noxious to the noxious range (20, 40, 50, 60, 70, and 80 mm Hg of 20 seconds in duration with 4-minute intervals between consecutive distensions). The corresponding EMG signal was recorded (NL100AK AC Preamplifier headstage; Digitimer Ltd, Welwyn Garden City, United Kingdom), amplified (gain 2k; NL104A AC Preamplifier; Digitimer Ltd), filtered (bandpass 50-5000 Hz, NL125/126 Band Pass Filter; Digitimer Ltd), digitized (CED Micro1401; Cambridge Electronic Design Ltd, Cambridge, United Kingdom), and stored for analysis.

2.2.3. Colonic compliance

Immediately after VMR assessment, the balloon was manually inflated with known volumes of air (0.25, 0.5, 0.75, 1.0, 1.25, and 1.5 mL for rat studies and 40, 80, 120, 160, and 200 μL for mouse studies), and the corresponding intracolonic pressure was recorded to construct volume–pressure curves as a measure of colonic compliance.11,12,15,24,35

2.2.4. Visceromotor response statistical analysis

The analog EMG signal obtained from the VMR recordings was rectified and integrated. To quantify the magnitude of the VMR at each distension pressure, the area under the curve (AUC) during the distension (20 seconds) was corrected for the baseline activity (AUC predistension, 20 seconds). Total AUC was quantified by adding the individual AUC at each distension pressure.15,35 Data are presented as mean ± SEM, where N represents the number of animals. Area under the curve data were statistically analyzed by the generalized estimating equations method followed by a least significant difference (LSD) post hoc test when appropriate using IBM SPSS Statistics 23.0. Total AUC data were analyzed using unpaired 2-tailed t tests (for differences between 2 groups) or one-way analysis of variance (ANOVA) with post hoc analysis conducted with the Tukey multiple comparisons test (for differences between more than 2 groups). Analysis and figures were prepared using GraphPad Prism version 7.0. Differences were considered significant at P < 0.05.

2.3. Ex vivo mouse colonic primary afferent preparation recording

Single-unit extracellular recordings from splanchnic colonic afferent nerves were made from healthy, colitis (IBD-like), and CVH (IBS-like) mice as previously described.6,14,31 The colon (5-6 cm) and mesentery (containing the lumbar colonic nerves)
were removed intact, along with either the attached neurovascular bundle containing the inferior mesenteric ganglion and splanchnic nerve. The tissue was transferred to ice-cold Krebs solution, and, following further dissection, the distal colon and rectum were opened longitudinally along the antimesenteric border to orientate lumbar colonic insertions to lie along the edge of the open preparation. The tissue was pinned flat, mucosal side up, in a specialized organ bath consisting of 2 adjacent compartments generated from clear acrylic (Danz Instrument Service, Adelaide, South Australia, Australia), the floors of which were lined with SYLGYARD 184 (Dow Corning Corp., Midland, MI). The neurovascular bundle containing the splanchnic nerve was extended from the tissue compartment into the recording compartment where they were laid onto a mirror. A movable wall with a small "mouse hole" was lowered into position to allow passage of the nerves and the recording chamber filled with paraffin oil. The colonic compartment was superfused with a modified Krebs solution (117.9 mM NaCl, 4.7 mM KCl, 25 mM NaHCO3, 1.3 mM NaH2PO4, 1.2 mM MgSO4(H2O)7, 2.5 mM CaCl2, 1.1 mM D-glucose), bubbled with carbogen (95% O2 and 5% CO2) at a temperature of 34˚C. All preparations contained the L-type calcium channel antagonist nifedipine (1 µM) to suppress smooth muscle activity and the prostaglandin synthesis inhibitor indomethacin (3 µM) to suppress potential inhibitory actions of endogenous prostaglandins. Under a dissecting microscope, the splanchnic nerve was dissected away from the neurovascular bundle and the nerve sheath surrounding the splanchnic nerve. Using fine forceps, the nerve trunk was teased apart into 6 to 10 bundles, which were individually placed onto a platinum recording electrode. A platinum reference electrode rested on the mirror in a small pool of Krebs solution adjacent to the recording electrode.

### 2.3.1. Characterization of colonic afferent properties

Receptive fields were identified by systematically stroking the mucosal surface and mesenteric attachment with a stiff brush. Once identified, receptive fields were assessed with 3 distinct stimuli to enable classification: focal compression of the receptive field with calibrated von Frey hairs (vfh; 2000 mg; each force applied 3 times for a period of 3 seconds with a 10-second interval between each application), mucosal stroking with calibrated vfh (10 mg force applied 10 times), and circular stretch (5 g for a period of 1 minute). Stretch was applied using a claw made from bent dissection pins attached to the tissue adjacent to the afferent receptive field and connected to a cantilever system through thread. Weights were applied to the opposite side of the cantilever system to initiate graded colonic stretch. Only circular, and not longitudinal, stretch was tested in this study. Categorization of afferent properties was accorded based on previously published classifications and consisted of colonic nociceptors responding to focal compression and high-intensity stretch (≥7 g), but not low-intensity stroking (10 mg) nor low-intensity circular stretch (≤5 g).

For colonic nociceptor recordings from healthy, colitis, and CVH mice, after the baseline firing rate was recorded in response to mechanical stimulation with vfh (2 g), orolinab (0.01, 0.1, 1.0, or 10 µM) and/or a CB2 antagonist (SR144528; 1.0 µM SR144528 alone or 1.0 µM SR144528 plus 1.0 µM orolinab) were applied for 10 minutes through a small metal ring to the surface of the mucosal epithelium of colonic nociceptors, as previously described. Measurement of the firing rate in response to mechanical stimulation with vfh (2 g) was repeated after drug application.

### 2.3.2. Colonic nociceptor statistical analysis

Electrical signals generated by nerve fibers were amplified, filtered, digitized, and stored as described previously. Action potentials were analyzed off-line using the Spike2 version 5.21 waveform function and discriminated as single units on the basis of distinguishable waveform, amplitude, and duration. Data are presented as spikes/second or total number of action potentials per response. Data are expressed as mean with the SEM, and n is the number of afferents. Data were analyzed using one-way ANOVA followed by Bonferroni post hoc tests to determine significance. Differences were considered significant at P < 0.05.

### 2.4. Quantitative reverse transcription polymerase chain reaction studies

#### 2.4.1. Isolation of mouse colonic tissue and dorsal root ganglia

Healthy, colitis, or CVH mice (N = 2-6 per group) were humanely killed using carbon dioxide asphyxiation followed by cardiac perfusion, and tissues were quickly isolated. From each group, 2 cm of the distal colon was isolated and placed in ice-cold phosphate-buffered saline (PBS). The colon was flushed with ice-cold PBS to remove remaining fecal pellets and was cut longitudinally to collect the mucosal layer by carefully scraping the top layer under a dissecting microscope. The mucosal layer and the remaining tissue (muscle + ENS) were separated into different tubes and snap-frozen in liquid nitrogen. Dorsal root ganglia (DRG) were isolated concurrently with the colon to minimize the isolation time and ensure high messenger RNA (mRNA) integrity. Colon-innervating thoracolumbar (TL; T10-L1) and lumbarosacal (LS; L6-S1) DRG were isolated using the last rib (T13) as a location marker and stored in 2 separate tubes. TL and LS DRG were snap-frozen in liquid nitrogen. All tissues were stored at −80˚C until use as described previously.

#### 2.4.2. Sample preparation

RNA from mouse colonic tissue was isolated using the PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA) and from mouse DRG using the PureLink RNA Micro Kit (Invitrogen) according to the manufacturer’s instructions without modifications as described previously. All samples underwent an on-column DNase treatment with the PureLink DNase Set (Invitrogen). The elution volumes were 60 µL for mouse colonic tissue RNA and 20 µL for mouse DRG RNA. RNA was aliquoted and stored at −80˚C until use. RNA quality was assessed using the 2100 Bioanalyzer Instrument (Agilent Technologies, Inc, Santa Clara, CA) or the 2200 TapeStation System (Agilent Technologies, Inc). All samples had an RNA integrity number of 6.5 or higher.

#### 2.4.3. Quantitative reverse transcription polymerase chain reaction and probes

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using the EXPRESS One-Step Superscript qRT-PCR Kit (Invitrogen) and commercially available hydrolysis probes (TaqMan, Thermo Fisher Scientific). Rodent CB2 genes have 2 distinct promoter regions resulting in differential tissue expression [CB2α and CB2β]. Therefore, 3 TaqMan probes were used to measure CB2 expression: CB2α (MM00438286_m1 [mouse Cnr2A exon 1 + 3]), CB2β (APELLUZ624 [mouse Cnr2b exon 2 + 3]), and CB2α+β (MM02620087_s1 [detects both mouse Cnr2a and Cnr2b isoforms]). Cannabinoid receptor 1 transcription expression was also
Olorinab reversed colitis-induced hypersensitivity to CRD in colitis animals but had no effect in healthy animals. (A) Representative examples of EMG signals in response to each distension pressure for all animal cohorts. (B) In healthy rats, compared with vehicle treatment, olorinab administration at the highest dose of 30 mg/kg failed to alter the VMR to CRD. Comparison was not significant based on the generalized estimating equation method followed by the LSD post hoc test (\( P > 0.05 \)). (C) Total AUC (sum of the AUC obtained at all distension pressures) of the VMR to CRD showed no difference in response between healthy rats treated with vehicle or the highest dose of olorinab (30 mg/kg). Comparison was not significant using a 2-tailed unpaired \( t \) test (\( P > 0.05 \)). (D) Vehicle-treated colitis rats exhibited significantly enhanced VMR to CRD compared with vehicle-treated healthy control rats. Significant increases in colitis rats were observed across all distension pressures from 40 mm Hg. Olorinab treatment of colitis rats with doses of either 3 mg/kg or 30 mg/kg significantly reduced VMR to CRD relative to vehicle-treated colitis rats. Comparisons were performed using the generalized estimating equation method followed by the LSD post hoc test. \( * P < 0.01; ** P < 0.001 \). (E) No significant changes in colonic compliance were observed between healthy rats treated with vehicle or olorinab at either dose. (G) No significant changes in colonic compliance were observed between healthy rats treated with vehicle or olorinab at either dose. All compliance comparisons were performed using the generalized estimating equation method followed by a Tukey multiple comparisons test. \( * P < 0.05; ** P < 0.01; *** P < 0.001 \). No significant changes in colonic compliance were observed between healthy rats treated with vehicle or olorinab at either dose. All compliance comparisons were performed using the generalized estimating equation method followed by the LSD post hoc test, and results were not significant (\( P > 0.05 \)). Data are presented as mean \( \pm \) SEM.
marker for T13 and L1 DRG, TL DRG (T9-L1) and LS DRG (L6-S1) were isolated, pooled separately for TL and LS, and placed in ice-cold 4% PFA. Spinal cord areas that corresponded to the isolated DRG sections and the entire spleen were also removed and placed in 4% PFA. All tissues were postfixed in 4% PFA at 4°C for 20 to 24 hours, then transferred to a series of graded sucrose/0.1 M PB solutions (10%, 20%, and 30%) each for 24 hours. Tissue was frozen in Optimal Cutting Compound (Tissue-Tek OCT Compound, Sakura Finetek, Alphen aan den Rijn, Netherlands) and stored at −80°C until use. Tissue sections were cut with a cryostat at 10-μm thickness. Sections were mounted in duplicate or triplicate for each sample of colon and DRG, respectively, with a randomly selected sample from each group per slide; some slides also contained a section of spleen (positive control). Slides were air dried at room temperature and washed in 7.4 pH PBS before the in situ hybridization (ISH) staining protocol.

In situ labeling was performed with the RNAscope 2.5 HD Manual Assay Brown (Advanced Cell Diagnostics, Newark, CA) kit. Briefly, slides were pretreated with H2O2 solution, washed, and submerged in prewarmed humidity chamber. RNAscope Probes for CB2 (NM_009924.3), a negative control (dihydrodipicolinate reductase gene [dapB]; EF191515), and a positive control (peptidylprolyl isomerase B gene; NM_011149.2) were used. Sections underwent amplification per the manufacturer’s protocol and were counterstained the following day with 25% hematoxylin solution, Gill No. 1 (Sigma-Aldrich, Saint Louis, MO), dipped in acid alcohol, and incubated in Scott’s water bluing agent (3.5 g/L NaHCO3 and 20 g/L DPX (Sigma-Aldrich), and dried overnight until imaged. Sections were imaged with a NanoZoomer Digital Slide Scanner (Hamamatsu Photonics, Shizoku, Japan) using 5× to 40× objectives, with no modifications made to the images.

2.5.1. In situ hybridization analysis
All images were imported into a QuPath image analysis program, allowing the regions of interest (ROIs) to be created onto the images covering only the colonic epithelium. The detect positive staining analysis module with standardized settings (downsample factor 2, Gaussian sigma 2, hematoxylin threshold “negative” 1 OD units, allowing the regions of interest (ROIs) to be created onto the images covering only the colonic epithelium. The detect positive staining analysis module with standardized settings (downsample factor 2, Gaussian sigma 2, hematoxylin threshold “negative” 1 OD units, DAB threshold “positive” 0.3-0.4 OD units) was then run for each ROI in each image. This produced measurements for the percentage of the total ROI stained with “positive pixels.” Measurements of percentages of the positive pixel area were collected from 3 to 4 ROIs per image and copied to an Excel spreadsheet. After all images were analyzed in this fashion, they were sorted into their experimental groups, and the percentages of the positive pixel area per ROI per section were plotted and analyzed using GraphPad Prism (version 8). Data are plotted as all ROIs per section, average area per section, and average area per mouse.

3. Results
3.1. Olorinab decreased colitis-induced acute visceral hypersensitivity
To determine the potential for olorinab to reduce acute visceral hypersensitivity, we evaluated whether olorinab affected visceral sensitivity to CRD in healthy rats in vivo by measuring the VMR to

Figure 3. Olorinab reversed hypersensitivity to CRD in CVH mice but had no effect in healthy mice. (A) Representative examples of EMG signals in response to each distension pressure for all mouse cohorts. (B) Vehicle-treated CVH mice exhibited significantly enhanced VMR to CRD compared with vehicle-treated healthy control mice. Significant increases in CVH mice were observed for distension pressures higher than 40 mm Hg. Olorinab treatment of CVH mice with doses of 3 mg/kg, 10 mg/kg, or 30 mg/kg significantly reduced VMR to CRD relative to vehicle-treated healthy mice. (C) Total AUC of the VMR to CRD relative to vehicle-treated healthy mice. Comparisons were performed using a one-way ANOVA followed by Tukey post hoc tests. *P < 0.05; †P < 0.001; ‡P < 0.0001. (D) No significant changes in colonic compliance were observed between healthy mice treated with vehicle and CVH mice treated with vehicle or olorinab at all doses. Data are presented as mean ± SEM. AUC was calculated as the difference of area values obtained predistension (20 seconds) minus those obtained during distension (20 seconds). ∗Sum of all distension pressures. ANOVA, analysis of variance; AUC, area under the curve; CRD, colorectal distension; CVH, chronic visceral hypersensitivity; EMG, electromyography; LSD, least squares difference; VMR, visceromotor response.
increasing CRD pressures by recording EMG activity from electrodes surgically implanted into the abdominal muscles. The abdominal EMG activity increased in a graded fashion with increasing levels of CRD (Fig. 2A). Olorinab 30 mg/kg, the highest dose tested in this study, had no effect on the VMR to CRD in healthy rats compared with vehicle-treated control animals (Figs. 2A–C). We and others have previously demonstrated that colitis in mice and rats induces pronounced visceral hypersensitivity to mechanical stimuli. We and others have previously demonstrated that colitis in mice and rats induces pronounced visceral hypersensitivity to mechanical stimuli.

3.2. Olorinab decreased colitis-induced chronic visceral hypersensitivity

We have previously demonstrated that after recovery from colitis, mice display pronounced visceral hypersensitivity to mechanical stimuli in the postinflammatory state. Here, we repeated those findings and found that 28 days after colitis induction, mice displayed CVH in vivo, as indicated by significantly elevated VMR to CRD, particularly at distension pressures from 40 mm Hg to 80 mm Hg, compared with control mice (Figs. 3A, B). We also found that the CVH mice displayed significantly increased total VMRs compared with control mice (Figs. 3A, C). We then treated mice with 4 different doses of olorinab (1, 3, 10, or 30 mg/kg) or vehicle and found that doses of 3 mg/kg, 10 mg/kg, or 30 mg/kg significantly reduced the VMR to CRD and the total AUC to CRD relative to vehicle-treated colitis mice (Figs. 3A–C). By contrast, olorinab 1 mg/kg was not effective at reducing the VMR to CRD in CVH mice (Figs. 3A–C). Notably, colonic compliance was unaltered in CVH mice compared with healthy control mice and was unaltered by olorinab treatment (Fig. 3D), further supporting the hypothesis that the analgesic effect of olorinab occurs at the level of the colonic afferent endings.

3.3. Olorinab reduced mechanical hypersensitivity of colonic nociceptors from colitis and chronic visceral hypersensitivity mice through a cannabinoid receptor 2–dependent mechanism

To determine whether olorinab alters colonic sensory function at the afferent level, ex vivo recordings of colonic nociceptors from healthy control, colitis, and CVH mice were assessed. We found that acute application of olorinab (0.01 μM-10 μM) to the mucosal surface surrounding the afferent ending had no effect on healthy colonic nociceptor responses to mechanical stimuli (Figs. 4A–C), which was consistent with our in vivo findings of olorinab administered to healthy rats. By contrast, olorinab (0.01 μM-10 μM) significantly and dose dependently decreased the activity of colonic nociceptors from colitis mice, with a maximum reduction in response to mechanical stimulation of approximately 50% at the highest concentration of olorinab tested.
Figure 5. Olorinab dose dependently inhibited colonic nociceptors from colitis mice through a CB2-dependent mechanism. (A) Application of increasing concentrations of olorinab to ex vivo colonic nociceptor endings isolated from colitis mice caused a dose-dependent decrease in action potential firing in response to mechanical stimulation (2 g vfh). Comparisons were performed using a one-way ANOVA followed by the Bonferroni post hoc test (*P < 0.05; **P < 0.01; †P < 0.001). (B) The change in colitis colonic nociceptor mechanosensitivity induced by olorinab compared with baseline responses indicated a dose-dependent decrease in nociceptor firing with increasing concentrations of olorinab. (C) Single-unit colonic nociceptor recordings from colitis mice showed mechanical responsiveness at baseline and dose-dependent inhibition with increasing concentrations of olorinab. (D) Application of the CB2 antagonist SR144528 had no effect on the baseline mechanosensitivity of colitis colonic nociceptors and prevented olorinab-induced inhibition of nociceptor action potential firing in response to mechanical stimulation (2 g vfh; †P > 0.05). (E) The change in colitis colonic nociceptor mechanosensitivity induced by olorinab compared with baseline demonstrated no inhibitory action of olorinab in the presence of the CB2 antagonist. (F) Single-unit colonic nociceptor recordings from colitis mice showed mechanical responsiveness was unchanged with olorinab application in the presence of the CB2 antagonist. Data are presented as mean ± SEM. ANOVA, analysis of variance; CB2, cannabinoid receptor 2; IBD, inflammatory bowel disease; vfh, von Frey hair.

Figure 6. Olorinab dose dependently inhibited colonic nociceptors from CVH mice through a CB2-dependent mechanism. (A) Ex vivo application of increasing concentrations of olorinab to ex vivo colonic nociceptor endings isolated from CFVH mice caused a decrease in action potential firing in response to mechanical stimulation (2 g vfh; *P < 0.05; **P < 0.01; †P < 0.001). (B) The change in CVH colonic nociceptor mechanosensitivity induced by olorinab compared with baseline responses indicated a dose-dependent decrease in nociceptor response with increasing concentrations of olorinab. (C) Single-unit colonic nociceptor recordings from CVH mice showed mechanical responsiveness at baseline and dose-dependent inhibition with increasing concentrations of olorinab. (D) Application of the CB2 antagonist SR144528 had no effect on the baseline mechanosensitivity of CVH colonic nociceptors and prevented olorinab-induced inhibition of nociceptor action potential firing in response to mechanical stimulation (2 g vfh; †P > 0.05). (E) The change in CVH colonic nociceptor mechanosensitivity induced by olorinab compared with baseline demonstrated no inhibitory action of olorinab in the presence of the CB2 antagonist. (F) Single-unit colonic nociceptor recordings from CVH mice showed the mechanical responsiveness was unchanged with olorinab application in the presence of the CB2 antagonist. Data are presented as mean ± SEM. ANOVA, analysis of variance; CB2, cannabinoid receptor 2; CVH, colonic visceral hypersensitivity; IBS, irritable bowel syndrome; vfh, von Frey hair.
Although olorinab treatment did not have a significant effect at a concentration of 0.01 μM, olorinab at concentrations of 0.1 μM, 1.0 μM, and 10 μM did result in a significant reduction in mechanosensitivity of colonic nociceptors from colitis mice (Figs. 5A–C). To confirm that the antinociceptive effect of olorinab is indeed mediated by CB2 in the colonic mucosa from healthy, colitis, and CVH mice, with CB2A as the most prevalent CB2 isoform. (B) In the colonic longitudinal and circular muscle also containing the myenteric plexus (ENS) from healthy, colitis, and CVH mice, CB1 had the highest relative abundance of all CB receptor transcripts compared with CB2A, CB2A, and CB2B. (C) In TL and LS DRG from healthy, colitis, and CVH mice, CB1 had the highest relative abundance of all CB receptor transcripts. CB2 mRNA was also detected in the TL and LS DRG, with the CB2A isoform as the predominantly expressed CB2 isoform. Expression profiles did not significantly differ between healthy, colitis, and CVH states in the (A) colonic mucosa, (B) colonic muscle + ENS, or (C) TL or LS DRG (P > 0.05). All comparisons shown were performed using a one-way ANOVA followed by the Tukey multiple comparison post hoc test (*P < 0.05; **P < 0.01; †P < 0.001; ‡P < 0.0001). CB1 and CB2 mRNA expression was measured relative to reference gene mRNA expression (Ppia, Gapdh, and β-actin) quantified by geometric mean. Data are presented as mean ± SEM. ANOVA, analysis of variance; CB1, cannabinoid receptor 1; CB2, cannabinoid receptor 2; DRG, dorsal root ganglia; ENS, enteric nervous system; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; LS, lumbosacral; mRNA, messenger ribonucleic acid; Ppia, peptidylprolyl isomerase A; TL, thoracolumbar.

(Figs. 5A–C). Although olorinab treatment did not have a significant effect at a concentration of 0.01 μM, olorinab at concentrations of 0.1 μM, 1.0 μM, and 10 μM did result in a significant reduction in mechanosensitivity of colonic nociceptors from colitis mice (Figs. 5A–C). To confirm that the antinociceptive effect of olorinab is indeed mediated by CB2, colonic nociceptors from mice with colitis were treated with the CB2 antagonist SR144528 with and without concurrent olorinab administration. SR144528 alone had no effect on the mechanosensitivity of colonic nociceptors from colitis mice, suggesting a lack of endogenous CB2 tone in this state (Figs. 5D–F). However, SR144528 blocked olorinab-induced inhibition of colonic nociceptors from colitis mice (Figs. 5D–F), confirming that CB2 mediates the antinociceptive effect of olorinab in colitis.
A significant reduction in mechanosensitivity was observed with olorinab at concentrations of 0.1 μM, 1.0 μM, and 10 μM. Colonic nociceptors from CVH mice were also treated with the CB2 receptor antagonist SR144528, with and without concurrent olorinab application. SR144528 alone had no effect on the mechanosensitivity of colonic nociceptors from CVH mice (Figs. 6D–F), suggesting a lack of endogenous CB2 tone in this CVH state. However, concurrent administration of SR144528 with olorinab attenuated the olorinab-induced reduction in mechanosensitivity of colonic nociceptors from CVH mice (Figs. 6D–F), confirming that the antinociceptive effect of olorinab in CVH states is mediated by CB2.

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3.4. Cannabinoid receptor 1 and cannabinoid receptor 2 mRNA expression within colonic tissue and dorsal root ganglia in control, colitis, and chronic visceral hypersensitivity states

Having shown that olorinab reduced colonic hypersensitivity in colitis and CVH but not in control states and that this effect was CB2 dependent, we wanted to determine whether CB2 was expressed at potential sites of olorinab activity and to evaluate whether CB2 was upregulated in disease states. To do so, qRT-PCR probes for the 2 CB2 isoforms (CB2A and CB2B) and a probe that spanned both isoforms (CB2A+C2) were used. A CB1 probe was also used for comparison.
mRNA expression of CB1 and CB2 was assessed in the colonic mucosa, the circular and longitudinal muscle of the colon including the myenteric plexus between them (colonic muscle + ENS), and DRG isolated from healthy control, colitis, and CVH mice. We found that CB2 was the more abundant transcript in the colonic mucosa and that there were no significant differences in CB1 or CB2 isoform expression between control, colitis, and CVH states (Fig. 7A). By contrast, CB1 was the predominant isoform in the colonic muscle + ENS, followed by CB2A (Fig. 7B). However, no significant differences in CB1 or CB2 expression levels were observed between healthy control, colitis, and CVH states (Fig. 7B). In the TL and LS DRG that innervate the colon, CB1 was the predominantly expressed cannabinoid receptor, whereas CB2A was the most abundantly expressed CB2 isoform (Fig. 7C). Overall, there was no significant difference in isoform expression between the DRG in control, colitis, and CVH states, suggesting a lack of change in transcription of these receptors in the DRG at the time points investigated (Fig. 7C).

3.5. Cannabinoid receptor 2 mRNA localization using in situ hybridization within colonic tissue and dorsal root ganglia in control, colitis, and chronic visceral hypersensitivity states

Olorinab reduced visceral hypersensitivity ex vivo and in vivo in colitis and CVH states but did not significantly change CB2 mRNA expression, as demonstrated by qRT-PCR. Therefore, we sought to determine the localization of CB2 in colonic tissue and DRG using ISH. First, the CB2 probe was validated using positive

Figure 9. ISH of CB2 mRNA expression in DRG and proposed mechanism of action of olorinab: (A–C) Representative images of sections of thoracolumbar DRG from healthy mice that underwent hematoxylin staining (blue) and ISH labeling for (A, B) CB2 (brown dots) or (C) the negative probe dapB. Representative images of CB2 labeling in sections of (D) thoracolumbar and (E) lumbosacral DRG from healthy, acute colitis, and CVH mice. Scale bars = 20 μm. (F) Hypothesized mechanism of action of olorinab, which is a highly selective full agonist for CB2 that exhibits low brain penetration. To modulate abdominal pain, olorinab may activate CB2 located on one or multiple cell types including epithelial cells, immune cells, and afferent nerves within the gastrointestinal wall. Downstream or direct effects of CB2 activation may then reduce action potential firing of colonic nociceptors. This would reduce the nociceptive signal being sent from the gastrointestinal tract to the spinal cord, where this nociceptive information is processed and then sent to the brain where pain is perceived. CB2, cannabinoid receptor 2; CVH, chronic visceral hypersensitivity; DRG, dorsal root ganglia; ISH, in situ hybridization; LS, lumbosacral; TL, thoracolumbar.
In colonic tissue, CB2 labeling was prominent within the epithelial cells lining the lumen and the crypts (Figs. 8B, C). In comparison, CB2 mRNA labeling was sparser within the lamina propria, muscularis mucosae, and myenteric plexus (Figs. 8B, C). Quantitative analysis showed that there was no significant difference in CB2 expression in the colons from healthy, colitis, and CVH mice (Figs. 8C–F). We also performed ISH in TL and LS DRG and found that CB2 mRNA localized in subsets of DRG neurons from healthy control, colitis, and CVH mice (Figs. 9A–E).

4. Discussion

Visceral hypersensitivity associated with colitis leads to substantial abdominal pain in patients with IBD. Colitis results in the release of neuroactive signaling molecules from a variety of cell types, including epithelial and immune cells. Numerous mediators can act directly on receptors expressed by afferent fibers innervating the colon, resulting in sensitization to mechanical and chemical stimuli. Importantly, activation of nociceptors can induce an inflammatory reaction (neurogenic inflammation) by releasing calcitonin gene-related peptide and substance P from their peripheral terminals, further increasing afferent sensitization. Similarly, visceral hypersensitivity is a key mechanism underlying abdominal pain in IBS and may result from increased intestinal permeability, altered gut microbiota, low-grade inflammation, and dysfunction of the brain–gut axis. Therefore, reducing visceral hypersensitivity is a targeted way to potentially alleviate abdominal pain in IBD and IBS.

In our study, intracolonic administration of TNBS or DNBS was used to induce colitis resulting in significantly enhanced VMR to CRD and colonic nociceptor activity compared with control animals, consistent with the induction of visceral hypersensitivity. Similarly, in the postinflammatory state, we observed CVH in ex vivo and in vivo studies. These observations are comparable with previous reports demonstrating inflammation-induced hypersensitivity of colonic afferents at acute and chronic time points. These findings support the validity of these preclinical models for the evaluation of the role of CB2 signaling in colitis-induced acute and chronic visceral pain.

Olorinab (3 and 30 mg/kg) significantly reduced the VMR to CRD in colitis rodents compared with vehicle treatment. In the presence of olorinab, VMR to CRD in animals with colitis was similar to responses in healthy control animals, and no effects on colonic compliance in any treatment group were observed. Furthermore, mechanosensory responses of colonic nociceptors were significantly and dose dependently reduced with olorinab in colitis animals. We found similar results in our CVH model when mice were administered olorinab at a time point when visceral hypersensitivity was already established. We extended these studies in CVH mice to include additional doses of olorinab and found that 3, 10, and 30 mg/kg reduced visceral hypersensitivity in vivo. In addition, mechanosensory responses of colonic nociceptors from CVH mice were significantly and dose dependently reduced by olorinab. In colitis and CVH states, the antinoceptive activity of olorinab was blocked by the CB2 antagonist SR144528.

Our data support a previous study using VMR to CRD in rodents with colitis induced by TNBS to assess the relative roles of CB1 and CB2. Agonists of both CB1 and CB2 have been shown to diminish the hypersensitivity caused by colitis, and a CB1 antagonist could enhance colitis-induced hyperalgesia. Similar protective effects of CB2 activation in preclinical models of colitis have been shown, and these effects were blocked in the presence of a CB2 antagonist and were absent in CB2-deficient mice. Therefore, the results of this study provide additional support for the role of CB2 in reducing visceral nociception during and after colitis and the specificity of olorinab in targeting CB2.

Our data are also in line with previous observations that CB2 activation is an important regulator of neuroimmune function in small intestinal nerves. Bradykinin-induced activation of mesenteric afferents in vivo was reversed by the selective CB2 agonist AM1241, and this effect was completely abolished by the CB2 antagonist AM630. Notably, Hillsley and colleagues showed that AM1241 also inhibited bradykinin effects in mesenteric afferents from healthy unsensitized animals, whereas in our study, CB2 activation with olorinab had no impact on VMR to CRD or mechano-sensitive nociceptors in tissue from healthy animals. Key differences between our study and this previously published work include ex vivo recordings from colonic afferents vs in vivo recordings from small intestinal afferents, as well as comparing the effects of CB2 agonists on mechanical vs chemical (bradykinin) stimulation of afferents. In addition, not all CB2 agonists activate CB2 in the same manner, with various agonists showing bias for different signal transduction pathways; therefore, AM1241 and olorinab may interact with CB2 differently resulting in these observations. Furthermore, olorinab is a full agonist of CB2, which is critical for efficient receptor internalization and prevention of tachyphylaxis; whereas AM1241 acts as a partial CB2 agonist, which may also contribute to these differences. Moreover, our previous research has identified other G-protein-coupled receptors that appear nonfunctional or dormant in healthy conditions but become responsive to selective agonists in disease states. This functional upregulation was observed with K-opioid receptors (KORs) and oxytocin receptors (OTRS), which only mediated antinoceptive effects in colonic nociceptors during colitis or postinflammatory CVH states, but not during healthy states. Transcriptional and/or posttranscriptional modifications may contribute to these observations; however, more studies are needed to understand the molecular mechanisms accounting for the functional upregulation of KORs, OTRs, and CB2 in diseased states.

Using qRT-PCR, we observed low but detectable levels of CB2 mRNA in the DRG of healthy, colitis, and CVH animals. We also showed that CB2 mRNA is expressed within the colonic mucosa and colonic muscle + ENS in healthy, colitis, and CVH states. Interestingly, although CB2 is expressed at higher abundance than CB1 in the DRG and colonic muscle + ENS, in the colonic mucosa, CB2—specifically the CB2A isoform—predominates. These findings confirm CB2 expression patterns observed in previous studies.

Our ISH studies further confirm these observations, with CB2 mRNA predominantly localized in colonic epithelial cells and some expression also observed in the lamina propria, muscularis mucosae, myenteric plexus, and subsets of neurons in the TL and LS DRG. This localization in DRG neurons confirms previous RNAseq analysis, indicating that some colon-innervating DRG neurons express CB2 mRNA.

Although previous work has shown increased expression of CB2 in preclinical models of gastrointestinal inflammation and disease and in colonic tissue from patients with IBD and IBS compared with healthy individuals, a significant increase in CB2 mRNA expression was not observed in colitis or CVH vs healthy states in the tissues investigated in our study. The lack of change in CB2 mRNA expression in colonic tissue in colitis or CVH mice we observed is consistent with other studies using a DNBS-induced colitis mouse model. Overall, these differences may be due to several factors. For example, CB2 expression was assessed at very specific time points.
points in the colitis and CVH profile when colonic hypersensitivity is known to be present. However, the kinetics of CB2 transcript upregulation in these acute VH and CVH rodent models may differ from other models or the longer-term chronic disease states associated with IBD and IBS. Overall, our current findings suggest CB2 expression is not dramatically upregulated after an inflammatory trigger, as seen with OTRs. Rather, CB2 appears to lie in a dormant state until it is sensitized, much like our previous findings with KORs.\(^6,52\)

In addition to promoting antinociceptive effects on colonic afferents, CB2 agonism has been reported to reduce inflammation in models of IBD.\(^5,9,52,54,57\) Activation of CB2 expressed on immune cells results in changes in the cytokine profile secreted,\(^57,59,60\) which can lead to the modulation of inflammation and can potentially reduce neuronal sensitization.\(^23,59,50,54\) This is further supported by increased susceptibility to TNBS-induced colitis and inflammation in CB2-knockout mice compared with wild-type mice.\(^22\) Furthermore, research using human explant tissue has shown that activation of CB2, but not CB1, may protect against cytokine-mediated inflammation and epithelial damage known to contribute to abdominal pain.\(^27\) Future studies will investigate whether orinab has potential anti-inflammatory actions that may mediate or contribute to its antinociceptive actions.

Overall, these data indicate that orinab, through selective activation of CB2, may provide a novel treatment for abdominal pain associated with IBD and IBS. These data provide further evidence of CB2-mediated control of visceral hypersensitivity during or after states of inflammation and increased function of CB2 in diseased states. We hypothesize that orinab might activate CB2 located on one or multiple cell types, including epithelial cells, immune cells, and nerves in the intestinal wall (Fig. 9F). After receptor activation, direct or indirect effects of CB2 activation may then suppress colonic nociceptors sending nociceptive signals to the central nervous system. It should be noted that although these data are derived from well-studied and validated rodent models of colitis and CVH, as in all animal models, there is no certainty that the model accurately reflects human disease in all conditions.

In conclusion, our data indicate that orinab, a selective full CB2 agonist, was effective at reducing visceral hypersensitivity in animal models of IBD and IBS. These preclinical data exploring the mechanism of action and impact of orinab on visceral hypersensitivity support the further evaluation of orinab in clinical settings. Orinab is currently in clinical development for abdominal pain associated with gastrointestinal conditions, including IBD (ClinicalTrials.gov identifier, NCT03155945) and IBS (NCT04043455).

Conflict of interest statement
S. Schmied, B. Lindstrom, and J. Adams are employees of Arena Pharmaceuticals, Inc. S.M. Brierley received research funding from Arena Pharmaceuticals to conduct the study. The remaining authors have no conflicts of interest to declare. Data from this study were previously presented in part at Digestive Disease Week (DDW) on May 2, 2020, as a virtual ePoster; DDW 2019 on May 18 to 21, 2019, in San Diego, CA; American Neurogastroenterology and Motility Society (ANMS) Annual Meeting on August 16 to 18, 2019, in Chicago, IL; United European Gastroenterology (UEG) Week on October 11, 2020, as a virtual presentation; and UEGW on October 19 to 23, 2019, in Barcelona, Spain.

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References
[1] Adam B, Liebregts T, Gschosssmann JM, Krippner C, Scholl F, Ruwee M, Holtmann G. Severity of mucosal inflammation as a predictor for alterations of visceral sensory function in a rat model. PAIN 2006;123: 179–86.
[2] Adams JW, Unett D, Anthony T, Galtlin J, Gaidarov I. AQP371: A Potent, highly selective, full agonist of the human CB2 receptor with sustained analgesic effects in rodents. J Pain 2018;19(52).
[3] American Gastroenterological Association. IBS in America. Survey summary findings. Vol. 2020. Bethesda, MD: American Gastroenterological Association, 2015.
[4] Bellino NW, Bayrer JR, Leitch DB, Castro J, Zhang C, O’Donnell TA, Brierley SM, Ingraham HA, Julius D. Enterochromaffin cells are gut chemosensors that couple to sensory neural pathways. Cell 2017;170: 185–98.e116.
[5] Borelli F, Romano B, Petrosino S, Pagano E, Capasso R, Coppola D, Battista G, Orlando P, Di Marzo V, Izzo AA. Palmitoylethanolamide, a naturally occurring lipid, is an orally effective intestinal anti-inflammatory agent. Br J Pharmacol 2015;172: 142–58.
[6] Brierley SM, Jones RC III, Gebhart GF, Blackshaw LA. Splanchnic and pelvic mechanosensory afferents signal different qualities of colonic stimuli in mice. Gastroenterology 2004;127:166–78.
[7] Brierley SM, Linden DR. Neuropathology and dysfunction after gastrointestinal inflammation. Nat Rev Gastroenterol Hepatol 2014;11: 611–27.
[8] Brust A, Croker DE, Colleiss B, Ragnansson L, Andersson A, Jain K, Garcia-Caraballo S, Castro J, Brierley SM, Alewod PF, Lewis RJ. Conopeptide-derived kappa-opioid agonists (conorphins): potent, selective, and metabolic stable dynorphin a mimetics with antinociceptive properties. J Med Chem 2016;59:2381–95.
[9] Cain KC, Headstrom P, Jarrett ME, Motzer SA, Park H, Burr RL, Surawicz CM, Heitkemper MM. Abdominal pain impacts quality of life in women with irritable bowel syndrome. Am J Gastroenterol 2006;101:124–32.
[10] Carstens BB, Berecki G, Daniel JT, Lee HS, Jackson KA, Tae HS, Sadeghi M, Castro J, O’Donnell T, Deiteren A, Brierley SM, Craik DJ, Adams DJ, Clark RJ. Structure-activity studies of cysteine-rich alpha-conotoxins that inhibit high-voltage-activated calcium channels via GABAB receptor activation reveal a minimal functional motif. Angew Chem Int Ed Engl 2016;55:4692–6.
[11] Caswell HO, Herrington AM, Hughes PA, Martin CM, Ge P, Shea CM, Jin H, Jacobson S, Hanning G, Mann E, Cohen MB, MacDougall JF, Lavins BJ, Kurtz CB, Silos-Santiago I, Johnston JM, Currie MG, Blackshaw LA, Brierley SM. Linaclootide inhibits colonic nociceptors and relieves
abdominal pain via guanylate cyclase-C and extracellular cyclic guanosine 3',5'-monophosphate. Gastroenterology 2013;145:1334-6.e1331-1311.

[32] Hughes PA, Castro J, Harrington AM, Isaacs N, Moretta M, Hicks GA, Urso DM, Brierley SM. Increased kappa-opioid receptor expression and function during chronic visceral hypersensitivity. Gut 2014;63:1199–200.

[33] Inserna MC, Israel MR, Caldwell A, Castro J, Devis JR, Harrington AM, Keramidas A, Garcia-Caraballo S, Maddern J, Erickson A, Grundy L, Rykovych GY, Zimmermann K, Lewis RJ, Brierley SM, Vetter I. Multiple subtypes of colonic and rectal GPR11, GPR115, and GPR17 potentiate the pathological effects of Pacific ciguatoxin-1. Sci Rep 2017;7:42810.

[34] Ivanova Y, Ando K, Taniguchi K, Koba N, Sugiya A, Sudo M. Identification of a highly potent and selective CB2 agonist, RQ-0020730, for the treatment of irritable bowel syndrome. Bioorg Med Chem Lett 2015;25:236–40.

[35] Iwamoto Vargas NN, Pattison LA, Zhao P, Lieu T, Latorre F, Jensen DD, Castro J, Aurelio L, Le GT, Flynn B, Herenbrink CK, Yeatman HR, Edington-Mitchell L, Porter CJH, Halls ML, Canals M, Veldhuis NA, Poole DP, McLean P, Hicks GA, Schef N, Chen E, Bhattacharya A, Schmidt BL, Brierley SM, Vanner SJ, Bunnell NW. Protease-activated receptor-2 in endosomes signals persistent pain of irritable bowel syndrome. Proc Natl Acad Sci U S A 2018;115: E7438–47.

[36] Ikouchi A, Kusuda K, Sugiyama M, Oumura H. Pharmacological evaluation of a novel cannabinoid 2 (CB2) ligand, PF-03550006, in vitro and in vivo by using a rat model of visceral hypersensitivity. J Pharmacol Sci 2008;106:219–24.

[37] Imamual ES, Schneller CR, Wallace NH, Hornby PJ. Agonists of cannabinoid receptor 1 and 2 inhibit experimental colitis induced by oil of mustard and by dextran sodium sulfate. Am J Physiol Gastrointest Liver Physiol 2006;291:G364–71.

[38] Krishna Kumar K, Shalev-Benami M, Robertson MJ, Hu H, Banister SD, Hollingsworth SA, Latorre T, Cenac N, Garcia-Caraballo S, Maddern J, Rychkov GY, Weyer AD, Dekan Z, Deiteren A. Selenenothiol oxytocin analogues have anagues properties in a mouse model of chronic abdominal pain. Nat Commun 2014;5:3165.

[39] Desormeaux C, Bautzova T, Garcia-Caraballo S, Rolland C, Barbero MR, Brierley SM, Barbara G, Vergnolle N, Cenac N. Protease-activated receptor 1 is implicated in irritable bowel syndrome mediators-induced signaling to thoracic human sensory neurons. PAIN 2018;159:1257–67.

[40] Doteh G, Chang L, Shih W, Barbero MR, Cremon C, Stanghellini V, De Porta F, Mayer EA. Randomised clinical trial: the anagues properties of dietary supplementation with palmitoylethanolamine and polydolamide in irritable bowel syndrome patients. Neurogastroenterol Motil 2019;31:e13688.

[41] Duncan M, Moulhate A, Mackie K, Keenan CM, Buckley NE, Davison JS, Patel KD, Pittman GJ, Sharkey KA. Cannabinoid CB2 receptors in the enteric nervous system mediate gastrointestinal contractility in lipopolysaccharide-treated rats. Am J Physiol Gastrointest Liver Physiol 2008;295:G78–87.

[42] Erk P, Aziz O, Barbara G, Farmer AD, Fukudo S, Mayer EA, Niesler B, Duncan M, Mouihate A, Mackie K, Keenan CM, Buckley NE, Davison JS, Patel KD, Pittman GJ, Sharkey KA. Cannabinoid CB2 receptors in the enteric nervous system mediate gastrointestinal contractility in lipopolysaccharide-treated rats. Am J Physiol Gastrointest Liver Physiol 2008;295:G78–87.

[43] Erk P, Aziz O, Barbara G, Farmer AD, Fukudo S, Mayer EA, Niesler B, Quigley EM, Rajic-Stojanov M, Schermann M, Schvile-Kuntuke J, Simren M, Zipfel S, Spiller RC. Irritable bowel syndrome. Nat Rev Dis Primers 2016;2:16014.

[44] Engel MA, Kellner RE, Emrick JJ, Zhang C, Castro J, 8224–56.

[45] Emmanuel A, Hungin APS, Layer P, Stanghellini V, Whorwell P, Zerbib F, Brierley SM, Barbara G, Vergnolle N, Cenac N. Protease-activated receptor-2 in endosomes signals persistent pain of irritable bowel syndrome. Proc Natl Acad Sci U S A 2018;115: E7438–47.

[46] Enck P, Aziz O, Barbara G, Farmer AD, Fukudo S, Mayer EA, Niesler B, Quigley EM, Rajic-Stojanov M, Schermann M, Schvile-Kuntuke J, Simren M, Zipfel S, Spiller RC. Irritable bowel syndrome. Nat Rev Dis Primers 2016;2:16014.

[47] Enck P, Aziz O, Barbara G, Farmer AD, Fukudo S, Mayer EA, Niesler B, Quigley EM, Rajic-Stojanov M, Schermann M, Schvile-Kuntuke J, Simren M, Zipfel S, Spiller RC. Irritable bowel syndrome. Nat Rev Dis Primers 2016;2:16014.

[48] Erk P, Aziz O, Barbara G, Farmer AD, Fukudo S, Mayer EA, Niesler B, Quigley EM, Rajic-Stojanov M, Schermann M, Schvile-Kuntuke J, Simren M, Zipfel S, Spiller RC. Irritable bowel syndrome. Nat Rev Dis Primers 2016;2:16014.

[49] Erk P, Aziz O, Barbara G, Farmer AD, Fukudo S, Mayer EA, Niesler B, Quigley EM, Rajic-Stojanov M, Schermann M, Schvile-Kuntuke J, Simren M, Zipfel S, Spiller RC. Irritable bowel syndrome. Nat Rev Dis Primers 2016;2:16014.

[50] Erk P, Aziz O, Barbara G, Farmer AD, Fukudo S, Mayer EA, Niesler B, Quigley EM, Rajic-Stojanov M, Schermann M, Schvile-Kuntuke J, Simren M, Zipfel S, Spiller RC. Irritable bowel syndrome. Nat Rev Dis Primers 2016;2:16014.
Sadeghi M, Erickson A, Castro J, Deiteren A, Harrington AM, Grundy L, Adams DJ, Brierley SM. Contribution of membrane receptor signalling to chronic visceral pain. Int J Biochem Cell Biol 2018;98:10–23.

Salvatierra J, Castro J, Erickson A, Li Q, Braz J, Gilchrist J, Grundy L, Rychkov GY, Deiteren A, Rais R, King GF, Slusher BS, Basbaum A, Pasricha PJ, Brierley SM, Bosmans F. NaV1.1 inhibition can reduce visceral hypersensitivity. JCI Insight 2018;3:e121000.

Sanson M, Bueno L, Fioramonti J. Involvement of cannabinoid receptors in inflammatory hypersensitivity to colonic distension in rats. Neurogastroenterol Motil 2006;18:949–56.

Sharkey KA, Wiley JW. The role of the endocannabinoid system in the brain–gut axis. Gastroenterology 2016;151:252–66.

Singh UP, Singh NP, Singh B, Price RL, Nagarkatti M, Nagarkatti PS. Cannabinoid receptor-2 (CB2) agonist ameliorates colitis in IL-10(-/-) mice by attenuating the activation of T cells and promoting their apoptosis. Toxicol Appl Pharmacol 2012;258:256–67.

Soethoudt M, Grether U, Fingerle J, Grim TW, Fezza F, de Petrocellis L, Ullmer C, Rothenschneider B, Perner C, van Gilis N, Finlay D, MacDonald C, Chicca A, Gene MD, Stuart J, de Vries H, Mastrandrello N, Xia L, Alachouzos G, Baggaelaar MP, Martella A, Mock ED, Deng H, Heitman LH, Connor M, Di Marzo V, Gertsch J, Lichtman AH, Maccarrone M, Pacher P, Glass M, van der Stelt M. Cannabinoid CB2 receptor ligand profiling reveals biased signalling and off-target activity. Nat Commun 2017;8:13958.

Spiegel BM, Bolus R, Harris LA, Lucak S, Chey WD, Sayuk G, Esralian E, Lembo A, Karsan H, Tillisch K, Talley J, Chang L. Characterizing abdominal pain in IBS: guidance for study inclusion criteria, outcome measurement and clinical practice. Aliment Pharmacol Ther 2011;32:1192–202.

Storr MA, Keenan CM, Zhang H, Patel KD, Makriyannis A, Sharkey KA. Activation of the cannabinoid 2 receptor (CB2) protects against experimental colitis. Inflamm Bowel Dis 2009;15:1678–85.

Turcotte C, Blanchet MR, Laviolette M, Flamand N. The CB(2) receptor and its role as a regulator of inflammation. Cell Mol Life Sci 2016;73:4449–70.

Weinland SR, Morris CB, Hu Y, Leserman J, Bangdiwala S, Grossman DA. Characterization of episodes of irritable bowel syndrome using ecological momentary assessment. Am J Gastroenterol 2011;106:1813–20.

Wright K, Rooney N, Feeney M, Tate J, Robertson D, Welham M, Ward S. Differential expression of cannabinoid receptors in the human colon: cannabinoids promote epithelial wound healing. Gastroenterology 2005;129:437–53.

Yacoobyn BR, Hansauer S, Klassen P, English BA, Staubert K, Barish CF, Gilder K, Turner S, Higgins PDR. Safety, pharmacokinetics, and efficacy of olorinab, a peripherally acting, highly selective, full agonist of the cannabinoid receptor 2, in a phase 2a study of patients with chronic abdominal pain associated with Crohn’s disease. Crohn’s Colitis 2020;360:3.