Co-expression of μ and δ opioid receptors by mouse colonic nociceptors

BACKGROUND AND PURPOSE
To better understand opioid signalling in visceral nociceptors, we examined the expression and selective activation of μ and δ opioid receptors by dorsal root ganglia (DRG) neurons innervating the mouse colon.

EXPERIMENTAL APPROACH
DRG neurons projecting to the colon were identified by retrograde tracing. δ receptor-GFP reporter mice, in situ hybridization, single-cell RT-PCR and μ receptor-specific antibodies were used to characterize expression of μ and δ receptors. Voltage-gated Ca\(^{2+}\) currents and neuronal excitability were recorded in small diameter nociceptive neurons (capacitance <30 pF) by patch clamp and ex vivo single-unit afferent recordings were obtained from the colon.

KEY RESULTS
In situ hybridization of oprm1 expression in Fast Blue-labelled DRG neurons was observed in 61% of neurons. μ and δ receptors were expressed by 36–46% of colon DRG neurons, and co-expressed by ~25% of neurons. μ and δ receptor agonists inhibited Ca\(^{2+}\) currents in DRG, effects blocked by opioid antagonists. One or both agonists inhibited action potential firing by colonic afferent endings. Incubation of neurons with supernatants from inflamed colon segments inhibited Ca\(^{2+}\) currents and neuronal excitability. Antagonists of μ, but not δ receptors, inhibited the effects of these supernatant on Ca\(^{2+}\) currents, whereas both antagonists inhibited their actions on neuronal excitability.

CONCLUSIONS AND IMPLICATIONS
A significant number of small diameter colonic nociceptors co-express μ and δ receptors and are inhibited by agonists and endogenous opioids in inflamed tissues. Thus, opioids that act at μ or δ receptors, or their heterodimers may be effective in treating visceral pain.

Abbreviations
DRG, dorsal root ganglia; DSS, dextran sulfate sodium; eGFP, enhanced green fluorescent protein; IBD, inflammatory bowel disease; TEA, tetraethylammonium; VGCC, voltage-gated calcium channels

DOI:10.1111/bph.14222 © 2018 The British Pharmacological Society
Introduction

Abdominal pain is a debilitating symptom for patients with chronic disorders such as inflammatory bowel disease (IBD), resulting in emotional suffering, physical disability and increased medical costs (Bielefeldt et al., 2009; Srinath et al., 2012). Opioid use has markedly increased in the past decade. Five to 13% of patients with IBD are prescribed chronic narcotics in the outpatient setting (Cross et al., 2005; Hanson et al., 2009; Targownik et al., 2014), and opioid use reaches 70% for inpatients (Long et al., 2012). Opioid drugs can be efficacious, but in some settings can also be detrimental due to escalating dosing with its attendant side effects (e.g. nausea, cognitive impairment, constipation) and, paradoxically, an opioid-driven increase in abdominal pain, referred to as narcotic bowel syndrome (Drossman et al., 2005; Stein and Machelska, 2011; Fichna et al., 2012). Understanding how opioids alter the excitability of peripheral dorsal root ganglia (DRG) neurons is particularly important because 50–100% of the analgesic effect of systemically administered opioids are mediated by opioid receptors on these neurons (Stein and Machelska, 2011).

These peripheral opioid mechanisms also become increasingly important as inflammation progresses, due to an enhanced endogenous opioid system (Stein and Machelska, 2011; Boue et al., 2014; Stein, 2016). During chronic inflammation, accumulating tissue CD4 + T cells secrete β-endorphin, [Met]enkephalin and dynorphin A, the endogenous ligands of opioid receptors. These endogenous opioids play an important role in enhancing analgesia and mitigating opioid tolerance by signalling to intestinal nerve terminals of DRG neurons (Verma-Gandhu et al., 2006; Stein and Machelska, 2011; Fichna et al., 2012; Stein, 2016). Given the importance of these actions, targeting this endogenous pathway could provide a means to enhance analgesia during inflammation. However, the opioid receptors mediating gastrointestinal analgesia have not been adequately described.

DRG neurons express δ, μ and κ opioid receptors, GPCRs that inhibit neuronal excitability and release of inflammatory neuropeptides (Stein, 2016). However, the functional expression of these receptors on specific subsets of DRG neurons has been an area of ongoing controversy (Scherrer et al., 2009; Woolf, 2009; Beaudry et al., 2011; Bardoni et al., 2014; Zhang et al., 2015). In the somatosensory system, recent studies (Bardoni et al., 2014) suggest a divergence of μ and δ receptor expression, with δ receptors being mainly expressed on myelinated A-type fibres and μ and δ receptor co-expression in less than 10% of DRG neurons. Compared with the somatosensory pathways, there is reportedly little expression of δ receptors on DRG neurons innervating the viscera, suggesting opioids only signal to μ receptors in the GI tract (Scherrer et al., 2009). Despite these potentially important differences, little is known about the expression of opioid receptors on DRG neurons that innervate the intestine. Such studies are needed to improve our understanding of the role of opioids in modulating visceral pain, particularly given the known interactions between μ and δ receptors, including heterodimerization, and the mechanisms of inflammation-induced plasticity of opioid signalling (Stein, 2016).

Technical issues have confounded the interpretation of studies of opioid receptor expression, particularly of δ receptors (Stein, 2016; Zhang et al., 2015). We therefore studied δ receptor-eGFP reporter mice, where expression of δ receptors has been previously validated in neurons (Scherrer et al., 2009; Poole et al., 2011). We used combined neuroanatomical and electrophysiological techniques to determine the expression of μ and δ receptors by colonic DRG neurons. Voltage-gated calcium currents and measures of neuronal excitability provide a functional measure of opioid receptor signalling given their key role in presynaptic inhibition of neurotransmitter release in central terminals in the dorsal horn of the spinal cord, and in generation of action potentials in DRG neurons. We found that μ and δ receptors were expressed on colonic DRG neurons and we examined the effects on nociceptive signalling of selective opioid agonists and endogenous opioids in inflamed colons in mice.

Methods

Animals and identification of colon DRG neurons

All animal care and experimental protocols were approved by the Animal Ethics Committee of Monash University, the University of Adelaide and the South Australian Health and Medical Research Institute (SAHMRI), and Queen’s University. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010; McGrath and Lilley, 2015). In all groups, mice were males that were group housed (four to five per cage) and maintained under temperature (22 ± 4°C) and light-controlled (12 h light/dark cycle) conditions with free access to food and water. Specific strain and housing details for each group are outlined below.

Chronic colitis was induced in C57Bl/6 male mice (Charles River Laboratories, Saint-Constant, Québec, Canada) by oral administration of 2% DSS in the drinking water for three cycles of 5 days alternating with 5 days of normal water, as previously described (Valdez-Morales et al., 2013). At day 30, control and DSS mice were killed by isoflurane overdose and cervical transection, and colons were excised. Colonic supernatants were generated by cutting the colon into 5 mm segments and incubating each of them overnight in 250 μL of RPMI medium with 10% fetal calf serum, penicillin/streptomycin and gentamycin/amphotericin B at 37°C, with 95% O2 and 95% CO2. Supernatants were removed, sterile-filtered and stored at −80°C. Our previous studies have shown that incubation of neurons overnight in supernatants evoke similar electrophysiological changes to those observed in retrogradely labelled neurons from the colon of DSS mice (Ibeakanma et al., 2011; Valdez-Morales et al., 2013).

To identify DRG neurons innervating the colon, Fast Blue injections were made into the wall of colon, as previously described (Ibeakanma et al., 2011), and Fast Blue retrograde labelled neurons in DRG were identified in subsequent imaging.

Immunofluorescence

Colonic DRG neurons were identified by Fast Blue-labelling in δ receptor-eGFP knock-in mice (male, 6–8 weeks) (Scherrer et al., 2006). Mice were caged with sawdust bedding and fed Barastoc chow (Ridley, AgrilProducts, Victoria, Australia).
After 7 days, mice were injected with the δ receptor agonist SNC80 (10 mg·kg⁻¹ s.c. in acidified saline solution, 30 min) and killed by cervical dislocation. Lumbosacral DRG were harvested and fixed in paraformaldehyde (4% solution in PBS, 2 h on ice). DRG were cleared of fixative (3× PBS washes), cryoprotected in 30% sucrose (w/v in PBS) and embedded in Optimal Cutting Temperature solution (Sakura Finetek, Torrance, California, USA). DRG were cryosectioned (12 μm) and every fifth section was collected onto positively charged slides (Superfrost Plus). Tissues were air-dried before blocking (5% normal horse serum, 0.1% Triton X-100 in PBS containing 0.1% sodium azide, 30 min, room temperature). Sections were incubated with primary antibodies: chicken anti-GFP, Abcam, ab13970, 1:500; rabbit anti-μ receptor (UMB3), Abcam, ab134054, 1:250 (Lupp et al., 2011), diluted in blocking buffer overnight at 4°C. Sections were washed (3× PBS), incubated with secondary antibodies (donkey anti-chicken Alexa Fluor 488, 1:500; donkey anti-rabbit Alexa Fluor 568, 1:500, Invitrogen/Thermofisher), washed (3× PBS) then mounted (Prolong Gold, Invitrogen/Thermofisher). Samples were imaged using a Leica TCS-SP8 confocal microscope using a 20× oil immersion objective. Images were of 1024×1024 pixel resolution and 16-bit depth.

In situ hybridization

Male mice were caged with sawdust bedding and fed Barastoc chow (Ridley, AgriProducts, Victoria, Australia). They were killed by cervical dislocation and trigeminal ganglia dissected. RNA was extracted from the ganglia using the Qagen (Charsworth, California, USA) RNAEasy kit and was reverse transcribed using Superscript III (Invitrogen, Victoria, Australia) (Bron et al., 2014). cDNA for mouse OPRM1 was amplified by RT-PCR. The following forward and reverse primers were used: TATAACAAATGAGACTGCCACA (mOPRM1_F352) and GATATTTAATACGACTCATATAAGAGAGAGATGTGGAATGTTTACATTCCAGG (mOPRM1_R1103_T7). The design of the reverse primer included the T7 promoter sequence (underlined), which allows the PCR products to be used directly for the generation of digoxigenin (DIG)-labelled antisense cRNA probes by in vitro transcription with T7 RNA polymerase (Roche Products, Dee Why, NSW, Australia). In situ hybridization combined with retrograde tracing and immunohistochemistry was performed on cryosections of mouse colonic DRG neurons, as described (Bron et al., 2014). An antibody against HuC/D was used as a pan-neuronal marker (mouse monoclonal, #A21207, Invitrogen; 1:1000), and labelling was detected using a donkey anti-mouse secondary antibody (Alexa Fluor488 conjugated, 1:500, Invitrogen).

Sections were scanned using 10× or 20× objective magnification with a VSlide Fluorescent Slide Scanner (MetaSystems, Carl Zeiss, North Ryde BC, NSW, Australia) and stitched together using the Metafer platform. Digitized images were exported as TIFF files and processed in Adobe Photoshop for panel preparation.

Image analyses

All DRG sections containing Fast Blue-positive neurons were imaged and analysed in an unblinded fashion. Fast Blue-positive neurons were identified from projected z-stacks and co-expression of GFP and μ receptor immunoreactivities were then determined. Images were thresholded to facilitate analysis. Oprm1 expression in Fast Blue-positive neurons was determined from fluorescence and bright field image overlays. Neuron sizes were determined using ImageJ (area, perimeter and length) and expressed in μm² or μm respectively. Data were expressed relative to the total number of Fast Blue-positive neurons.

Isolation of DRG neurons for electrophysiological studies and single-cell PCR

Male mice (C57Bl/6, Charles River Laboratories) were fed Purina lab chow (Purina USA) mouse #5015 and cage bedding contained a combination corn cob bedding that was changed every 14 days. DRG neurons were acutely dissociated as described (Ibeakanma et al., 2011; Valdez-Morales et al., 2013). C57Bl/6 mice (Charles River) were killed with i.p. ketamine/xylazine and DRG from T9-T13 were dissected. Neurons were dispersed and suspended in DMEM (pH 7.2–7.3, 10% FCS, 1000 U·mL⁻¹) (named Biomaterials, Fremont, CA, USA) cover slips and incubated in 95% O₂ and 5% CO₂. Two hours after cells were applied to cover slips in 24 well plates, the well plates were incubated with the supernatant/F12 medium (mixture 1:10) and incubated overnight (16–23 h) until retrieval for electrophysiological studies. Acute application of drugs was applied the day following acute dissociation of the neurons. Electrophysiologists were not blinded to the application of drug or supernatant.

Single-cell RT-PCR

Single neurons were processed to obtain cDNA using SuperScript III Cells Direct cDNA Synthesis Kit (Invitrogen) according to the manufacturer’s instructions. Briefly, we used single Fast Blue-labelled small DRG neurons (<30 μm) from primary cultures. Each neuron was harvested under into the patch pipette containing 6 μL of 10× resuspended buffer and 1 μL of RNaseout (2 U·μL⁻¹) by applying negative pressure. The content of the patch pipette was expelled into a PCR-tube containing 4 μL of 10× resuspended buffer, 2 μL Oligo dT₂₀ and 1 μL dNTP (10 mM), and the reaction was incubated at 70°C for 5 min and then 2 min on ice. After adding 6 μL 5× RT buffer, 1 μL of RNaseout (2 U·μL⁻¹), 1 μL of SuperScript III reverse transcriptase (200 U·μL⁻¹) and 1 μL DTT (0.1 M), the sample was transferred to 50°C for 50 min, the reaction was inactivated by heating to 85°C for 5 min and placed on ice. As negative controls, fluid from the vicinity of the collected cells was amplified or template was omitted. PCR reaction contained 0.2 μM primers, 0.5 U Taq Polymerase, 2.0 mM MgCl₂, 10 mM dNTP, and 10× PCR buffer (Qiagen) (25 μL final volume). PCR reaction conditions were 40 cycles of initial activation at 95°C for 5 min, denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 5 min. PCR was performed using the following intron-spanning mouse primers: DOR (NM_013622.3) outer forward, 5’-TTCTGGGCAACGCTCTGGTCGTCTGCT-3’; DOR outer reverse 5’-CATAAGCACACCGTGATGATG-3’ (510-bp product); DOR inner forward, 5’-TGTTGTCGATGCTCGGTAC-3’; DOR inner reverse 5’-TGAAGCAAGACCAGGATGC-3’ (320-bp product).
and the action potential discharge at twice rheobase, as previously described using perforated patch clamp recordings with amphotericin B (0.24 mg·mL⁻¹) (Ibeakanma and Vanner, 2010; Cattaruzza et al., 2011). The extracellular solution was 140 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, 10 D-glucose, pH 7.4. The pipette solution was 110 K-gluconate, 30 KCl, 2 CaCl₂, pH 7.25. The final pipette resistance was 2–5 MΩ in bath solution, and the liquid junction potential was corrected (12 mV).

### Ex vivo colonic afferent recording studies

*Ex vivo* single-unit extracellular recordings of action potential discharge were made from splanchnic colonic afferents from C57BL/6 male mice (Brierley et al., 2004; Hughes et al., 2009; Castro et al., 2013; de Araujo et al., 2014; Hughes et al., 2014). Mice were acquired from an in-house C57BL/6j breeding program (from strain #000664 originally purchased from Jackson Laboratories) within SAHMRI’s specific and opportunistic pathogen free animal care facility. Cages were filled with aspen wood chip bedding. Food consisted of the Jackson lab diet 5K52 JL RAT and MOUSE/AUTO 6F DIET (Cat# ASSPECIAL) and cages contained Jackson lab bedding: Jackson lab diet 5K52 JL RAT and MOUSE/AUTO 6F DIET from Charles River were fed PMI lab chow (Purina USA) and were blinded to the specific drug being tested. Following baseline measurements, mechanosensitivity was then retested after the application of either (Cat# ASPSPECIAL) and cages contained Jackson lab bedding: CA PURA CHIP ASPEN COARSE (Cat# ASPMAEB). Animals were killed by CO₂ inhalation. In this study, we focused on high-threshold nociceptive afferents, also termed serosal or vascular afferents. These nociceptive afferents have high-mechanical activation thresholds and respond to noxious distension (40 mmHg), stretch (≥7 g) or von Frey hair filaments (2 g) but not to fine mucosal stroking (10 mg von Frey hairs) (Hughes et al., 2009). These afferents also become hypersensitive during and after resolution of colonic inflammation (Hughes et al., 2009; Brierley and Linden, 2014). Baseline mechanosensitivity was determined in response to application of a 2 g von Frey hair probe to the afferent receptive field for 3 s. This process was repeated three to four times, separated each time by 10 s.

In the first series of experiments, electrophysiologists were blinded to the specific drug being tested. Following baseline measurements, mechanosensitivity was then retested after the application of either (Ibeakanma and Vanner, 2010). The extracellular solution was 140 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, 10 D-glucose, pH 7.4. The pipette solution was 110 K-gluconate, 30 KCl, 2 CaCl₂, pH 7.25. The final pipette resistance was 2–5 MΩ in bath solution, and the liquid junction potential was corrected (12 mV).

**Opioid signalling in colonic DRG neurons**

**British Journal of Pharmacology (2018) 175** 2622–2634 2625

---

**Acknowledgments**

This study was supported by grants from the Australian Research Council and the South Australian Health and Medical Research Institute (SAHMRI). The authors would like to thank Rachel Coates and the SAHMRI animal care facility for their expert assistance. The authors declare no conflict of interest.

---

**References**


---

**Supplementary Information**

Supplementary material associated with this article can be found, in the online version, at doi:10.1038/nature.2018.23422.

---

**Open Access**

This is an open access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.
application of either DADLE (100 nM) or DAMGO (100 nM) to the mucosal surface for 5 min via the small metal ring placed over the receptive field of interest. Following a 30 min washout period, mechanosensitivity was retested for reversibility. The other agonist was then applied to the receptive field for 5 min followed by testing of the mechanosensitive response. Reversibility was rechecked after a 30 min washout period. To ensure stability of the unit and adequate washout of the agonist, a unit was considered to be inhibited by either the μ or δ receptor agonist if the mechanosensitive response in the presence of drug was less than 75% of the baseline response and the response following washout was within 25% of the baseline response. To further mitigate potential bias from run-down or incomplete washout of the agonist, the order of the application of DADLE and DAMGO was altered for each experiment. Investigators were not blinded to the drug being tested.

Data and statistical analyses
The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015). Data were analysed using Prism 6 or 7 Software (GraphPad Software, California, USA), and results were expressed as mean ± SEM. Differences between groups were examined by paired or unpaired $t$-tests. Differences between multiple groups were examined using ANOVA and a Bonferroni or Dunnett’s post hoc test. The post hoc test was only applied when the $F$ statistic indicated significance. Statistical analysis was applied to experiments where neurons were obtained from at least five animals. The afferent nerve recordings (1–2 single units obtained per colon of each animal; 1 unit = 1 single axon recording) shown in Figure 3B–D were obtained from fewer than five animals for each drug tested, and therefore, no statistical analysis was performed. A $P$ value <0.05 was considered to be significant. In patch clamp studies, the number of neurons and animals differed per dataset within a given experiment because each drug concentration and/or antagonist was tested each day plus the accompanying controls. Drugs or supernatants were added to separate wells containing the isolated neurons on small glass plates. Consequently, the numbers varied depending on the success of viable recordings from each plate that had been taken from the corresponding wells.

Materials
[D-Ala$^2$, D-Leu$^3$]-enkephalin (DADLE), (+)-4-[(aR)-α-(2S,5R,4-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylenazamide (SNC 80), and [D-Ala$^2$, N-MePhe$^4$, Gly-$\text{o}$-$\text{r}$]-enkephalin (DAMGO) were all purchased from Tocris Bioscience (UK) [extracellular recordings or Sigma (patch clamp recordings)]. (4bS,8R,8aS,14bR)-5,6,7,8,14b-Hexahydro-7-(2-methyl-2-propenyl)-4,8-methanobenzofuro[2,3-a]pyrido[4,3-b]carbazole-1,8a(9H)-diol (SDM25N) and D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH$_2$ (CTOP) were purchased from Tocris Bioscience.

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 et al., 2017).

Results
Expression of μ and δ receptors by colonic afferent neurons
Expression of oprm1 mRNA, the gene for the μ receptor, by Fast Blue-positive colonic DRG neurons was examined by in situ hybridization (Figure 1A). Positive staining for oprm1 was detected in approximately 61% of all Fast Blue neurons (62/101 neurons from three animals). In contrast, μ receptor-immunoreactivity (IR) was detected in only 36% of all Fast Blue-labelled neurons (75/210 neurons) (Figure 1B). δ receptor-IR labelling was detected in 46% of colonic afferents (190/411 neurons from three animals). Approximately a quarter of all Fast Blue-labelled neurons were positive for both δ receptor GFP-IR and μ receptor-IR (24%, 58/245 neurons). Colonic afferents that co-expressed μ and δ receptors had an average soma area of 482 ± 27 μm$^2$ and average maximum soma perimeter of 83 ± 2.4 μm and a length (measured in sections containing nuclei) of 27.95 μm ± 0.84 μm.

To confirm the expression of μ and δ receptors on DRG neurons innervating the colon, small diameter neurons (<25 μm) labelled with Fast Blue from C57BL/6 mice (13 neurons from six animals) were individually selected, and expression of μ and δ receptor mRNAs was examined by single-cell nested PCR. The agarose gel electrophoresis of 13 different neurons detecting expression of μ and δ receptors is shown in Figure 1C. Among 13 Fast Blue-labelled small diameter DRG neurons, 38% expressed μ receptor mRNA (320 bp), 46% expressed δ receptor mRNA (272 bp) and 23% co-expressed μ and δ receptor mRNAs.

Agonists at μ and δ receptors inhibit Ca$^{2+}$ currents in small diameter DRG neurons
To determine whether μ and δ receptors regulate voltage-gated Ca$^{2+}$ channels, we measured $I_{\text{Ba}}$ in small DRG neurons in response to acute (min) and chronic (overnight) exposure to μ and δ receptor agonists and their antagonists (DRG neurons obtained from 27 mice). Preliminary experiments were conducted to address the contribution of T-type Ca$^{2+}$ channels to whole cell $I_{\text{Ba}}$ in DRG neurons by comparing currents evoked from holding potentials of −60 and −90 mV. No difference in current amplitude or time course was observed between holding potentials of −60 and −90 mV, suggesting that low voltage-activated T-type channels did not contribute to $I_{\text{Ba}}$ under our recording conditions.

Consistent with previous findings (Schroeder et al., 1991; Moises et al., 1994), superfusion of the μ receptor agonist DAMGO (100 nM) for 5 min led to a significant (63.5%) reduction in $I_{\text{Ba}}$ currents (data not shown). Similar reductions in $I_{\text{Ba}}$ currents were observed (63.2%) with overnight incubation of DAMGO (100 nM; Figure 2.). In all subsequent experiments, neurons were incubated overnight with the μ receptor agonist (DAMGO) or the δ receptor agonist (DADLE). Figure 2A shows examples of currents recorded from a DRG...
neuron. DAMGO (0.01–1 μM) and DADLE (0.01–1 μM) reduced the amplitude of IBa in a concentration-dependent manner (Figure 2B). Statistically significant inhibition of these currents was observed in DRG neurons incubated overnight with 0.1 μM DAMGO or 0.1 and 1 μM DADLE (Figure 2B). DAMGO and DADLE inhibited IBa with similar potency in small DRG neurons (Figure 2B). There were no changes in the activation (DADLE – $k = 5.0 \pm 0.47$ mV, $V_h = -19.6 \pm 0.54$ mV; DAMGO – $k = 4.5 \pm 0.4$ mV, $V_h = -19.0 \pm 0.44$) and inactivation (DADLE – $k = 38.4 \pm 0.45$ mV, $V_h = -61.8 \pm 4.0$ mV; DAMGO – $k = 23.7 \pm 5.0$, $V_h = -61.5 \pm 6.0$ mV). A scatter plot from all cells incubated with a maximum concentration (1 μM) of DAMGO and DADLE showed that DAMGO inhibited IBa in 7/9 cells and DADLE inhibited IBa in 17/20 cells (data not shown). The inhibition of IBa evoked by DAMGO and DADLE was blocked by CTOP and SDM25N respectively (Figure 2C). The antagonists alone had no effect.

**Agonists at μ and δ receptors inhibit colonic nociceptors**

We first examined whether μ and δ receptor agonists inhibited neuronal excitability of isolated DRG neurons using patch clamp recordings. We found that an acute (5 min) application of either DAMGO or DADLE (100 nM) applied to neurons increased the rheobase and increased the input resistance (Figure 3A). They also decreased the membrane potential from baseline (DAMGO = −44.4 ± 0.8 to −49.9 ± 0.8 mV, $P < 0.05$; DADLE −44.2 ± 0.7 to −47.2 ± 1.0 mV). To determine if μ or δ receptor agonists modify colonic nociceptor function in intact tissue, we made

---

**Figure 1**

Expression of μ and δ receptors on Fast blue-labelled DRG neurons. (A) Representative photomicrographs of *in situ* hybridization of oprm1 expression in Fast Blue-labelled DRG neurons (white arrows) and non-Fast Blue-labelled neurons (yellow arrow). Hu antibody staining identifies all neurons in the ganglia. 61% of Fast Blue-labelled neurons expressed oprm1 mRNA. Scale = 100 μm. (B) Representative photomicrographs of δ receptor expression in ganglia from δ receptor-eGFP (DOR-eGFP) knock-in mouse and double labelling with μ receptor (MOR) immunoreactivity (merge). Colonic neurons identified by Fast Blue retrograde labelling. 46% of Fast Blue-labelled neurons expressed δ receptors and 24% were co-immunoreactive for μ and δ receptors. Scale = 150 μm. (C) A representative agarose gel electrophoresis (2%) stained with ethidium bromide to assess expression of mRNA for μ and δ receptors in small diameter Fast Blue-labelled DRG neurons. PCR products 272 bp, 320 bp and 164 bp corresponding to μ receptor (MOR), δ receptor (DOR) and β-actin cDNAs respectively. Lane 1, exACTGene 50 bp Mini DNA Ladder; Lane 2–13, PCR products from 13 individual small Fast Blue-labelled DRG neurons. Neurons 4, 8 and 13 co-expressed μ and δ receptor mRNAs. No transcripts were amplified from bath fluid or without template (Lanes 14 and 15). Of the 13 neurons, 23% co-expressed μ and δ receptor mRNAs.
Figure 2

DAMGO (μ receptor agonist) and DADLE (δ receptor agonist) inhibited \( I_{\text{Ba}} \) through voltage-gated calcium channels on small DRG neurons. (A) Representative whole-cell recording of calcium currents induced by depolarization from the holding potential of \(-90 \text{ to } 0 \text{ mV} \) for 100 ms, represented in the inset above. The currents were obtained from different small DRG neurons incubated overnight with δ receptor agonist (DADLE 0.1 μM) or μ receptor agonist (DAMGO 0.1 μM). (B) Concentration dependency of inhibition of \( I_{\text{Ba}} \) by DADLE (0.01–1 μM) or (C) DAMGO (0.01–1 μM) on small DRG neurons. Incubated overnight. Treatment with DADLE or DAMGO produced a marked decrease in \( I_{\text{Ba}} \). The amplitude of inward currents was normalized to cell capacitance. Data shown are means ± SEM from the number of cells in each bar. * \( P < 0.05 \), significantly different from control (Med); one-way ANOVA with Dunnett’s post test. (C) Effects of μ and δ receptor antagonists on \( I_{\text{Ba}} \) through voltage-gated Ca\(^{2+} \) channels in DRG neurons. Inhibition of \( I_{\text{Ba}} \) by DADLE (100 nM) and DAMGO (100 nM), shown in (B) were blocked by SDM25N (1 μM) and CTOP (1 μM) respectively. SDM25N and CTOP alone had no effect on \( I_{\text{Ba}} \). Data shown are means ± SEM from the number of cells in each bar.
Figure 3

Patch clamp and ex vivo single-unit afferent recordings showing μ and δ receptor agonists inhibit colonic afferents. (A) Acute application of μ and δ receptor agonists (100 nM; applied for 5 min) inhibits the excitability of isolated DRG neurons (increase rheobase) and increases input resistance. Data shown are means ± SEM; n = 9. *P < 0.05, significantly different as indicated; one-way ANOVA and Bonferroni post test. (B) Representative recording (left panel) of a colonic nociceptor response to von Frey hair (vfh) probing before and after administration of δ receptor agonist DADLE (100 nM). Summary data (means ± SEM; right panel) showing DADLE inhibited splanchnic colonic nociceptors from healthy mice, n = 5 single afferent recordings from axons innervating the colons from three mice. (C) Representative recording (left panel) of a colonic nociceptor response to von Frey hair (vfh) probing before and after administration of δ receptor agonist SNC 80 (1000 nM). Summary data (means ± SEM; right panel) showing SNC 80 inhibited splanchnic colonic nociceptors from healthy mice, n = 5 single afferent recordings from axons innervating the colons from three mice. (D) Representative recording (left panel) of a colonic nociceptor response to von Frey hair (vfh) probing before and after administration of the μ receptor agonist DAMGO (100 nM). Summary data (means ± SEM; right panel) showing DAMGO inhibited splanchnic colonic nociceptors from healthy mice, n = 7 single afferent recordings obtained from axons innervating the colons from four mice. Note as the data in B, C and D are from less than five animals, no statistical analysis was carried out.

Opioid signalling in colonic DRG neurons

*ex vivo* colonic afferent recordings from mouse splanchnic high-threshold nociceptors, which respond to focal compression and noxious stretch/distension. The δ receptor agonist DADLE (100 nM; 5 units from three animals) inhibited colonic nociceptor action potential firing in response to noxious mechanical stimuli (Figure 3B). Another highly selective δ receptor agonist, SNC 80 (1000 nM; 5 units from three animals), also inhibited nociceptor mechanosensitivity (Figure 3C). The μ receptor agonist DAMGO (100 nM; 7 units from four animals) also inhibited colonic nociceptors (Figure 3D). The percent inhibition by the μ and δ receptor agonists was similar (DADLE = 21 ± 6, SNC 80 = 26 ± 8, DAMGO = 25 ± 4).

We next examined whether μ and δ receptor agonists could inhibit mechanosensitivity in the same unit. In these experiments, 6/8 units obtained from eight animals were inhibited by either DAMGO (100 nM) or DADLE (100 nM) (Figure 4), and of these, three were inhibited by both DAMGO and DADLE. In units that were inhibited by DADLE, afferent firing was reduced from 9.55 ± 3.33 to 6.03 ± 2.50 spikes s⁻¹. In units that were inhibited by DAMGO, afferent firing was reduced from 14.22 ± 2.72 to 8.59 ± 1.5 spikes s⁻¹.

**Endogenous opioids released from inflamed colon inhibit colonic nociceptors**

Neurons incubated overnight with supernatants from DSS-inflamed colons (Figure 5A) exhibited a significant decrease in amplitude of I_R compared with I_R in neurons incubated with supernatants from uninflamed colon or control supernatants. In neurons incubated with supernatants from inflamed colon, neither DADLE nor DAMGO had an additional inhibitory effect on I_R (Figure 5B).

Dissociated neurons were preincubated with the μ receptor antagonist (CTOP 1 μM) or the δ receptor antagonist (SDM25N 1 μM) 30 min before incubation with colonic supernatants from control and chronic DSS mice and the effects on I_R and neuronal excitability examined. In control studies (Figure 5C), the application of CTOP or SDM25N alone had no effect on I_R. DAMGO (100 nM) and DADLE (100 nM). In studies of the chronic DSS supernatant (Figure 5C), the inhibitory effect of the chronic DSS supernatant on I_R was suppressed by pretreatment with the μ receptor antagonist CTOP, but not with δ receptor antagonist SDM25N (Figure 5C).

Given that the inhibition of I_R by endogenous opioids was mediated predominantly by μ receptor activation, we examined whether a similar receptor selectively applied to modulation of action potential electrogenesis. We have previously shown that the rheobase of DRG neurons incubated in chronic DSS supernatant is increased and the action potential discharge is decreased compared with the effects of control supernatant on neuronal excitability and that this effect is blocked by naloxone (Valdez-Morales et al., 2013). In the current study (Figure 5D), both CTOP and SDM25N decreased the rheobase (29% each) and CTOP increased action potentials.
potential discharge (1.9×) in neurons incubated in cDSS supernatant.

**Discussion**

Therapeutic opioids such as morphine and related compounds act predominantly on μ receptors to treat acute and chronic abdominal pain in patients with disorders such as IBD. However, these agents exhibit a significant adverse side effect profile, and consequently, there is a need for alternative therapies and a renewed interest in δ receptor-targeted agents (Woolf, 2009; Stein, 2016; Zhang et al., 2015). A fundamental step towards understanding how opioids can be exploited in this clinical setting is to examine the functional expression of μ and δ receptors in DRG neurons, and in particular whether they are expressed on small neurons including nociceptors innervating the colon. Co-expression of these receptors on the same neuron has important implications for understanding opioid signalling, given the potential for physical interactions and the formation of heterodimers (Targownik et al., 2014; Geppetti et al., 2015; Stein, 2016).

We found that μ and δ receptors are co-expressed on a substantial proportion of colonic DRG neurons and that...
activation of each of these receptors by endogenous opioids can inhibit sensory signalling from the intestine (Ibeakanma et al., 2011).

There has been considerable controversy regarding the expression of $\mu$ and $\delta$ receptors in DRG neurons and whether these receptors are co-expressed in neurons (Rau et al., 2005; Scherrer et al., 2009; Stein, 2016; Woolf, 2009; Joseph and Levine, 2010; Wang et al., 2010; Beaudry et al., 2011; Normandin et al., 2013; Bardoni et al., 2014; Zhang et al., 2015), and if so, in which populations of neurons. Furthermore, differences may exist between the somatosensory and visceral pain pathways (Scherrer et al., 2009). Much of this controversy has been attributed to the lack of specificity of receptor antibodies, particularly to $\delta$ receptors, and non-selective effects of high concentrations of opioid agonists (Woolf, 2009; Bardoni et al., 2014; Zhang et al., 2015). We used $\delta$ receptor-eGFP knock-in mice, in situ hybridization and single-cell RT-PCR to enable specific detection of $\delta$ receptor expression (Scherrer et al., 2006; Scherrer et al., 2009; Bardoni et al., 2014). In the somatosensory system, recent studies using this mouse model have shown that only 17% of DRG neurons express $\delta$ receptors and that expression was confined to the larger myelinated CGRP positive DRG neurons (Bardoni et al., 2014). Only a small proportion co-expressed $\mu$ and $\delta$ receptors (<5%). Visceral afferents account for <10% of all DRG neurons (Beayk and Grundy, 2005), and therefore, we used retrograde tracing techniques to establish the organ specificity of the neurons. Using this approach, our immunohistochemical studies found that about 36% of the neurons that innervated the colon expressed $\mu$ receptors and 46% expressed $\delta$ receptors. Moreover, we found that 24% of neurons co-expressed $\mu$ and $\delta$ receptors, and many were small diameter neurons, a proportion considerably higher than found in the somatosensory system. Similar proportions were observed in our single-cell PCR studies of $\mu$ and $\delta$ receptor mRNAs. Thus, our studies suggest a much higher proportion of neurons co-express $\mu$ and $\delta$ receptors in the visceral sensory system, including small diameter DRG neurons that have properties of nociceptors (Moore et al., 2002; Stewart et al., 2003).

Voltage-gated Ca$^{2+}$ channels play a central role in peripheral and central presynaptic modulation of neurotransmitter release from DRG neurons (Beyak and Vanner, 2005; Stein, 2016). We recorded from small DRG neurons and found that both $\mu$ and $\delta$ receptor agonists inhibited currents and neuronal excitability in a significant proportion of neurons. While our patch clamp studies (overnight incubation) did not allow sequential testing of opioid agonists to test for functional co-expression in the same neuron, we showed that a significant subset of DRG neurons projecting to the colon co-expressed mRNA and immunoreactivity for $\mu$ and $\delta$ receptors, as described above. Moreover, we examined this question directly using single unit afferent recordings from the colon to measure the inhibition evoked by selective opioid agonists on action potential generation on visceral afferents in response to noxious mechanical stimulation (Hughes et al., 2009). Here, we found that both $\mu$ and $\delta$ receptor agonists cause inhibition of action potential firing to these stimuli and that a similar proportion of single units responded to both agonists. We showed that the inhibitory action of the $\delta$ receptor agonist DADLE (100 nM) on $I_{Na}$ was completely blocked by the selective $\delta$ receptor antagonist SDM25N and that the highly selective $\delta$ receptor agonist SNC80 (Loriga et al., 2013) evoked a similar degree of inhibition of single colonic afferent units to that observed with DADLE. Together, these findings and the functional studies of others (Beaudry et al., 2011) suggest small diameter visceral DRG neurons co-express $\mu$ and $\delta$ receptors in a significant proportion of neurons. Interestingly, these findings from our single-unit recordings contrast with our recent reports using the same colonic afferent recording preparation, whereby $\kappa$ receptor agonists only inhibited colonic nociceptors from mice during or following a bout of colonic inflammation (Hughes et al., 2014). These contrasting observations highlight the respective roles played by the $\delta$, $\mu$ and $\kappa$ receptors in opioid signalling, in different pathological conditions.

Understanding the expression of $\mu$ and $\delta$ receptors on nociceptive neurons in the GI tract under healthy conditions is important for interpreting the effects of inflammation. Inflammation leads to significant plasticity of opioid signalling (Zhang et al., 2015), affecting receptor expression, G-protein signalling and receptor trafficking. The effect of inflammation on opioid signalling pathways can vary considerably between opioid receptor subtype signalling pathways. For example, $\mu$ receptors are up-regulated (Stein and Machelska, 2011) in inflammation, including studies of human IBD (Philippe et al., 2006), whereas $\delta$ receptors appear to be unchanged or down-regulated (Ji et al., 1995). We and others (Verma-Gandhu et al., 2006; Valdez-Morales et al., 2013; Boue et al., 2014) have shown in models of chronic IBD that endogenous opioids released from CD4$^+$ T cells exert an important analgesic action. Moreover, administration of opioid analgesics is needed for IBD patients for the management of severe symptoms and complications. Here, we show that during chronic inflammation endogenous opioids inhibit Ca$^{2+}$ currents and neuronal excitability, but in contrast to control neurons where activation of both $\mu$ and $\delta$ receptors has similar effects, the inhibitory effect on Ca$^{2+}$ currents was restricted to $\mu$ receptor signalling. Further studies are needed to determine the mechanism underlying this altered signalling but these findings may suggest that drugs targeting $\delta$ receptors alone in chronic intestinal inflammation will exert little direct effect on opioid-induced analgesia mediated by voltage-gated Ca$^{2+}$ currents.

In summary, our study has shown that DRG neurons innervating the colon express $\mu$ or $\delta$ receptors and that they are co-expressed on a substantial subpopulation of nociceptors. We also found that selective activation of both $\mu$ and $\delta$ receptors inhibits Ca$^{2+}$ currents and mechanically evoked action potential discharge from the colon. Further studies are needed in human tissues to confirm that these findings in mice translate to humans. Such findings in humans are important for the understanding of the mechanism of drugs that are targeting several peripheral opioid receptors in the gut, such as the $\mu$ receptor agonist and $\delta$ receptor antagonist, eluxadoline, for the treatment of irritable bowel syndrome (Dove et al., 2013). Furthermore, this co-expression is a prerequisite for the formation of $\mu$-$\delta$ receptor heterodimers, and therefore, these heterodimers may be expressed on a significant number of colonic nociceptors. If so, this could have important implications for understanding the actions of drugs designed to disrupt $\delta$ receptor signalling.
in the GI tract, as this may increase analgesia and mitigate tolerance (Geppetti et al., 2015).

**Acknowledgements**

This work was funded by an operating grant from Crohn’s and Colitis Canada (CCC), awarded to S.V. and A.L. N.W.B. is supported by National Health and Medical Research Council, Australian Research Council Centre of Excellence in Convergent Bio-Nano Science and Technology and Monash University. Work in the authors’ laboratory is funded in part by Takeda Pharmaceuticals Inc. This work was also funded in part by the National Health and Medical Research Council (NHMRC) of Australia Project Grant # 1083480 awarded to S.M.B. and D.P. and by NIH grants R01NS102722-01 and R01DE026806-01A1 (N.B.). S.M.B. and P.A.H. are NHMRC R.D. Wright Biomedical Research Fellows, NHMRC APP1049730 and NHMRC X (NB).

**Author contributions**


**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 recommended by funding agencies, publishers and other organisations engaged with supporting research.

**References**


updates and expansion to encompass the new guide to IMMUNOPHARMACOLOGY. Nucl Acids Res 46: D1091–d1106.


