CO-EXPRESSION OF \( \mu \) AND \( \delta \) OPIOID RECEPTORS BY MOUSE COLONIC NOCICEPTORS

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ABSTRACT

Background and Purpose: To better understand opioid signaling in visceral nociceptors, we examined the expression and selective activation of mu (MOR) and delta opioid receptors (DOR) by dorsal root ganglia (DRG) neurons innervating the mouse colon.

Experimental Approach: DRG neurons projecting to the colon were identified by retrograde tracing, DOR-GFP reporter mice, in situ hybridization, single-cell RT-PCR, and MOR-specific antibodies were used to characterize expression of MOR and DOR. Voltage-gated Ca²⁺ currents and neuronal excitability were recorded in small diameter nociceptive neurons (capacitance < 30pF) 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-labeled DRG neurons was observed in 61% of neurons. MOR and DOR were expressed by 36-46% of colon DRG neurons, and co-expressed by ~25% of neurons. MOR and DOR agonists inhibited Ca²⁺ currents in DRG and these effects were 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 Ca²⁺ currents and neuronal excitability. The MOR but not the DOR antagonist, inhibited the supernatant effects on Ca²⁺ currents, whereas both antagonists inhibited their actions on neuronal excitability.

Conclusions and Implications: A significant number of small diameter colonic nociceptors co-express MOR and DOR and are inhibited by agonists and endogenous opioids in inflamed tissues. Thus, opioids that act at MOR or DOR or their heterodimers may be effective in the treatment of visceral pain.
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, Davis & Binion, 2009; Srinath, Walter, Newara & Szegethy, 2012). Opioid use has markedly increased in the past decade. Five -13% of patients with IBD are prescribed chronic narcotics in the outpatient setting (Cross, Wilson & Binion, 2005; Hanson, Loftus, Harmsen, Diehl, Zinsmeister & Sandborn, 2009; Targownik, Nugent, Singh, Bugden & Bernstein, 2014), and opioid use reaches 70% for inpatients (Long, Barnes, Herfarth & Drossman, 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., 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 & Machelska, 2011).

These peripheral opioid mechanisms also become increasingly important as inflammation progresses, due to an enhanced endogenous opioid system (Boe et al., 2014; Stein, 2015; Stein & Machelska, 2011). During chronic inflammation, accumulating tissue CD4+T cells secrete ß-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 signaling to intestinal nerve terminals of DRG neurons (Fichna et al., 2012; Stein, 2015; Stein & Machelska, 2011; Verma-Gandhu et al., 2006). 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 delta, mu, and kappa (DOR, MOR, KOR) G protein-coupled receptors that inhibit neuronal excitability and release of inflammatory neuropeptides (Stein, 2015). However, the functional expression of these receptors on specific subsets of DRG neurons has been an area of ongoing controversy (Bardoni et al., 2014; Beaudry, Dubois & Gendron, 2011; Scherrer et al., 2009; Woolf, 2009; Zhang, Bao & Li, 2015). In the somatosensory system, recent studies (Bardoni et al., 2014) suggest a divergence of MOR and DOR expression, with DOR being mainly expressed on myelinated A-type fibers and MOR-DOR co-expression in less than ten percent of DRG neurons. Compared to the somatosensory pathways, there is reportedly little expression of DOR on DRG neurons innervating the viscera, suggesting opioids only signal to MOR 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 MOR and DOR including heterodimerization, and the mechanisms of inflammation-induced plasticity of opioid signaling (Stein, 2015).

Technical issues have confounded the interpretation of studies of opioid receptor expression, particularly of DOR (Stein, 2015; Zhang, Bao & Li, 2015). We therefore studied DOR eGFP reporter mice, where DOR expression has been previously validated in neurons (Poole, Pelayo, Scherrer, Evans, Kieffer & Bunnett, 2011; Scherrer et al., 2009). We used combined neuroanatomical and electrophysiological techniques to determine the expression of MOR and DOR by colonic DRG neurons. Voltage-gated calcium currents and measures of neuronal excitability provide a functional measure of opioid receptor signaling 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 MOR and DOR were expressed on colonic DRG neurons and examined the effects on nociceptive signaling of selective opioid agonists and endogenous opioids in inflamed colons.

METHODS

Animals and identification of colon DRG neurons

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 approved procedures using animals. Investigators adhered to the ARRIVE and BJP guidelines for animal experimentation. In all groups, mice were males that were group housed (4-5 per cage) and maintained under temperature (22±4°C) and light- (12 h light/dark cycle) controlled conditions with free access to food and water. Specific strain and housing details for each group are outline below.

Chronic colitis was induced in C57Bl/6 male mice (Charles River Laboratories) 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 labeled neurons from the colon of cDSS 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 labeled neurons in DRG were identified in subsequent imaging.

**Immunofluorescence**

Colonic DRG neurons were identified by Fast Blue-labeling in DOReGF knock-in mice (male, 6-8 weeks) (Scherrer et al., 2006). Mice caged with sawdust bedding and fed Barastoc chow (Ridley, AgriProducts, Victoria, Australia). After 7 days, mice were injected with the DOR agonist SNC80 (10 mg/kg 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 x PBS washes), cryoprotected in 30% sucrose (w/v in PBS), and embedded in Optimal Cutting Temperature solution (Sakura Finetek). 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, RT). Sections were incubated with primary antibodies: chicken anti-GFP, Abcam, ab13970, 1:50; rabbit anti-MOR (UMB3), Abcam, ab134054, 1:250, Lupp et al. 2011 http://www.ncbi.nlm.nih.gov/pubmed/20851148) diluted in blocking buffer overnight at 4°C. Sections were washed (3 x 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 x PBS) then mounted (Prolong Gold, Invitrogen/Thermofisher). Samples were imaged using a Leica TCS-SP8 confocal microscope using a 20x oil immersion objective. Images were of 1024x1024 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 Qiagen (Charsworth, California, USA) RNEasy kit
and was reverse transcribed using Superscript III (Invitrogen, Victoria, Australia) (Bron, Wood, Brock & Ivanusic, 2014). cDNA for mouse OPRM1 was amplified by RT-PCR. The following forward and reverse primers were used: TATACCAAAATGAAGACTGCAACCA (mOPRM1_F352) and GAATTCTAATACGACTCACTATAGGGAGACATCGTTTGAAGTTTTCATCCAGG (mOPRM1_R1103_T7). The design of the reverse primer includes the T7 promoter sequence (underlined), which allows the PCR products to be used directly for the generation of digoxigenin (DIG)-labeled 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, Wood, Brock & Ivanusic, 2014). An antibody against HuC/D was used as a pan-neuronal marker (mouse monoclonal, #A21207, Invitrogen; 1:1000) and labeling was detected using a donkey anti-mouse secondary antibody (Alexa Fluor488 conjugated, 1:500, Invitrogen).

Sections were scanned using 10x or 20x 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 analyzed in an unblinded fashion. Fast Blue-positive neurons were identified from projected z-stacks and coexpression of GFP and MOR 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 PMI 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) (Inamed 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-labeled small DRG neurons (≤30 pF) from primary cultures. Each neuron was harvested under into the patch pipette containing 6 μl of 10X resuspended buffer and 1 μl of RNAseout (2U/μL) by applying negative pressure. The content of the patch pipette was expelled into a PCR-tube containing 4 μl of 10X resuspended buffer, 2 μl Oligo dT(20) 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 5X RT buffer, 1 μl of RNAseout (2U/μL), 1 μl of Superscript III reverse transcriptase (200U/μl) and 1 μl DTT (0.1M). 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 minutes, denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 1 minute, and final extension at 72°C for 5 minutes. PCR was performed using the following intron-spanning mouse primers: DOR (NM_013622.3) outer forward, 5'-TTCTGGGCAAGTGCTGTC-3'; DOR outer reverse 5'-CATAGCACACCTGTAGATG-3' (510-bp product); DOR inner forward, 5'-TGTTTGGCATCGTCCGGTAC-3'; DOR inner reverse 5'-TGAAGCCAAGACCAGGTCCTGTC-3' (320-bp product); MOR (NM_001039652.1) outer forward 5'-TTCTGGGCAAGTGCTGTC-3'; MOR outer reverse 5'-CATAGCACACCTGTAGATG-3' (510-bp product); MOR inner forward 5'-AGGCCCTGGATTTCCGTACC-3'; MOR inner reverse 5'-CATCGGGACTCTTGCTGTC-3' (272-bp product); Actin (NM_007393.3) outer forward 5'-GCCAACCGTGAAAAGATGAC-3'; Actin outer reverse 5'-GGCTGTGGCTGGCGAG-3' (556-bp product); Actin inner forward 5'-GGCTGTGGCTGGCGAG-3'; Actin inner reverse 5'-CTTCATGAGGTAGTCTGCA-3' (164-bp product, Invitrogen). Nested PCR reactions were performed with 1 μl of a 1:10 dilution of the
first PCR. The amplification products were analyzed by ethidium bromide staining subsequent to agarose gel electrophoresis (2%).

**Patch clamp recordings**

All experiments were performed at room temperature as previously described (Valdez-Morales et al., 2013). Patch pipettes were fabricated from borosilicate glass capillaries (Warner Instruments, Connecticut, USA) and had resistances between 2-4 MΩ. Voltage-clamp recording was performed by use of Multiclamp 700B or Axopatch 200B amplifiers, digitized by Digidata 1440A or 1322A AD converters, and recorded using pClamp 10.2 software (all by Molecular Devices, California, USA). Acutely dissociated neurons plated on coverslips were incubated overnight (37°C) with colonic supernatants from control and DSS mice with and without selective mu and delta agonists and antagonists. Only cells with a resting membrane potential more negative than -40 mV were analyzed. We used only small neurons that exhibit properties of nociceptors (Capacitance ≤30 pF).

Barium currents (I_{Ba}) flowing through voltage-gated calcium channels (VGCC) were recorded using the whole-cell configuration and an extracellular solution consisting of (in mM): 140 tetraethylammonium (TEA)-Cl, 2 MgCl₂, 5 BaCl₂, 10 Glucose, 10 HEPES (pH 7.4 adjusted with TEA-OH). The pipette solution contained (in mM): 120 CsCl, 1 MgCl₂, 10 HEPES, 10 EGTA, 4 Mg-ATP and 0.3 Na-GTP (pH 7.2 adjusted with CsOH). A calculated liquid junction potential (Clampex Junction Potential Assistant) of 10 mV was digitally compensated. After whole-cell configuration, the cell membrane capacitance and series resistance were electronically compensated 80-85%. For whole-cell I_{Ba}, neurons were held at -90 mV and depolarized to 0 mV for 100 ms, leaving an interval of 15 s to help minimize rundown. Neurons that showed >10% rundown of current amplitude over 10 min were discarded. The whole-cell current was measured in DRG neurons held at -90 mV in response to 100 msec voltage steps ranging from -60 mV to +55 mV in 5 mV increments, once every 5 sec. The steady-state inactivation of VGCCs was measured by depolarizing cells to a series of prepulse potentials from -90 to 55 mV for 400 ms followed by a command potential to 0 mV for 100 ms. Activation and inactivation curves were fitted with a single Boltzmann function of the form \( G/G_{\text{max}} = 1/(1 + \exp(V_{50} - V_m/k)) \), where \( G \) is conductance, \( V_m \) is membrane voltage, \( k \) is the slope factor, and \( G_{\text{max}} \) is maximal conductance. The amplitude of inward currents was normalized to cell capacitance.

Changes in neuronal excitability in response to acute (min) or chronic (overnight) application of agonists or colonic supernatants were measured by recording the rheobase and the action
potential discharge at twice rheobase, as previously described using perforated patch clamp recordings with amphotericin B (0.24 mg/ml) (Cattaruzza et al., 2011; Ibeakanma & Vanner, 2010). The extracellular solution was: 140 NaCl, 5 KCl, 1 MgCl$_2$, 2 CaCl$_2$, 10 HEPES, 10 D-glucose, pH 7.4. The pipette solution was: 110 K-gluconate, 30 KCl, 10 HEPES, 1 MgCl$_2$, 2 CaCl$_2$, 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, Jones (III), Gebhart & Blackshaw, 2004; Castro et al., 2013; de Araujo et al., 2014; Hughes, Brierley, Martin, Brookes, Linden & Blackshaw, 2009; 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: CA PURA CHIP ASPEN COARSE (Cat# ASPJMAEB). Animals were killed by CO$_2$ 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 (40mmHg), stretch (≥7g) or von Frey hair filaments (2g) but not to fine mucosal stroking (10 mg von Frey hairs) (Hughes, Brierley, Martin, Brookes, Linden & Blackshaw, 2009). These afferents also become hypersensitive during and after resolution of colonic inflammation (Brierley & Linden, 2014; Hughes, Brierley, Martin, Brookes, Linden & Blackshaw, 2009).

Baseline mechanosensitivity was determined in response to application of a 2 g vfh probe to the afferent receptive field for 3 s. This process was repeated 3–4 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 the DOR agonists; DADLE (100nM) or SNC 80 (1000nM) or the MOR agonist DAMGO (100nM). These compounds were applied to the mucosal surface of the colon for a period of 5 minutes at each concentration via a small metal ring placed over the receptive field of interest. Action potentials were analyzed off-line using the Spike 2 waveform function (version 5.21; Cambridge Electronic Design, Cambridge, UK) and discriminated as single units on the basis of distinguishable waveform, amplitude and duration. Data are presented as spikes/s and are expressed as mean ± SEM.
In the second series of experiments, C57BL/6 male mice from Charles River were fed PMI lab chow (Purina USA) mouse #5015 and cage bedding contained a combination corn cob bedding that was changed every 14 days. They were killed with i.p. ketamine/xylazine and the colons were excised. Both DAMGO and DADLE were tested on the same colonic afferent unit (serosal or mesenteric unit). Baseline mechanosensitivity was determined in response to a 1g vfh probe to the afferent receptive field for 3s. The process was repeated 3-4 times, separated by 10s. Mechanosensitivity was then retested after the application of either DADLE (100nM) or DAMGO (100nM) to the mucosal surface for 5 minutes via the small metal ring placed over the receptive field of interest. Following a 30 minute washout period, mechanosensitivity was retested for reversibility. The other agonist was then applied to the receptive field for 5 minutes followed by testing of the mechanosensitive response. Reversibility was rechecked after a 30 minute washout period. To ensure stability of the unit and adequate washout of the agonist, a unit was considered to be inhibited by either the MOR or DOR 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 rundown 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.

Statistical analyses

Data were analyzed 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 5 animals. The afferent nerve recordings (1-2 single units obtained per colon of each animal; 1 unit = 1 single axon recording) shown in Fig. 3B-D were obtained from fewer than 5 animals for each drug tested and therefore no statistical analysis was performed. A p value < 0.05 was considered to be significant and only reported as *P <0.05 as per British Journal of Pharmacology guidelines. 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\(^5\)]-enkephalin (DADLE), (+)-4-[(\(\alpha\)R)-\(\alpha\)-[(2S,5R)-4-Allyl-2,5-dimethyl-1-piperazinyl]-3-methoxybenzyl]-N,N-diethylbenzamide (SNC\(_{80}\)), and [D-Ala\(^2\), N-MePhe\(^4\), Gly-ol\(^5\)]-enkephalin (DAMGO) were all purchased from Tocris Bioscience (UK) (extracellular recordings or Sigma (patch clamp recordings). \((4bS,8R,8aS,14bR)\)-5,6,7,8,14,14b-Hexahydro-7-(2-methyl-2-propenyl)-4,8-methanobenzofuro[2,3-a] pyrido[4,3-b] carbazole-1,8a(9H)-dil (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

MOR and DOR are expressed by colonic afferent neurons

Expression of oprm1 mRNA, the gene for the MOR, by Fast Blue-positive colonic DRG neurons was examined by in situ hybridization (Fig. 1A). Positive staining for oprm1 was detected in approximately 61% of all Fast Blue neurons (62/101 neurons from 3 animals). In contrast, MOR-IR was detected in only 36% of all Fast Blue-labeled neurons (75/210 neurons) (Fig. 1B). DOR-GFP labeling was detected in 46% of colonic afferents (190/411 neurons from 3 animals). Approximately a quarter of all Fast Blue-labeled neurons were positive for both DOR GFP-IR and MOR-IR (24%, 58/245 neurons). Colonic afferents that co-expressed DOR and MOR had an average soma area of 482±27 \(\mu\)m\(^2\) and average maximum soma perimeter of 83±2.4 \(\mu\)m and a length (measured in sections containing nuclei) of 27.95 \(\mu\)m ± 0.84 \(\mu\)m.

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

**MOR and DOR agonists inhibit Ca\(^{2+}\) currents in small diameter DRG neurons**

To determine whether MOR and DOR regulate voltage-gated Ca\(^{2+}\) channels, we measured I\(_{Ba}\) in small DRG neurons in response to acute (min) and chronic (overnight) exposure to MOR and DOR 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\(_{Ba}\) in DRG neurons by comparing currents evoked from holding potentials of -60 mV 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\(_{Ba}\) under our recording conditions.

Consistent with previous findings (Moises, Rusin & Macdonald, 1994; Schroeder, Fischbach, Zheng & McCleskey, 1991), superfusion of the MOR agonist DAMGO (100 nM) for 5 min led to a significant (63.5%) reduction in I\(_{Ba}\) currents (data not shown). Similar reductions in I\(_{Ba}\) currents were observed (63.2%) with overnight incubation of DAMGO (100 nM; Fig. 2.). In all subsequent experiments, neurons were incubated overnight with MOR agonist (DAMGO) or DOR agonist (DADLE). Fig. 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 I\(_{Ba}\) in a concentration-dependent manner (Fig. 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 (Fig. 2B). DAMGO and DADLE inhibited I\(_{Ba}\) with similar potency in small DRG neurons (Fig. 2B). There were no changes in the of activation (DADLE - k = 5.0 ± 0.47 mV, V\(_h\) = -19.6 ± 54 mV; DAMGO - k = 4.5 ± 0.4 mV, V\(_h\) = -19.0 ± 0.44 ) and inactivation (DADLE - k = 38.4 ± mV, V\(_h\) = -61.8 ± 4.0mV ; DAMGO - k = 23.7±5.0, V\(_h\) = -61.5 ± 6.0 mV). A scatter plot from all cells incubated with a maximum concentration (1 μM) of DAMGO and DADLE showed that DAMGO inhibited I\(_{Ba}\) in 7/9 cells and DADLE inhibited I\(_{Ba}\) in 17/20 cells (data not shown). The inhibition of I\(_{Ba}\) evoked by DAMGO and DADLE was blocked by CTOP and SDM25N, respectively (Fig. 2C). The antagonists alone had no effect.

**DOR and MOR agonists inhibit colonic nociceptors**

We first examined whether MOR and DOR agonists inhibited neuronal excitability of isolated
DRG neurons using patch clamp recordings. We found that an acute (10 min) application of either DAMGO or DADLE (100nM) applied to neurons increased the rheobase and increased the input resistance (Fig. 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, p <0.05). To determine if MOR or DOR agonists modify colonic nociceptor function in intact tissue, we made ex vivo colonic afferent recordings from mouse splanchnic high-threshold nociceptors, which respond to focal compression and noxious stretch/distension. The DOR agonist DADLE (100nM; 5 units from 3 animals) inhibited colonic nociceptor action potential firing in response to noxious mechanical stimuli (Fig. 3B). Another highly selective DOR agonist, SNC 80 (1000 nM; 5 units from 3 animals), also inhibited nociceptor mechanosensitivity (Fig. 3C). The MOR agonist DAMGO (100nM; 7 units from 4 animals) also inhibited colonic nociceptors (Fig. 3D). The percent inhibition by the DOR and MOR agonists was similar (DADLE = 21 ± 6, SNC 80 = 26 ± 8, DAMGO = 25 ± 4).

We next examined whether MOR and DOR agonists could inhibit mechanosenstivity in the same unit. In these experiments, 6/8 units obtained from 8 animals were inhibited by either DAMGO (100nM) or DADLE (100nM) (Fig. 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. In units that were inhibited by DAMGO, afferent firing was reduced from 14.22 ± 2.72 to 8.59 ± 1.5 spikes/s.

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

Neurons incubated overnight with supernatants from DSS-inflamed colons (Fig. 5A) exhibited a significant decrease in amplitude of $I_{Ba}$ compared with $I_{Ba}$ in neurons incubated with supernatants from uninflamed colon or control supernatants ($P<0.05$, one-way ANOVA with Bonferroni post-test). In neurons incubated with supernatants from inflamed colon, neither DADLE nor DAMGO had an additional inhibitory effect on $I_{Ba}$ (Fig. 5B).

Dissociated neurons were preincubated with the MOR antagonist (CTOP 1 μM) or the DOR antagonist (SDM25N 1 μM) 30 min before incubation with colonic supernatants from control and chronic DSS mice and the effects on $I_{Ba}$ and neuronal excitability examined. In control studies (Fig. 5C), the application of CTOP or SDM25N alone had no effect on $I_{Ba}$. DAMGO (100 nM) and DADLE (100 nM). In studies of the chronic DSS supernatant Fig. 5C), the inhibitory effect of the chronic DSS supernatant on $I_{Ba}$ was supressed by pretreatment with the MOR
antagonist CTOP, but not with DOR antagonist SDM25N (Fig. 5C) (One-way ANOVA with Dunnett’s post-test).

Given that the inhibition of $I_{Na}$ by endogenous opioids was mediated predominantly by MOR 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 to 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 (Fig. 5D), both CTOP and SDM25N decreased the rheobase (29% each, $p < 0.05$) and CTOP increased action potential discharge (1.9X, $p<0.05$) in neurons incubated in cDSS supernatant.

**DISCUSSION**

Therapeutic opioids such as morphine and related compounds act predominantly on MORs 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 need for alternative therapies and there is a renewed interest in DOR targeted agents (Stein, 2015; Woolf, 2009; Zhang, Bao & Li, 2015). A fundamental step towards understanding how opioids can be exploited in this clinical setting is to examine the functional expression of MOR and DOR 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 signaling, given the potential for physical interactions and the formation of heterodimers (Geppetti, Veldhuis, Lieu & Bunnett, 2015; Stein, 2015; Targownik, Nugent, Singh, Bugden & Bernstein, 2014). We found that MOR and DOR 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 signaling from the intestine. (Ibeakanma et al., 2011)

There has been considerable controversy regarding the expression of MOR and DOR in DRG neurons and whether these receptors are co-expressed in neurons (Rau, Caudle, Cooper & Johnson, 2005) (Scherrer et al., 2009; Stein, 2015) (Bardoni et al., 2014; Beaudry, Dubois & Gendron, 2011; Joseph & Levine, 2010; Normandin, Luccarini, Molat, Gendron & Dallel, 2013; Wang et al., 2010; Woolf, 2009; Zhang, Bao & Li, 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 DOR, and non-selective effects of high concentrations of opioid agonists (Bardoni et al., 2014; Woolf, 2009; Zhang, Bao & Li, 2015). We used DOReGFP knock-in mice, in situ hybridization and single-cell RT-PCR to enable specific detection of DOR expression (Bardoni et al., 2014; Scherrer et al., 2009; Scherrer et al., 2006). In the somatosensory system, recent studies using this mouse model have shown that only 17% of DRG neurons express DOR, and that expression was confined to the larger myelinated CGRP positive DRG neurons (Bardoni et al., 2014). Only a small proportion co-expressed MOR and DOR (~5%). Visceral afferents account for <10% of all DRG neurons (Beyak and Grundy, 2006) 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 MOR and 46% expressed DOR. Moreover, we found that 24% of neurons co-expressed MOR and DOR 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 MOR and DOR mRNAs. Thus, our studies suggest a much higher proportion of neurons co-express MOR and DOR in the visceral sensory system, including small diameter DRG neurons that have properties of nociceptors (Moore, Stewart, Hill & Vanner, 2002; Stewart, Beyak & Vanner, 2003).

Voltage-gated Ca$^{2+}$ play a central role in peripheral and central presynaptic modulation of neurotransmitter release from DRG neurons (Beyak & Vanner, 2005; Stein, 2015). We recorded from small DRG neurons and found that both DOR and MOR 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 MOR and DOR, 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, Brierley, Martin, Brookes, Linden & Blackshaw, 2009). Here we found that both MOR and DOR 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 DOR agonist DADLE (100nM) on $I_{\text{Ba}}$ was completely blocked by the selective DOR antagonist SDM25N and that the highly selective DOR agonist SNC 80 (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, Dubois & Gendron, 2011), suggest
small diameter visceral DRG neurons co-express MOR and DOR 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 KOR 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 the DOR, MOR and KOR play in opioid signaling in different pathological conditions.

Understanding the expression of MOR and DOR 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 signaling (Zhang, Bao & Li, 2015), affecting receptor expression, G-protein signaling and receptor trafficking. The effect of inflammation on opioid signaling pathways can vary considerably between opioid receptor subtype signaling pathways. For example, MOR is upregulated (Stein & Machelska, 2011) in inflammation, including studies of human IBD (Philippe et al., 2006), whereas DOR appears to be unchanged or down-regulated (Ji, Zhang, Law, Low, Elde & Hokfelt, 1995). We and others (Boue et al., 2014; Valdez-Morales et al., 2013; Verma-Gandhu et al., 2006) 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 Ca²⁺ currents and neuronal excitability, but in contrast to control neurons where activation both MOR and DOR have similar effects, the inhibitory effect on Ca²⁺ currents is restricted to MOR signaling. Further studies are needed to determine the mechanism underlying this altered signaling but these findings may suggest that drugs targeting DOR alone in chronic intestinal inflammation will exert little direct effect on opioid-induced analgesia mediated by voltage-gated Ca²⁺ currents.

In summary, our study has shown that DRG neurons innervating the colon express MOR or DOR and they are co-expressed on a substantial subpopulation of nociceptors. We also found that selective activation of both MOR and DOR receptors inhibits Ca²⁺ 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 multiple peripheral opioid receptors in the gut, such as the MOR agonist and DOR antagonist, eluxadoline, for the treatment of irritable bowel syndrome (Dove et al., 2013). Furthermore, this co-expression is a prerequisite for the formation of MOR-DOR 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
DOR signaling in the GI tract, as this may increase analgesia and mitigate tolerance (Geppetti, Veldhuis, Lieu & Bunnett, 2015).

Contributions:

Designed research: SV and AL conceived the study. All authors designed components of the research studies.

Performed research: RG, EM, NJ, RB, DP, JC, MC, PH conducted the experiments.

Analyzed data: RG, EM, RB, DP, JC, NJ, MC, PH analyzed the data.

Wrote the paper: SV, AL, SB, NB wrote the paper.

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Figure 1. MOR and DOR expression on Fast blue-labeled DRG neurons. (A) Representative photomicrographs of *in situ* hybridization of oprm1 expression in Fast Blue-labeled DRG neurons (white arrows) and non-Fast Blue-labeled neurons (yellow arrow). Hu antibody
staining identifies all neurons in the ganglia. 61% of Fast Blue-labeled neurons expressed oprm1 mRNA. Scale = 100 μm. (B) Representative photomicrographs of DOR expression in ganglia from DORéGFP knock-in mouse and double labeling with MOR immunoreactivity (merge). Colonic neurons identified by Fast Blue retrograde labeling. 46% of Fast Blue-labeled neurons expressed DOR and 24% were co-immunoreactive for MOR/DOR. Scale = 150 μm. (C) A representative agarose gel electrophoresis (2%) stained with ethidium bromide to assess MOR and DOR mRNA expression in small diameter Fast Blue-labeled DRG neurons. PCR products 272 bp, 320 bp and 164 bp corresponding to MOR, DOR and β-actin cDNAs, respectively. Lane 1, exACTGene 50bp Mini DNA Ladder; Lane 2-13, PCR products from 13 individual small Fast Blue-labeled DRG neurons. Neurons 4, 8 and 13 co-expressed MOR and DOR mRNAs. No transcripts were amplified from bath fluid or without template (Lane 14 and 15). Of the 13 neurons, 23% co-expressed MOR and DOR mRNAs.
**Figure 2.** DAMGO (MOR agonist) and DADLE (DOR 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 $-90$ to $0$ mV for $100$ ms.
represented in the inset above. The currents were obtained from different small DRG neurons incubated overnight with DOR agonist (DADLE 0.1 μM) or MOR agonist (DAMGO 0.1 μM). (B) Concentration dependency of inhibition of $I_{Ba}$ 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 $I_{Ba}$ (*p < 0.05, *p < 0.05). Number of cells appears in each bar. Bars represent mean ± SEM. * denote statistical significance by one-way ANOVA with Dunnett’s post-test. The amplitude of inward currents was normalized to cell capacitance. (C) Effects of MOR and DOR antagonists on $I_{Ba}$ through voltage-gated Ca$^{2+}$ channels in DRG neurons. Inhibition of $I_{Ba}$ 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 $I_{Ba}$. Bars represent mean ± SEM. Number of cells appears in each bar * significant difference from control ($P < 0.05$, one-way ANOVA with Dunnett’s post-test).
**Figure 3.** Patch clamp and *ex vivo* single-unit afferent recordings showing DOR and MOR agonists inhibit colonic afferents. (A) Acute application of MOR and DOR agonists (100nM; applied for 10 min) inhibits the excitability of isolated DRG neurons (increase rheobase) and
increases input resistance (*p<0.05). (B) Representative recording (left panel) of a colonic nociceptor response to von Frey hair probing before and after administration of DOR agonist DADLE (100nM). Summary data (right panel) showing DADLE inhibits splanchnic colonic nociceptors from healthy mice, n=5 single afferent recordings from axons innervating the colons from 3 mice. (C) Representative recording (left panel) of a colonic nociceptor response to von Frey hair probing before and after administration of DOR agonist SNC 80 (1000nM). Summary data (right panel) showing SNC 80 inhibits splanchnic colonic nociceptors from healthy mice, n = 5 single afferent recordings from axons innervating the colons from 3 mice. (D) Representative recording (left panel of a colonic nociceptor response to von Frey hair probing before and after administration of MOR agonist DAMGO (100nM). Summary data (right panel) showing DAMGO inhibits splanchnic colonic nociceptors from healthy mice, n = 7 single afferent recordings obtained from axons innervating the colons from 4 mice.
Figure 4. Ex vivo single-unit recordings showing inhibit DOR and MOR agonists inhibit same colonic afferent unit. (A) Representative recording of a colonic nociceptor response to von Frey probing before and after administration of MOR agonist DAMGO (100nM). Following washout
of DAMGO, von Frey probing is applied to the same unit before and after administration of DADLE (100nM). **(B)** Proportion of single-unit afferents responding to either DADLE (100nM) or DAMGO (100nM) alone, or both (n = 8 units).
Figure 5. The effects of chronic DSS supernatant on $I_{Ba}$ and neuronal excitability (A) Bars represent summary data for $I_{Ba}$ in cultured DRG neurons incubated overnight with medium, control or chronic DSS supernatants. The amplitude of $I_{Ba}$ inward currents in DRG neurons
incubated with medium was not significantly different from those incubated in control supernatant (open bar vs. grey bar). Chronic DSS supernatants produced a marked decrease in $I_{Ba}$ (*$p < 0.05$) compared with medium and control supernatants. Number of cells appears in each bar. Bars represent mean ± SEM. *denote statistical significance by one-way ANOVA with Bonferroni’s post-test. The amplitude of inward currents was normalized to cell capacitance. (B) Dissociated DRG neurons were incubated overnight with chronic DSS supernatants plus DAMGO (0.1 μM) or DADLE (0.1 μM). Neither DADLE nor DAMGO had not additional inhibitory effect on $I_{Ba}$. (C) Dissociated neurons were preincubated with MOR antagonist (CTOP 1 μM) or DOR antagonist (SDM25N 1 μM) 30 min before incubation with control (Ctl Sup) (left panel) or chronic DSS supernatants (cDSS Sup) (right panel). CTOP reversed the inhibitory effect of chronic DSS supernatants on $I_{Ba}$ in cultured DRG neurons but SDM25N has no effect. (D) Rheobase recorded following incubation with cDSS supernatant is inhibited by both SDM25N and CTOP (left panel, *$p < 0.05$) and action potential discharge by CTOP (right panel, *$p < 0.05$). Bars represent mean ± SEM. Number of cells appears in each bar; * significant difference from control ($P < 0.05$, one-way ANOVA with Dunnett’s post-test).