Inflammation-associated changes in DOR expression and function in the mouse colon

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DiCello JJ, Saito A, Rajasekhar P, Eriksson EM, McQuade RM, Nowell CJ, Sebastian BW, Fichna J, Veldhuis NA, Canals M, Bunnett NW, Carbone SE, Poole DP. Inflammation-associated changes in DOR expression and function in the mouse colon. Am J Physiol Gastrointest Liver Physiol 315: G544–G559, 2018. First published June 21, 2018; doi:10.1152/ajpgi.00025.2018.—Endogenous opioids activate opioid receptors (ORs) in the enteric nervous system to control intestinal motility and secretion. The μ-OR mediates the deleterious side effects of opioid analgesics, including constipation, respiratory depression, and addiction. Although the δ-OR (DOR) is a promising target for analgesia, the function and regulation of DOR in the colon are poorly understood. This study provides evidence that endogenous opioids activate DOR in myenteric neurons that may regulate colonic motility. The DOR agonists DADLE, deltorphin II, and SNC80 inhibited electrically evoked contractions and induced neurogenic contractions in the mouse colon. Electrical, chemical, and mechanical stimulation of the colon evoked the release of endogenous opioids, which stimulated endocytosis of DOR in the soma and proximal neurites of myenteric neurons of transgenic mice expressing DOR fused to enhanced green fluorescent protein. In contrast, DOR was not internalized in nerve fibers within the circular muscle. Administration of dextran sulfate sodium induced acute colitis, which was accompanied by DOR endocytosis and an increased density of DOR-positive nerve fibers within the circular muscle. The potency with which SNC80 inhibited neurogenic contractions was significantly enhanced in the inflamed colon. This study demonstrates that DOR-expressing neurons in the mouse colon can be activated by exogenous and endogenous opioids. Activated DOR traffics to endosomes and inhibits neurogenic motility of the colon. DOR signaling is enhanced during intestinal inflammation. This study examines functional expression of DOR by myenteric neurons and supports the therapeutic targeting of DOR in the enteric nervous system.

ENDOCYTOSIS; ENTERIC NERVOUS SYSTEM; G PROTEIN-COUPLED RECEPTOR; INTRODUCTION

Opioids are a leading treatment for severe chronic pain. Although highly effective, their usefulness is limited by the side effects of tolerance, dependence, and respiratory depression (38). The gastrointestinal tract (GIT) is a major target of opioid drugs, and their use can lead to development of opiate-induced bowel dysfunction, including nausea, vomiting, visceral pain, and constipation (19). Intractable opioid-induced constipation (OIC) affects more than 40% of patients receiving opioid analgesics and is a major cause of noncompliance (13, 36). In contrast to other GIT effects, the severity of OIC persists throughout treatment and does not diminish with the development of analgesic tolerance. Moreover, OIC can occur at lower doses than those required for effective analgesia.

Both the analgesic and constipatory actions of opioids are mediated through activation of the μ-opioid receptor (MOR) (77), a member of the G protein-coupled receptor (GPCR) family. In the GIT, MOR is expressed primarily by neurons of the enteric nervous system (ENS) (40). The ENS controls the coordinated contraction and relaxation of the muscularis externa as well as secretomotor activity. Opioids mediate their effects through inhibition of neuronal firing, resulting in both...
dysmotility and inhibition of secretion, ultimately leading to constipation (46, 77).

The δ-opioid receptor (DOR) has been proposed as an alternative therapeutic target to MOR for the treatment of chronic pain. DOR agonists suppress chronic inflammatory and neuropathic pain in rodents, although translation to human studies is lacking (68, 75). Whether DOR agonists also inhibit colonic motility leading to constipation and the mechanism by which DOR controls intestinal motility are not fully understood. DOR agonists have been reported to inhibit colonic transit (10, 31). Other studies have described limited effects but were restricted to short-term measurements of small intestinal transit (15, 23, 56). Although these in vivo studies suggest a central mechanism of action, functional expression of DOR by enteric neurons has been demonstrated by electrophysiology (21). Furthermore, DOR-selective agonists inhibit neurotransmitter transmission in isolated intestinal preparations from a range of species, including humans (3, 14, 17, 34, 48).

By using transgenic mice expressing DOR fused to enhanced green fluorescent protein (eGFP), we previously reported that DOR is expressed by 50% of myenteric neurons of the colon and is localized to ascending and descending interneurons and excitatory and inhibitory motoneurons involved in generating propulsive motility (60). The majority of DOR-eGFP positive neurons in this region are nitricergic (~70%), suggesting that DOR can influence the inhibitory control of colonic motility (60). DOR agonists induced endocytosis of DOR-eGFP in myenteric neurons, indicating functional expression. DOR-eGFP was not detected in intestinal smooth muscle or in nonneuronal cells associated with the control of intestinal motility. These observations suggest that DOR agonists are likely to inhibit colonic motility directly through actions at myenteric neurons. In support of this hypothesis, DOR-eGFP-positive neurons are closely associated with enkephalin-immunoreactive nerve fibers (60), and enkephalins expressed by descending and ascending interneurons of the mouse colon suppress transmission to motor neurons via a naltrindole-sensitive and DOR-dependent mechanism (29). This observation is consistent with the prokinetic action of the DOR antagonist naltrindole, which effectively removes an inhibitory opioidergic influence on intestinal motility (24). These studies suggest that DOR is functionally expressed by myenteric neurons and support our hypothesis that DOR is of physiological and therapeutic importance in the regulation of intestinal motility.

An understanding of the mechanism by which DOR controls the GIT is important because DOR agonists and antagonists are potential therapies for intestinal disorders. DOR is functionally expressed by myenteric neurons of the human colon and inhibits excitatory neurotransmission to smooth muscle (3, 14, 34, 44). Eluxadoline (Viberzi), currently approved for the treatment of diarrhea-predominant IBS-D, is a mixed agonist of MOR and the δ-opioid receptor (KOR) and a DOR antagonist (41, 76). Eluxadoline has been proposed to exert its therapeutic actions by targeting MOR-DOR heteromers (25). Racedadotril (acetorphan) is a peripherally acting enkephalinase inhibitor that exerts its anti-diarrheal and anti-nociceptive effects by preventing degradation of the endogenous DOR ligand enkephalin (7).

The function of opioid receptors may also be altered by chronic therapy or disease. Chronic morphine treatment induces MOR endocytosis in myenteric neurons, which may be related to upregulation of dopamine 2 (52). Long-term morphine exposure also affects MOR signaling in myenteric neurons (20) and expression of β-arrestin 2, a master regulator of GPCR signaling and trafficking (37). We recently reported that the neurokinin 1 receptor (NK1R) is internalized in the inflamed intestine (59), which may similarly reflect chronic exposure to endogenous agonists and altered expression of key regulatory proteins such as dynamin and β-arrestin. Reduced levels of GPCRs at the surface of cells may lead to a corresponding decrease in responsiveness to an agonist (53). However, we have recently reported that GPCRs can continue to signal in endosomes of neurons to control excitability and pain transmission (35, 78). Whether changes in the subcellular distribution of DOR in enteric neurons correlates with altered function is unknown. An upregulation of opioids and their receptors may also correlate with altered function. DOR is upregulated at the transcript and protein level in intestinal inflammation (39, 55). Furthermore, DOR function in the small intestine is enhanced during inflammation, with increased potency of DOR agonists in both anti-transit and anti-secretory assays (56, 72). This potentiation of DOR-dependent effects is likely to be mediated through a “sensitization” of DOR and is similar to the increased analgesic effects of DOR agonists in inflammatory pain (62). The cellular and subcellular distribution of DOR in the inflamed intestine was not determined in these studies.

In the present study, we demonstrate DOR-dependent effects on neuromuscular transmission and functional innervation of DOR-expressing myenteric neurons and characterize changes in DOR distribution and function during acute colitis.

MATERIALS AND METHODS

Mice

DOR-eGFP (male and female, 6–12 wk; see Ref. 63) and C57BL/6J (male, 6–12 wk) mice were obtained from the Monash Animal Research Platform. Mice were maintained under temperature (22 ± 4°C) and light-controlled (12-h:12-h light-dark cycle) conditions with free access to food and water. All procedures were approved by the Monash Institute of Pharmaceutical Sciences animal ethics committee.

Motility

Mice were euthanized by cervical dislocation. The large intestine was removed and placed in Krebs solution (in mM: 118 NaCl, 4.7 KCl, 1.1 H2O, 1.18 KH2PO4, 25 NaHCO3, 11.6 glucose, and 2.5 CaCl2; 95% O2-5% CO2). Luminal contents were flushed with Krebs solution, and the mesentery and fat were removed by sharp dissection. Segments of distal colon (~10 mm) were suspended by cotton ligatures to a Grass FT03 force displacement transducer (Grass Instruments, Quincy, MA). Specimens were placed into 10-ml organ baths containing Krebs solution (37°C, 95% O2-5% CO2). Preparations were orientated to measure contractions of the circular muscle and placed under a basal tension of 0.5–1 g. Following a 1-h equilibration, isometric contractions were measured and recorded using a PowerLab 4SP acquisition system and LabChart software version 5 (ADInstruments, Castle Hill, NSW, Australia).

Electrically evoked contractions. Neurogenic contractions were evoked using electrical field stimulation (EFS; 60 V, 0.5-ms duration, 3 pulses/s; Grass S88 stimulator) via two parallel platinum electrodes incorporated into the tissue holder. Unless otherwise stipulated, tissues were stimulated three times to establish a baseline response, and tissues not exhibiting consistent increases in tension were discarded.
DOR agonists were administered in a cumulative manner (1 nM to 10 μM), with 2- to 5-min exposure before responses to EFS were measured (3 stimuli at 2- or 5-min intervals). The average amplitude of EFS contractions at each concentration was normalized to the mean amplitude of basal responses and expressed as a percentage inhibition of baseline. Data were fitted to three-parameter nonlinear regression curves using GraphPad Prism 7.0 (GraphPad Software), and EC50 and Emax values were determined. Statistical analysis between two curves was performed using Student’s t-test for parametric data or Mann-Whitney U-test for nonparametric data.

Agonist-evoked contractions. The direct addition of DOR agonists evokes a tonic contraction. To determine whether SNC80-evoked contractions were inhibited by naltrexol (1 μM), changes in the amplitude of the basal tone were measured after cumulative drug addition. To define the mechanisms through which SNC80 evoked contractions, tissues were prepared as described above. Following a 30-min equilibration period, tissues were electrically stimulated using the parameters outlined above (2 repeats, 2-min intervals) and then incubated for 20 min with vehicle (0.1% DMSO), tetrodotoxin (TTX; 1 μM), Nω-nitro-L-arginine (t-NNa; 100 μM), or hexamethonium (HEX; 1 μM). SNC80 was then added in a cumulative manner (1 nM to 10 μM; 2-min exposures between subsequent additions). Changes in the amplitude of contractions associated with each SNC80 concentration were measured. The effects of inhibitors on the response to 100 nM SNC80 were determined. Data were statistically analyzed using Student’s t-test for parametric data or Mann-Whitney U-test for nonparametric data.

DOR Endocytosis

Exogenous agonists. DOReGFP endocytosis was examined in organotypic preparations as described (60). The distal colon from DOReGFP mice was excised and placed in ice-cold Krebs buffer (including 10 μM nicardipine and 100 nM TTX). Tissue was cut along the mesenteric border, and wholemount preparations were pinned and stretched, with the mucosa downward onto silicone elastomer-lined (Sylgard 184; Dow Corning, Midland, MI) cell culture dishes. Preparations were equilibrated in Krebs (37°C, 95% O2-5% CO2, 1-h recovery). Tissues were then cooled with ice-cold Hanks’ balanced salt solution (HBSS), washed (3 × 5 min) to reduce membrane fluidity, and then incubated in HBSS containing DADLE (1 nM to 10 μM) for 1 h, 4°C. After washing (3 × 5 min, Krebs buffer) to remove unbound agonist, preparations were warmed in Krebs buffer (37°C, 30 min) before fixation (4% paraformaldehyde, overnight, 4°C).

Endogenous agonists. To examine the effects of EFS, colon preparations from DOReGFP mice were placed unstretched in water-jacketed organ baths (Krebs, 37°C, 95% O2-5% CO2, 1-h recovery) and then electrically stimulated (60 V, 0.5-ms duration, 3 pulses/s, every min for 10 min, followed by a 20-min recovery period without stimulation) to promote release of endogenous transmitters. Control preparations from the same tissue were also mounted into organ baths but were not subjected to EFS. Following treatment, preparations were placed into ice-cold Krebs (containing nicardipine and TTX) and then pinned and fixed as wholemounts. The effect of veratridine on DOReGFP localization was examined in organotypic preparations of the colon, as described above. Preparations were exposed to veratridine (10 μM) for 30 min and then fixed. The effect of DOR inhibition was determined using naltrexol (1 μM) with a 10-min preincubation and inclusion throughout the treatment period. Reflex activation of DOR was examined in the isolated colon, using increased intraluminal pressure as a stimulus. The whole colon was placed in a horizontal organ bath superfused with warmed Krebs buffer, and cannulae were connected to the proximal and distal ends of the colon using cotton ligatures. The proximal cannula was connected to an inflow reservoir (20 ml of Krebs solution-2 cm H2O). Adjustment of the height of this reservoir was used to change intraluminal pressure. Back pressure was adjusted using an outflow tube connected to the distal cannula (maintained at 2 cm H2O). Following a 30-min equilibration, intraluminal pressure was elevated to a point where persistent propulsive contractions were initiated (defined as the “pressure threshold”). Control preparations were retained at basal intraluminal pressure. Colonic motility patterns were video recorded using a webcam (8 megapixels, 6 fps capture rate), and spatiotemporal maps were generated based on colon diameter (“Dmap”) using custom software (16).

Real-Time Imaging of Endocytosis

The distal colon from DOReGFP mice was removed and placed in Krebs buffer. The mucosa, submucosa, and longitudinal muscle were removed by sharp dissection, resulting in a circular muscle-myenteric plexus wholemount preparation. EGFP fluorescence was detected by confocal microscopy (Leica TCS SP8, HC PLAN APO 0.95 NA ×25 water immersion objective; 488 nm excitation and 530 ± 20 nm emission). All imaging was performed at 37°C. Full Z-stacks (line averaging of 4) encompassing the myenteric plexus and circular muscle layers were captured every 3 min (1,024 × 1,024 pixels, scan speed of 600 Hz). Veratridine (10 μM) or DMSO vehicle were administered directly into the bath (1:8 dilution to ensure adequate mixing), and DOReGFP trafficking was imaged for 30 min post-addition. Following the completion of experiments, preparations were fixed and post-stained with antibodies against GFP and ENK (see Immunofluorescence and Microscopy below) and then imaged by confocal microscopy (Leica TCS SP8, HC PLAN APO 0.75 NA ×20 oil objective), as outlined below. Images were realigned using key landmarks and overlaid in FIJI using UnwarpJ (2) and Linear Stack Alignment with SIFT (42) functions.

Immunofluorescence and Microscopy

Procedures for immunolabeling of tissues have been described in detail (40, 59). Tissues for sectioning were embedded in OCT (Sakura Finetek, Torrance, CA) and cut on a cryostat (16-μm thickness, nonsequential sections). Circular muscle-myenteric plexus wholemounts were labeled by indirect immunofluorescence to detect immunoreactivities for EGFP (rabbit polyclonal anti-EGFP, Thermofisher no. A11122, 1:1,000 dilution), ENK [mouse anti-enkephalin, clone NOC1; Santa Cruz Biotechnology, Dallas, TX, USA (18), 1:200 dilution], HuC/D [human anti-Hu (43); 1:25,000 dilution], lysosomal-associated membrane protein 1 [LAMP1; rat anti-mouse LAMP1, clone 1D4B; Developmental Studies Hybridoma Bank (60), 1:400 dilution], NK-R [rabbit anti-rat NK-R, CURE no. 94168, 1:1,000 dilution (59)], NOS (goat polyclonal anti-nNOS, 1:1,000 dilution; GeneTex no. 89962, Irvine, CA), and TGN38 [sheep anti-TGN38, NB1-20263, 1:100 dilution; Novus Biologicals, Littleton, CO (79)]. Preparations were incubated with primary antibodies in blocking buffer (5% normal horse serum in PBS containing 0.1% sodium azide and 0.1% Triton X-100) at 4°C either overnight (sections) or for 48 h (wholemounts). Primary antibody labeling was detected using donkey secondary antibodies conjugated to Alexa Fluor 405, 488, 568, or 647 (1:500 to 1:1,000 dilution, 1-h incubation at room temperature; Thermofisher). Preparations were mounted using ProLong Diamond anti-fade mountant (ThermoFisher). Images were captured using a Leica TCS SP8 confocal system as described (59). Images for quantitative analysis of the subcellular distribution of DOReGFP were captured at 1,024 × 1,024 pixel resolution and 16-bit depth using ×40 or ×63 objectives (≥2.0 zoom). Images used for determining cell counts were captured using equivalent settings with ×20 oil immersion objective and pinhole of two airy units. Five fields of view, including full thickness Z-stacks of ganglia (0.75 zoom), were acquired per preparation for counts. Images for improved resolution through deconvolution (~120-nm resolution) were captured with HyD hybrid detectors using a ×63 objective (1.4 NA), 2,048 × 2,048 pixel resolution, pinhole of 0.5, and a z-step of 0.15 μm (pixel size <30 nm). Images were deconvolved with Huygens Professional
MW, "colitis grade") was MP Biomedicals, and TTX citrate was from described (59). Significant weight loss occurred on assessment was performed in a treatment-blinded manner, as described (59). DOReGFP trafficking, resulting in a binary image with positive or negative pixels. The relative percentages of total positive pixels (i.e., DOReGFP) at the cell surface and cytosol were determined. Regions of interest were defined based on Hu and NOS immunoreactivities. The relative proportion and type of neurons that expressed DOReGFP under normal and diseased conditions were determined from captured images, as we have described for MOR (40). Changes in innervation were determined by measuring the relative density of immunolabeling per area. Positive labeling was determined using the threshold function, as described above. Enteric ganglia were excluded from analysis of labeling in sections. Comparison between conditions was conducted using Student’s t-test for parametric data or Mann-Whitney U-test for nonparametric data.

Dextran Sulfate Sodium Colitis

Dextran sulfate sodium (DSS) colitis was induced as previously described (59). Mice (C57BL/6J and DOReGFP) were administered DSS (2% wt/vol in drinking water) over a 5-day period. Control mice received normal drinking water. Mice were monitored daily for weight loss, presence of fecal blood, and signs of distress. Tissues harvested for analysis of the subcellular localization of DOReGFP were collected into ice-cold Krebs containing nicardipine and TTX to minimize additional DOReGFP trafficking. Tissues harvested for localization of DOReGFP or for contraction assays were harvested for contraction assays

DOR Agonists Inhibit Neurogenic Contractions

Many activated GPCRs, including ORs, internalize upon sustained agonist stimulation (60, 71). DADLE-evoked DOReGFP endocytosis was quantified to directly demonstrate functional activation of DOR in myenteric neurons. DADLE (1 nM to 10 μM, 30 min) promoted robust and concentration-dependent endocytosis of DOReGFP from the cell surface to endosome-like structures in myenteric neurons (pEC50 = 7.92 ± 0.17, Emax = 57.31 ± 1.97, n = 41–57 neurons per concentration from n = 6 mice, Fig. 4, A–C), with maximal responses observed at concentrations ≥100 nM. DOReGFP was internalized in both the soma and ganglion-associated

DOR Agonists Evoke Circular Muscle Contractions Through a Neurogenic Mechanism

To determine whether DOR agonists affect basal contractility of the colon, agonists were applied to unstimulated tissues. SNC80 evoked sustained, concentration-dependent contractions of the circular muscle (Fig. 2). These responses were maximal at 10 nM, suggesting that these were either biphasic or rapidly desensitized (Fig. 2A). SNC80-evoked contractions were effectively blocked by naltrindole (1 μM), consistent with a DOR-dependent mechanism (Fig. 2, A and B). Contractions to SNC80 were significantly reduced by the NOS inhibitor l-NNA (100 μM), which was indicative of a mechanism involving modulation of nitricergic signaling (Fig. 3, A and B). SNC80-evoked contractions were abolished by TTX (1 μM; Fig. 3C) and significantly attenuated by HED (1 μM; Fig. 3D), consistent with a neurogenic mechanism of action. Quantitative data are presented in Fig. 3, E and F. These data indicate that DOR agonists can both promote and inhibit contractile activity of the colon through similar mechanisms as reported for MOR (51) and are consistent with the established expression of DOReGFP in both cholinergic and nitricergic neuronal populations and absence of DOReGFP expression by smooth muscle and other cellular mediators of contractile activity (60).

The Prototypical DOR Agonist DADLE Evokes DOReGFP Endocytosis

DOR Agonists Inhibit Neurogenic Contractions

To determine whether DOR influences neurogenic contractions of the mouse colon, we examined the effect of three DOR-selective agonists (DADLE, deltorphin II, SNC80) on the amplitude of electrically evoked contractions. EFS contractions were inhibited in a concentration-dependent manner by the prototypical DOR agonist DADLE (pEC50 = 8.22 ± 0.41, Emax = 87.48 ± 5.36%, n = 6; Fig. 1, A and B) and by the highly selective DOR agonist deltorphin II (pEC50 = 9.26 ± 0.15, Emax = 73.45 ± 3.03%, n = 5; data not shown) and SNC80 (pEC50 = 6.33 ± 0.29, Emax = 96.75 ± 2.26%, n = 6; Fig. 1, C and D). The effects of DADLE were competitively inhibited by the DOR selective antagonist naltrindole (100 nM), consistent with an effect mediated through DOR (DADLE + NLT: pEC50 = 7.22 ± 0.15, Emax = 80.53 ± 3.17%, n = 8, P = 0.02; Fig. 1, B and E). Although there was a rightward shift in the SNC80 curve, neither the potency nor the efficacy of SNC80 was significantly reduced by naltrindole (1 μM; SNC80 + NLT: pEC50 = 5.67 ± 0.47, Emax = 102.3 ± 4.65%, n = 6, P = 0.12; Fig. 1, D and F). To account for potential prolonged inhibitory actions of SNC80 associated with cumulative agonist addition, the inhibitory effect of a single concentration of SNC80 (EC50: 275 nM) was examined in the presence or absence of NLT (300 nM). Under control conditions, SNC80 inhibited electrically evoked contractions by 57.2 ± 5.7%. Pre-exposure to NLT significantly reduced the effects of SNC80 (23.6 ± 3.0%, P = 0.001, unpaired t-test; n = 5 preparations), consistent with an action mediated through DOR. These data confirm that DOR is functionally expressed by myenteric neurons of the mouse colon and support the hypothesis that DOR agonists can inhibit colonic motility through peripheral actions.

DOR Agonists Evolve Circular Muscle Contractions Through a Neurogenic Mechanism

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proximal neurites of all positive neurons. These data demonstrate that DOR is functionally expressed by myenteric neurons and that all DOR-eGFP-positive neurons respond equivalently to the prototypical DOR agonist DADLE.

**DOR Positive Neurons are Functionally Innervated in the ENS**

Endocytosis of GPCRs has been used to identify sites of neuropeptide release and receptor activation associated with pathophysiology (1, 8, 45, 59). Functional innervation of DOR-eGFP-positive myenteric neurons was determined through examination of drug-evoked and electrically and mechanically evoked endocytosis. ENK-immunoreactive varicosities were in close apposition to both the soma and neurites of DOR-eGFP-positive and other neurons, including nerve fibers within the circular muscle layer (Fig. 5A).

The voltage-activated sodium channel opener veratridine causes hyperexcitability of enteric neurons and spasmodic contractions of the colon (33) and promotes release of neuropeptides, including opioids (67). Internalization of DOR-eGFP in response to release of endogenous DOR agonists was examined by quantitative microscopy. Under unstimulated control conditions, DOR-eGFP was localized mainly to the cell surface of myenteric neurons [79.00 ± 0.87%, 95% confidence intervals].

**Fig. 1.** δ-Opioid receptor (DOR) agonists inhibit electrically evoked contractions of the mouse colon. A and B: DADLE reduced the amplitude of electrically evoked contractions in a concentration-dependent manner. C and D: SNC80 also inhibited neurogenic contractions but was less potent than DADLE. B and E: DADLE-mediated inhibition was significantly reduced by naltrindole (NLT). D and F: In contrast, SNC80-mediated effects were not significantly inhibited by naltrindole. Data points represent means ± SE; n = 5–8 mice/group.
interval (CI): 77.27–80.73%, n = 92 neurons, n = 6 mice). Veratridine treatment (10 μM, 30 min) resulted in significant internalization of DOReGFP [%total DOReGFP at the cell surface: 63.38% ± 0.95 (95% CI: 61.50–65.26%), n = 146 neurons, n = 6 mice, P = 0.0001] relative to untreated controls (Fig. 5, B and C). This occurred in both the soma and proximal neurites but not in nerve fibers within the circular muscle. DOReGFP endocytosis in response to veratridine was blocked by naltrindole [79.80 ± 0.70% (95% CI: 78.41–81.19%), n = 126 neurons, n = 6 mice; Fig. 5, B and C], consistent with the activation of DOR in response to release of an endogenous agonist. NK₁R internalization was examined in submucosal neurons from the same preparations to confirm activation and specificity. NK₁R immunoreactivity was localized to the plasma membrane of the majority of submucosal neurons [77.55% ± 0.88, (95% CI: 75.58–79.33%), n = 53 neurons, n = 3 mice; images not shown] (54). Treatment with veratridine resulted in significant NK₁R internalization [60.40 ± 1.8% (95% CI: 56.76–64.04%), n = 33 neurons, n = 3 mice]. Naltrindole had no significant effect on veratridine-evoked NK₁R endocytosis [58.60 ± 1.26% (95% CI: 56.07–61.13%), n = 49 neurons, n = 3 mice; Fig. 5C].

Electrical field stimulation (10 min, with 20-min recovery, Fig. 6A) was associated with a significant increase in intracellular DOReGFP in the soma and neurites within ganglia and a corresponding reduction in cell surface-associated labeling [47.93 ± 1.30% (95% CI: 45.35–50.50%), n = 88 neurons, n = 6 mice, P < 0.0001; Fig. 6, B and C]. This is consistent with activation of DOR by endogenous ligands. The selective DOR antagonist naltrindole (100 nM) used to confirm that DOReGFP endocytosis was due to release of endogenous DOR agonists and not through nonspecific effects associated with EFS. Naltrindole significantly reduced EFS-evoked DOReGFP endocytosis [66.53 ± 1.51% (95% CI: 63.53–69.53%), n = 75 neurons, n = 6 mice, P < 0.0001; Fig. 6, B and C], confirming the requirement for agonist binding to the orthorhochic site of DOR for internalization to occur.

Pharmacological studies using naltrindole indicate that DOR is involved in the enkephalinergic suppression of colonic motility (24, 29). The release of endogenous DOR ligands during colonic motility patterns was determined using DOReGFP endocytosis as a marker of DOR activation. Endocytosis of DOReGFP was compared in neurons from colons stimulated with low basal intraluminal pressure or in colons in which the intraluminal pressure was raised to the mechanical threshold required to elicit propagating contractions (Fig. 7A).

Under low stimulus conditions, DOReGFP was localized to both the cell surface and to endosome-like structures in the soma and proximal neurites within myenteric ganglia [69.13 ± 1.13% (95% CI: 68.59–70.73%), n = 127 neurons, n = 6 mice; Fig. 7B]. The relative percentage of cell surface-associated DOReGFP in myenteric neurons was significantly decreased in preparations exposed to elevated intraluminal pressures [47.82 ± 1.11% (95% CI: 45.62–50.01%), n = 119 neurons, n = 6 mice, P = 0.0001 vs. basal control, 2-tailed t-test; Fig. 7, B and C]. Endocytosis was observed in all neurons examined and was restricted to the soma and proximal neurites and not in nerve fibers associated with the circular muscle layer (Fig. 7D). These data indicate that DOReGFP-positive myenteric neurons are functionally innervated and are activated during complex reflexes associated with propagating motility patterns in the colon. These findings support a potential role for DOR in the regulation of colonic motility, a complex physiological process that is coordinated largely by neuronal networks located in the myenteric plexus (69).

**DOReGFP Trafficking in Myenteric Neurons Is Spatially Dependent**

DOReGFP internalization did not occur in nerve fibers associated with the circular muscle in response to either stimulation with exogenous agonists or release of endogenous transmitters. This observation suggests that DOR endocytosis occurs in compartmentally distinct regions within a neuron. DOReGFP endocytosis was examined in real time in circular muscle-myenteric plexus wholemount preparations to determine both the kinetics and subcellular sites of trafficking. Under basal conditions, DOReGFP was localized to the plasma membrane of the soma and neurites of myenteric neurons and to nerve fibers within the circular muscle layer, where it was closely associated with enkephalin-positive varicose fibers (Fig. 8A). Treatment with veratridine (10 μM) resulted in the appearance of intracellular vesicular DOReGFP labeling in the soma and proximal neurites (Fig. 8B). Initial changes were first evident within 9 min of addition. In contrast, no endocytosis was detected in nerve fibers within the circular muscle over the duration of imaging (30 min). No redistribution of DOReGFP occurred over time in vehicle-treated preparations (Fig. 8C). The apparent lack of DOReGFP endocytosis in these nerve fibers confirmed the observation that agonist-evoked endocytosis was restricted to the soma and proximal neurites during

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**Fig. 2.** SNC80 evoked tonic contractions of colonic circular muscle through a DOR-dependent mechanism. A: quantitative analysis of the amplitude of contractions to SNC80 demonstrating a biphasic response that peaked at a concentration of 10 nM. B: SNC80 (10 nM) produced a rapid and sustained contraction that was abolished by naltrindole (NLT; 1 μM). *P < 0.05 and **P < 0.01 compared with the NLT treatment group. Statistical analyses were performed using the Mann-Whitney U-test. Data are expressed as means ± SE; n = 7 mice/group.
the time periods examined and was not a transient event associated with rapid recycling back to the cell surface.

**Acute colitis is Associated With a Change in the Number or Phenotype of DOReGFP-Positive Myenteric Neurons**

Intestinal inflammation is associated with reduced neuronal numbers, neuronal hyperexcitability, and altered neuronal phenotype (9, 47). Changes to the cellular and subcellular distribution of DOR have not been directly examined in the inflamed intestine. Quantitative analysis of the number and the neurochemical coding of DOReGFP-positive neurons indicated that there was a significant increase in the relative number of Hu-immunoreactive neurons that were DOReGFP positive in the DSS group compared with the healthy control group

**Fig. 3.** SNC80-evoked contractions of the colon are mediated through direct actions on myenteric neurons. A: SNC80 evoked robust concentration-dependent contractions, which were abolished by L-N^(-)^-nitro-L-arginine (L-NNA; B) and tetrodotoxin (TTX; C) and reduced by hexamethonium (HEX; D). SNC80 was applied 22 min after inclusion of vehicle, L-NNA, TTX, or HEX. E: quantitative analysis of SNC80-evoked responses demonstrating effects of the various inhibitors. F: responses to 100 nM SNC80 were significantly attenuated by all inhibitors. *P < 0.05 and ***P < 0.001 compared with vehicle control, Kruskal-Wallis 1-way ANOVA. Data are expressed as means ± SE and are derived from n = 5–11 mice/treatment group.
neurons analyzed, n = 9 mice; P = 0.03). There was a significant increase in the proportion of DOReGFP neurons that were also nNOS immunoreactive (nNOS-positive neurons: DSS: 73 ± 2%, n = 1,030 neurons analyzed, n = 9 mice vs. control: 66 ± 2%, n = 1,079 neurons analyzed, n = 9 mice; P = 0.027). Analysis of the density of enkephalin immunoreactivity in myenteric ganglia indicated that there was no significant alteration in the density of enkephalin immunoreactivity during DSS inflammation, consistent with a previous report (6) [%total ganglion area: control 10.74 ± 0.83% (95% CI: 8.95–12.54%), n = 15 ganglia; DSS 9.89 ± 1.02% (95% CI: 7.76–12.01%), n = 22 ganglia; P = 0.551, 2-tailed unpaired t-test].

The density of DOReGFP-positive innervation of the circular muscle layer was detected in sections using immunofluorescence and quantified relative to vehicle controls. DSS colitis was associated with a significant increase in the density of DOReGFP-positive nerve fibers in the circular layer of the muscularis externa (1.82 ± 0.13-fold increase relative to vehicle control, P < 0.001, 5 nonsequential sections/mouse, n = 6 mice/group. Fig. 9, A and B). Collectively, these data indicate that acute inflammation is associated with changes in the number and phenotype of DOR-positive neurons and with increased DOR-positive nerve fiber density.

Acute Colitis is Associated With DOReGFP Endocytosis

Previous studies, including our own, have demonstrated that the neurokinin 1 receptor (NK1R) is internalized in myenteric neurons of the inflamed intestine (45, 59). Similarly, NK1R is internalized in dorsal horn neurons of the spinal cord following peripheral inflammation (1). Colitis is associated with increased expression of endogenous opioids by colitogenic T cells (6) and by myenteric neurons (74), suggesting that the inflamed colon is an environment in which there is chronic exposure of GPCRs to agonists. The subcellular distribution of DOReGFP in myenteric neurons of the colon was examined in tissues from mice with acute colitis. There was a significant reduction in cell surface DOReGFP (54.55%; 95% CI: 49.10–60.01, n = 5) relative to healthy controls (77.37%; 95% CI: 73.09–81.65, n = 5; P < 0.0001; Fig. 10, A and B). Co- staining studies demonstrated that DOReGFP labeling was partly coincident with LAMP-1 immunoreactivity in myenteric neurons of the inflamed colon, suggesting an association with lysosomes (Fig. 10 C).

Acute Colitis is Associated With a Change in DOR Function

Intestinal inflammation is associated with increased expression of the Oprd1 and Oprml genes, increased levels of endogenous enkephalins, and enhanced MOR- and DOR-dependent signaling (6, 39, 56, 57, 74). Inflammation is also associated with enhanced MOR signaling in the peripheral terminals of somatic nociceptors (80) and with increased surface expression and function of DOR in analgesic pathways (12). However, receptor endocytosis is generally associated with desensitization of responses to agonists and with the promotion of unique cellular signaling from within endosomes (35, 49, 53, 78). We tested whether DOR-dependent inhibition of neurogenic contractions of the colon was altered in acute inflammation using SNC80 as a representative DOR agonist.
SNC80 inhibited EFS-evoked contractions of the circular muscle in a concentration-dependent manner (pEC\textsubscript{50} = 6.23 ± 0.21, \(E_{\text{max}} = 91.14 ± 8.54\), \(n = 6\)). Acute DSS colitis was associated with a significant increase in the potency at which SNC80 inhibited EFS-evoked contractions (pEC\textsubscript{50} = 7.15 ± 0.24, \(n = 5\), \(P = 0.04\)), but the magnitude of responses to EFS was generally reduced. Inflammation did not significantly affect the \(E_{\text{max}}\) of responses to SNC80 (control: 91.14 ± 8.54%; DSS: 86.02 ± 4.36%; \(P = 0.59\); Fig. 10D). Internalization of DOR in the soma and proximal neurites of myenteric neurons of the inflamed colon would be predicted to effectively remove cell surface receptor and diminish responses to DOR agonists. However, the experimental data presented above demonstrate that this was not the case, with inflammation associated with a significant enhancement of responses to SNC80. This is consistent with the previous studies outlined above.

**DISCUSSION**

We report that DOR is functionally expressed by myenteric neurons of the mouse colon. DOR agonists can both inhibit and promote neurogenic contractions of colonic smooth muscle, and this is presumably related to the different sites of neuronal expression, as previously characterized (60). DOR-positive neurons are closely associated with enkephalin-containing nerve fibers, and DOR is activated upon generalized stimulation of the ENS, suggesting functional innervation. Inflammation is associated with endocytosis of DOReGFP in the soma and neurites of myenteric neurons and with increased DOReGFP-positive innervation of the colon. These changes in receptor distribution are associated with a corresponding enhancement of responses to the DOR-selective agonist SNC80.
DOR Inhibits Colonic Motility Through a Peripheral Mechanism

Our results suggest that DOR is expressed by inhibitory and excitatory motoneurons of the mouse colon, where activation evokes contractions and inhibits EFS-stimulated contractions, respectively. The role of DOR in the neurogenic control of intestinal contractility was determined pharmacologically using the agonists DADLE, deltorphin II, and SNC80. These agonists exhibit high selectivity for DOR over MOR and KOR but differ markedly in their affinities for DOR ($pK_i$: DADLE = 9.1 and SNC80 = 7.2) (5). In our experiments, SNC80 was also less potent than DADLE, and this may have compromised our ability to demonstrate effective blocking of SNC80-evoked contractions and agonist-evoked DOReGFP endocytosis by naltrindole. Our data demonstrate that naltrindole significantly attenuates the inhibitory effect of a single application of 275 nM SNC80 on EFS contractions. This suggests that sustained DOR signaling may occur upon activation, which is not effectively blocked by naltrindole in assays involving cumulative SNC80 addition. There is pharmacological evidence to support the existence of two DOR subtypes that are likely to reflect differences in posttranslational processing or interaction with other proteins, including MOR (73). Therefore, it is also conceivable that differences that we report are due to actions at distinct DOR subtypes or reflect differences in DOR interaction with other proteins in the different neuronal populations examined.

It has been reported that DOR agonists inhibit small intestinal and colonic transit primarily through a central action (10, 11, 31, 50), whereas other studies suggest that DOR agonists

![Diagram A](image1)

![Diagram B](image2)

![Diagram C](image3)
have minimal impact on intestinal transit (15, 23). Although it has previously been concluded that peripheral (i.e., enteric) DOR does not mediate effects on transit (65), the fact remains that DOR is functionally expressed by a high proportion of excitatory and inhibitory myenteric neurons of the mouse colon (60), supporting a peripheral mechanism of action. Moreover, there is a clear DOR-dependent inhibitory effect on neurogenic contractions of the isolated mouse colon (Ref. 48 and present study), consistent with functional expression by myenteric neurons. These observations derived from the mouse colon are in accordance with data from the human (3, 14, 34, 44) and rat (28, 30) intestine but differ from the guinea pig intestine, where DADLE has minimal effect on neurogenic contractions (22, 61).

**Evidence for Functional Innervation of DOR-Expressing Neurons**

Three lines of functional evidence support our conclusion that DOR-positive neurons are functionally innervated by opioid-containing nerve fibers. First, electrically evoked activation of neurons resulted in significant internalization of DOReGFP, and this was effectively blocked by preincubation with naltrindole. Thus, DOReGFP is activated and internalized through release of and interaction with an endogenous ligand. Second, there is a clear DOR-dependent inhibitory effect on neurogenic contractions of the isolated mouse colon (Ref. 48 and present study), consistent with functional expression by myenteric neurons. These observations derived from the mouse colon are in accordance with data from the human (3, 14, 34, 44) and rat (28, 30) intestine but differ from the guinea pig intestine, where DADLE has minimal effect on neurogenic contractions (22, 61).

**The DOR-Enkephalin Axis in Colitis**

Cutaneous and intestinal inflammation are associated with an increase in immune- and neuron-derived enkephalins (6, 70, 74), which has been proposed to have an endogenous analgesic and anti-inflammatory function. Our observation that acute inflammation is associated with a loss of cell surface DOR and a corresponding increase in intracellular receptor in the soma is consistent with these studies demonstrating an increase in the amount and/or release of endogenous agonist. Intracellular DOReGFP was associated with LAMP1-positive lysosomes, and there was a corresponding reduction in cell surface receptor. These data are consistent with receptor endocytosis, rather than accumulation of DOReGFP due to defective trafficking or overexpression. Inflammation is associated with endocytosis of NK_1R in spinal and myenteric neurons (1, 45, 59). Chronic exposure to the MOR agonist morphine is associated with altered expression of key regulatory proteins by enteric neurons, including β-arrestins and dynamin (37, 52), and with altered MOR signaling (20). These changes may also
occur during inflammation, as there is likely to be chronic exposure to endogenous agonists. The enhancement of responses to SNC80 suggests that endocytosis, or effective loss, of DOR from the soma and associated proximal neurites has little impact on electrically evoked contractions. These are presumably mediated by neurotransmitter release from nerve terminals and consistent with the DOReGFP trafficking studies, where there was no evidence for endosomal DOReGFP in nerve fibers in the circular muscle layer. Whether inflammation-induced DOR endocytosis has any effect on DOR-dependent inhibition of more complex coordinated motor patterns, which presumably require activation of soma-associated DOR as part of a reflex circuit, remains to be determined. However, we anticipate that the results of this type of study will be confounded by the established dysmotility associated with the inflamed and postinflamed states (9, 47).

**DOR Function is Enhanced in the Inflamed Colon**

DOR agonists are only highly efficacious as analgesics during chronic inflammatory or neuropathic pain states (62). Previous studies using Complete Freund’s Adjuvant-induced paw inflammation demonstrated increased expression of DOR at the plasma membrane of spinal and dorsal root ganglion neurons, which was associated with enhanced analgesic effects of DOR agonists (12, 26). Although enhanced inhibitory effects of SNC80 were detected in the inflamed colon, DOReGFP was largely internalized in the soma and proximal neurites under these conditions, and DOReGFP endocytosis in nerve fibers within the muscularis externa was not observed. The increased intracellular localization of DOR in addition to the minimal change in the number and neurochemical coding of DOReGFP-positive neurons suggests that increased DOR signaling was most likely through actions at nerve fibers associated with the muscularis externa. This is supported by the significantly increased density of DOReGFP-positive nerve fibers in the muscularis externa in the inflamed colon. Intestinal

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Fig. 8. Real-time imaging of δ-opioid receptor/enhanced green fluorescent protein (DOReGFP) endocytosis in organotypic preparations of the colon. A and B: overview image (A) indicating regions of interest in the myenteric and circular muscle (CM) layers (presented in B). Veratridine treatment resulted in DOReGFP internalization in myenteric neurons (arrowheads) but not in nerve fibers of the circular muscle (arrows). C: vehicle treatment was not associated with a redistribution of DOReGFP in either region. Scale, 20 (veratridine) and 50 μm (vehicle). Representative examples from 4 independent repeats/treatment. ENK, enkephalin.

Fig. 9. Colitis increases δ-opioid receptor (DOR) expression and function. A and B: dextran sulfate sodium (DSS) colitis was associated with a significant increase in the density of δ-opioid receptor/enhanced green fluorescent protein (DOReGFP)-positive nerve fibers in the muscularis externa relative to untreated controls. Scale, 100 μm. ****P < 0.0001. Unpaired 2-tailed Student’s t-test; n = 6 mice/group. DAPI, 4',6-diamidino-2-phenylindole.
inflammation is associated with neuronal loss, and it is possible that the changes in both the relative number and altered neurochemistry of DOReGFP-positive neurons could reflect reduced susceptibility of DOR-expressing neurons to neurodegenerative changes or result from phenotypic plasticity (47). Collectively, these findings suggest that increased expression of DOR at the cell surface or changes to the DOR-expressing population are unlikely to be the driver of these functional changes. It is also likely that DOR-dependent G protein-coupling and signaling are amplified under inflammatory conditions. Our data are also consistent with previous studies that demonstrated that the potency of DOR agonists to inhibit intestinal transit and permeability is augmented in inflammation (56, 72). These authors attributed these changes to increased DOR expression and to the unmasking of a distinct DOR subpopulation (58, 72). Both DOR and MOR mRNA and protein expression are increased in intestinal inflammation (39, 57), and our data indicate that this is reflected in increased innervation and a shift in the DOR-expressing neuronal population. Peripheral analgesic actions of MOR agonists are also enhanced during inflammation (64, 81), and this has been attributed to increased receptor density associated with enhanced trafficking to terminals and with more effective G protein-coupling. MOR-dependent inhibition of intestinal function is similarly augmented in intestinal inflammation (56).

**Spatial Regulation of DOR Endocytosis**

We have used antibody labeling and transgenic mice expressing fluorescently tagged DOR and MOR to demonstrate in the intestine that key opioid receptors are expressed only by neurons (Refs. 40 and 60 and DiCello JJ, Massotte D, and Poole DP, unpublished observations). Thus, enkephalinergic nerve fibers within the circular muscle are likely to functionally innervate opioid receptor-expressing nerves, rather than smooth muscle or interstitial cells. The lack of DOReGFP endocytosis in circular muscle nerve fibers in response to electrical or drug stimulation or in inflammatory disease supports the hypothesis that key regulatory mechanisms differ between neuronal compartments. The hypothesis is also supported by functional evidence for enhanced responses to SNC80 in tissues from mice with acute colitis, where DOReGFP is significantly internalized within the soma and proximal neurites. The assumption that there is effective delivery of agonists to this layer of the colon is supported by the internalization of DOReGFP in submucosal neurons of the same preparation.

To our knowledge, this is the first direct evidence for spatially dependent regulation of GPCR trafficking in enteric neurons. This observation has implications for the type, magnitude, and duration of GPCR signaling in different neuronal compartments. Polarized endocytosis of GPCRs has been described in central neurons, where there can be distinct differences between the somatic, dendritic, and axonal compartments (4, 66). Our observations indicate that the initial distribution of DOReGFP throughout the neuron is indiscriminate, with subsequent endocytosis occurring within specific regions. The importance of endocytosis for GPCR signaling and function in the enteric nervous system has not been defined in depth. Endocytosis is important for the full repertoire of signaling by GPCRs (27, 35, 78) and is required for MOR-dependent MAPK and CREB signaling in enteric neurons (20). DOR is generally considered to be a nonrecycling GPCR, as it...

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**Fig. 10. Δ-Opioid receptor (DOR)/enhanced green fluorescent protein (DOReGFP) is internalized in myenteric neurons of the acutely inflamed colon, and DOR function is enhanced.**

A: DOReGFP was localized to the cell surface of myenteric neurons under control conditions. B: acute colitis was associated with increased intracellular DOReGFP and a corresponding reduction in cell surface labeling. C: DOReGFP was coincident with immunoreactivity for the lysosomal marker lysosomal-associated membrane protein 1 (LAMP1) in myenteric neurons of the inflamed colon. D: The potency at which SNC80 inhibited electrical field stimulation (EFS)-evoked neurogenic contractions was significantly enhanced in colons from dextran sulfate sodium (DSS)-treated mice. Scale, 10 μm. ****P < 0.0001, 1-way ANOVA; n = 5–6 mice/group.
is ubiquitinated and targeted to lysosomes for degradation following agonist-induced endocytosis (e.g., see Refs. 32 and 60). However, this has not been examined in myenteric neurons following physiologically relevant stimulation or in a broad range of DOR agonists. The observation that DOReGFP endocytosis does not occur in more distal processes of myenteric neurons suggests that DOR may be retained and resensitized at the cell surface, allowing more rapid responses to subsequent agonist stimulation.

Conclusion

In summary, we have demonstrated that DOR is functionally expressed in the mouse colon, where it promotes inhibitory effects on neuromuscular function. DOR expression, distribution, and function are significantly altered during inflammation. These findings have important implications for the use of the mouse as a preclinical model for the study of DOR in gastrointestinal disease and for our basic understanding ofGPCR function in the ENS.

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AUTHOR CONTRIBUTIONS


REFERENCES


DELTA OPIOID RECEPTORS IN THE ENTERIC NERVOUS SYSTEM


48. Pelayo JC, Scherrer G, Evans CJ, Kieffer BL, Bunnett NW. Localization and regulation of fluorescently labeled delta opioid receptor,


