MONASH University

# *Advancing the understanding of opioid receptor biology and function in the enteric nervous system*


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Drug Discovery Biology

Monash Institute of Pharmaceutical Sciences

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# Contents

## Abstract

Opiates which target the μ opioid receptor (MOR) are highly effective analgesics and anti-diarrheals. However, their use is severely limited due to the development of intractable constipation which mainly arises through their sustained inhibitory effects on myenteric neurons. Recent efforts to develop safer opiate therapeutics without GI-associated side effects have failed (Altarifi et al., 2017; Kliewer et al., 2019). This is attributed to fundamental knowledge of opioid receptor biology in the enteric nervous system (ENS). Thus, there is an unmet need for a greater understanding of how opioid receptor regulation and signalling underlies the control of physiological processes, including GI motility.

Although the δ opioid receptor (DOR) is a promising therapeutic target for chronic pain and psychiatric disorders, the function and regulation of DOR in myenteric neurons are poorly understood. In **Chapter 2**, we demonstrated that DOR agonists inhibit neurogenic contractions and elevate the basal tone of the colon, confirming functional expression of DOR in the ENS. Furthermore, the generation of complex colonic motor patterns which contribute to content movement are associated with DOR endocytosis in myenteric neurons. We also found that DOR function and expression are enhanced during colitis, a condition which is associated with dysmotility. Thus, this study supports the therapeutic targeting of DOR expressed by myenteric neurons.

In **Chapter 3**, we examined whether the colon develops tolerance to DOR-mediated responses. We established using spatiotemporal mapping of the intact colon that the frequency of motor patterns is reduced following either an acute (3 h) or chronic (3 d) exposure to SNC80, but not ARM390. The ability of ARM390 or SNC80 to inhibit motility correlated with their DOR internalizing properties. SNC80-mediated inhibition of motility correlated with DOR internalization in both the nerve fibers innervating the muscle and the soma, whereas DOR was mainly retained at the surface of myenteric neurons following treatment with ARM390. These data indicate the potential importance of endocytosis to the functional effects of DOR agonists in the colon.

In **Chapter 4**, we determined the utility of commonly used endocytic inhibitors to probe endosomal signalling in the ENS at the cellular and physiological level. Although inhibitors of clathrin-mediated endocytosis (PitStop2; PS2) or GRK2/3-dependent phosphorylation (Compound101; Cmpd101) were effective at blocking DOR internalization in myenteric neurons, we established using tissue contraction assays and calcium imaging that PS2 suppresses neurogenic processes (PS2), whereas Cmpd101 directly inhibits muscle contractility. In addition, the ability of either morphine (weak internalizer of MOR) or SNC80 (strong internalizer of DOR) to produce a tonic contraction was blocked by PS2. Our data highlights the unsuitability of these inhibitors for the study of neurally-mediated gut function.

The MOR and DOR heteromer is the proposed target of the IBS-D drug eluxadoline. Currently, sites of coexpression and potential interactions between MOR and DOR are largely undefined in the ENS. In **Chapter 5**, we demonstrated using transgenic MORmCherry/DOReGFP mice that there is extensive overlap between MOR and DOR in myenteric neurons. We determined that MOR and DOR internalize independently in myenteric neurons. In addition, MOR and DOR displayed no functional cooperativity. The prior administration of strong internalizers of DOR desensitized MOR-dependent responses in a unidirectional manner. Thus, MOR and DOR functionally interact through a heteromer-independent mechanism.

# Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one original paper published in a peer reviewed journal, two submitted publications and one narrative. The core theme of the thesis is *'Advancing the understanding of opioid receptor biology and function in the enteric nervous system'*. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Drug Discovery Biology theme under the supervision of Dr Daniel Poole, Dr Simona Carbone and Professor Nigel Bunnett.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of *Chapters 2-4* my contribution to the work involved the following:

| Thesis Chapter | Publication Title | Status | Nature and % of student contribution | Co-author name(s) Nature and % of Co-author's contribution* | Co-author(s), Monash student |
|---|---|---|---|---|---|
| *Chapter 2* | *Inflammation-associated changes in DOR expression and function in the mouse colon.* | *Accepted* | *50%.( Figure 9 completed during my Honours year) conceived and designed research, analysed data, interpreted results. Drafted, edited and revised manuscript* | *1) AS-performed experiments and analyzed data (1%)*<br>*2) PR-performed experiments, analysed data, designed research-1%*<br>*3) CJN-designed research (1%)*<br>*4) RMM-performed experiments (1%)*<br>*5) BWS-performed experiment and analysed data-1%*<br>*6) EME-interpreted results-1%*<br>*7) NAV-interpreted results-1%*<br>*8) MC-interpreted results-1%*<br>*9) JF-interpreted results-1%*<br>*10) NWB revised manuscript-1%*<br>*11) SEC designed research, interpreted results, drafted manuscript-10%*<br>*12) DPP conceived and designed research, analysed data, interpreted results, edited and revised manuscript- 30%* | *No* |

| | | | | | |
|---|---|---|---|---|---|
| *Chapter 3* | *Agonist-dependent development of delta opioid receptor tolerance in the colon* | *Revision submitted to journal.* | *60% - conceived, designed, conducted, analyzed and interpreted experiments, supervised the study and wrote the manuscript* | *1) AS conducted and analyzed experiments-2 %*<br>*2) BWS and RM conducted experiment.-2%*<br>*3) PR assisted with experimental analysis and made figure 9-1%*<br>*4) NAV, AB and MC assisted with interpretation of data-3%*<br>*5) SEC conducted experiments, supervised the study, and assisted with drafting of the manuscript- 2 %*<br>*6) DPP conceived, designed, conducted and analyzed experiments, supervised the study, and wrote the manuscript- 30%* | *No* |
| *Chapter 4* | *Clathrin and GRK2/3 inhibitors block delta opioid receptor internalization in myenteric neurons and inhibit neuromuscular transmission in the colon* | *Submitted* | *50%- conceived, designed, conducted, analyzed and interpreted experiments, supervised the study and assisted in writing the manuscript* | *1) AS conducted and analyzed experiments-1%*<br>*2) PR conducted experiments and assisted in writing the manuscript-10%*<br>*3 )EE assisted with experimental analysis 5%*<br>*4) NAV, AB, MC assisted with interpretation of data-2%*<br>*5) SEC conducted experiments, supervised the study-2 %*<br>*6) DPP conceived, designed, conducted and analyzed experiments, supervised the study, and wrote the manuscript- 30%* | *No* |

I have not renumbered sections of submitted or published papers in an effort to generate a consistent presentation within the thesis.

**Student signature:** **Date:**

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

**Main Supervisor signature:** **Date:**

# Publications during enrolment

## Research Articles

Ang, S. Y., Evans, B. A., Poole, D. P., Bron, R., **DiCello, J. J.**, Bathgate, R. A. D., Summers, R. J. (2018). INSL5 activates multiple signalling pathways and regulates GLP-1 secretion in NCI-H716 cells. *J Mol Endocrinol, 60*(3), 213-224. doi:10.1530/jme-17-0152

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 (2018) Inflammation-associated changes in DOR expression and function in the mouse colon. Am J Physiol Gastrointest Liver Physiol 315 (4):G544-g559. doi:10.1152/ajpgi.00025.2018.

Lay, J., Carbone, S. E., **DiCello, J. J.**, Bunnett, N. W., Canals, M., & Poole, D. P. (2016). Distribution and trafficking of the mu-opioid receptor in enteric neurons of the guinea pig. *Am J Physiol Gastrointest Liver Physiol, 311*(2), G252-266. doi:10.1152/ajpgi.00184.2016

## Conference Abstracts

**DiCello, J.J**., Ceredig R.A., Carbone, S.E., Bron R, Bunnett N.W., Massotte, D., Poole D.P. *Evidence for mu and delta opioid receptor coexpression and functional interactions in the mouse colon* (2016). ASCEPT Annual Scientific Meeting (Melbourne, Australia). *Oral and Poster Presentation (Prize for best poster presentation).*

**DiCello, J.J**., Ceredig R.A., Carbone, S.E., Bron R, Bunnett N.W., Massotte, D., Poole D.P. *Evidence for mu and delta opioid receptor coexpression and functional interactions in the mouse colon* (2016). 2nd Meeting of the Federation of Neurogastroenterology and Motility (San Francisco, USA). *Poster Presentation*.

**DiCello, J.J**., Ceredig R.A., Carbone, S.E., Bron R, Bunnett N.W., Massotte, D., Poole D.P. *Evidence for mu and delta opioid receptor coexpression and functional interactions in the mouse colon* (2016). Advances in Urogenital and Gut Research Symposium (Melbourne, Australia). *Oral Presentation*.

**DiCello, J.J.**, Saito A, McQuade, R, Sebastian, B.W., Carbone, S.E., Poole, D.P. *Do delta opioid receptor agonists promote debilitating constipation?* (2017). Advances in Urogenital and Gut Research Symposium (Gold Coast, Australia). ***Invited Speaker***.

**DiCello, J.J.**, Carbone, S.E., Sebastian, B.W., Saito A, Rajasekhar, P, Canals, M, Poole, D.P. *Enhancement of delta opioid receptor signalling by a positive allosteric modulator reduces the velocity of colonic motility without contipatory effects.* (2017). Australasian Neurogastroenterology and Motility Association (Melbourne, Australia). *Poster Presentation.*

**DiCello, J.J.**, Saito A, McQuade, R, Sebastian, B.W., Carbone, S.E., Poole, D.P. *The effects of prolonged dosage of delta opioid receptor agonists on colonic motility in the mouse: Analgesics without GI motility-associated side effects?* (2018) 3rd Meeting of the Federation of Neurogastroenterology and Motility (Amsterdam, The Netherlands). *Poster Presentation*.

**DiCello, J.J.**, Saito, A, Ceredig R.A., Canals, M, Carbone, S.E, Massotte, D., Poole D.P. *Anatomical and functional evidence for interactions between mu and delta opioid receptors in myenteric neurons of the mouse colon.* (2018) Molecular Pharmacology of G Protein Coupled Receptors Biennial Scientific Meeting (Melbourne, Australia). *Poster Presentation.*

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## Abbreviations

| | |
|---|---|
| **5-HT** | 5-hydroxytryptamine or Serotonin |
| **5-HT$_3$** | 5-HT 3 Receptor |
| **5-HT$_4$** | 5-HT 4 Receptor |
| **AC** | Adenylate Cyclase |
| **AHP** | Afterhyperpolarization |
| **AP2** | Adaptor Protein Subunit 2 |
| **ATP** | Adenosine Triphosphate |
| **BMP-2** | Bone Morphogenetic Protein 2 |
| **cAMP** | Cyclic Adenosine Monophosphate |
| **CCR** | Chemokine receptor |
| **CD** | Crohn's Disease |
| **ChAT** | Choline Acetyltransferase |
| **CMMC** | Colonic Migrating Motor Complex |
| **CNS** | Central Nervous System |
| **Cmpd101** | Compound101 |
| **DAMGO** | [D-Ala$^2$,*N*-MePhe$^4$, Gly-ol]-enkephalin |
| **DOR** | δ Opioid Receptor |
| **DOReGFP** | DOR tagged with GFP at the C-terminus |
| **DPDPE** | [D-Pen$^{2,5}$] Enkephalin |
| **DRG** | Dorsal Root Ganglia |
| **DSS** | Dextran Sodium Sulphate |
| **ECE-1** | Endothelin Converting Enzyme-1 |
| **EC** | Enterochromaffin Cells |
| **EFS** | Electrical Field Stimulation |
| **ENS** | Enteric Nervous System |
| **ERK** | Extracellular-signal Regulated Kinases |
| **GFP** | Green Fluorescent Protein |
| **GI** | Gastrointestinal |
| **GIRK** | G protein Inwardly Rectifying Potassium |
| **GIT** | Gastrointestinal Tract |
| **GPCR** | G Protein-Coupled Receptor |
| **GRK** | G Protein-Coupled Receptor Kinase |
| **GRK6-/-** | GRK6 knockout mouse |

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|---|---|
| **GRPR** | Gastrin-Releasing Peptide Receptor |
| **IBD** | Inflammatory Bowel Disease |
| **IBS** | Irritable Bowel Syndrome |
| **IBS-C** | IBS-Constipation Predominant Form |
| **IBS-D** | IBS-Diarrhea Predominant Form |
| **IBS-M** | IBS-Mixed Form |
| **ICC** | Interstitial Cells of Cajal |
| **ICC-IM** | Intramuscular Interstitial Cells of Cajal |
| **IPAN** | Intrinsic Primary Afferent Neuron |
| **KOR** | κ Opioid Receptor |
| **L-NA** | $N^G$-Nitro-L-arginine |
| **$M_3R$** | Muscarinic Acetylcholine Receptor 3 |
| **MAPK** | Mitogen-Activated Protein Kinase |
| **mCherry** | A form of Red Fluorescent Protein |
| **MOR** | μ opioid receptor |
| **MOR-/-** | MOR knockout mouse |
| **MORmCherry** | MOR tagged with mCherry at the C-terminus |
| **NFM** | Neurofilament Medium |
| **NK** | Neurokinin |
| **$NK_1R$** | Neurokinin 1 Receptor |
| **$NK_3R$** | Neurokinin 3 Receptor |
| **nNOS** | neuronal Nitric Oxide Synthase |
| **NO** | Nitric Oxide |
| **NOR** | Nociceptin/Orphanin FQ Opioid Receptor |
| **NOS** | Nitric Oxide Synthase |
| **OBD** | Opioid-induced Bowel Dysfunction |
| **OIC** | Opioid-induced Constipation |
| **PDGFRα+** | Platelet-Derived Growth Factor α-positive Receptor |
| **PI-IBS** | Post-Infectious IBS |
| **PKA** | Protein Kinase A |
| **PKC** | Protein Kinase C |
| **PS2** | Pitstop® 2 |
| **SP** | Substance P |
| **STC** | Slow-Transit Constipation |

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|---|---|
| **TNBS** | Trinitrobenzene Sulfonic Acid |
| **TTX** | Tetrodotoxin |
| **UC** | Ulcerative Colitis |
| **VIP** | Vasoactive Intestinal Peptide |
| **VTA** | Ventral Tegmental Area |
| **βArr1** | β Arrestin-1 |
| **βArr2** | β Arrestin-2 |
| **βArr2-/-** | β Arrestin-2 knockout mouse |

# Chapter 1
# *General Introduction*

## 1.1 The gastrointestinal tract

The gastrointestinal tract (GIT) is a highly complex organ that permits the digestion of food, the control of water balance and electrolytes, and the expulsion of waste. The GIT forms a long and continuous tube that can be separated into upper and lower components. The upper GIT extends from the mouth to the stomach and is mainly involved in the physical and chemical breakdown of food. The lower GIT comprises of the small intestine and colon and is the major site of nutrient and water absorption and waste expulsion (Greenwood-Van Meerveld, Johnson, & Grundy, 2017). This PhD thesis will mainly focus on the colon which is the site most affected in functional motility disorders associated with irritable bowel syndrome (IBS), slow-transit constipation (STC) and opioid-induced constipation (OIC). A major function of the colon is to absorb or secrete minerals including magnesium and calcium, electrolytes and water from ingested matter.

### 1.1.1 The enteric nervous system

In contrast to other peripheral organs, the GIT regions can function autonomously from input by the central nervous system (CNS) (Bayliss & Starling, 1899). The reflex control of gastrointestinal (GI) function is mediated by an intrinsic neural network known as the enteric nervous system (ENS). The ENS consists of two ganglionated plexuses which regulate several GI processes (**Figure 1.1**). The myenteric plexus lies between the longitudinal and circular muscle layer of the *muscularis externa* and has a primary role in the coordination of GI motility. The submucosal plexus is located within the submucosal region and can have different roles depending on the animal species. In smaller mammals, the submucosal plexus mainly regulates the secretion of water and electrolytes, whereas in humans it is also involved in coordinating motor functions (Furness et al., 2014). The cell bodies of enteric neurons are protected by surrounding enteric glial cells. Recent evidence indicates that these cells may also influence GI functions, including motility and secretion (McClain, Fried, & Gulbransen, 2015). This thesis will mainly focus on myenteric neurons and colonic motility.

The autonomy of the ENS was first demonstrated by Bayliss and Starling in the isolated small intestine (1899) and colon (1901) of dogs. It is now appreciated that the ENS is the principal driver of colonic functions including motility and secretion. The importance of the ENS is demonstrated by intestinal disorders including Chagas disease and Hirschsprung disease. The acquired Chagas disease is caused by the parasite *Trypanosoma cruzi* and 10-21 % of patients present with megacolon and/or megaoesophagus. These manifestations cause prolonged constipation and achalasia, respectively, and are a result of loss of enteric neurons (Perez-Molina & Molina, 2018). The congenital Hirschsprung disease is characterized by the failed migration of the ENS into distal segments of the colon. Patients present with megacolon and are unable to pass stools (Heuckeroth, 2018). In both diseases, the surgical removal of the aganglionic region is critical for survival.

Despite the autonomic nature of the ENS, effective control of motility requires input from the CNS (Furness, 2012; Shimizu et al., 2006). The importance of the CNS to motility reflexes is highlighted in patients who suffer an injury to the central defecation centre in the spinal cord. These patients present with dysmotility and faecal incontinence which further impacts their quality of life (Lynch & Frizelle, 2006). The CNS also has a modulatory role in motility in smaller animals. Parasympathetic denervation of the rat colon delays colonic transit *in vivo*, whereas transection of sympathetic innervation accelerates motility (Ridolfi et al., 2011). Gribovskaja-Rupp *et al.* (2012) also demonstrated that the stimulation of parasympathetic fibers enhanced propulsive motility in the isolated guinea pig colon, whereas sympathetic denervation delayed transit. This modulatory influence of extrinsic nerve fibers complicates *in vivo* investigation of ENS-dependent effects on GI motility.

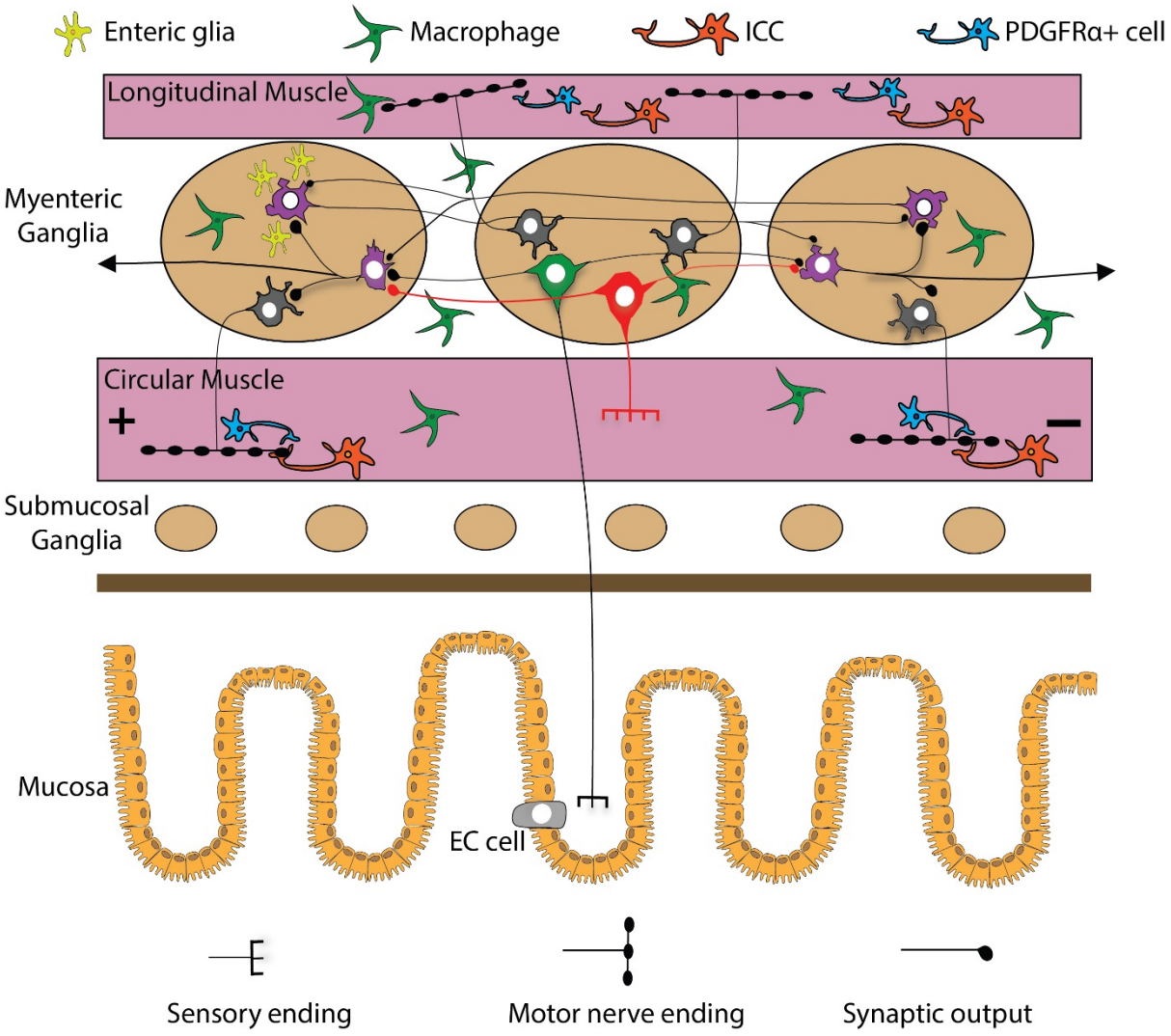


**Figure 1.1 Myenteric pathways and non-neuronal cells involved in the motility reflex (Adapted from Spencer et al., 2016)**. Intrinsic primary afferent neurons (IPANs) project to the intestinal muscle (red neuron) to detect mechanical distortion. Enterochromaffin cells (EC cells) release serotonin following chemical changes in the lumen. Serotonin activates 5-HT receptors expressed by mucosally-projecting IPANs (green neuron). IPANs initiate the motility reflex through the coordinated activation of ascending excitatory and descending inhibitory neural pathways. Both ascending and descending interneurons (purple) receive input from IPANs, and provide synaptic output to excitatory and inhibitory motoneurons, respectively (grey). Excitatory motoneurons release acetylcholine as a major neurotransmitter to produce an oral contraction (+), whereas inhibitory motoneurons release nitric oxide to promote aboral relaxation (-). Other cell types which modulate myenteric responses or contribute to neuromuscular transmission include enteric glia, muscularis macrophages, interstitial cells of Cajal (ICC) and PDGFRα+ cells.

### 1.1.2 Local non-neuronal cells and their involvement in motility

Although the ENS is the principal driver of motility, other local cell types may influence its output (**Figure 1.1**; comprehensively reviewed by Schneider, Wright, & Heuckeroth, 2018). Glial cells surround enteric neurons and provide neuroprotection. The selective activation of enteric glia enhances colonic motility both *in vitro* and *in vivo* (McClain et al., 2015; Rao et al., 2017). McClain *et al.* (2015) used a chemogenetic approach to demonstrate that the activation of glia cells enhances neurogenic contractions, but not relaxations, of the mouse colon. In addition, the activation of enteric glia enhanced the number of peristaltic contractions in the intact colon. These effects are abolished by the neuronal blocker tetrodotoxin (TTX), highlighting the importance of the ENS for glia-mediated effects.

Furthermore, there are two types of interstitial cells which are interspersed within the smooth muscle and myenteric region. Interstitial cells of Cajal (ICC) and platelet-derived growth factor α-positive (PDGFRα+) cells (or fibroblast-like cells) facilitate responses to myenteric motor neurons and mediate changes in smooth muscle excitability through coupling via gap junctions. Cobine *et al.* (2011) used immunohistochemistry to demonstrate close apposition of inhibitory nerve fibers with ICCs situated within the muscular layer (ICC-IM) and PDGFRα+ cells in the mouse internal anal sphincter. Mutant mice lacking ICCs exhibited reduced inhibitory neuron-mediated responses. In contrast, excitatory neurotransmission was enhanced, and this correlated with an increase in muscarinic acetylcholine receptor 3 ($M_3R$) expression (Sanders et al., 2014). ICCs are also pacemaker cells which produce slow wave electrical activity in smooth muscle cells (Sanders et al., 2012). Slow waves generate rhythmic TTX-insensitive contractions which propagate a short distance and are identified as ripples (Lentle et al., 2008). The short propagation distance of ripples suggests they are important for mixing of contents rather than motility. An elegant study by Baker *et al.* (2015) provided detail on the myenteric innervation of PDGFRα+ cells by measuring changes in intracellular calcium levels following electrical field stimulation (EFS) of the mouse colon. Calcium transients are a measure of cellular activation. Stimulation produced sequential calcium transients in nerve fibers, followed by PDGFRα+ cells and then smooth muscle cells. This identified a highly-ordered sequence of activation between these cell types. Antagonists of the $P2Y_1$ purinoceptor blocked EFS-evoked calcium transients in PDGFRα+ and smooth muscle cells, indicating purine-dependent activation of these cells. Interestingly, gap junction inhibitors blocked EFS-evoked calcium transients exclusively in smooth muscle cells, suggesting direct innervation of PDGFRα+ cells by motoneurons (Baker et al., 2015).

Resident macrophages may also play a role in the homeostasis of enteric neurons and motility (De Schepper et al., 2018). Resident colonic macrophages enhanced motility through the local release of bone morphogenetic protein 2 (BMP-2) (Muller et al., 2014). The authors suggest that BMP-2 exerts direct effects on enteric neurons to modulate motility. Moreover, macrophages and enteric neurons secrete factors, including BMP-2 and macrophage colony-stimulating factor, required for the maintenance of each other. However, Luo *et al.* (2018) used both optogenetic and chemogenetic approaches and demonstrated that selective activation of colonic macrophages leads to increases in motility mediated through an ENS-independent manner. These conflicting results may be due to different subsets of resident macrophages targeted in their methodological approaches. Further clarification is needed in this area since resident macrophages may be an attractive therapeutic target for the treatment of dysmotility and inflammation.

### 1.1.3 Neurochemistry and functional subtypes of myenteric neuron

The ENS contains distinct neuronal subtypes that are collectively required for the reflex control of GI function. The neurochemical coding of these neurons has been well characterized and is conserved across species including the mouse, guinea pig and human (Brehmer, 2006; Costa & Brookes, 2008;

Sang & Young, 1996; Schemann & Neunlist, 2004). The neurochemistry is also matched to morphology, electrophysiological properties and axonal projections (Costa & Brookes, 2008). Neurochemical coding is commonly used to identify functional subtypes of myenteric neurons and their role on motility. Myenteric neurons can be functionally classified as motoneurons, interneurons or intrinsic primary afferent neurons (IPANS). A list of the functional subtypes and their matched neurochemical properties is provided in **Table 1.1**. Enteric neurons are classified based on their morphology (Dogiel classification; Dogiel 1899) or electrophysiological properties (S or AH type) (Hirst, Holman, & Spence, 1974). Defining the neurochemistry of myenteric neurons has been instrumental in understanding motility-associated changes in GI diseases and disorders (Brierley & Linden, 2014; Mawe, 2015; Schemann & Neunlist, 2004; Wattchow et al., 2008). It has also enabled us to understand or predict the neural pathways responsible for drug-mediated effects on motility. For example, the neurochemical coding of μ opioid receptor (MOR)-expressing neurons was recently characterized in the guinea pig colon and ileum (Lay et al., 2016). A high proportion of inhibitory motoneurons expressed MOR which matches known opiate-mediated effects on inhibitory neurotransmission and muscle contractions (Wood & Galligan, 2004; refer to ***1.2.4 Distribution and endogenous regulation of MOR in the ENS***).

### 1.1.3.1 Motoneurons

Motoneurons are uniaxonal with multiple dendrites (Dogiel type I morphology) and receive fast synaptic inputs from other neurons (S type). Studies using DiI retrograde labelling from muscle layers visualized the axonal projections of motoneurons to smooth muscle cells (Brookes & Costa, 1990; Brookes, Steele, & Costa, 1991; Costa et al., 1992). Pharmacological studies have been instrumental in identifying the major neurotransmitters involved in motility (Tonini & Costa, 1990; Waterman & Costa, 1994).

Excitatory motoneurons have ascending projections to smooth muscle cells and they release neurotransmitters to evoke tissue contractions. Tonini and Costa (1990) used a pharmacological approach to determine the major excitatory neurotransmitter released by ascending motoneurons. They used a partitioned organ bath to divide the isolated guinea pig ileum into oral, intermediate and aboral components, and a motility reflex was evoked by distending the intestinal wall. The addition of the muscarinic antagonist hyoscine to the oral component almost completely inhibited the reflex contraction, indicating acetylcholine is the major excitatory neurotransmitter. Hyoscine had little effect on the distention-evoked contraction when administered to the intermediate or aboral segments, confirming these pathways are ascending and oral to the mechanical stimulus. The projections of these neurons were confirmed and visualized using DiI retrograde labelling combined with choline acetyltransferase (ChAT)-immunolabelling as a marker of cholinergic motoneurons (Brookes et al., 1991). Moreover, both electrically-stimulated and distension-evoked contractions were inhibited by a non-selective antagonist for neurokinin (NK) receptors. These data were important for the confirmation of a tachykinergic component of the excitatory pathways (Costa et al., 1985). Retrograde tracing combined with immunolabelling for the endogenous tachykinin substance P (SP) demonstrated these are ascending excitatory motoneurons.

Inhibitory motoneurons have descending projections to the muscle and they mediate smooth muscle relaxation. This relaxation facilitates the movement of the bolus along the gut, and also maintains basal tone (Brierley et al., 2001). Electrophysiological recordings from smooth muscle cells of the guinea pig colon provided insight into the neurotransmitters responsible for aboral relaxation. Distension of flat sheet preparations triggered smooth muscle hyperpolarization aboral to the stimulus. This response was blocked by the nitric oxide synthase (NOS) inhibitor $N^G$-Nitro-L-arginine (L-NA), implicating the involvement of nitric oxide (NO) (Spencer & Smith, 2001). Purinergic and vasoactive

intestinal peptide (VIP) signalling are also important for the aboral relaxation (Grider & Makhlouf, 1986; Smith & Furness, 1988). Importantly, Foxx-Orenstein and Grider (1996) found that artificial pellets do not traverse past the initiation site following exposure to a VIP antagonist or NOS inhibitor. This demonstrates the importance of the aboral relaxation for the motility reflex (see ***1.1.4 Peristalsis***). In the myenteric plexus of the guinea pig, descending motoneurons are immunoreactive for both nitric oxide synthase (NOS) and VIP (Costa et al., 1992).

### 1.1.3.2 Intrinsic primary afferent neurons

IPANs are the sensory arm of the myenteric plexus and are important for initiating motility reflexes (Furness et al., 1998). They are round with multiple axons (Dogiel type II morphology), and their electrophysiological properties are defined by a long after-hyperpolarizing potential (AH type) that follows the action potential. IPANs can receive synaptic input thus evoking slow excitatory post synaptic potentials (Furness et al., 1998). IPANs may innervate the muscle or mucosa to sense mechanical and chemical changes, respectively. Mechanical distortion is transduced by mechanosensitive ion channels located on the endings of IPANs (Kunze et al., 2000). A potent stimulant of mucosal-projecting IPANS is 5-hydroxytryptamine (5-HT) which is released from enterochromaffin cells (ECs) (Foxx-Orenstein & Grider, 1996). The importance of this proposed pathway has recently been challenged (see ***1.1.4 Peristalsis***). The detection of mucosal stimuli is thought to be mediated indirectly via the activation of ECs (Heredia et al., 2009).

### 1.1.3.3 Interneurons

Interneurons form chains along the gut and relay messages between motoneurons and IPANs. They typically have Dogiel type I morphology and S type electrophysiological properties. They can be divided into ascending and descending neurons based on their projection to neighbouring motoneurons. Typically, ascending interneurons are directed orally and synapse with excitatory motoneurons. In a study conducted by Tonini and Costa (1990), the addition of the nicotinic receptor antagonist hexamethonium to the oral, intermediate or anal compartment reduced distension-evoked contractions, indicative of cholinergic transmission in ascending interneurons. The activation of the neurokinin 3 receptor ($NK_3R$) in ileal muscle strips produced an atropine- and TTX-sensitive contraction, implicating tachykinins as neurotransmitters in this pathway (Costa et al., 1985). Generally, descending interneurons project to inhibitory motoneurons and involve cholinergic, purinergic and tachykinergic signalling for effective neurotransmission (Johnson et al., 1998).

**Table 1.1 Neurochemistry of myenteric neurons (Adapted from Furness, 2012)**

| Type of neuron | Electrophysiological properties | Key neurochemical markers |
|---|---|---|
| Excitatory motoneuron | S type | ChAT, calretinin |
| Inhibitory motoneuron | S type | nNOS, VIP, enkephalin |
| Ascending interneuron | S type | ChAT, SP, ATP, calretinin |
| Descending interneuron | S type | ChAT, ATP, nNOS, VIP, 5-HT, somatostatin |
| Intrinsic primary afferent neuron | AH type | ChAT, calretinin, NFM (mouse), calbindin, CGRP, NeuN (guinea pig) |

*Choline acetyltransferase (ChAT), neuronal nitric oxide synthase (nNOS), vasoactive intestinal peptide (VIP), substance P (SP), adenosine triphosphate (ATP), 5-hydroxytryptamine (5-HT), neurofilament M (NFM), calcitonin gene-related peptide (CGRP).*

### 1.1.4 Peristalsis

The expulsion of faecal matter is essential for survival and is tightly controlled to maximise the absorption and secretion of water, minerals and electrolytes. Smooth muscle layers provide the mechanical apparatus for a series of coordinated motility patterns, collectively known as peristaltic contractions. Peristaltic contractions are pivotal for the propulsion of content along the GIT, where contraction oral to the site of a bolus provides force to move content forward, and aboral relaxation permits passage of content to the next region. Peristalsis is achieved by the coordinated interplay of myenteric pathways (Spencer et al., 2016) and was first described as a 'reflex' by Bayliss and Starling (1899). Recent evidence suggests that the neural circuitry that drives peristalsis occurs via a 'neuromechanical loop' (Costa et al., 2015; Dinning et al., 2014). The neuromechanical loop hypothesis states that local distension of the colon by a bolus simultaneously activates oral excitatory and aboral inhibitory motor pathways to allow its movement to the next section of intestine (**Figure 1.1**). As the bolus enters, it distends the gut wall in this region subsequently activating local mechanoreceptors within the myenteric plexus to trigger the following set of contractions and relaxations (Dinning et al., 2014). As sensory input is the initiator of this neurochemical loop, peristaltic contractions are largely influenced by the physical properties of the luminal contents. For example, faecal pellets which stretch a small surface area of the colon migrate at a slower rate than those that cover a larger surface area (Costa et al., 2015). Moreover, different grades of mechanical stretch alter the rate of peristaltic contractions. Increasing the intraluminal pressure of guinea pig ileum enhanced the number of propulsive motor patterns generated (Gwynne et al., 2014). This change was unaffected by selective 5-HT receptor antagonists suggesting this process is dependent on stretch rather than the mucosal release

of serotonin. In an earlier study by Arkwright *et al.* (2012), the relationship between the change in intraluminal pressure and the contraction rate in the isolated rabbit proximal colon was examined by manometry. A step-wise elevation in intraluminal pressure produced a linear increase in contraction rate, highlighting the importance of mechanical distortion to peristaltic contractions.

There is still debate about whether the release of 5-HT from ECs is necessary for the initiation and propagation of peristaltic contractions (Heredia et al., 2009; Keating & Spencer, 2010; Spencer et al., 2011). Heredia *et al.* (2009) used both a pharmacological approach and mucosa-free colons to examine the contribution of serotonin release to the generation of peristaltic contractions. The addition of the selective 5-$HT_3$ receptor (5-$HT_3$R) antagonist ondansetron, which blocks serotonin activity at IPANs, reduced the amplitude and tension of propulsive contractions to a fixed pellet. Intracellular recordings of smooth muscle activity were made following distension of both normal and mucosa-free colons by a fixed pellet. This also provided detail of the contribution of serotonin to peristaltic contractions. The action potentials generated in smooth muscle cells were effectively abolished in mucosa-free colons, whereas myogenic slow waves were unaffected. These electrophysiological recordings were conducted in flat sheet preparations. Keating and Spencer (2010) directly measured serotonin release following the generation of complex motor patterns. However, propagating contractions were still recorded even after removing the mucosa, indicating 5-HT released from EC cells is not necessary for peristaltic contractions. Moreover, Spencer *et al.* (2011) used both video mapping and contraction measurements of the isolated guinea pig colon to demonstrate a negligible role for mucosal serotonin in the generation of peristaltic contractions. The fine dissection of the mucosa did not influence the propagation of a free pellet along the colon nor the generation of fluid distension-evoked peristaltic contractions. There was a decrease in the velocity of propagating contractions in the mucosa-free preparations which suggests 5-HT may be involved in the regulation, rather than the generation, of peristalsis. This is an area of research which needs further clarification.

### 1.1.4.1 Colonic migrating motor complex

A neurally-mediated cyclical motor pattern has been reported *in vivo* (Ehrlein, Reich, & Schwinger, 1982; Hipper & Ehrlein, 2001) and in the *isolated* colon (Spencer et al., 2016). These are defined as colonic migrating motor complexes (CMMCs), and they propagate in an aboral direction along the colon. CMMCs are conserved across mammalian species including mice, rats, guinea pigs, rabbits and humans (Costa et al., 2013; Spencer et al., 2012), and they require the coordinated interplay between inhibitory and excitatory myenteric motor pathways (Costa et al., 2017; Spencer, 2013). Although CMMCs can be observed in the non-distended colon (Costa et al., 2015), their frequency and propagation length increases by constant distention. Thus, a mechanical input is necessary for their organization (Barnes et al., 2014; Costa et al., 2017; Gwynne et al., 2014). CMMCs may also travel through peristaltic contractions that are actively propelling a bolus, which suggests that they assist with driving the propulsion of luminal contents (Costa et al., 2015). This also indicates that the colon may require a combination of different motor patterns to achieve successful expulsion of faeces. CMMCs are easily recorded from isolated colons which makes them an ideal measurement for motility. CMMC parameters are commonly measured for the determination of drug-mediated effects to colonic motility.

### 1.1.5 Dysmotility during inflammation and infection

Dysmotility is prevalent in a range of GI diseases and functional disorders including inflammatory bowel disease (IBD), IBS and post infectious-IBS (PI-IBS). IBD is a debilitating condition which can be separated into Crohn's disease (CD) and ulcerative colitis (UC). CD is characterized by transmural inflammation which can occur anywhere in the GIT. UC is restricted to the colon and is limited to mucosal inflammation (Xavier & Podolsky, 2007). IBS is a functional disorder

with no pathological hallmarks. Patients can present predominantly with diarrhea (IBS-D), constipation (IBS-C) or a mix of both (IBS-M). Interestingly, a subset of patients with resolved IBD experience IBS-related symptoms which suggests these two conditions are closely linked (Teruel, Garrido, & Mesonero, 2016). A bout of enteritis may also alter motility and secretion, and these changes may persist even after the removal of the infectious agent. PI-IBS was first described in 1950 by G.M. Stewart, and a large cohort study conducted by Marshall *et al.* (2006) showed that after the waterborne bacterial dysentery outbreak in Walkerton, Canada (May 2000) approximately 26 % of those infected later developed IBS. Although it is difficult to ensure that individuals in the study did not suffer from IBS prior to the outbreak, this was the first study that looked at PI-IBS in a cohort that had a clear trigger.

Animal models of colitis and enteritis have helped determine the contribution of enteric neurons to dysmotility. Neuronal plasticity occurs in both animal models of inflammation and enteritis, and this leads to their hyperexcitability (Brierley & Linden, 2014; Mawe, 2015). The AH potential of IPANs is significantly reduced following trinitrobenzene sulfonic acid (TNBS)-induced colitis or infection with the nematode *Trichinella spiralis* (*T. spiralis*) (Linden et al., 2003; Palmer, Wong-Riley, & Sharkey, 1998). Synaptic facilitation also occurs in interneurons during colitis. This is mediated through an increase in the readily releasable pool of neurotransmitters (Krauter et al., 2007). Hoffman *et al.* (2011) provided a mechanistic understanding of the contribution of myenteric neuroplasticity to motility. In this study, the authors used pharmacological approaches to inhibit the AHP and enhance synaptic transmission, thus mimicking the hyperexcitability induced by inflammation or enteritis. In healthy colons, the addition of these compounds reduced the rate of pellet propulsion. This was similar to the motility disturbances evident in the inflamed colon. Furthermore, agents which effectively enhanced the magnitudes of AHPs normalised motility in the inflamed colon, confirming the contribution of hyperexcitable IPANs to colitis-induced dysmotility (Hoffman et al., 2011; Linden et al., 2003).

Interestingly, changes in neuroplasticity are evident even after the resolution of inflammation or the removal of the infectious agent (Krauter et al., 2007; Lomax et al., 2007). Post-infectious and –inflammatory models are typically used to mimic IBS symptoms. In addition to altered neurotransmission, other factors may also play a role in dysmotility. TNBS-induced colitis is associated with approximately a 20% loss in myenteric neurons as assessed by immunofluorescence (Sarnelli et al., 2009). In dextran sodium sulphate (DSS)- and TNBS-induced colitis, there is also a significant reduction in the expression of the serotonin reuptake inhibitor in the mucosa. This also coincides with enhanced mucosal 5-HT which may act to increase propulsive motility (Linden et al., 2005; Oshima et al., 1999). Moreover, inflammation and infection alter neuromuscular transmission. Colonic muscle strips from *T. spiralis*-infected rats displayed reduced contraction amplitudes following the addition of TTX and a NOS inhibitor. There was also a decrease in the duration of electrically evoked-inhibitory junction potentials in smooth muscle cells. Diminished smooth muscle contractions and relaxations to cholinergic and nitrergic stimulation, respectively, were also recorded in muscle strips of colons from transgenic mouse models of chronic inflammation (Robinson et al., 2016). These data demonstrate an impairment of inhibitory neurotransmission in inflammation.

Pharmacological interventions are the main treatment options for GI dysmotility. A list of current and emerging treatment options has been comprehensively reviewed by Simren & Tack (2018). Constipation may be targeted by the 5-$HT_4$ receptor ($5HT_4R$) agonist prucalopride. Prucalopride acts through the myenteric plexus to enhance motor patterns. There are limited pharmacological options for the treatment of diarrhea and most therapies target an opioid receptor-mechanism. These treatments include the peripherally restricted MOR agonist loperamide and the mixed MOR agonist/ δ opioid receptor (DOR) antagonist eluxadoline. These agents will be described in ***1.2 Opioid receptors***. Opioids act to dampen signalling in the ENS (Wood & Galligan, 2004) and may be a potential mechanism to

counteract hyperexcitability that can underlie dysmotility. To date, we only have a basic understanding of opioid receptor biology in the ENS. Therefore, this thesis focuses on improving our knowledge of opioid receptors at the neurophysiological level in the ENS and determining associated changes in signalling and function at the cellular and organ level during healthy and disease states. Conceptual advancements in opioid receptor signalling are also explored in the ENS and the implications of these for motility.

## 1.2 Opioid receptors

G protein-coupled receptors (GPCRs) are the largest class of cell surface proteins and consist of approximately 800 members. They respond to a diverse range of environmental stimuli and are involved in all physiological and pathophysiological processes. Their biological importance makes them attractive therapeutic targets for many diseases, and they are targeted by more than 30 % of clinically-approved drugs (Hauser et al., 2017a). Unfortunately, the translation of drugs from the laboratory to the clinic has a high failure rate mainly due to a lack of efficacy in humans and the development of on-target side effects.

Opioid receptors are members of the class A (rhodopsin-like) GPCR superfamily. Opioid receptors can be divided into classical or non-classical subtypes. The naloxone-sensitive classical subtypes consist of MOR, DOR and the κ opioid receptor (KOR). These three receptors were cloned in the early 1990s and share approximately 60 % sequence homology. Moreover, each individual receptor is highly conserved across species. MOR, DOR and KOR are encoded by the *oprm1*, *oprd1* and *oprk1* genes, respectively (Stevens, 2009). The non-classical nociceptin/orphanin FQ opioid receptor (NOR) was cloned in 1994 and shares high sequence homology with the classical opioid receptors (Mollereau et al., 1994). However, NOR is insensitive to naloxone. This thesis will focus on MOR and DOR because the pharmacological and genetic tools needed to improve our knowledge of their functional roles in the ENS are more readily available. Genetic tools to examine NOR and KOR function with high specificity have only recently become available (Ozawa et al., 2015; Snyder et al., 2018).

### 1.2.1 MOR as a therapeutic target

Opiates, which mainly target MOR, have been have been used for millennia for their pain-relieving and anti-diarrheal properties. Today, their analgesic and anti-diarrheal efficacies remain unparalleled. In addition, there are currently 26 MOR-targeted drugs in clinical trials for the treatment of pain (Hauser et al., 2017a). Despite their established therapeutic importance, opiates produce detrimental side-effects including respiratory depression, dependence and tolerance. In Western societies, opioid misuse and abuse is a major cause of accidental death (Hauser et al., 2017b). In addition, opiates produce adverse GI-associated side effects collectively known as opioid-induced bowel dysfunction (OBD). OBD occurs in more than 40 % of patient's administered opiates and is a persistent issue throughout the duration of treatment (Ketwaroo, Cheng, & Lembo, 2013). Furthermore, OBD greatly reduces patient quality of life and is a major cause of non-compliance to analgesic regimens. The most common and debilitating symptom of OBD is intractable constipation which is prevalent in more than 25 % of patients receiving opioid therapy (Holzer, 2009). Novel approaches for developing MOR analgesics without constipating side effects include identification of ligands which display signalling bias for G protein-dependent pathways (DeWire et al., 2013; Manglik et al., 2016; discussed in ***1.2.7 Opioid receptor regulation and trafficking***). However, these advances have been incremental and the long-term administration of these ligands still produce constipation in rodents (Altarifi et al., 2017). Therefore, there is a need to better understand how MOR expression and signalling underlies the control of GI motility.

### 1.2.2 DOR as a therapeutic target

DOR is an emerging therapeutic target for a range of CNS disorders including chronic pain, migraine, anxiety and depression (Gendron et al., 2015). However, translation to humans is lacking. The analgesic efficacy of the selective DOR agonist ADL5747 did not significantly differ from the placebo in patients with postherpetic neuralgia (https://clinicaltrials.gov/ct2/show/NCT0105864). Similar results were reported for the pain-relieving properties of the DOR agonist ADL5859 in patients with rheumatoid arthritis (https://clinicaltrials.gov/ct2/show/NCT00626275) and diabetic peripheral neuropathy (https://clinicaltrials.gov/ct2/show/NCT00603265). The DOR agonist NP2 recently finished phase II trials for the treatment of pain in patients with malignant tumours, but the results have not been published (Spahn & Stein, 2017). Experimental evidence also suggests that unlike MOR, DOR is not associated with adverse side effects including abuse, dependence and respiratory depression (Gendron et al., 2015). However, the physiological role DOR plays in the colon and the potential for OIC is largely undetermined or contradictory. The selective DOR agonist SNC80 inhibited colonic transit in rats through a central mechanism (Broccardo, Improta, & Tabacco, 1998). In direct contrast, colonic transit in mice was unaffected by the selective DOR agonist JNJ-20788560 (Codd et al., 2009). However, results from this study may be misleading because the established constipatory opiate morphine did not significantly inhibit motility (Codd et al., 2009). Other studies have demonstrated that DOR agonists either do not impact GI transit, or inhibit small intestinal transit through a central mechanism (Broccardo et al., 1998; Codd et al., 2009; Gallantine & Meert, 2005; Petrillo et al., 2003). Despite this, there is comprehensive evidence supporting the functional and anatomical expression of DOR in the ENS (see ***1.2.5 Distribution and endogenous regulation of DOR in the ENS***). Therefore, a better understanding of whether DOR activation in the ENS influences colonic motility is needed. This will help determine whether emerging therapeutics targeting DOR may produce constipation and the potential for modulation of DOR for the treatment of intestinal disorders.

### 1.2.3 Endogenous opioids in the GIT

Endogenous opioid peptides are important for the regulation of GI processes including motility (Thompson, Canals, & Poole, 2014). Opioids are 'receptor-preferring' ligands because they display varying affinities for each receptor subtype. Endorphins (α and β) are derived from proopiomelanocortin and display similar affinity for MOR and DOR but have low affinity for KOR (Goldstein & Naidu, 1989). In the GIT, endorphins are mainly confined to enteroendocrine cells (Kokrashvili et al., 2009), tuft cells (Gerbe et al., 2011) and are released by immune cells (Verma-Gandhu et al., 2007). Enkephalins are cleaved from proenkephalin and there are at least 10 endogenous biologically active products. They display slightly higher affinity for DOR over MOR but have lower affinity for KOR (Goldstein & Naidu, 1989). Enkephalins are mainly produced by enteric neurons and are detected in nerve fibers innervating the smooth muscle and mucosa (Furness et al., 1993; Poole et al., 2011; Thompson et al., 2014). They are also localized to enteroendocrine cells (Kokrashvili et al., 2009; Nihei & Iwanaga, 1985) and CD4+ T cells (Boue et al., 2014). Dynorphins are derived from prodynorphin and have higher affinity for KOR over MOR and DOR (Goldstein & Naidu, 1989). Dynorphins are expressed by both myenteric and submucosal neurons (Furness et al., 1985; Steele & Costa, 1990). Endomorphin exhibits high affinity and selectivity for MOR. The coding DNA and precursor for this opioid has not been identified. A recent study using immunofluorescence techniques localized endomorphin-2, but not endomorphin-1, to myenteric and submucosal neurons. However, the specificity of the antibody used in this study has not been adequately tested (Li et al., 2014). NOR is primarily activated by the endogenous ligand nociceptin which is generated from the precursor prepronociceptin (Mollereau et al., 1996). The localization of nociceptin within the GIT has yet to be determined.

The endogenous opioid system may be clinically targeted for disorders of motility and secretion. Racecadotril (Acetorphan) is an enkephalinase inhibitor which is administered to patients with acute secretory diarrhea. The inhibition of enkephalinase activity enhances signalling through DOR. The effect of racecadotril on chronic conditions including IBS-D has not been examined (Szymaszkiewicz et al., 2018). Despite the localization of enkephalins in the myenteric plexus, colonic motility is unaffected in healthy humans following acute treatment with racecadotril (Bergmann et al., 1992). Preventing the breakdown of enkephalins is expecting to dampen myenteric signalling and reduce motility. However, whether this drug is effectively delivered to the colon and can penetrate the muscular layer to reach the myenteric plexus is unknown. In addition, whether DOR expressed by myenteric neurons has a role in motility needs to be confirmed. Another pharmacological approach which may enable the enhancement of opioidergic signalling in the GIT is 'allosteric modulation'. Allosteric modulators bind to non-conserved sites on GPCRs which are topographically distinct to the orthosteric site where endogenous ligands bind. They act to either enhance or diminish responses to orthosteric ligands. Pure allosteric modulators display no intrinsic efficacy which preserves the spatiotemporal signalling profile of endogenous ligands. This is in direct contrast to orthosteric agonists which produce a generalised effect (Christopoulos & Kenakin, 2002). Allosteric modulators for MOR and DOR have recently been characterised (Burford et al., 2013; Burford, Traynor & Alt, 2015). Examining whether these receptors are endogenously activated in the myenteric plexus and contribute to reflex control of gut function will be the first step in determining the potential utility of these modulators for motility disorders.

### 1.2.4 Distribution and endogenous regulation of MOR in the ENS

MOR is functionally expressed in the ENS of various mammalian species. Opiates, including morphine, exert their actions on the ENS directly through MOR as demonstrated by a loss of response in mice with a global deletion of the receptor (MOR-/-; Roy, Liu, & Loh, 1998). The importance of MOR in the regulation of GI motility is also demonstrated by the clinical use of peripherally-restricted MOR antagonists and agonists for the treatment of OIC and acute diarrhea, respectively (Holzer, 2009). The administration of the peripherally-restricted MOR antagonist Alvimopan alone accelerates colonic transit in human patients, highlighting an intrinsic role of MOR in the regulation of motility (Gonenne et al., 2005). This is in direct contrast to a study conducted by Roy *et al.* (1998) which demonstrated a slowing of GI transit in MOR-/- mice. MOR agonists may also modulate intestinal motility through a central mechanism of action in mice (Mori et al., 2013). Therefore, results reported using these genetically modified mice may be confounded due to a loss in central-mediated regulation of motility. Other centrally-mediated behaviours may also affect these results. MOR-/- mice exhibit reduced food intake and high depressive-mood-like behaviours (Lutz & Kieffer, 2013; Nogueiras et al., 2012). Mice with a conditional knockout of the *oprm1* gene in the ENS may be useful for specifically defining the intrinsic role of the receptor in motility. There is evidence that MOR is intrinsically innervated in the ENS. The electrical stimulation of myenteric preparations from the guinea pig ileum produces a neurogenic contraction and endocytosis of MOR. Internalization was also effectively inhibited by the MOR antagonist β-CAN (Sternini et al., 2000). These studies confirm that endogenous opioids are released and activate MOR during neurogenic processes.

Mapping the functional identities of neurons which express MOR is important for understanding the neural pathways responsible for opiate-mediated effects on motility. The neurochemical coding of enteric neurons which express MOR in the guinea pig intestine has been comprehensively characterised by immunolabelling with validated primary antibodies (Ho et al., 2003; Lay et al., 2016; Lupp et al., 2011). MOR was mainly localized to the inhibitory nitrergic population in the myenteric plexus of the ileum and colon, whereas only a small proportion of cholinergic neurons

express MOR. MOR was also expressed in submucosal neurons which demonstrates a potential role in secretory functions. Although commonly used for studies of ENS function, the neurochemical coding of neurons expressing MOR in the mouse is unknown. A mouse with a red fluorescent protein (mCherry) tagged to the C terminus of MOR (MORmCherry) was recently used to map the distribution of the receptor in the CNS. These mice were generated by homologous recombination, as was used to develop mice expressing DOR with a C-terminal enhanced green fluorescent protein (GFP) tag (DOReGFP) (Erbs et al., 2015; Scherrer et al., 2006). This provided a tool with which a detailed examination on the pathways involved in MOR-mediated control of CNS processes could be performed.

### 1.2.5 Distribution and endogenous regulation of DOR in the ENS

The distribution and function of DOR in the ENS has not been examined in detail relative to MOR. The expression of DOR in the mouse GIT was characterised with high specificity using DOReGFP mice (Poole et al., 2011). DOR was expressed by both myenteric and submucosal neurons in the ileum and colon, indicating a potential role in both motility and secretion (Poole et al., 2011). Like MOR, DOR was abundantly expressed in the nitrergic population of myenteric neurons. A small proportion of excitatory motoneurons also expressed DOR. Detecting DOR is confounded by concerns regarding the specificity of commercially available DOR antibodies (Scherrer et al., 2009). DOR is also responsive to enkephalins in the myenteric plexus which indicates a potential endogenous role in regulating motility. The exogenous application of met-enkephalin induced DOReGFP internalization, and enkephalinergic nerve fibers are in close apposition to the soma of DOReGFP-positive neurons (Poole et al., 2011). The DOR antagonist naltrindole enhances the velocity at which pellets are propelled in small segments of the guinea pig colon (Foxx-Orenstein, Jin, & Grider, 1998). This suggests that DOR has an inhibitory influence on colonic motility under basal conditions. However, the same concentration of naltrindole also reduced the inhibition of neurogenic contractions by DAMGO [D-Ala$^2$,*N*-MePhe$^4$, Gly-ol]-enkephalin (DAMGO) (Breslin et al., 2012; Wade et al., 2012). This indicates that naltrindole-mediated effects reported in the study by Foxx-Orenstein and Grider (1998) may be DOR-independent. Therefore, the precise role of DOR on motility reflexes is still undetermined.

DOR agonists also exert direct physiological effects on the ENS, supporting a role in the peripheral control of motility. Selective DOR agonists inhibit neurogenic contractions and reduce inhibitory neurotransmission in isolated segments of the human and non-human primate colon and jejunum (Bauer, Sarr, & Szurszewski, 1991; Chamouard et al., 1994; Hoyle et al., 1990). DOR agonists also inhibit neurogenic contractions of the rat ileum (Gray, White, & Coupar, 2005). The functional expression of DOR in the intestine is also dependent on the species and region examined. Egan and North (1981) demonstrated expression of DOR by myenteric neurons of the guinea pig ileum using electrophysiology. However, this study did not determine the selectivity of the DOR agonist DADLE which also displays affinity for MOR in this tissue (Porreca, LoPresti, & Ward, 1990). In a separate set of studies, both neurogenic contractions and peristaltic pressure recordings from guinea pig ileum were unaltered following the addition of selective DOR agonists (Leedham et al., 1991; Shahbazian et al., 2002; Taylor, 2011). This is in marked contrast to the guinea pig colon, where selective DOR agonists reduce mechanically-evoked propulsive contractions (Foxx-Orenstein et al., 1998). Although the mouse is commonly used in preclinical studies of DOR therapeutics (Wade et al., 2012; Pradhan et al., 2009), there is a clear lack of understanding of the functional role of DOR in the mouse colon.

### 1.2.6 Canonical opioid receptor signalling

The canonical signalling pathway for opioid receptors involves the recruitment of inhibitory $G_{i/o}$ proteins (Al-Hasani & Bruchas, 2011). Agonist binding results in a conformational change in the transmembrane domains of the receptor. This allosterically activates the G protein complex consisting

of the $G_{i/o}$ and $G_{\beta\gamma}$ subunits. These subunits dissociate and act on different intracellular proteins to alter cellular activity. Translocation of the $G_{i/o}$ subunit inhibits adenylate cyclase (AC) activity and subsequent cyclic adenosine monophosphate (cAMP) production. This subunit may also promote the opening of G protein-gated inwardly rectifying potassium (GIRK) channels which results in neuronal hyperpolarization. The $G_{\beta\gamma}$ subunit directly inhibits voltage gated-calcium channels which suppresses neurotransmitter release (Syrovatkina et al., 2016). G protein signalling also activates downstream signalling molecules including, but not limited to, extracellular signal-regulated kinases (ERKs) (Belcheva et al., 1998; Duraffourd et al., 2014; Halls et al., 2016).

Studies examining the cellular signalling of opioid receptors in the ENS have mainly focussed on ion channel coupling in guinea pig neurons (Galligan & Akbarali, 2014). Opioids influence ion channel conductance through a G protein-dependent mechanism (Johnson, 1990; Karras & North, 1979). MOR may also couple to sodium channels in cultured myenteric neurons from the mouse (Smith et al., 2012). The inhibition of sodium channels also produces hyperpolarization. **Figure 1.2** illustrates established opioid receptor signalling and ion channel modulation in enteric neurons.

The inhibitory effects of opiates on gut motility was first described by Trendelenburg (1917) using the guinea pig ileum. Ultimately, the suppression of neuronal activity accounts for the observed physiological effects of opioids on GI motility. Opioid agonists enhance circular muscle tone via the disinhibition of the inhibitory input. This leads to spasmodic contractions of the muscle and disrupts the coordinated peristaltic movements needed to effectively expel contents (Wood & Galligan, 2004). Moreover, the activation of opioid receptors on excitatory pathways reduces the neurogenic contractions needed to propel contents. Inhibition of submucosal neurons suppresses the movement of water and electrolytes across the lumen and contributes to constipation.

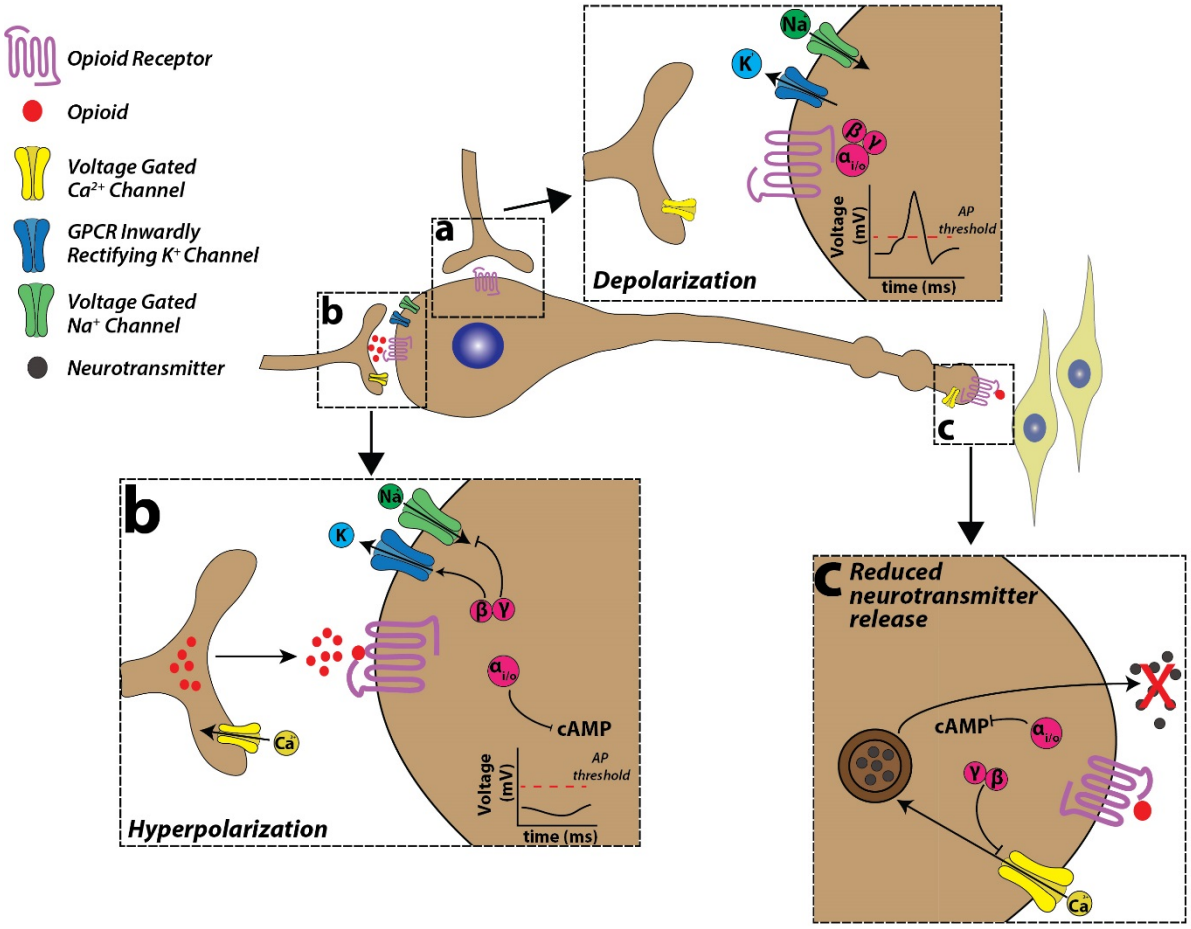


**Figure 1.2 Established molecular mechanisms underlying opioid receptor modulation of enteric neurotransmission. (a)** At the postsynaptic level, the opening of $Na^+$ channels (green) results in neuronal depolarization. After the action potential is triggered, $K^+$ channels open (blue) which leads to hyperpolarization and restoration of the membrane potential to its resting state. (**b**) The activation of opioid receptors results in the dissociation of the heterotrimeric G protein into $G\alpha_{i/o}$ and βγ subunits. The $G\alpha_{i/o}$ subunit inhibits adenylate cyclase and subsequent cAMP activity. The βγ directly activates $K^+$ channels leading to hyperpolarization and reduced neuronal excitability. Although morphine blocks $Na^+$ channels, the exact underlying molecular mechanism is unknown. (**c**) At the presynaptic level, the βγ subunit directly inhibits $Ca^{2+}$ channels (yellow) and the subsequent influx of $Ca^{2+}$ ions into the nerve terminal, ultimately suppressing neurotransmitter release.

### 1.2.7 Opioid receptor regulation and trafficking

GPCR signalling is a tightly controlled process and must be regulated for normal physiological functions. Signalling at the plasma membrane is terminated by two major processes; desensitization and endocytosis. Desensitization can be subdivided into homologous (agonist-dependent) and heterologous (agonist-independent; discussed in ***1.3.1 Heterologous desensitization of GPCRs***). Following receptor activation, G protein receptor kinases (GRKs) are recruited to the activated receptor and promote phosphorylation of GPCRs at the C terminus and intracellular loop regions (Ferguson, 2001). This uncouples G proteins from the receptor and also sterically hinders any further G protein interaction, resulting in receptor desensitization. There are 7 GRKs encoded in the human genome which can be divided into 3 families; GRK-like 1 (GRK1 and 7), GRK2-like (GRK2 and GRK3) and GRK-4 like (GRK 4, GRK5 and GRK6) (Ribas et al., 2007). Receptor phosphorylation increases the affinity of β-arrestins for the receptor. β-arrestins are adaptor molecules which serve as a scaffolding platform for various proteins involved in endocytosis and signalling. Although there are 4 arrestin isoforms (arrestin 1 to 4), only arrestin 2 (β-arrestin 1; βArr1) and arrestin 3 (β-arrestin 2; βArr2) are ubiquitously expressed throughout the body (Kang et al., 2014). Molecules which are implicated in endocytosis include the adaptor protein subunit 2 (AP2) and the heavy chain of clathrin. These two proteins form the core structure of the clathrin-coated pit. The large GTPase dynamin mediates internalization through the severing of the invaginated clathrin vesicle. Although GRK-mediated phosphorylation is critical for the initial termination of signalling, endocytosis represents a more prolonged phase of desensitization since G protein activation at the cell surface is hindered (Rajagopal & Shenoy, 2018). Moreover, both desensitization and endocytosis are implicated in the development of GPCR tolerance (Allouche, Noble, & Marie, 2014).

Following endocytosis, GPCRs can be sorted into a recycling or degradation pathway. GPCRs in the recycling pathway are resensitized and reinserted back into the plasma membrane, whereas GPCRS targeted for lysosomal degradation are downregulated. GPCRs which are targeted for degradation can be recovered by either de novo synthesis or via the recruitment of preformed stores. Downregulation leads to a more prolonged inhibition of signalling and potential cellular tolerance (Allouche et al., 2014). It is now acknowledged that this canonical view of GPCR regulation is oversimplified. GPCRs may be homologously desensitized through a GRK- and endocytosis-independent manner including via protein kinase C (PKC) (Bailey et al., 2009; Miess et al., 2018). In addition to their role in GPCR trafficking, β-arrestins may also function as scaffolds for the recruitment of signalling complexes to the activated receptor (Luttrell et al., 1999).

#### 1.2.7.1 MOR regulation and trafficking in enteric neurons

The first demonstration of MOR trafficking in any neuron was by indirect immunofluorescence in the myenteric plexus of the guinea pig ileum (Sternini et al., 1996). MOR is internalized in an agonist-dependent manner and recycled back to the cell surface (Lay et al., 2016; Minnis et al., 2003; Sternini et al., 1996). Prolonged treatment with MOR agonists may also induce cellular adaptations which alter receptor trafficking. Acute exposure to morphine failed to induce MOR-endocytosis in myenteric neurons (Sternini et al., 1996). However, chronic morphine treatment induced internalization which correlated with enhanced levels of phosphorylated dynamin (Patierno et al., 2011). This may have important implications for the development of OIC. MOR is distributed at both the plasma membrane and within intracellular regions in enteric neurons (Lay et al., 2016; Poole et al., 2011).

The nature of MOR desensitization in the ENS is complex. Moreover, studies in the ENS contradict findings in the CNS (Akbarali, Inkisar, & Dewey, 2014; Williams et al., 2013). The regulation of MOR in the ENS has mainly been examined at the functional level. Sternini *et al.* (2000)

found that a 15 min pre-exposure to the high-internalizing agonist DAMGO, but not morphine, desensitized MOR-mediated inhibition of electrically-stimulated contractions of the ileum. This study suggests that receptor internalization is correlated with the desensitization of MOR-mediated responses. However, other studies have reported no correlation between receptor internalization and desensitization of MOR-mediated responses. Morphine-mediated inhibition of neurogenic contractions were reduced 30 minutes after a single addition of the same agonist (Kang et al., 2012; Ross et al., 2008). This suggests that MOR is desensitized in an endocytosis-independent manner. Interestingly, morphine-mediated effects on the guinea pig colon were retained, highlighting potential region-bias in the regulation of MOR (Kang et al., 2012; Ross et al., 2008). Similar region-specific effects were reported for equivalent DAMGO- or morphine-evoked contractions in the mouse. However, responses to other high-internalizing agonists including fentanyl and etorphine were desensitized in both regions. Thus, desensitization of MOR-evoked contractions also exhibit ligand bias. Furthermore, these studies confirm that desensitization of MOR in the ENS is internalization-independent.

β-arrestins may play a role in the desensitization of MOR-mediated responses in the intestine. DAMGO and morphine-evoked contractions are desensitized in colons from mice with a global deletion of βArr2 (βArr2-/-). The responses to fentanyl and etorphine were still effectively desensitized in both the ileum and colon of βArr2-/- mice, suggesting that desensitization of these responses may involve other regulatory proteins (Maguma, Dewey, & Akbarali, 2012). This also suggests that the importance of βArr2 for MOR signalling in the colon is ligand specific. The deletion of only one form of β-arrestin may be insufficient to abrogate desensitization, with reports showing that both β-arrestins can bind to agonist-activated MOR (Groer et al., 2011). The contribution of βArr1 to the regulation of MOR-mediated responses in the ENS has not been examined.

The recruitment of βArr2 to MOR is proposed to have important implications for physiological processes including pain and GI functions. The observation that morphine analgesia was both enhanced and prolonged in a βArr2-/- mouse suggested that biased signalling towards G proteins may enhance the analgesic efficacy of opioids (Bohn et al., 1999). Furthermore, the βArr2 pathway may also be responsible for adverse side effects (Bohn et al., 2000; Raehal, Walker, & Bohn, 2005). The development of analgesic tolerance, acute respiratory depression, and constipation were reduced in βArr2-/- mice. Based on these studies, drug discovery efforts for safer opioids have been largely focused around developing ligands which preferentially activate the G protein-dependent signalling pathway (DeWire et al., 2013; Manglik et al., 2016; Schmid et al., 2017). The phenomenon that selective ligands for the same GPCR can signal through distinct pathways is known as biased agonism. An example of a G protein-biased MOR agonist is oliceridine (TRV130) which recently failed to progress past phase III clinical trials due to safety concerns with dosing and lack of efficacy over morphine. Initial studies found that TRV130 produced robust acute analgesia in both rats and mice. TRV130-treated mice also exhibited reduced constipation compared to those administered with morphine. However, this study was limited to measurements of up to 4 hours following the initial administration of TRV130 (DeWire et al., 2013). A more comprehensive study which examined the direct effects of TRV130 on colonic motility found this compound still produced constipation (Altarifi et al., 2017). Daily administrations of either morphine or TRV130 inhibited bead expulsion time to a similar degree in mice, indicating that both compounds have constipating actions. In addition, a recent study by Kliewer *et al.* (2019) demonstrated that constipation still develops to fentanyl and morphine using transgenic mice expressing MOR lacking key phosphorylation sites required for effective β-arrestin recruitment (Miess et al., 2018). This suggests that there are other important mechanisms other than biased signalling away from β-arrestins which may contribute to the adverse effects of opioids in the GIT. Therefore, there is a need to better understand opioid receptor expression and signalling in the myenteric plexus.

### 1.2.7.2 DOR regulation and trafficking in the ENS

DOR is targeted for lysosomal degradation in enteric neurons following stimulation with met-enkephalin or the synthetic agonist SNC80. Furthermore, *de novo* synthesis is required for the replenishment of DOR at the cell surface (Poole et al., 2011). The trafficking route for DOR in other cell types is ligand-dependent. Treatment with SNC80 leads to lysosomal degradation in cortical neurons and recombinant cells, whereas [D-Pen$^{2,5}$] Enkephalin (DPDPE) can promote both DOR recycling and degradation (Audet et al., 2012; Zhang et al., 2008). DOR may also be recycled back to the cell surface in cortical neurons via an intracellular retrieval complex even after being targeted to lysosomes (Charfi et al., 2018). These studies detail the complexity of DOR trafficking and demonstrates that system and ligand bias may significantly influence the regulation of receptor-mediated responses.

The desensitization of DOR-mediated responses in the ENS has not been examined. There are also conflicting data in the literature regarding the molecular mechanisms underlying the desensitization of DOR in distinct cell types. Pradhan *et al.* (2009) demonstrated that G protein-dependent signalling in membrane preparations of the brain and spinal cord is effectively desensitized by agonists that strongly, but not weakly, internalize DOR (SNC80 and ARM390, respectively). Equivalent observations were made for two agonists with distinct internalizing strengths in recombinant cells (Hong et al., 2009). In these studies, the authors conclude that desensitization is dependent on DOR internalization. However, other studies have reported an endocytosis-independent mechanism of desensitization. Navratilova *et al.* (2007) found that mutating the major GRK2 phosphorylation sites on the C-terminus of DOR inhibited desensitization without affecting β-arrestin recruitment and DOR internalization. In addition, ARM390-mediated inhibition of cAMP was effectively desensitized in recombinant cells without inducing receptor internalization (Marie et al., 2003). Although the exact mechanisms underlying DOR desensitization are still unclear, it appears to be both a cell type- and ligand-dependent process. This also supports our argument that the desensitization of DOR-mediated responses needs to be directly studied in the ENS.

## 1.2.8 Endosomal and location-biased signalling

A major conceptual advancement in the GPCR biology field is the demonstration that activated and internalized receptors may continue to signal from within endosomes (Murphy et al., 2009). The multi-protein complex containing the activated GPCR, β-arrestins, G protein and signalling proteins (i.e. mitogen-activated protein kinases; MAPKs) is known as a 'signalosome' (Ellisdon & Halls, 2016). Signalosomes emit a sustained response that is both spatially and temporally distinct from plasma membrane-derived signals (Eichel & von Zastrow, 2018). Therefore, the nature and duration of the cellular response may be defined by endosomal signalling (Cottrell et al., 2009; **Figure 1.3**). The recent development of biosensors which readily detect activated states of GPCRs or G proteins in real time have provided direct evidence of endosomal signalling in both native and recombinant cells (Irannejad et al., 2013). In addition to endosomal signalling, specific ligands can display 'location-bias' whereby they are able to cross the plasma membrane and directly activate GPCRs in discrete intracellular sites (Irannejad et al., 2017). A recent study by Stoeber *et al.* (2018) used a fluorescently-labelled nanobody to establish that endogenous opioids and related peptides drive MOR or DOR signalling at the cell surface and within endosomes of recombinant cells and striatal neurons. In direct contrast, clinical opiates or synthetic opioid receptor agonists, such as morphine or SNC80, also cross the plasma membrane and activate their respective receptors located in the Golgi network. Therefore, the activation of a single GPCR subtype in different locations may drive unique signalling through distinct pathways with significant implications for their effects on physiological processes.

Only a limited number of studies have examined the ability of GPCRs to signal from within endosomes in enteric neurons. Moreover, location-biased signalling by GPCRs in enteric neurons has not been examined, confirming the limited understanding of GPCR biology in the neurogastroenterology field. Agonist-evoked internalization of the $NK_1R$ results in the activation of ERK1/2 in cultured myenteric neurons (Pelayo et al., 2011). The duration of this signal is regulated by the endosomal endopeptidase endothelin converting enzyme-1 (ECE-1). The inhibition of ECE-1 prolongs SP-evoked endosomal signalling which leads to enhanced LDH release, indicating that endosomal signalling of $NK_1R$ needs to be tightly regulated for protection against apoptosis. MOR may also signal through endosomes in rat myenteric neurons. Acute treatment with DAMGO induced ERK1/2 phosphorylation, which was effectively blocked by two distinct inhibitors of endocytosis; hypertonic sucrose and the dynamin inhibitor Dynasore. Chronic morphine treatment enhanced both MOR internalization and the activation of ERK1/2. This suggests that chronic exposure to morphine induces adaptive changes to MOR-mediated signalling in myenteric neurons (Duraffourd et al., 2014). However, the direct role of endosomal signalling to physiological processes in the GIT including motility remains unknown.

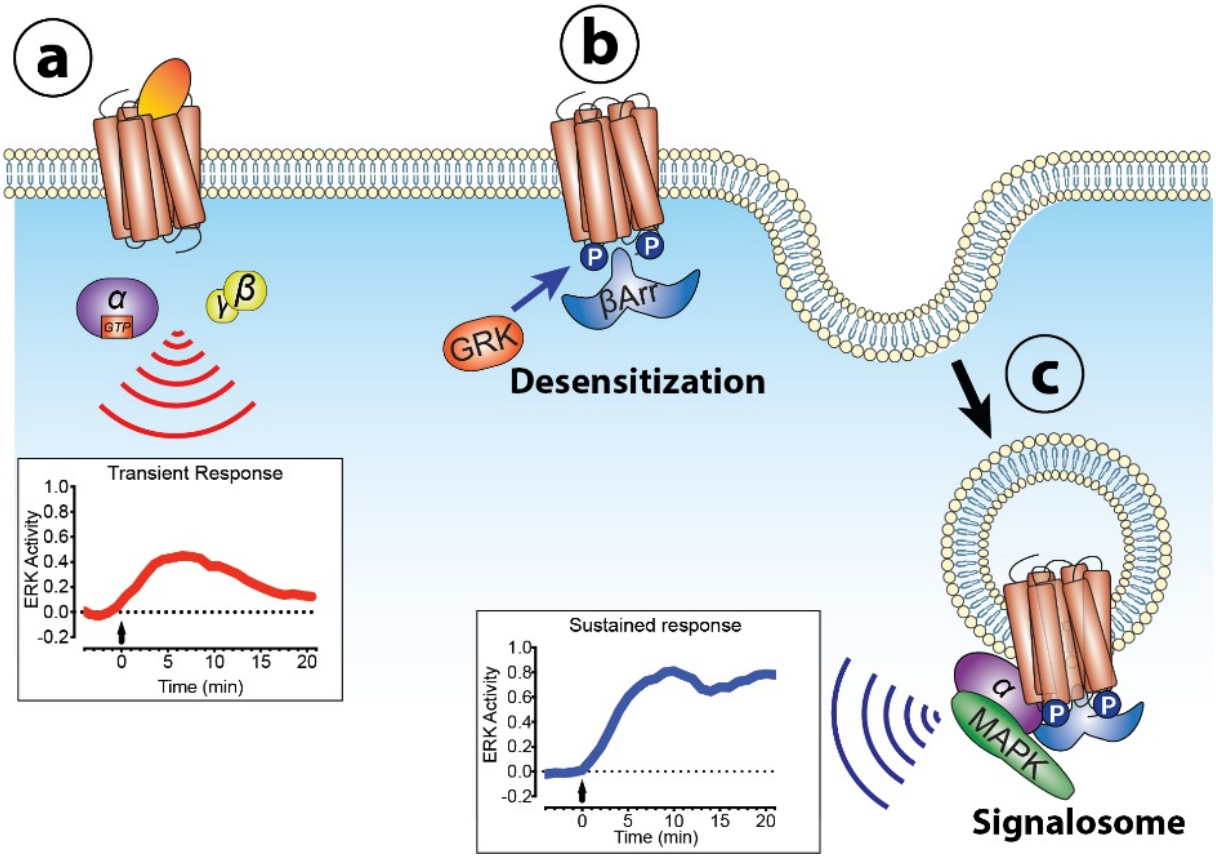


**Figure 1.3 The conceptual advancement of endosomal signalling. (a)** The activation of GPCRs at the plasma membrane results in the dissociation of the heterotrimeric G protein complex and the regulation of specific signalling pathways. (**b**) Following prolonged stimulation, G protein receptor kinases (GRKs) phosphorylate the GPCR and enhance the affinity for β-arrestins (βArrs). This regulatory event promotes desensitization leading to a transient signalling response. (**c**) βArrs are also adapters for endocytic machinery including dynamin and clathrin. Following endocytosis, βArrs act as a scaffold for the formation of a multiprotein signalling complex known as a signalosome. Signalosomes comprise of the GPCR, the activated G protein and a signalling molecule. Signalosomes emit a sustained wave of intracellular signalling.

### 1.2.9 Development of tolerance to opioid receptor-mediated responses in the ENS

An issue with opiate use is the development of analgesic tolerance. Tolerance is defined as the loss of response following continued exposure to a drug. Acute tolerance may occur after short-term exposure, whereas long-term exposure may lead to chronic tolerance (Williams et al., 2013). Tolerance is complex and multifaceted, and it is generally accepted that the regulation of receptor signalling including phosphorylation, G protein uncoupling, desensitization, endocytosis and down-regulation are involved in the underlying mechanism (Akbarali et al., 2014; Allouche et al., 2014). Therefore, mechanistic studies examining tolerance at the behavioural level have mainly focused on the contribution of some of these events. For example, Bohn *et al.* (2000) demonstrated that morphine retains its analgesic efficacy in βArr2-/- mice, suggesting that βArr2 mediates the development of tolerance to opiate effects on pain. In addition, Kliewer *et al.* (2019) established that analgesic tolerance to both morphine and fentanyl was reduced in transgenic mice which express phosphorylation-deficient MOR, implicating GRK and β-arrestin recruitment to tolerance.

The inhibitory effects of opiates on GI motility are retained with sustained exposure and this may underlie OIC (Leedham et al., 1991). OIC is mediated through the prolonged activation of MOR expressed by enteric neurons. In addition to the system-biased development of opioid tolerance (CNS vs ENS), there are also region-specific differences in the loss of opioid responsiveness in the intestine. Tolerance develops to the inhibitory effects of morphine in the mouse and guinea pig ileum (Kang et al., 2012; Leedham et al., 1991). The responses to opiates are retained in the colon with repeated exposure, implicating this region of the GIT in OIC (Kang et al., 2012; Ross et al., 2008). The differential development of tolerance in the ileum and colon is suggested to be mediated by the levels of βArr2. Morphine responses diminished after sequential administration to colons from βArr2-/- mice (Kang et al., 2012). Furthermore, βArr2 is downregulated in the guinea pig ileum following chronic morphine treatment, whereas levels were unchanged in the colon. The ability of morphine to reduce faecal output over a 6 h period was lost in βArr2-/- mice (Raehal et al., 2005). Morphine-mediated inhibitory effects on faecal output over 6 h were also reduced, but not reversed, in mice lacking GRK6 (GRK6-/-; Raehal et al., 2009). These findings indicate that βArr2 is implicated in the sustained inhibitory effects of opiates in the colon. However, ligands which are poor recruiters of βArr2, including TRV130, still produce constipation (Altarifi et al., 2017). Moreover, their inhibitory effects on neurogenic contractions of the colon are retained following repeated administrations (Altarifi et al., 2017). This suggests that there are other factors besides the recruitment of βArr2 which are involved in the sustained actions of opiates on colonic motility. Whether DOR agonists also produce sustained inhibitory effects on GI motility is undetermined. This provides an opportunity to compare the prolonged effects of DOR activation in the ENS with MOR. This may help predict motility-associated side effects of emerging DOR therapeutics and provide mechanistic insight into the factors governing opioid receptor tolerance.

## 1.3 Functional interactions between MOR and DOR

It is generally accepted that distinct GPCRs can directly and indirectly interact at the cellular level to modulate their responses. GPCRs may physically interact to form heteromers. These functional interactions between GPCRs are proposed to be therapeutic targets for a range of disorders including IBS-D and pain. Receptors may also indirectly interact through the sharing or recruitment of the same signalling or regulatory molecules. However, the precise mechanisms by which GPCRs interact is poorly understood. Furthermore, there is a limited understanding of whether GPCRs may functionally interact in the ENS.

### 1.3.1 Heterologous desensitization of GPCRs

A common mechanism by which cross-talk between GPCRs can occur is via heterologous desensitization. This is defined as the inhibition of signalling by a GPCR following the activation of a different GPCR. The molecular mechanisms underlying this process include the activation of kinases including PKC and protein kinase A (PKA) (Zhang et al., 2003), phosphorylation of GPCRs by GRKs (Mouledous et al., 2012), the recruitment of arrestins to the inactive receptor (Toth et al., 2018) and the sharing of intracellular signalling molecules such as G proteins (Alt et al., 2002).

Heterologous desensitization of GPCR-mediated responses by opioid receptors is implicated in certain physiological processes. Zhang *et al.* (2003) found that the activation of MOR on monocytes desensitizes both chemokine receptor (CCR)-1 and CCR2-mediated chemotaxis through a PKC-dependent mechanism. This may contribute to the immunosuppressive actions of opioids. The cross-desensitization of specific CCRs and MOR is bi-directional. The activation of a subset of CCRs (CCR5, CCR2, CCR7 and CXCR4) by their respective proinflammatory mediators desensitized MOR-induced chemotaxis of cultured monocytes (Szabo et al., 2002). Furthermore, the activation of CCR5 induced phosphorylation of MOR in cell lines which correlated with reduced DAMGO-mediated analgesia. These findings highlight a cross-talk mechanism potentially involved in inflammatory pain. Activation of KOR desensitized the gastrin-releasing peptide receptor (GRPR) in dorsal horn neurons leading to an inhibition of non-histaminergic itch. Mechanistic studies using transgenic mice and heterologous cell lines expressing both receptors demonstrated that this was through a PKC-dependent mechanism (Munanairi et al., 2018). Although not directly examined, this finding suggests that the dysregulation of this pathway may be involved in chronic itch conditions and targeting this receptor-receptor interaction is a potential therapeutic approach for the treatment of pruritus.

Only a limited number of studies have directly examined the heterologous desensitization of GPCR-responses in the ENS (Frieling et al., 1999; Schmidlin et al., 2002). The activation of $NK_1R$ on cultured myenteric neurons inhibited agonist-induced internalization of $NK_3R$. This is proposed to be mediated through a strong association of βArr1 with $NK_1R$ which affects the recruitment of βArr-2 to the activated receptor and the signalling of $NK_3R$. The heterologous desensitization of NKR-responses in the SMP influences secretory processes. Frieling *et al.* (1999) demonstrated that neurogenic secretion evoked by $NK_3R$ activation was effectively desensitized by preincubation with a $NK_1R$ agonist, but not vice versa. To our knowledge, it is unknown whether this form of GPCR cross-talk is involved in normal reflex activation of motor patterns or occurs in pathology leading to dysmotility.

#### 1.3.1.1 Heterologous desensitization of MOR- and DOR-mediated responses

Despite the physiological importance of cross-talk between opioid receptors and other GPCRs, electrophysiological evidence suggests that MOR and DOR do not interact in central neurons. Wang *et al.* (2010) found that the inhibition of calcium currents in small diameter DRG neurons by DAMGO were unaffected by preincubation with DOR agonists. In addition, Wang *et al.* (2018) used a similar approach to establish that activation of GIRK channels by MOR agonists in lamina projection neurons was unaltered by DOR agonists. In addition, a subset of neurons in the ventral tegmental area (VTA), an area of the brain which is important for motivational and rewarding behaviours, responded to both DOR and MOR agonists (Margolis et al., 2017). However, whether DOR-mediated responses were desensitized by MOR agonists was not examined. Furthermore, is it not known whether MOR and DOR can functionally interact in the ENS.

### 1.3.2 GPCR heteromerization

GPCRs were first thought to function as single signalling entities. Over the past decade, a plethora of studies have demonstrated that GPCRs can physically interact to form heteromers. The first identification of a physiologically-relevant GPCR heteromer was the class C $GABA_B$ receptor. This receptor is made up of two distinct, but similar, subunits, and interaction between these subunits is necessary for the receptors to function in native tissue (White et al., 1998). This represents a well-established example of a GPCR heteromer. However, the physiological-relevance of class A GPCR heteromers is controversial, mainly because most studies have used heterologous systems. Only a few heteromers have been characterised in native tissue, including the MOR-DOR heteromer. GPCR heteromers represent novel drug targets and have been researched extensively for this reason (Gomes et al., 2016). However, conflicting findings question the existence of class A GPCR heteromers *in vivo*. To address areas of debate, experts in the GPCR heteromer field recently defined strict classification criteria for the definitive demonstration of heteromers (Gomes et al., 2016; Pin et al., 2007). These are outlined in **Table 1.2**.

**Table 1.2 Criteria for the recognition of GPCR heteromers in tissue (Adapted from Pin et al., 2007).**

| |
|---|
| ***Criterion 1:*** Receptors should colocalize and physically interact at the cellular level. |
| ***Criterion 2:*** Heteromers should exhibit pharmacological properties distinct from those of the individual receptors. |
| ***Criterion 3:*** Heteromer disruption should lead to a loss of heteromer-specific properties. |

#### 1.3.2.1 The case for the MOR-DOR heteromer.

The proposed MOR-DOR heteromer is one of the most extensively studied of all possible class A GPCR pairs (Gomes et al., 2016). This is mainly because novel pharmacological tools have been developed to selectively probe for this interaction both *in vitro* and *in vivo*. However, there is still debate regarding the existence of this interaction *in vivo*. To address criterion 1 (**Table 1.2**) several groups have demonstrated using *in situ* hybridisation or double knockin transgenic mice that MOR and DOR are abundantly coexpressed in the CNS (Erbs et al., 2015; Wang et al., 2010). Moreover, they are co-immunoprecipitated in membrane preparations of hippocampal neurons and heterologous cell lines (Erbs et al., 2015; George et al., 2000; Gomes et al., 2004), indicating direct interaction or indirect interaction within a complex. Furthermore, structural studies have identified the transmembrane regions of the receptors where potential interactions may occur (Granier et al., 2012).

In addition, the MOR-DOR heteromer exhibits a unique pharmacological profile (Criterion 2 from **Table 1.2** and **Figure 1.4**). The potential physical interaction between MOR and DOR was proposed following a study demonstrating attenuation in the development of morphine tolerance in DOR-/- mice (Abdelhamid et al., 1991). The co-administration of a selective DOR antagonist also reduced morphine tolerance (Zhu et al., 1999). Several studies have demonstrated that these receptors

may form allosteric interactions since the occupation of one receptor leads to an enhanced signalling and binding profile of the other (Gomes et al., 2011; Gomes et al., 2000). This potentially explains the findings of Gomes *et al.* (2004) who demonstrated that morphine-mediated thermal analgesia was enhanced following the antagonism of DOR. Thus, targeting the MOR-DOR heteromer may be a more suitable drug target for pain than MOR. This unique pharmacological property is also evident in VTA neurons which coexpress both receptors (Margolis et al., 2017). Furthermore, these receptors can co-internalize in an agonist-dependent manner in recombinant cell lines (Milan-Lobo & Whistler, 2011). DOR agonists and morphine promoted the trafficking of MOR to lysosomes in cell lines expressing both receptors, leading to enhanced MOR degradation (Bao et al., 2018; He et al., 2011). The co-trafficking of MOR and DOR to lysosomes may be responsible for the development of analgesic tolerance to morphine. The administration of a TAT-conjugated peptide which disrupts the formation of the MOR-DOR heteromer reduced morphine tolerance *in vivo* (He et al., 2011). The endocytic profile of the MOR-DOR heteromer following the addition of MOR agonists, including morphine, is contradictory. Most studies highlight that morphine is low-internalizing, whereas others demonstrate it internalizes MOR at high concentrations (Bao et al., 2018; Milan-Lobo & Whistler, 2011). Therefore, although the co-internalization of MOR and DOR may indicate a physical interaction between receptors, the functional relevance of this observation is still unclear.

Novel tools were developed to address criterion 3 (**Table 1.2**) for the MOR-DOR heteromer. This includes an antibody selective for the MOR-DOR heteromer (Gupta et al., 2010). This antibody was used to demonstrate enhanced expression of the MOR-DOR in the brains of morphine tolerant mice. In addition, a small molecule compound named CYM51010 was identified to be a biased agonist for the heteromer (Gomes et al., 2013). This compound exhibited a similar analgesic profile to morphine, but without side effects including analgesic tolerance. However, the specificities of CYM51010 has been questioned (see ***1.3.2.2 The case against the MOR-DOR heteromer***).

### 1.3.2.2 The case against the MOR-DOR heteromer

The existence of the MOR-DOR heteromer has been questioned. Alt *et al.* (2002) found that these receptors may not necessarily physically interact *in vitro*, rather they share the same pool of G proteins which may be responsible for the novel pharmacological profile. Scherrer *et al.* (2009) and Wang *et al.* (2018) demonstrated with high specificity using transgenic and knockout mice that MOR and DOR are predominantly expressed in distinct pain pathways. Contradictory findings have been reported in regard to the role of DOR in MOR-mediated analgesia and tolerance. Scherrer *et al.* (2009) demonstrated that morphine tolerance still develops in DOR-/- mice. In addition, MOR-mediated thermal analgesia is unaffected by the antagonism of DOR (Gomes et al., 2013; Scherrer et al., 2009). Wang *et al.* (2018) also found that these receptors are not co-internalized in central neurons co-expressing both receptors. Finally, questions have been raised regarding the selectivity of the pharmacological tools which have been used to probe the MOR-DOR heteromer in native tissues. Immunofluorescent labelling by the MOR-DOR-selective antibody was still evident in tissues from both DOR-/- and MOR-/- mice. This indicates that it is likely that this antibody does not exclusively detect MOR-DOR in tissues (Gupta et al., 2010). The proposed MOR-DOR heteromer biased agonist CYM51010 still activated G-protein signalling in membrane preparations of neurons from DOR-/- mice. Its analgesic efficacy was partially blocked by MOR selective antagonists, and it also internalized MOR in neurons from DOR-/- mice. These findings suggest that CYM51010 is a non-specific MOR agonist (Gomes et al., 2013; Wang et al., 2018). Collectively, these studies indicate that although the MOR-DOR heteromer has been identified as a novel and highly specific therapeutic target, definitive evidence for its existence is lacking.

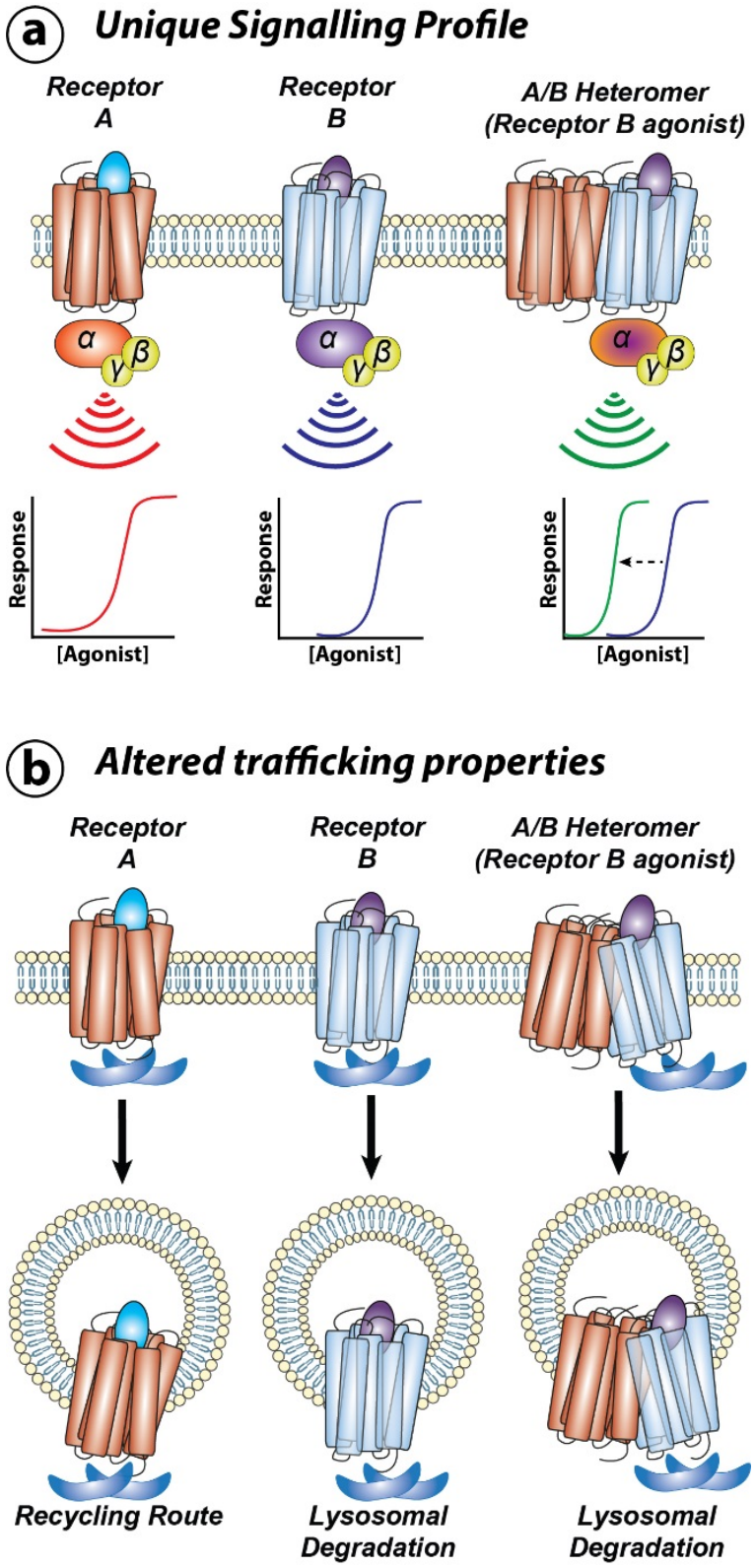


**Figure 1.4 The unique signalling and trafficking properties of GPCR heteromers. (a)** Two distinct GPCRs (Receptor A and B) may physically interact to form a heteromer (A/B heteromer). This produces allosteric interactions between the receptors and results in changes to their signalling profile. For example, the potency of an agonist for Receptor A is enhanced at the A/B heteromer. (**b**) GPCR heteromers also exhibit distinct trafficking properties compared to the individual receptors. For example, the recycling Receptor A is targeted for lysosomal degradation following heteromerization with Receptor B.

#### 1.3.2.3 Is there evidence to support the existence of the MOR-DOR heteromer in the ENS?

GPCR heteromerization is a largely unexplored area of research in the ENS field. The first evidence of MOR and DOR coexpression in any system was demonstrated in myenteric neurons of the guinea pig ileum (Egan & North, 1981). Using electrophysiological and pharmacological techniques, they found that both receptors coexist in a subset of neurons. More recently, DOR was extensively characterised with high specificity in the ENS using DOReGFP mice (Poole et al., 2011). A large proportion of DOR-positive neurons coexpressed MOR, suggesting that they may interact in the ENS. However, this study did not use pharmacological approaches to examine whether MOR and DOR may functionally interact, and this was not the primary focus of the research. A recent report by Fujita *et al.* (2014) used the MOR-DOR heteromer selective antibody to probe for expression in the mouse ileum. Although a relatively low abundance of protein was detected by the antibody, we predict this may potentially be a result of non-specific binding (Gupta et al., 2010). In **Chapter 5** of this thesis we report that there is substantial overlap between MOR and DOR in the ENS. This provides an excellent opportunity to directly examine possible MOR-DOR function in tissues.

Recently, a mixed MOR agonist/ DOR antagonist named eluxadoline was approved for the treatment of IBS-D. Based on a study by Fujita and colleagues (2014), eluxadoline partially acts through the MOR-DOR heteromer to inhibit motility without promoting constipation. However, eluxadoline still inhibited motility in DOR-/- mice which demonstrates that its actions may be mediated solely through MOR (Wade et al., 2012; Fujita et al., 2014). Therefore, we hypothesise that this compound may be acting through MOR to inhibit motility, whereas the reduced constipation may be a result of increased motility through DOR antagonism and not necessarily a direct physical interaction between the two receptors. Based on current research in the GPCR field, it is still largely unknown whether MOR and DOR functionally interact, particularly in the ENS.

## 1.4 Scope of this thesis

The general aim of this thesis is to advance the understanding of opioid receptor signalling and function in the ENS. In addition, we examined whether conceptual advances in the GPCR field, including endosomal signalling and heteromerization, are applicable to neurogenic processes of the colon. Determining this will help broaden our knowledge of how GPCRs signal and function within the ENS and may have important implications for drug discovery.

DOR is an emerging therapeutic target for chronic pain and psychiatric disorders. DOR agonists are also proposed to have a safer side effect profile than drugs that act through MOR (Gendron et al., 2015). Despite the clear anatomical and functional evidence for expression of DOR by myenteric neurons, the role of peripheral DOR in the regulation of colonic motility is still unclear. The aim of **Chapter 2** was to re-examine whether the peripheral activation of DOR influences neurogenic processes of the colon. In addition, we investigated whether DOR-expressing myenteric neurons are activated during motility reflexes. Lastly, we examined inflammation-associated changes to DOR expression and function in the colon.

A major issue with opiates is the system-dependent development of tolerance to their responses. Central neurons become tolerant to opiate-mediated effects leading to reduced analgesic efficacy, whereas their ability to inhibit colonic motility is retained throughout treatment (Ross et al., 2008). Whether the colon becomes tolerant to the inhibitory effects of DOR agonists is unknown. In **Chapter 3**, we determined whether acute and chronic tolerance develops to DOR-mediated responses by the colon. We also examined whether the development of tolerance is correlated with the ability of the agonist to internalize DOR in myenteric neurons.

It is now widely accepted that GPCRs can continue to signal from within endosomes. Endosomal signalling is associated with a unique signalling profile which is both spatially and temporally distinct from plasma membrane-derived signals (Murphy et al., 2009). Few studies have examined endosomal signalling in enteric neurons. In addition, the contribution of endosomal signalling to the reflex control of colonic function has not been examined. Small molecules which selectively disrupt different stages of endocytosis are commonly used to examine endosomal signalling in cells including enteric neurons (Carbone et al., 2019; Pelayo et al., 2011). In **Chapter 4**, we determined whether these small molecule endocytic inhibitors are suitable for use in studies of the contribution of endosomal signalling to the neurogenic control of GI motility.

An emerging therapeutic target for pain and IBS-D is the MOR-DOR heteromer. The development of pharmacological tools to probe for the MOR-DOR heteromer in the ENS include biased agonists and MOR-DOR selective antibodies. However, issues concerning the specificity of these tools have been raised, and a recent study by Wang *et al.* (2018) used a systematic approach to demonstrate that these receptors do not interact in pain pathways. Therefore, a comprehensive examination of this interaction in the ENS is warranted. This will help determine whether the MOR-DOR heteromer is a possible therapeutic target for the treatment of GI diseases or disorders. In **Chapter 5**, we first determined whether MOR and DOR are coexpressed in the ENS. We then comprehensively examined whether MOR and DOR functionally interact in the ENS and the mechanisms through which this was mediated.

# Chapter 2
# *Inflammation-associated changes in DOR expression and function in the mouse colon.*

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**RESEARCH ARTICLE** | *Hormones, Neurotransmitters, Growth Factors, Receptors, and Signaling*

# 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 demonstrates functional expression of DOR by myenteric neurons and supports the therapeutic targeting of DOR in the enteric nervous system.

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**NEW & NOTEWORTHY** DOR is activated during physiologically relevant reflex stimulation. Agonist-evoked DOR endocytosis is spatially and temporally regulated. A significant proportion of DOR is internalized in myenteric neurons during inflammation. The relative proportion of all myenteric neurons that expressed DOR and the overlap with the nNOS-positive population are increased in inflammation. DOR-specific innervation of the circular muscle is increased in inflammation, and this is consistent with enhanced responsiveness to the DOR agonist SNC80.

endocytosis; enteric nervous system; G protein-coupled receptor, intestinal motility; opioid 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 neuromuscular 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 DOReGFP positive neurons in this region are nitrergic (~70%), suggesting that DOR can influence the inhibitory control of colonic motility (60). DOR agonists induced endocytosis of DOReGFP in myenteric neurons, indicating functional expression. DOReGFP 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, DOReGFP-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 management of diarrhea-predominant irritable bowel syndrome (IBS-D), is a mixed agonist of MOR and the κ-OR (KOR) and a DOR antagonist (41, 76). Eluxadoline has been proposed to exert its therapeutic actions by targeting MOR-DOR heteromers (25). Racecadotril (acetorphan) is a peripherally acting enkephalinase inhibitor that exerts its anti-diarrheal and antinociceptive 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 dynamin 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 ($NK_1R$) 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*

DOReGFP (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, 7 $MgSO_4$, 1.1 $H_2O$, 1.18 $KH_2PO_4$, 25 $NaHCO_3$, 11.6 glucose, and 2.5 $CaCl_2$; 95% $O_2$-5% $CO_2$). 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% $O_2$-5% $CO_2$). 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 4/SP 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- or 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 $EC_{50}$ and $E_{max}$ 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 naltrindole (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), $N^G$-nitro-L-arginine (L-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% $O_2$-5% $CO_2$, 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% $O_2$-5% $CO_2$, 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 naltrindole (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 $H_2O$). 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 $H_2O$). 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 SP8-MP, 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 bUnwarpJ (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-GFP; 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], $NK_1R$ [rabbit anti-rat $NK_1R$, 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, NBP1-20263, 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 a ×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

(version 17.10), using a backprojected pinhole radius of 135 nm, signal-to-noise ratio of 10, and quality cutoff of 0.01.

*Image Analysis*

The subcellular distribution of DOReGFP was analyzed from captured images using the FIJI distribution of ImageJ [version 1.51g, Wayne Rasband, National Institutes of Health (NIH)], as previously described (59, 60). Briefly, single optical sections, including the cell nucleus, were used. The threshold was set based on a region without GFP labeling, 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 were harvested for localization of DOReGFP or for contraction assays of DOR function. 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 associated with activation of reflex pathways (59). Colon length was determined as a measure of disease severity. Tissue was processed for hematoxylin and eosin staining, and histological damage assessment was performed in a treatment-blinded manner, as described (59). Significant weight loss occurred on *days 4* ($P = 0.046$) and *5* ($P = 0.001$) relative to the vehicle control. DSS treatment was associated with significant colon shortening relative to mean control length (vehicle = 1.0 ± 0.04, DSS = 0.81 ± 0.02, $P < 0.001$) and combined histological and macroscopic damage score (mean score: vehicle = 0.80 ± 0.37, DSS = 11.31 ± 1.05, $P < 0.001$).

*Reagents*

DADLE, hexamethonium chloride, L-NNA, naltrindole hydrochloride, and nicardipine hydrochloride were from Sigma-Aldrich. [D-Ala2]-deltorphin II and veratridine were from Abcam. SNC80 was from Cayman Chemical, dextran sulfate sodium salt (36,000–50,000 MW, "colitis grade") was MP Biomedicals, and TTX citrate was from Alomone.

## RESULTS

*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 ($pEC_{50} = 8.22 \pm 0.41$, $E_{max} = 87.48 \pm 5.36\%$, $n = 6$; Fig. 1, *A* and *B*) and by the highly selective DOR agonists deltorphin II ($pEC_{50} = 9.26 \pm 0.15$, $E_{max} = 73.45 \pm 3.03\%$, $n = 5$; data not shown) and SNC80 ($pEC_{50} = 6.33 \pm 0.29$, $E_{max} = 96.75 \pm 2.26\%$, $n = 6$; 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: $pEC_{50} = 7.22 \pm 0.15$, $E_{max} = 80.53 \pm 3.17\%$, $n = 8$, $P = 0.02$; 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: $pEC_{50} = 5.67 \pm 0.47$, $E_{max} = 102.3 \pm 4.65\%$, $n = 6$, $P = 0.12$; 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 ($EC_{40}$: 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 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. 2*A*). 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 nitrergic signaling (Fig. 3, *A* and *B*). SNC80-evoked contractions were abolished by TTX (1 μM; Fig. 3*C*) and significantly attenuated by HEX (1 μM; Fig. 3*D*), 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 nitrergic 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*

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 ($pEC_{50} = 7.92 \pm 0.17$, $E_{max} = 57.31 \pm 1.97$, $n = 41$–$57$ 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

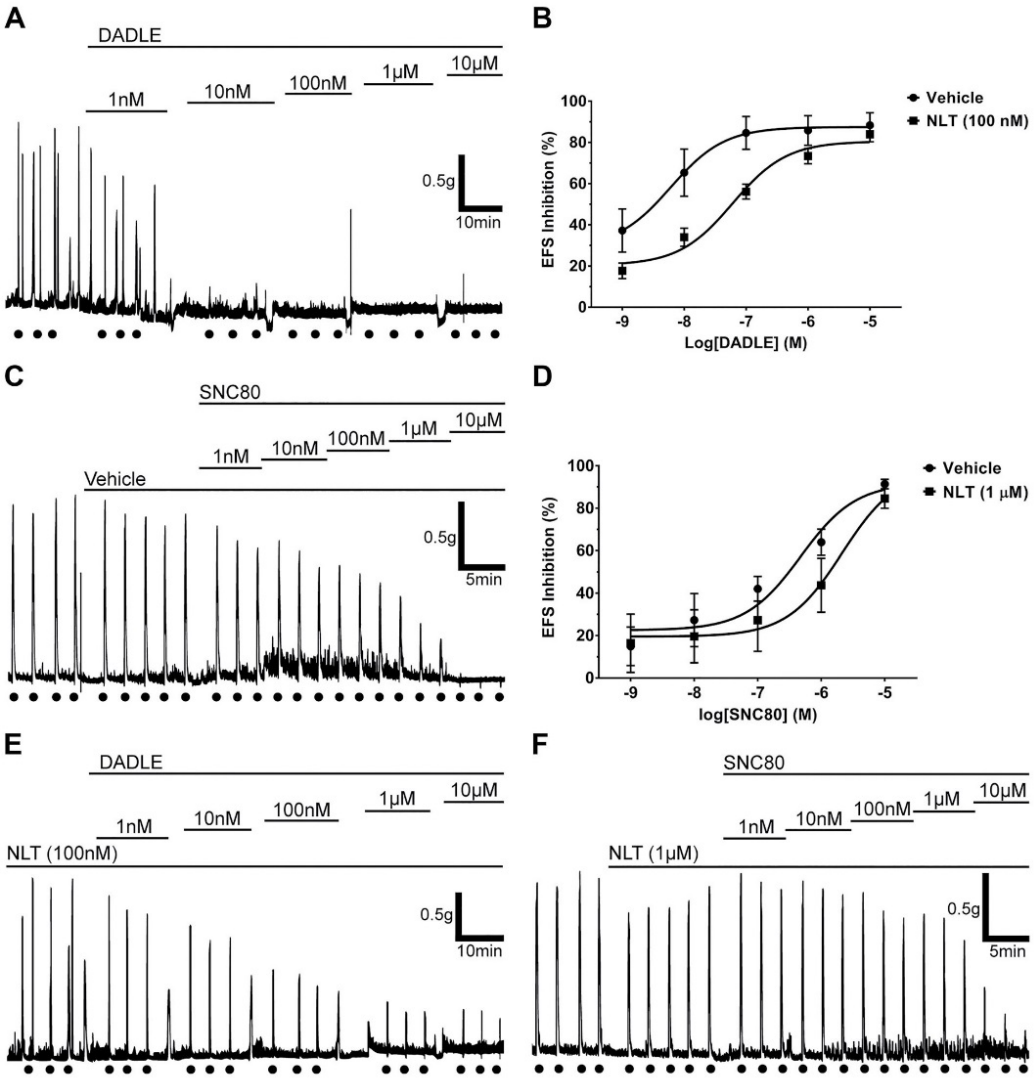


Fig. 1. δ-Opioid receptor (DOR) agonists inhibit electrically evoked contractions of the mouse colon. *A* and *B*: DADLE reduced the amplitude of electrically evoked contractions in a concentration-dependent manner. *C* and *D*: SNC80 also inhibited neurogenic contractions but was less potent than DADLE. *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.

proximal neurites of all positive neurons. These data demonstrate that DOR is functionally expressed by myenteric neurons and that all DOReGFP-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 DOReGFP-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 DOReGFP-positive and other neurons, including nerve fibers within the circular muscle layer (Fig. 5*A*).

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 DOReGFP in response to release of endogenous DOR agonists was examined by quantitative microscopy. Under unstimulated control conditions, DOReGFP was localized mainly to the cell surface of myenteric neurons [79.00 ± 0.87%, 95% confidence

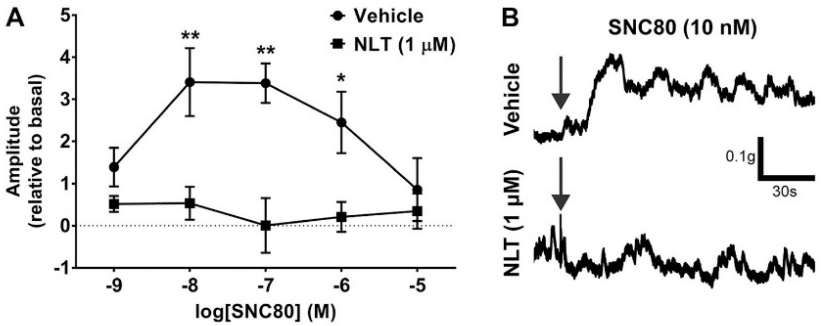


Fig. 2. SNC80 evoked tonic contractions of colonic circular muscle through a DOR-dependent mechanism. *A*: quantitative analysis of the amplitude of contractions to SNC80 demonstrating a biphasic response that peaked at a concentration of 10 nM. *B*: SNC80 (10 nM) produced a rapid and sustained contraction 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.

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. $NK_1R$ internalization was examined in submucosal neurons from the same preparations to confirm activation and specificity. $NK_1R$ 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 $NK_1R$ 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 $NK_1R$ endocytosis [58.60 ± 1.26% (95% CI: 56.07–61.13%), $n = 49$ neurons, $n = 3$ mice; Fig. 5*C*].

Electrical field stimulation (10 min, with 20-min recovery, Fig. 6*A*) 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 orthosteric 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. 7*A*). 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: 66.89–71.36%), $n = 127$ neurons, $n = 6$ mice; Fig. 7*B*]. 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. 7*D*). 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. 8*A*). Treatment with veratridine (10 μM) resulted in the appearance of intracellular vesicular DOReGFP labeling in the soma and proximal neurites (Fig. 8*B*). 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. 8*C*). 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

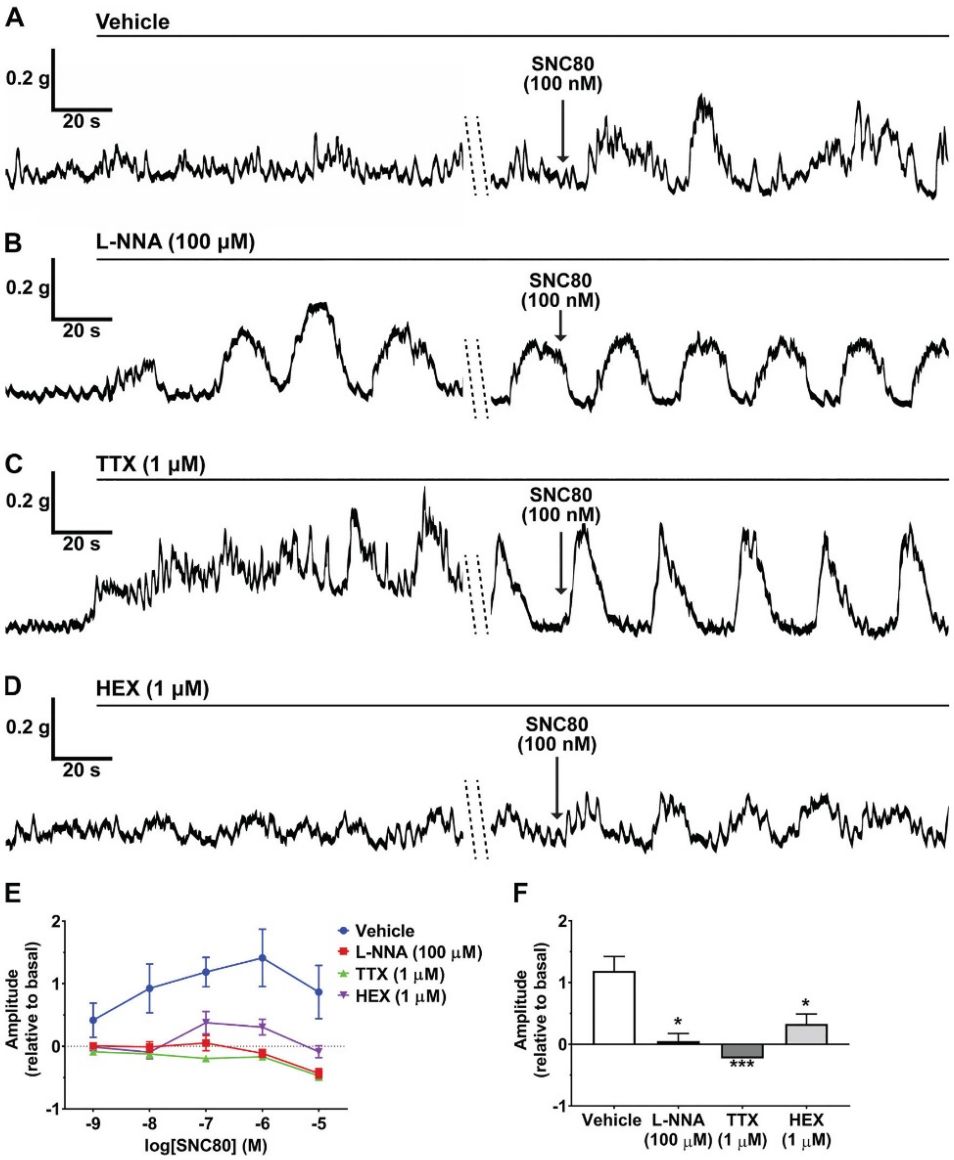


Fig. 3. SNC80-evoked contractions of the colon are mediated through direct actions on myenteric neurons. *A*: SNC80 evoked robust concentration-dependent contractions, which were abolished by $N^G$-nitro-L-arginine (L-NNA; *B*) and tetrodotoxin (TTX; *C*) and reduced by hexamethonium (HEX; *D*). SNC80 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.

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 (Hu+/DOReGFP+ neurons: DSS: 46 ± 1%, $n = 2{,}061$ neurons analyzed, $n = 9$ mice vs. control: 42 ± 1%, $n = 2{,}393$

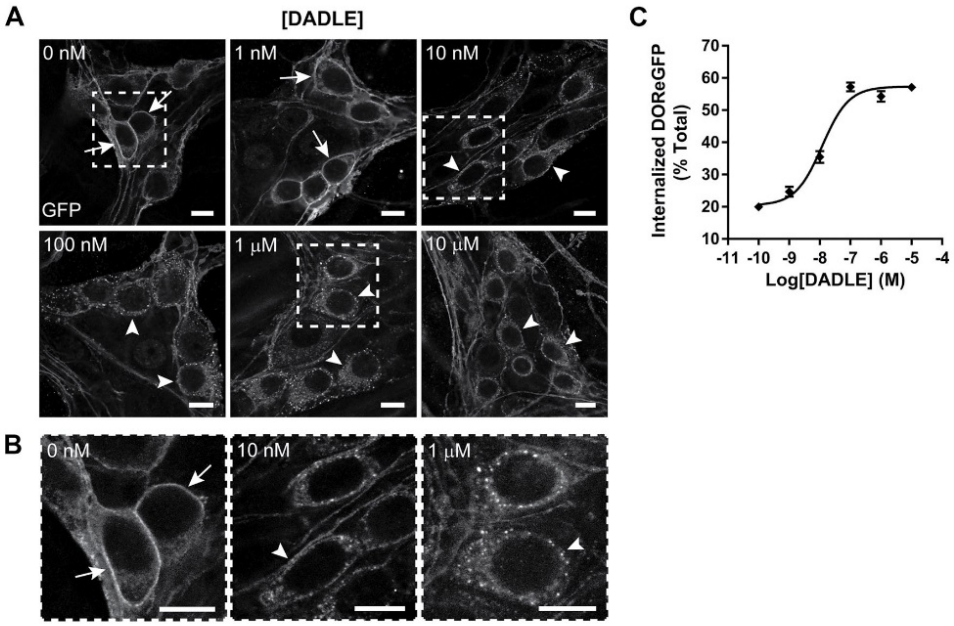


Fig. 4. DADLE promotes δ-opioid receptor/enhanced green fluorescent protein (DOReGFP) internalization. *A*: DOReGFP was localized to the surface of myenteric neurons of the colon under basal conditions (arrows). Treatment with DADLE (1 nM to 10 μM, 30 min) resulted in a significant redistribution of DOReGFP to endosomes (arrowheads). *B*: zoomed images of regions indicated by dashed-line boxes. *C*: quantitative analysis of DOR internalization in response to DADLE. Data represented as means ± SE. Scale, 10 μm.

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 ($NK_1R$) is internalized in myenteric neurons of the inflamed intestine (45, 59). Similarly, $NK_1R$ 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*). Costaining 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 *Oprm1* 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.

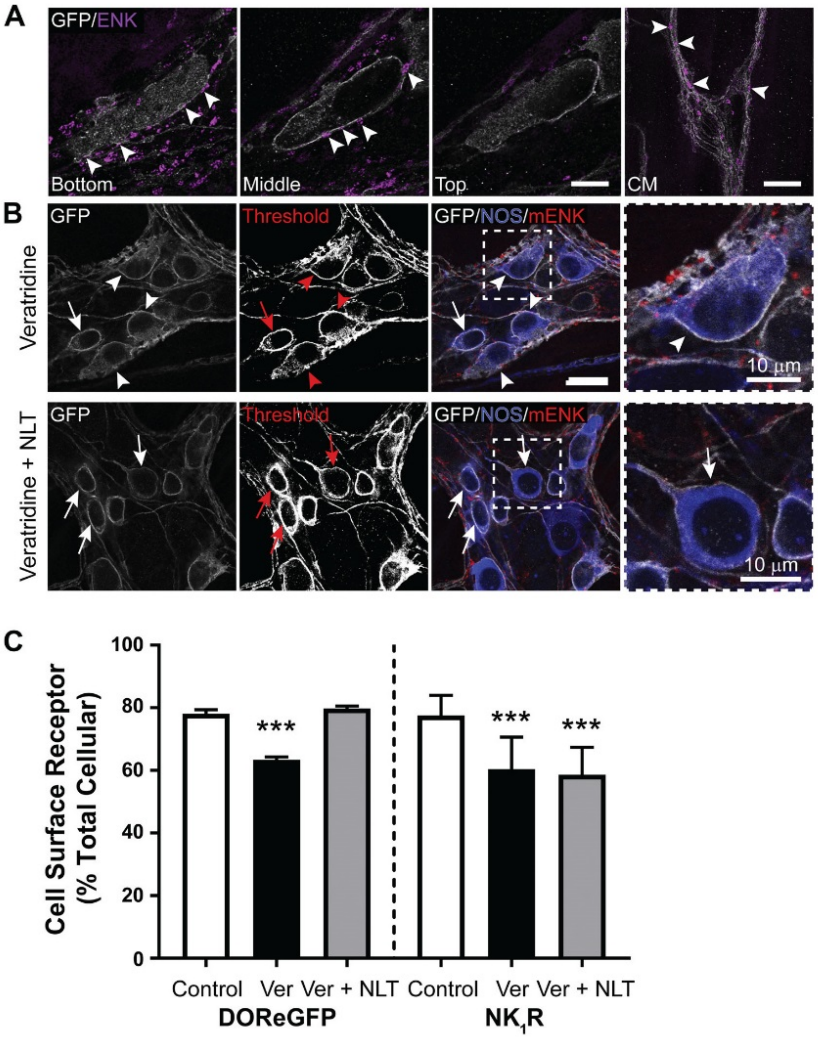


Fig. 5. δ-Opioid receptor/enhanced green fluorescent protein (DOReGFP) is internalized in response to chemically evoked release of endogenous neurotransmitters. *A*: enhanced-resolution images demonstrating close association of enkephalin (ENK)-immunoreactive varicosities with a DOReGFP-positive neuron at different levels of the soma and with nerve fibers within the circular muscle (CM) layer (arrowheads). *B*: treatment of preparations with veratridine (10 μM) promoted DOReGFP endocytosis, which was effectively blocked by naltrindole (NLT). *C*: quantitative analysis of DOReGFP internalization in response to veratridine (Ver). Submucosal neurokinin 1 receptor ($NK_1R$) also internalized in response to Ver but was unaffected by NLT (Ver + NLT). Scale, 20 μm. $^{***}P < 0.001$, 1-way ANOVA; $n = 6$ mice.

SNC80 inhibited EFS-evoked contractions of the circular muscle in a concentration-dependent manner ($pEC_{50} = 6.23 \pm 0.21$, $E_{max} = 91.14 \pm 8.54$, $n = 6$). Acute DSS colitis was associated with a significant increase in the potency at which SNC80 inhibited EFS-evoked contractions ($pEC_{50} = 7.15 \pm 0.24$, $n = 5$, $P = 0.04$), but the magnitude of responses to EFS was generally reduced. Inflammation did not significantly affect the $E_{max}$ of responses to SNC80 (control: 91.14 ± 8.54%; DSS: 86.02 ± 4.36%; $P = 0.59$; Fig. 10*D*). 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.

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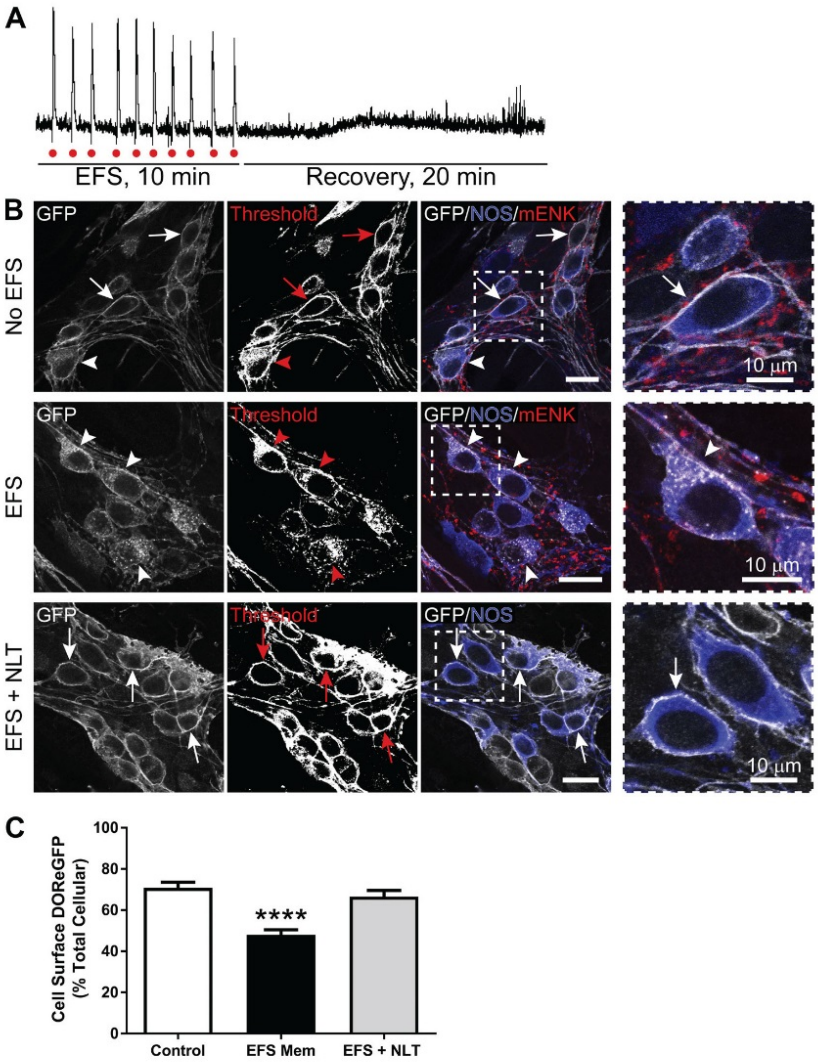


Fig. 6. δ-Opioid receptor/enhanced green fluorescent protein (DOReGFP) is internalized in response to electrically evoked release of endogenous neurotransmitters. *A* and *B*: electrical field stimulation (EFS; 10 min with 20-min recovery; *A*) resulted in internalization of DOReGFP, which was abolished by naltrindole (NLT; *B*). *C*: quantitative analysis of DOReGFP internalization in response to EFS. Scale, 20 μm. ****$P <$ 0.0001, 1-way ANOVA; $n = 6$ mice/group. ENK, enkephalin; GFP, green fluorescent protein; NOS, nitric oxide synthase.

*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 (pKi: 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-dependent inhibition of EFS contractions using naltrindole. However, this does not adequately explain the block 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

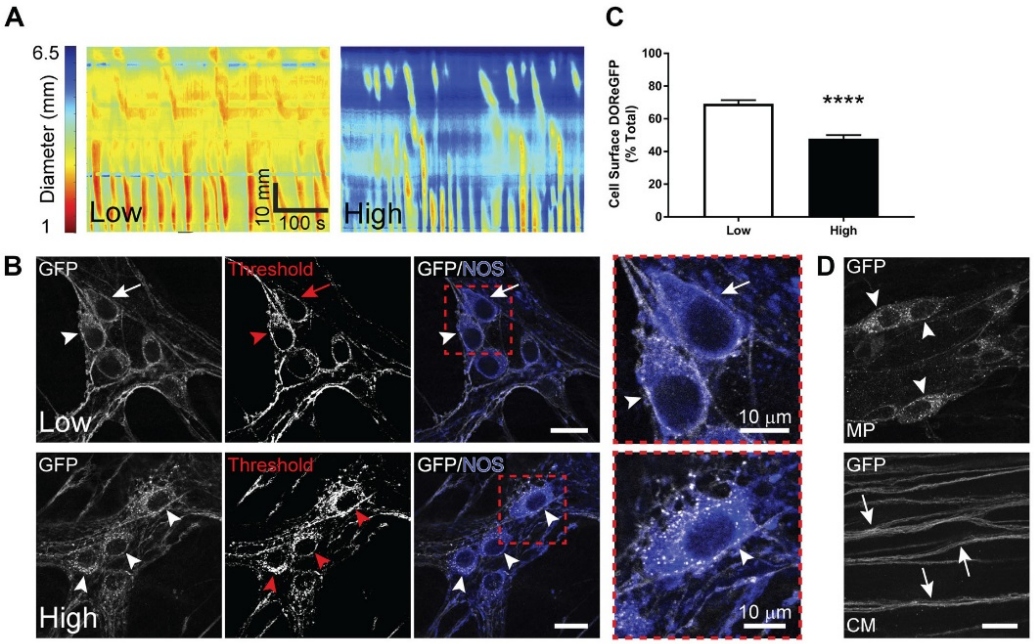


Fig. 7. δ-Opioid receptor/enhanced green fluorescent protein (DOReGFP) is internalized in response to reflex release of endogenous neurotransmitters. *A*: representative density maps showing contractile activity of the intact colon under conditions of low and high intraluminal pressure. *B*: DOReGFP was internalized when pressure in the lumen was raised ("high"). *C*: quantitative analysis of DOReGFP endocytosis under each stimulus condition. *D*: pressure-evoked DOReGFP endocytosis occurred in myenteric neurons (arrowheads), but not in nerve fibers (arrows), associated with the circular muscle (CM) layer of the same preparation. Scale, 20 µm. ****$P < 0.0001$, 2-tailed *t*-test; $n = 6$ mice/group.

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, nonselective activation of neurons by the sodium channel opener veratridine similarly resulted in significant DOReGFP endocytosis. This was also inhibited by naltrindole. Finally, mechanical activation of myenteric reflex pathways through elevated intraluminal pressure promoted significant DOReGFP endocytosis, consistent with the proposed modulatory role of enkephalins and DOR in the control of intestinal motility (24, 29). Thus, we conclude that DOR-expressing myenteric neurons of the mouse colon are functionally innervated by endogenous opioid-containing nerve fibers and are likely to contribute to the normal physiological control of colonic motility. Modulation of enkephalinergic signaling through DOR using, e.g., allosteric modulators may represent a novel therapeutic approach for the treatment of motility disorders.

### *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 have 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

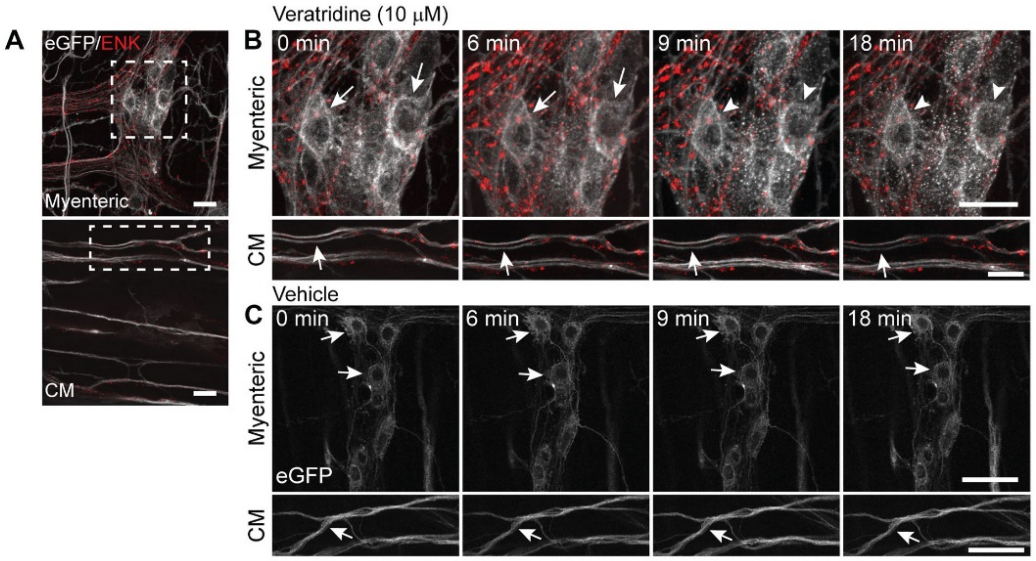


Fig. 8. Real-time imaging of δ-opioid receptor/enhanced green fluorescent protein (DOReGFP) endocytosis in organotypic preparations of the colon. *A* and *B*: overview image (*A*) indicating regions of interest in the myenteric and circular muscle (CM) layers (presented in *B*). Veratridine treatment 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.

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).

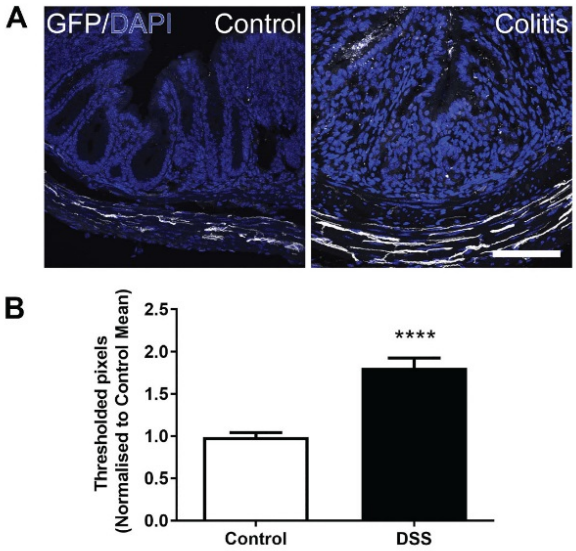


Fig. 9. Colitis increases δ-opioid receptor (DOR) expression and function. *A* and *B*: dextran sulfate sodium (DSS) colitis was associated with a significant increase in the density of δ-opioid receptor/enhanced green fluorescent protein (DOReGFP)-positive nerve fibers in the muscularis externa relative 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.

### *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 circular muscle layer. This is supported by the significantly increased density of DOReGFP-positive nerve fibers in the muscularis externa in the inflamed colon. Intestinal

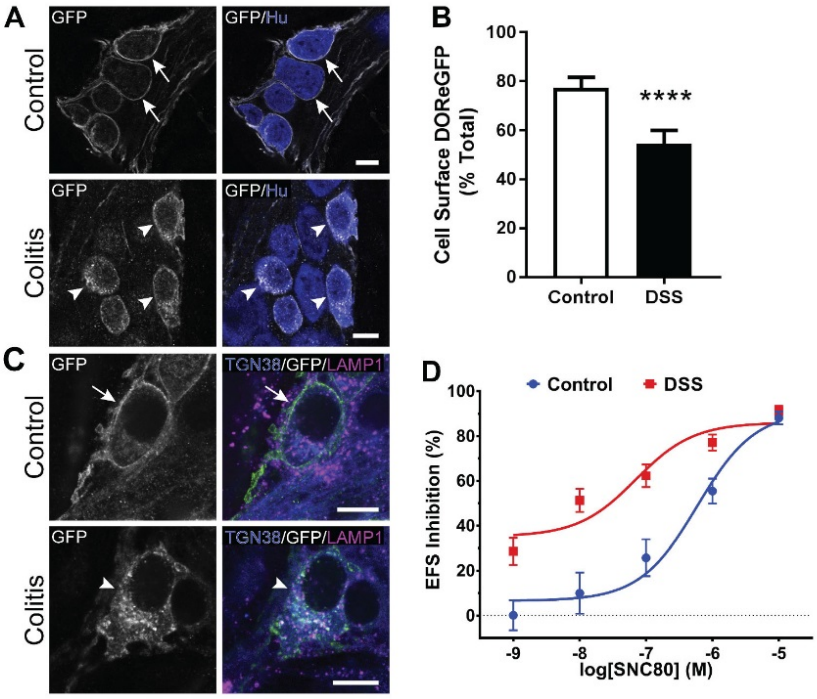


Fig. 10. δ-Opioid receptor (DOR)/enhanced green fluorescent protein (DOReGFP) is internalized in myenteric neurons of the acutely inflamed colon, and DOR function is enhanced. *A*: DOReGFP was localized to the cell surface of myenteric neurons under control conditions. *B*: acute colitis was associated 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.

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

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 of GPCR function in the ENS.

### ACKNOWLEDGMENTS

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### DISCLOSURES

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### AUTHOR CONTRIBUTIONS

J.J.D., P.R., C.J.N., S.E.C., and D.P.P. conceived and designed research; J.J.D., A.S., P.R., R.M.M., B.W.S., S.E.C., and D.P.P. performed experiments; J.J.D., A.S., R.M.M., B.W.S., S.E.C., and D.P.P. analyzed data; J.J.D., E.M.E., J.F., N.A.V., M.C., S.E.C., and D.P.P. interpreted results of experiments; J.J.D., P.R., and D.P.P. prepared figures; J.J.D., S.E.C., and D.P.P. drafted manuscript; J.J.D., M.C., N.W.B., S.E.C., and D.P.P. edited and revised manuscript; J.J.D., A.S., P.R., J.F., N.A.V., M.C., S.E.C., and D.P.P. approved final version of manuscript.

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# Chapter 3
# *Agonist-dependent development of delta opioid receptor tolerance in the colon.*

**Agonist-Dependent Development of Delta Opioid Receptor Tolerance in the Colon.**

***Running Title: DOR tolerance in the gut***

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**Contributions.** JJD conceived, designed, conducted, analyzed and interpreted experiments, supervised the study and wrote the manuscript. AS conducted and analyzed experiments. BWS and RM conducted experiments. PR assisted with experimental analysis and made figure 9. NAV, AB and MC assisted with interpretation of data. SEC conducted experiments, supervised the study, and assisted with drafting of the manuscript. DPP conceived, designed, conducted and analyzed experiments, supervised the study, and wrote the manuscript.

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**Key Words:** Enteric Nervous System, Opioid Receptor, Colon Motility, Endocytosis, GPCR Regulation

**Abstract**

The use of opioid analgesics is severely limited due to the development of intractable constipation, mediated through activation of mu opioid receptors (MOR) expressed by enteric neurons. The related delta opioid receptor (DOR) is an emerging therapeutic target for chronic pain, depression and anxiety. Whether DOR agonists also promote sustained inhibition of colonic transit is unknown. This study examined acute and chronic tolerance to SNC80 and ARM390, which were full and partial DOR agonists in neural pathways controlling colonic motility, respectively. Excitatory pathways developed acute and chronic tolerance to SNC80, whereas only chronic tolerance developed in inhibitory pathways. Both pathways remained functional after acute or chronic ARM390 exposure. Propagating colonic motor patterns were significantly reduced after acute or chronic SNC80 treatment but not by ARM390 pretreatment. These findings demonstrate that SNC80 has a prolonged inhibitory effect on propagating colonic motility. ARM390 had no effect on motor patterns and thus may have fewer GI side-effects.

## Introduction

Opioid receptors are members of the class A G protein-coupled receptor (GPCR) family. They comprise of three classical subtypes; the mu (MOR), delta (DOR) and kappa (KOR) receptors, all of which are expressed by the intrinsic neurons of the gastrointestinal (GI) tract [1]. The fourth member of the opioid receptor family is the nociceptin (NOP) receptor, which has high sequence homology to the classical receptors but no affinity for endogenous opioids [2]. The clinical use of MOR agonists, including morphine and fentanyl, is limited by the onset of debilitating side-effects including addiction, analgesic tolerance, respiratory depression and opioid-induced bowel dysfunction (OBD). OBD is a collection of GI side-effects, of which intractable constipation is the most common. Opioid-induced constipation (OIC) is prevalent in a significant portion of patients receiving opioid therapy and is a major cause of patient non-compliance with treatment [3].

In addition to GI-related side-effects, a significant limitation to prolonged opioid use is the development of analgesic tolerance, whereby higher doses of the drug are required to produce their desired effect. Tolerance is defined as the loss of response following *continued* exposure to a drug. Acute tolerance may occur after short-term exposure to a drug (hours), whereas long-term administration (daily) may lead to chronic tolerance. Desensitization usually precedes the development of tolerance and is characterized by the loss of response following acute *intermittent* exposures to an agonist (seconds to minutes). This is mediated through receptor uncoupling from G protein effectors, which occurs immediately after stimulation and initial signaling events [4]. In marked contrast to the development of analgesic tolerance, the inhibitory effects of opioids on GI motility are retained and may underlie the prolonged constipating actions of these drugs [5]. Thus, the development of tolerance to the opioid-mediated inhibition of GI motility is a desirable outcome associated with reduced potential for OIC. The distinct mechanisms involved in the development of opioid tolerance in the gut and brain are poorly defined.

The enteric nervous system (ENS), which is contained within the wall of the GI tract, is a major regulator of GI functions. It is comprised of the myenteric and submucosal plexuses, which control motility and secretomotor functions, respectively [6]. Activation of MOR expressed by enteric neurons inhibits action potential firing and associated neurotransmitter release. This disrupts the tightly coordinated neural reflexes, resulting in dysmotility and the inhibition of secretion, ultimately leading to sustained constipation [1]. The inhibitory actions of opioids, such as morphine, on the GI tract do not diminish with repeated administrations [7]. This highlights the complex nature of MOR signaling and regulation in the ENS and the central nervous system (CNS).

Although MOR remains the primary target for the development of opioid analgesics, recent studies highlight the potential of DOR as an emerging therapeutic target for chronic pain and emotional disorders [8]. DOR is also expressed by enteric neurons throughout the GI tract and DOR agonists can act directly on myenteric neurons to inhibit colonic motility [9-11]. Upon activation with high efficacy agonists such as SNC80, DOR is trafficked to lysosomes of neurons both in the CNS and in the ENS, effectively reducing functional receptors at the cell surface [11,12]. In the CNS, acute analgesic tolerance developed to the strongly internalizing agonist SNC80, while the weakly internalizing agonist ARM390 retained its efficacy [12]. However, the development of chronic analgesic tolerance to DOR agonists differed between species and pain models [13-15]. SNC80 produced tolerance in both mouse and rat models of inflammatory pain, whereas tolerance only developed to ARM390-mediated effects in the mouse. Furthermore, both agonists retained their analgesic efficacies in preclinical models of migraine pain [14]. However, drugs were administered every 48 hours in the latter study, making it difficult to directly compare the development of chronic tolerance to DOR-mediated effects between pain models. For emotional behaviors, chronic tolerance only developed to SNC80-mediated locomotor and anxiolytic effects [13,16]. Although DOR activation influences GI function, we are not aware of any studies that have examined the prolonged effects of DOR agonists on colonic motility. Furthermore, whether a correlation exists between the internalizing properties of DOR agonists and the regulation of their inhibitory actions in the colon is unknown. In the present study, our aim was to examine the short and long-term regulation of DOR-mediated responses by the mouse colon. We found that the development of both acute (3h) and chronic (3d) tolerance to DOR-mediated effects in *segments* of colon correlated with the subcellular distribution of DOR in myenteric neurons. However, the strongly DOR-internalizing agonist SNC80 had a prolonged inhibitory effect on *whole* colon motility. This study highlights the importance of investigating the prolonged effects of emerging opioid therapeutics using an intact system.

## Methods

***Animals.*** C57Bl/6J mice were obtained from the Monash Animal Research Platform (Clayton, VIC, Australia). Mice expressing DOR with a C-terminal enhanced green fluorescent protein (eGFP) tag were as described [17]. Male and female mice (6-12 weeks of age, 20-30g) were used. Mice were given free access to food and water and were housed in a light and temperature-controlled environment (24°C, 12 h light/dark cycle). All procedures involving mice were approved by the Monash Institute of Pharmaceutical Sciences animal ethics committee.

***Drugs.*** Drugs were obtained from the following sources: SNC80 (Cayman Chemicals, Ann Arbor, MI); A-RM100390 (ARM390) and SB 205607 (TAN67; Tocris, Ellisville, MO); loperamide hydrochloride, [D-Ala$^2$, D-Leu$^5$]-Enkephalin (DADLE), [D-Ala$^2$, N-Me-Phe$^4$, Gly$^5$-ol]-enkephalin acetate salt (DAMGO), and nicardipine hydrochloride (Sigma-Aldrich, Castle Hill, NSW, Australia). All drugs were diluted in DMSO for *in vitro* experiments with a maximum bath concentration of ≤0.1 %. For *in vivo* treatments, drugs were diluted in saline (SNC80 at pH 5.5; [18]) and administered either *per os* (p.o.) or by intraperitoneal injection (i.p.). Dosing was as follows: SNC80 (10 mg/kg, i.p.), ARM390 (10 mg/kg, p.o.), loperamide (1 mg/kg, i.p.). Vehicle controls received saline (0.9 %, i.p. or p.o.).

***Tissue contraction assays.*** Mice were euthanized by cervical dislocation and segments of distal colon were prepared as previously decribed [9]. Briefly, muscle strips were placed in 10 mL water-jacketed organ baths containing Krebs solution (in mMol $L^{-1}$; NaCl 118; KCl 4.70, $NaH_2PO_42H_2O$ 1; $NaHCO_3$ 25; $MgCl_26H_2O$ 1.2; D-Glucose 11; $CaCl_22H_2O$ 2.5) maintained at 37°C and bubbled with carbogen (95% $O_2$/5% $CO_2$). Isometric contractions of the circular muscle were measured using a force displacement transducer (FT03, Grass Instruments, Quincy, MA). Data were acquired with a PowerLab

4/SP system and viewed using LabChart software (v.5; AD Instruments Pty. Ltd., Castle Hill, NSW, Australia). Tissues were placed under a resting tension of 0.5-1 g and equilibrated for 30 min prior to use. Following the completion of each experiment, 10 μM carbamoylcholine (carbachol) was added to evaluate tissue viability. Tissues that were unresponsive to carbachol were omitted from analyses.

***Desensitization of agonist effects on electrically-evoked contractions.*** Neurogenic contractions were evoked by transmural electrical field stimulation (EFS; 0.5 msec duration, 3 pulses $s^{-1}$, 60V), which was applied through platinum electrodes [9]. Once reproducible baseline EFS responses were maintained (applied at 5 min intervals), tissues were incubated with either SNC80, ARM390 or DAMGO (1 μM, 5 min). Preparations were then electrically-stimulated (3 repeats, 5 min intervals) and the amplitudes of responses were averaged. This procedure was repeated three times, each separated by a washout period (3 washes, 5 min intervals). The amplitudes of EFS-evoked contractions were compared to baseline responses (i.e. in the absence of DOR agonists), and data were expressed as the percentage inhibition of the baseline EFS-evoked contraction. In a separate set of experiments, the recovery of EFS-evoked contractions was measured. Tissues were exposed once to either SNC80, ARM390, DADLE, or DAMGO (1 μM, 5 min), electrically-stimulated (3 repeats, 5 min intervals), and then washed (3 repeats, 5 min intervals). The average amplitude of EFS-evoked contractions was measured at defined time-points to determine the recovery time following the initial drug addition (15, 45, 75 min).

***Desensitization of agonist-evoked contractions.*** Bath addition of opioid-receptor agonists produces a tonic, neurogenic contraction of colonic circular muscle [9]. Tissues were incubated with either SNC80, ARM390, TAN67 or DAMGO (1 μM, 5 min) and then washed (3 washes, 5 min intervals). This procedure was repeated 3 times. The area under the curve (AUC) was measured for a 2 min period immediately following the point of each drug addition. The pre-exposure period (2 min) was subtracted from this value to account for variability in basal contractile activity.

***Measurement of acute and chronic tolerance to DOR-mediated effects on colonic contractility.*** To measure tolerance to acute or chronic exposure of DOR agonists, we adapted an existing protocol to assess analgesic tolerance to the same DOR agonists [12,13]. Mice were administered a single dose of either SNC80, ARM390, or saline (acute: 3 h; chronic: once daily for 3 d, then euthanized 1 d after final drug administration). Segments of distal colon were prepared as described above. After recording baseline EFS-evoked contractions, tissues were treated with SNC80 (1 μM; 5 min), then electrically-stimulated (3-6 repeats, 5 min intervals). Experiments were analyzed as described above. Data obtained from saline-treated mice were pooled, as there was no significant difference between p.o. and i.p. administration routes (Student's t-test, $p=0.96$, n=5-7 mice per group).

***Measurement of acute and chronic tolerance to DOR-mediated effects on propagating colonic motility.*** Mice were administered a single dose of either SNC80, ARM390, or saline as described above. In a separate set of experiments, mice were administered a single dose of the MOR agonist loperamide (3 h). Mice were euthanized, and the entire colon was placed into organ-bath chambers to record motor patterns by video imaging [19]. Following equilibration (30 min), contractile activity of each segment was recorded with a camera (Logitech QuickCam Pro) positioned 7–8 cm above the preparation. Videos were captured (6 frames/s) and saved in *.avi* format using VirtualDub software (version 1.9.11). Colonic activity was recorded in the absence of drug (basal; 20 min) and following bath addition of SNC80 (1 μM final bath concentration; 20 min). Video recordings were used to construct spatiotemporal maps using in-house edge detection software (developed by Dr Lukasz Wiklendt, Flinders University, Adelaide, Australia) using MATLAB software [20]. Colonic migrating motor complexes (CMMCs) were specifically analyzed. These were defined as propagating contractions directed from the proximal to the distal end of the colon, which travelled more than 40 % of the colon length [20].

***Receptor trafficking studies.*** DOReGFP mice were administered DOR agonists or saline, as described above for tolerance studies, and were euthanized after 3 h or 3 d. The distal colon was excised and placed in ice-cold Krebs buffer containing nicardipine (10 μM). Tissue was cut along the mesenteric border and wholemount preparations were pinned and stretched mucosa downwards onto silicone elastomer-lined dishes then fixed (4% PFA, overnight, 4˚C). In a separate series of experiments, organotypic preparations of the colon from DOReGFP mice were treated with either SNC80, ARM390 (both 1 μM) or TAN67 (1 nM-10 μM) (1 h, 4˚C), washed (3 washes, cold Krebs) and recovered (Krebs, 37˚C, bubbled with carbogen) for 30 min prior to fixation.

Circular muscle-myenteric plexus wholemounts were labeled by indirect immunofluorescence to detect GFP (rabbit polyclonal anti-GFP, ThermoFisher #A11122, 1:1,000 dilution), nNOS (goat polyclonal anti-nNOS, GeneTex #89962, 1:1,000 dilution), and the pan-neuronal marker HuC/D (Human anti-Hu [21], 1:25,000 dilution). Labeling was detected using donkey secondary antibodies conjugated to Alexa Fluor 488, 568, or 647 (1:500 dilution, ThermoFisher). Images were captured using a Leica TCS-SP8 confocal system as described [22]. Images for quantitative analysis of the subcellular distribution of DOReGFP in the soma were captured at 1024 x 1024 pixel resolution, whereas images of nerve fibers were at 2048 x 2048 pixel resolution. All images were captured at 16-bit depth using a 40x objective (1.3 NA). Five fields of view including ganglia (>2x zoom) were acquired per preparation. Images used for characterization of the neurochemical coding of DOReGFP labeling were taken using a 20x objective (0.75 NA; 0.75x zoom).

***Image analysis.*** The subcellular distribution of DOReGFP in the soma was analyzed from captured images as previously described [11, 22]. Briefly, single optical sections including the nucleus were used. The threshold was set based on a region without GFP labeling, resulting in a binary image with either 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 subcellular distribution of DOReGFP in nerve fibers was analyzed from projected z-stacks. The total area of the nerve fibers in the field of view was determined by applying a threshold, based on regions outside of the nerve fiber. DOReGFP positive puncta were measured using the ImageJ 'Find Maxima' plugin (15,000 noise tolerance) applied to the original stacked image (Gaussian blur of 1.00) and expressed as puncta/1000 $\mu m^2$.

***Statistical analysis.*** Data were expressed as mean ± standard error of the mean (s.e.m.). All graphs were constructed in Prism (v7.02, GraphPad Software Inc, CA, USA). Specific statistical analyses used for each experiment are indicated in the respective figure legends. $P<0.05$ was considered statistically significant at the 95% confidence level.

## Results

In the current study, the regulation of DOR-mediated responses in different motor pathways was examined using the following approaches: 1) an EFS-protocol using muscle strips to assay effects in excitatory pathways, 2) a direct agonist-contraction protocol using muscle strips to determine responses in inhibitory motor pathways, and 3) an *in vitro* whole colon motility assay to examine the development of tolerance in complex motor patterns using an intact system.

**DOR agonists inhibit excitatory neuromuscular transmission in the colon.** SNC80 and ARM390 inhibited EFS-evoked contractions in a concentration-dependent manner. SNC80 was more potent and efficacious than ARM390 ($pEC_{50}$ 7.1 ± 0.2 vs 5.8 ± 0.3 and $E_{max}$ 90.0 ± 5.5 % vs 69.3 ± 10.1 %,

respectively), suggesting that ARM390 is a partial agonist of DOR in the colon (**Fig. 1a**). TAN67 also exhibited equivalent inhibitory effects in this assay, consistent with partial agonism ($pEC_{50}$ 6.9 ± 0.2, $E_{max}$ 73.4 ± 4.4 %; **Supplementary Fig. 1a**).

MOR-dependent inhibition of EFS-evoked contractions is transient, allowing for the measurement of acute desensitization to repeated agonist addition as previously described [23]. To examine whether DOR-mediated effects on EFS-evoked contractions are either transient or sustained, the recovery of electrically-stimulated contractions to baseline levels was measured following a single exposure to agonist. SNC80-mediated responses were sustained since EFS-evoked contractions failed to recover to pre-exposure amplitudes following a washout and 75 min recovery period (SNC80: 66.9 ± 6.5 % inhibition vs post-recovery: 69.3 ± 5.7 % inhibition, n=4; **Fig. 1b, c**). Similar results were obtained for ARM390 (ARM390: 45.4 ± 5.8 % inhibition vs post-recovery: 48.6 ± 12.0 % inhibition, n=5; **Fig. 1b, d**) and the peptidic DOR agonist DADLE (85.0 ± 3.9 % inhibition vs post-recovery: 64.3 ± 7.9 % inhibition, n=5; **Fig. 1b, e**). In contrast, DAMGO-dependent inhibition of EFS-evoked contractions was transient since pre-exposure levels were partially restored when examined 15 min after the first washout (76.5 ± 4.7 % inhibition vs 15 min recovery: 29.7 ± 3.9 % inhibition, $p<0.001$, n=5; **Fig. 1b, f**) and were largely restored within 45 min post-washout. Thus, DOR-dependent inhibition of EFS-evoked contractions are sustained, whereas MOR-mediated inhibitory effects are transient. Furthermore, these data indicate that it is not possible to measure the acute desensitization of DOR-mediated inhibition of EFS-evoked contractions using standard approaches due to sustained receptor activation (**Supplementary Fig. 2a-f**).

**DOR-evoked contractions are desensitized.** DOR agonists evoke a tonic contraction of the isolated colon, which is primarily mediated through the hyperpolarization of inhibitory motor neurons [1,9]. We determined whether these contractions were acutely desensitized following repeated exposures to agonist using an approach that has been commonly used for MOR-agonists [7,23,24]. SNC80 and ARM390 (both 1 μM) evoked rapid, tonic contractions of colonic circular muscle (17.3 ± 2.9 g.s. and 6.2 ± 1.8 g.s., respectively, n=5-6). These contractions were desensitized, since all responses to subsequent additions of SNC80 and ARM390 were significantly diminished ($p<0.001$ and $p<0.05$, respectively; **Fig. 2a-d)**. TAN67 (1 μM) also evoked a similar contraction (14.2 ± 3.8 g.s.) that was effectively desensitized with repeated applications (**Supplementary Fig. 1b**).

DAMGO (1 μM) similarly evoked a robust tonic contraction of the colon (8.9 ± 2.4 g.s., n=5). In contrast to DOR agonists, DAMGO retained its efficacy to evoke tonic contractions following the second and third application. There was a small, but significant reduction in the contractile response following the fourth addition (6.8 ± 2.0 g.s., $p<0.05$; **Fig. 2e, f**). These data demonstrate that DOR and MOR differ markedly in their desensitization profiles following repeated agonist exposure.

**Myenteric excitatory pathways become tolerant to SNC80.** The development of tolerance to the effects of opioids in the ENS is predicted to reduce their constipatory actions and thereby improve their clinical utility. We examined whether excitatory motor pathways develop either acute (3 h) or chronic tolerance (3 d) to DOR agonists (SNC80 or ARM390) administered *in vivo* (**Fig. 3a, b**). The development of tolerance was indicated by a reduction in the ability of SNC80 (1 μM, applied *in vitro*) to inhibit EFS-evoked contractions. Acute treatment with ARM390 (10 mg/kg, 3h) did not alter the subsequent inhibitory effects of SNC80 when compared to vehicle (vehicle: 64.6 ± 3.1 % inhibition, n=13; **Fig. 3c;** ARM390: 66.1 ± 2.8 % inhibition, n=7; **Fig. 3g**). In contrast, acute treatment with SNC80 produced partial tolerance (50.2 ± 1.5 % inhibition, n=5, $p<0.05$; **Fig. 3e**). These data are summarized

in **Fig. 3i**. Similar results were obtained following chronic treatment with DOR agonists. SNC80-mediated inhibitory effects were equivalent in the vehicle and ARM390 pre-treated groups (vehicle: 70.0 ± 4.4 % inhibition, n=5; **Fig. 3d**; ARM390: 65.5 ± 3.1 % inhibition, n=5; **Fig. 3h**). Partial tolerance was developed following chronic treatment with SNC80 (52.0 ± 4.7 % inhibition, n=6, p< 0.05; **Fig. 3f**). A summary of these findings is presented in **Fig. 3j**. These data demonstrate that the development of tolerance to DOR agonists in the excitatory pathways involved in generating neurogenic contractions of the colon is highly dependent on the agonist used.

**Myenteric inhibitory pathways develop acute tolerance to SNC80.** To examine whether myenteric inhibitory pathways develop either acute (3 h) or chronic tolerance (3 d) to DOR agonists, we measured the ability of SNC80 (1 μM; applied *in vitro*) to produce a robust contraction following *in vivo* pretreatment with either SNC80 or ARM390. Tolerance will manifest as a loss in the ability of SNC80 to evoke contractions.

SNC80 produced robust contractions in colons from the acute vehicle (18.3 ± 3.0 g.s., n=12**; Fig. 4a**) and ARM390 pre-treated groups (14.8 ± 4.45 g.s., n=7**; Fig. 4e**). These contractions failed to develop following acute pre-treatment with SNC80 (0.9 ± 0.3 g.s., n=5, p<0.01 compared to vehicle; **Fig. 4c**). These results highlight that inhibitory pathways develop acute tolerance to SNC80, but not to ARM390, and are consistent with effects observed for excitatory pathways.

SNC80-evoked contractions were present following chronic treatment with either vehicle- (17.1 ± 2.3 g.s., n=6; **Fig. 4b**) or ARM390 (17.3 ± 3.9 g.s., n=6; **Fig. 4f**). Colons from the chronic SNC80 pre-treated group also exhibited an agonist-evoked contraction (13.7 ± 2.1 g.s., n=7; **Fig. 4d**), in direct contrast to the respective acute SNC80 pre-treatment group. Graphs summarizing these observations are presented in **Fig. 4g** and **Fig. 4h**. These data indicate that while acute tolerance is developed to the DOR internalizing ligand SNC80, chronic tolerance is not developed in inhibitory motor pathways upon longer-term exposure.

***In vivo* exposure to SNC80 has prolonged inhibitory effects on propagating motility.** Colonic motility involves the complex interplay between long enteric neural pathways [25]. Lesioning the colon into smaller segments disrupts the synchronization of these pathways. Therefore, assays using small segments of muscle strips may not adequately measure changes to complex motility patterns underlying propagating motility [26]. Colonic migrating motor complexes (CMMC) are the major propulsive motor pattern in mammalian species. Changes in the frequency of CMMCs were measured *in vitro* to examine the effects of prolonged (3 h and 3 d) exposure to DOR agonists on propagating motility patterns.

First, we assessed the changes in CMMCs following pre-exposure to DOR agonists or vehicle. Under basal conditions, CMMC frequency was unaltered in the acute ARM390 pre-treated group (7.7 ± 1.3 CMMCs/10 min, n= 10 mice; **Fig. 5c**) compared to vehicle (9.5 ± 1.3, n=10, p=0.4; **Fig. 5a**), whereas CMMCs were significantly reduced following acute pre-treatment with SNC80 (5.7 ± 1.0, n=10, p<0.05; **Fig. 5b).** The subsequent bath addition of SNC80 (1 μM) significantly reduced CMMC frequency in the vehicle (5.1 ± 1.2; **Fig. 5a**) and ARM390 pre-treated groups (4.4 ± 1.2; **Fig. 5c**) compared to their respective basal conditions (p<0.001 for both treatment groups), indicating that DOR retained function. In contrast, SNC80 had no significant effect on CMMC frequency in colons from the SNC80 pre-treated group (4.4 ± 0.7, n=10, p=0.38; **Fig. 5b**), consistent with a prolonged inhibitory effect. Colons from mice pre-treated with the antidiarrheal MOR agonist loperamide (1 mg/kg, i.p.; 3 h) generated significantly fewer CMMCs (2.8 ± 0.7, n=8; **Supplementary Fig. 3a)** compared to the SNC80 pre-treated group under basal conditions (p<0.05; **Supplementary Fig. 3b**). This result

demonstrates that the colon retains the ability to generate propagating contractions after acute SNC80 treatment.

To investigate chronic effects of DOR agonists on CMMCs, mice were treated daily with either ARM390 or SNC80 for 3 days. Colons from mice treated with ARM390 generated a similar number of CMMCs under basal conditions (6.9 ± 1.3, n=9; **Fig. 6c**) compared to the vehicle pre-treated group (8.3 ± 1.8, n=8, p=0.66; **Fig. 6a**). In contrast, basal CMMC frequency was significantly decreased after chronic pre-treatment with SNC80 (3.8 ± 0.8, n=10; $p<0.05$; **Fig. 6b**). SNC80 (1 μM) significantly reduced CMMC frequency in the vehicle- (4.6 ± 1.4, $p<0.001$; **Fig. 6a**) and ARM390-treated groups (4.7 ± 1.4, $p<0.05$; **Fig. 6c**) compared to their respective basal conditions but did not affect the number of CMMCs in the SNC80 pre-treated group (2.9 ± 0.7, p=0.42). These results suggest that the inhibitory effects of SNC80 are sustained over time, with no further effect following subsequent *in vitro* drug exposure. In contrast, prolonged exposure to ARM390 had no effect on basal CMMC generation and DOR function was retained. Chronic exposure to either DOR agonist did not alter either the neurochemical coding or relative proportion of myenteric neurons that expressed DOReGFP (**Table 1**). This indicates that functional changes observed are unlikely to be related to alterations in the number or the phenotype of neurons expressing DOR.

**Acute and chronic exposure to DOR agonists alters the subcellular distribution of DOReGFP in myenteric neurons.** Agonist-evoked trafficking of DOR from the cell surface into endosomes and lysosomes reduces the availability of functional receptors at the plasma membrane. This is linked to the development of tolerance to central behaviors [12]. We investigated whether there was a correlation between DOR internalization in myenteric neurons and the regulation of agonist-mediated responses by the colon. To determine the subcellular distribution of DOR under the same treatment conditions used in the desensitization assays, organotypic wholemount preparations from DOReGFP mice were treated with agonists (SNC80, ARM390, or TAN67) for 30 min. Under control conditions DOReGFP was mainly confined to the plasma membrane of the soma (71.8 ± 1.4 % cell surface DOReGFP, n=59 neurons from 4 mice), proximal neurites and nerve fibers within the circular muscle (29.2 ± 3.8 puncta/ 1000 μm², n= 28 fields of view; **Fig. 7a, d, e**). *In vitro* treatment with SNC80 (1 μM, 30 min) resulted in DOReGFP redistribution to endosome-like structures in the soma (42.9 ± 2.3 %, n=24 neurons from 3 mice) and proximal neurites, but not in nerve fibers (31.1 ± 6.4 puncta/ 1000 μm², n= 19 fields of view; **Fig. 7a, d, e**). In contrast, DOReGFP was retained at the cell surface of the soma (69.5 ± 2.3 %, n=34 neurons from 4 mice), proximal neurites, and nerve fibers (26.7 ± 3.7 puncta/ 1000μm², n= 12 fields of view) following treatment with ARM390 (1 μM, 30 min; **Fig. 7a, d, e**). TAN67 only weakly internalized DOReGFP at the highest concentration tested (10 μM; 66.0 ± 1.7 %, n= 37 neurons from 4 mice; **Supplementary Fig. 1c**) and was not used for subsequent acute and chronic treatment studies.

To examine the subcellular distribution of DOR under the conditions used in the acute (3 h) and chronic (3 d) tolerance assays, DOReGFP mice were administered agonists and receptor distribution was determined in myenteric wholemount preparations. DOReGFP was internalized in the soma (24.8 ± 1.0 %, n=91 neurons from 4 mice), proximal neurites and nerve fibers (92 ± 11.46 puncta/ 1000 μm², n= 15 fields of view) in preparations from the acute SNC80-treated group (**Fig. 7b, d, e**). In preparations from the chronic SNC80-treated group, DOReGFP was also associated with endosome-like structures within the soma (40.3 ± 1.4 %, n=165 neurons from 5 mice) and proximal neurites. There was a significant reduction in intracellular DOReGFP relative to the respective acute SNC80 treatment group ($p<0.001$). However, DOReGFP internalization in the nerve fibers was significantly increased (160.3 ± 12.66 puncta/ 1000μm², n= 21 fields of view; $p<0.001$; **Fig. 7c, d, e**). The acute administration

of ARM390 resulted in DOReGFP internalization in the soma (58.9 ± 1.5 %, n=87 neurons from 4 mice; $p<0.001$) and proximal neurites, although DOReGFP remained at the surface of the nerve fibers (26.4 ± 6.4 puncta/ 1000μm², n= 15 fields of view; **Fig. 7b, d, e**). In preparations from the chronic ARM390-treatment group, DOReGFP was confined to the plasma membrane of the soma (67.5 ± 1.1 %, n=130 neurons from 5 mice), proximal neurites, and nerve fibers (29.3 ± 3.1 puncta/ 1000μm², n= 24 fields of view; **Fig 7c, d, e**). These data demonstrate that there is distinct spatial and temporal regulation of agonist-evoked DOReGFP trafficking in myenteric neurons.

## Discussion

In the present study, multiple approaches were used to measure the desensitization and development of tolerance to DOR agonists in the colon. Our findings indicate that 1) DOR-mediated responses are desensitized in inhibitory, but not excitatory motor pathways; 2) Acute, but not chronic, tolerance develops to SNC80 in inhibitory pathways measured from muscle strips, whereas both forms of tolerance develop in excitatory pathways; 3) Tolerance did not develop to ARM390 in either pathway assayed from muscle strips; 4) Acute and chronic exposure to SNC80 had a prolonged inhibitory effect on whole colon motility, whereas equivalent exposure to ARM390 did not influence motility and DOR activity was retained. These results highlight key differences between tolerance assays using muscle strips and the whole colon. Finally, changes in the DOR endocytosis profile were evident in different myenteric neuronal compartments following acute and chronic exposure to agonists. The key findings of this study are summarized in **Fig. 8** and in **Supplementary Table 1**.

***Desensitization of DOR in the ENS is motor pathway specific.*** DOR-mediated effects on neurogenic contractions were examined following repeated exposures to agonist, with this protocol consistent with receptor 'desensitization', rather than tolerance [4]. The desensitization of DOR-mediated responses differed markedly when examined in either inhibitory or excitatory motor pathways. Our results demonstrate that DOR-mediated effects on EFS-evoked contractions are resistant to desensitization, whereas agonist-evoked contractions are effectively desensitized. EFS-evoked contractions arise through the release of excitatory neurotransmitters from the prejunctional nerve fibers [27], whereas DOR agonists evoke a tonic contraction mainly through the activation of receptors located on the soma [1]. However, they may also act through receptors located on inhibitory motor nerve terminals to influence this contraction [10]. DOR agonists acting at the soma hyperpolarize the neuron to reduce excitability, whereas activation of DOR on nerve terminals directly inhibits neurotransmitter release [1]. This suggests that desensitization is not only determined by the type of neuron that expresses DOR, but also by the compartment in which the receptor is located [28].

DOR-mediated effects on EFS-evoked contractions were sustained following a single exposure to agonist. The MOR agonists DAMGO and morphine retained their inhibitory effects following repeated additions [7,23,24]. Inhibition of EFS-evoked contractions by DAMGO was transient since baseline contractions were partially restored following washout, highlighting differences in the regulation of MOR and DOR in enteric neurons. ARM390 is a partial agonist in assays of enteric neuron function. Since ARM390 had a prolonged inhibitory effect, the significant increase in the inhibition of EFS-evoked contractions following each subsequent exposure is most likely mediated through activation of spare receptors [29]. The properties of the DOR agonists chosen, including their high affinity or possible slow dissociation rate, may be responsible for their prolonged effect. Neurogenic contractions are restored within 15 min following exposure to highly selective MOR agonists, including DAMGO and morphine [7,23,24]. Therefore, it is possible that regulatory proteins involved in MOR

resensitization are present in the nerve fibers, whereas those specific for DOR desensitization are absent or not recruited to the receptor. DOReGFP is retained at the plasma membrane of nerve fibers following exposure to either ARM390 or SNC80 for 30 minutes, suggesting that DOR remains in a functionally competent state. In contrast, both SNC80 and ARM390-evoked contractions were desensitized, indicating a possible dissociation between DOR internalization at the soma and desensitization of the response. It is now widely accepted that this correlation does not exist for other GPCRs including MOR, where, for example, morphine can induce desensitization without promoting receptor internalization [30,31]. However, there is still a discrepancy in the literature regarding the relationship between the internalization and desensitization of DOR. In cell lines that endogenously express DOR, ARM390-mediated signaling was desensitized despite DOR remaining at the plasma membrane [32]. However, Pradhan et al. [12] previously reported that ARM390 had no effect on G-protein coupling to DOR in purified membrane preparations of the brain and spinal cord. However, it should be noted that membrane preparations may lack key proteins involved in desensitization [33]. Furthermore, conflicting results have been reported in studies examining this relationship in heterologous expression systems [34-37]. Although these studies measured the same output (changes in adenylate cyclase activity), the differences in results may be attributed to the agonists tested or approaches used to inhibit endocytosis. Other factors may also play a role in the regulation of DOR-mediated responses in the colon. These include the recruitment of, or dissociation from, key signaling molecules at the cell surface [38].

Ultimately, the regulation of DOR-mediated responses observed depends on the neuronal compartment and motor pathways being studied. Moreover, the prolonged effect of SNC80 on EFS-evoked contractions correlates with its sustained inhibition of colonic motor patterns. Therefore, the electrical-stimulation assay may provide the best predictive capacity for the effects of agonists on motility. It is important to note that although ARM390 has maximal efficacy in cell lines endogenously expressing DOR and in behavioral assays measuring anti-nociception [12,32,39], it acts as a partial agonist in both assays used to measure colonic contractility. This may account for its limited effects on colonic motor patterns and suggests that any GI side-effects associated with this compound may be less pronounced.

***Motor pathways develop tolerance to SNC80, but not to ARM390.*** Tolerance can develop following continued exposure to an agonist [4]. Tolerance to clinical opioids does not develop in the colon, leading to persistent and debilitating constipation. Therefore, it is essential to examine whether DOR-targeted therapeutics will have similar properties. To assess tolerance development, DOR agonists were administered *in vivo* and DOR-mediated tissue responses were subsequently measured *in vitro* [7,40].

The acute administration of ARM390 (3 h) was associated with partial internalization of DOReGFP in the soma and proximal neurites of myenteric neurons. However, DOReGFP was confined to the plasma membrane of the soma following the chronic administration of ARM390 (3 d). DOReGFP remained at the surface of nerve fibers innervating the circular muscle for both treatment durations. The significant retention of DOReGFP at the cell surface is consistent with the subsequent activation and magnitude of DOR-mediated responses in both motor pathways. This is also consistent with studies which examined the development of tolerance to the DOR-mediated anxiolytic and anti-depressant effects following exposure to ARM390 and other agonists that weakly internalize DOR [13,16]. However, chronic administration of ARM390 produces analgesic tolerance [13,41]. This suggests that there are region specific differences in the regulation of the same receptor. Alternatively, this difference may arise from the different coupling efficiencies of the agonists used. Furthermore, the same concentration and administration route of ARM390 did not induce internalization at similar time points

in central neurons [12,13]. Receptor trafficking can differ between enteric and central neurons, potentially due to relative expression levels of key regulatory proteins [42].

DOR is trafficked to lysosomes in the soma of myenteric neurons following activation by SNC80 [11]. Acute administration of SNC80 (3 h) promoted DOReGFP internalization in the soma and nerve fibers. These data highlight that GPCR trafficking is a polarized process in enteric neurons and that the rate of endocytosis is reduced in nerve fibers, consistent with observations in central neurons [43]. The prolonged administration of MOR agonists alters receptor trafficking and signaling in enteric neurons [44]. Whether the increase in DOR trafficking in nerve fibers similarly changes signaling in enteric neurons is unknown.

There was a significant increase in DOReGFP located at the cell membrane after chronic administration of SNC80 (3 d) compared to the respective 3 h treatment group. In central neurons, DOReGFP is expressed at the cell surface following 24 h recovery from an acute or chronic administration regime [12,13]. The apparent increase in membrane-associated DOReGFP that we detect following chronic treatment with SNC80 is likely to arise from *de novo* synthesis [11]. The subcellular distribution of DOReGFP is consistent with the development of tolerance to SNC80 in the individual motor pathways. These results indicate that the inhibitory motor pathways likely developed acute tolerance to SNC80 based on a lack of functional receptor available at the soma, whereas DOR agonist-evoked contractions were restored following the chronic administration of SNC80. DOR-mediated effects on EFS-evoked contractions were partially tolerant following both acute (3 h) and prolonged (3 d) SNC80 treatment, potentially possibly due to internalization of receptors on the nerve terminals. These results are summarized in **Fig. 8**.

DOR can be recycled or degraded [45]. Although DOReGFP endocytosis and the development of tolerance are correlated, trafficking and sorting of DOR may influence these effects. Tissues that are treated with DOR agonists that promote recycling are less susceptible to the development of tolerance as compared to those that target DOR for degradation, including SNC80 [46]. The subsequent sorting of DOR following ARM390-induced internalization has not been examined in the ENS.

***SNC80, but not ARM390, has a prolonged inhibitory effect on propagating motor patterns.***

Excitatory and inhibitory motor pathways differ in their relative contribution to the regulation of CMMCs. The release of excitatory neurotransmitters is directly involved in generating CMMCs, whereas the ongoing firing of inhibitory neurotransmitters regulates their timing and frequency [47-49]. Acute (3 h) and chronic (3 d) treatment with ARM390 did not alter the number of CMMCs generated. Partial agonism may account for the negligible effects of ARM390 on CMMC frequency. Acute and chronic treatment with SNC80 significantly reduced CMMC frequency under basal conditions, indicating a prolonged inhibitory effect. This reduction may be mediated by its prolonged effects on excitatory motor pathways. Alternatively, since either an acute or chronic administration of SNC80 internalized DOReGFP in the nerve fibers, there is the possibility that the receptor can continue to signal through endosomes or other intracellular compartments [50,51]. While direct contraction assays on colon segments are effective for the measurement of tolerance to opioids, our data indicate that the capacity of this assay to accurately predict effects on propagating motility is low.

The inhibitory effects of acute SNC80 treatment on CMMC generation were comparatively lower than the effects of loperamide. Loperamide inhibits colonic transit, and in the current study it diminished the number of complex motor patterns. The relative extent to which SNC80 and loperamide inhibit CMMC frequency may be related to the respective distributions of DOR and MOR in the myenteric plexus. Most cholinergic excitatory myenteric neurons express MOR in the mouse colon,

with relatively less coexpression with nNOS-positive inhibitory neurons compared to DOR (DiCello et al. *unpublished*). Thus, loperamide is predicted to inhibit a greater proportion of excitatory neurons involved in generating CMMCs. In contrast, the majority of nitrergic inhibitory neurons express DOReGFP (73 %), whereas a relatively smaller proportion of cholinergic motor neurons express the receptor (14 %; [11]). Therefore, DOR agonists can only *directly* influence activity of 14 % of excitatory neurons, potentially leaving a large proportion available to generate CMMCs

The subsequent exposure of colons isolated from ARM390-pretreated mice (3 h or 3 d) to SNC80 significantly reduced the number of CMMCs compared to their respective controls. These results suggest that functionally competent DOR remained at the plasma membrane. However, equivalent treatment of colons isolated from SNC80 pre-treated mice (3 h and 3 d) with SNC80 had no further effect on CMMC frequency. Although other studies have defined this as tolerance, our data suggest that the drug has reached its maximal inhibitory capacity. Firstly, the number of basal CMMCs generated in the SNC80-pretreated groups (3 h and 3 d) was equivalent to the vehicle pre-treated group following bath exposure to SNC80. Secondly, there is functionally competent DOR present on myenteric neurons from the 3 d SNC80 pre-exposed mice, as DOR-mediated effects on neurogenic contractions were observed.

***Concluding remarks.*** DOR is an emerging therapeutic target for the treatment of chronic pain, depression and anxiety. A better understanding of potential side-effects is essential for drug development and to minimize issues commonly associated with MOR-targeted drugs. DOR agonists have relatively minimal GI motility-associated side-effects in animal-based and clinical studies [8]. In the present study, ARM390 did not directly affect colonic motility, suggesting it may have fewer GI motility-associated side effects relative to other MOR agonists. Moreover, DOR-dependent effects were retained over time, suggesting that ARM390 will not have a sustained negative impact on colonic function. However, some DOR agonists can inhibit colonic motility through central pathways [52]. Other agonists that weakly internalize DOR have no effect on colonic motility *in vivo* [53,54], although a direct correlation between internalization capacity and efficacy is difficult to determine. We propose that these compounds may be acting as partial agonists in the myenteric plexus. Tolerance develops to SNC80 in animal models of pain, anxiety and depression [13]. In marked contrast, SNC80 had a prolonged inhibitory effect on colonic motility, which may be associated with reduced transit. CMMCs were retained in these preparations, suggesting that a full DOR agonist will not promote constipation through a peripheral action. This observation is consistent with Gallantine and Meert [55], who found that the same dose of SNC80 given subcutaneously had reduced motility-associated effects compared to the clinical MOR agonist morphine. Thus, these data correlate with the direct inhibitory effects of SNC80 on colonic motility that we report. However, SNC80 still produces undesirable centrally-mediated side effects including analgesic tolerance. There is still an unmet need for chronic pain therapeutics without adverse and limiting side effects [56]. Taken together, the present study indicates that ARM390 or agonists with similar pharmacological properties, such as TAN67, may be ideal therapeutics for the treatment of pain, depression and anxiety without associated constipating side-effects. The findings of this study also highlight the unique nature of GPCR signaling and regulation in neurons of the ENS compared to the CNS, and the need to characterize emerging therapeutics in the relevant cells and tissues of interest, using the most appropriate assays.

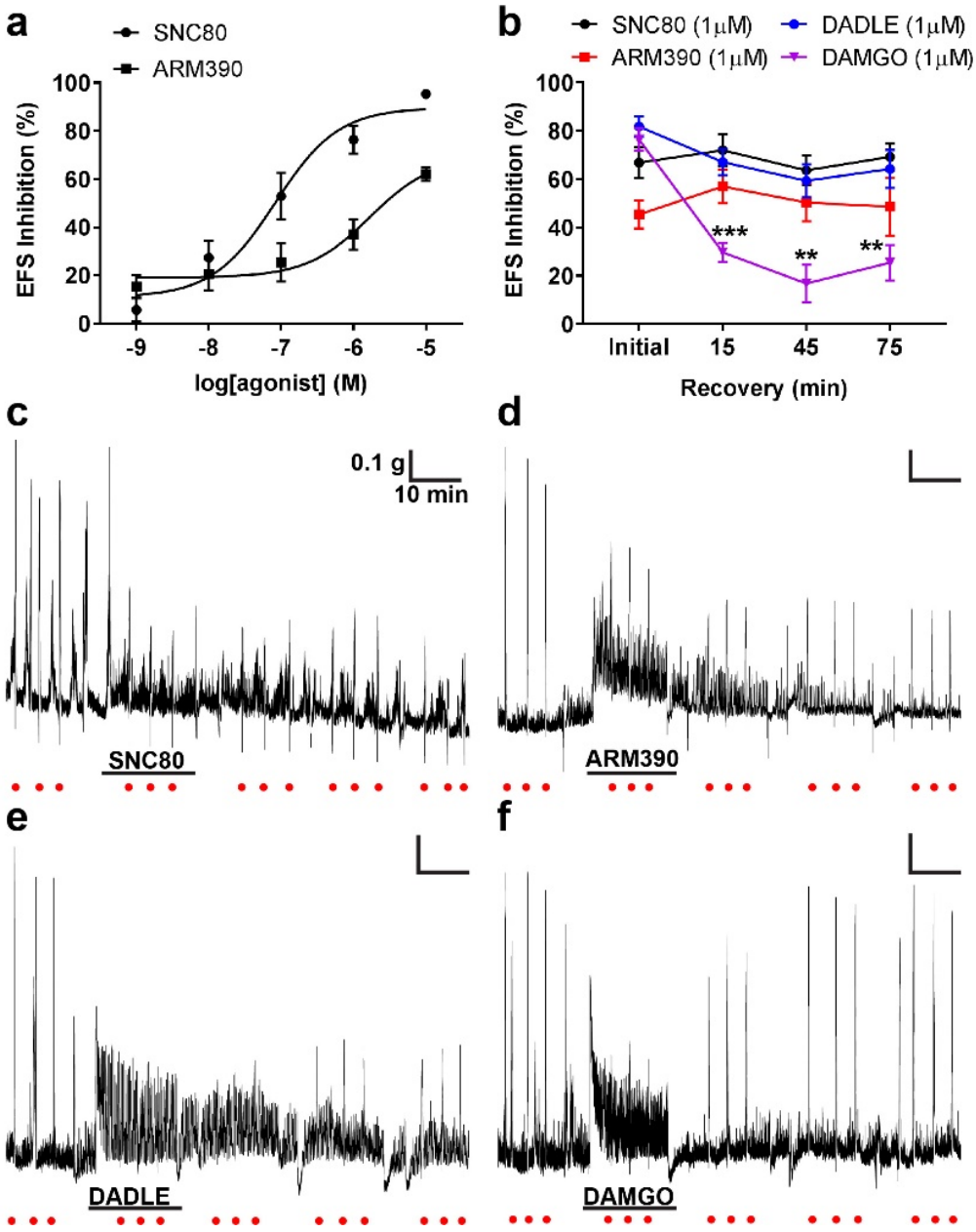



**Fig. 1** DOR agonists have a prolonged inhibitory effect on electrically-stimulated contractions of the colon. (**a**) SNC80 and ARM390 inhibited EFS-evoked contractions of the colon in a concentration-dependent manner. A single addition of the DOR agonists SNC80 (**c**), ARM390 (**d**) or DADLE (**e**) suppressed EFS-evoked contractions for the duration of the experimental time course (75 min). In contrast, EFS-evoked contractions were partially recovered after a 15-minute recovery period following DAMGO exposure (**f**). Arrows indicate drug addition. Red dots represent when EFS was applied. (**b**) Quantitative analysis demonstrating the recovery of EFS-evoked contractions over time. Data points are expressed as mean ± s.e.m., n=4-6 mice per treatment. Statistical comparisons for the SNC80, DADLE and DAMGO data were conducted using one-way repeated measures ANOVA followed by Dunnett's post-hoc test (**p<0.01 and ***p<0.001 compared to the initial response by agonist). Statistical analyses for the ARM390 data were performed using Friedman's test followed by Dunn's post-hoc analysis.

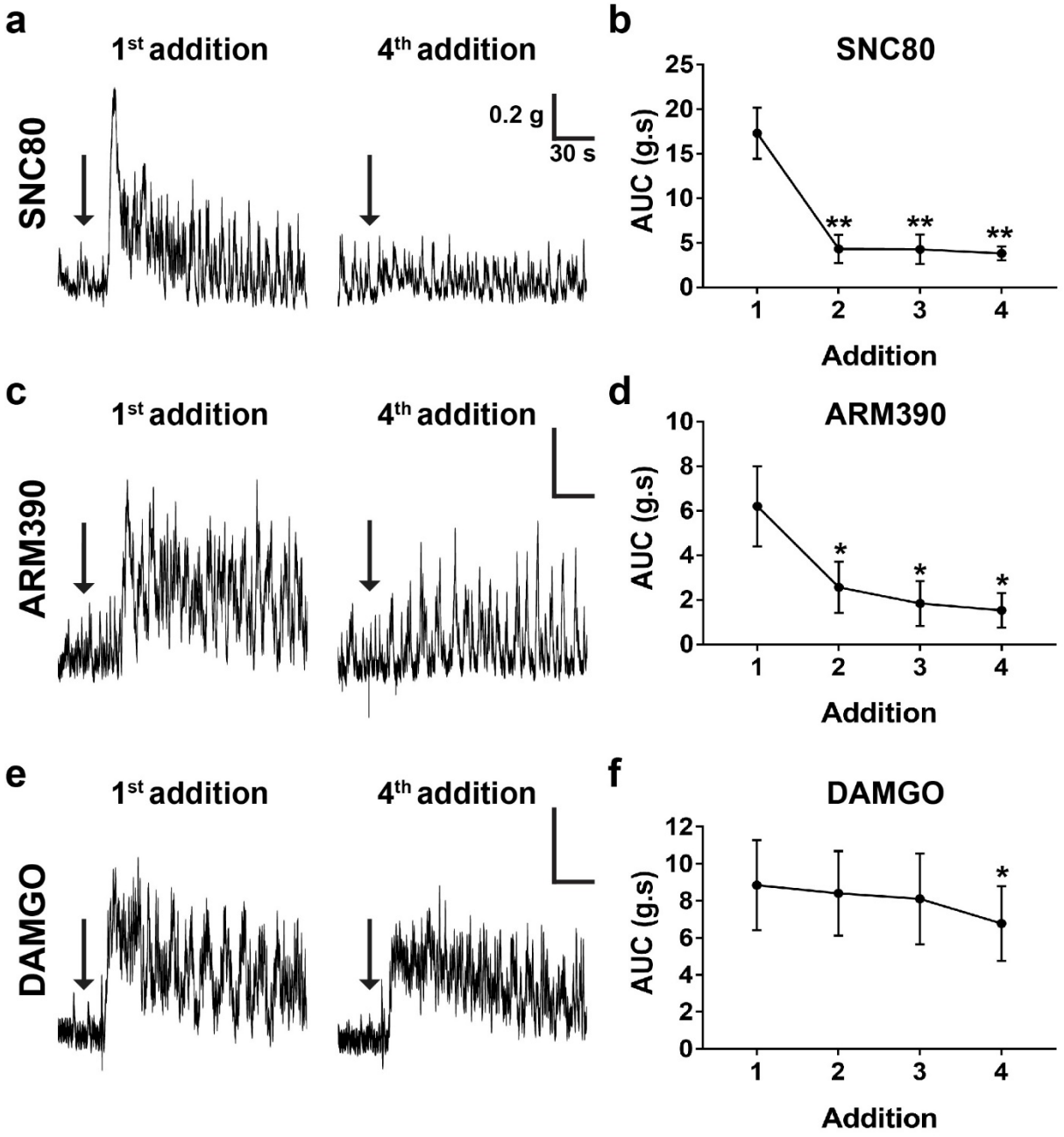



**Fig. 2** DOR agonist-evoked contractions are desensitized. (**a, b**) SNC80- and (**c, d**) ARM390-evoked contractions were significantly reduced following repeated exposures to agonists. (**e, f**) In contrast, contractions evoked by the MOR agonist DAMGO were retained with subsequent DAMGO additions. Arrows indicate agonist addition. Data points represent mean ± s.e.m., n=5 mice per treatment. Statistical comparison performed by one-way repeated measures ANOVA followed by Dunnett's post-hoc test (*$p<0.05$ and **$p<0.01$ compared to the initial addition).

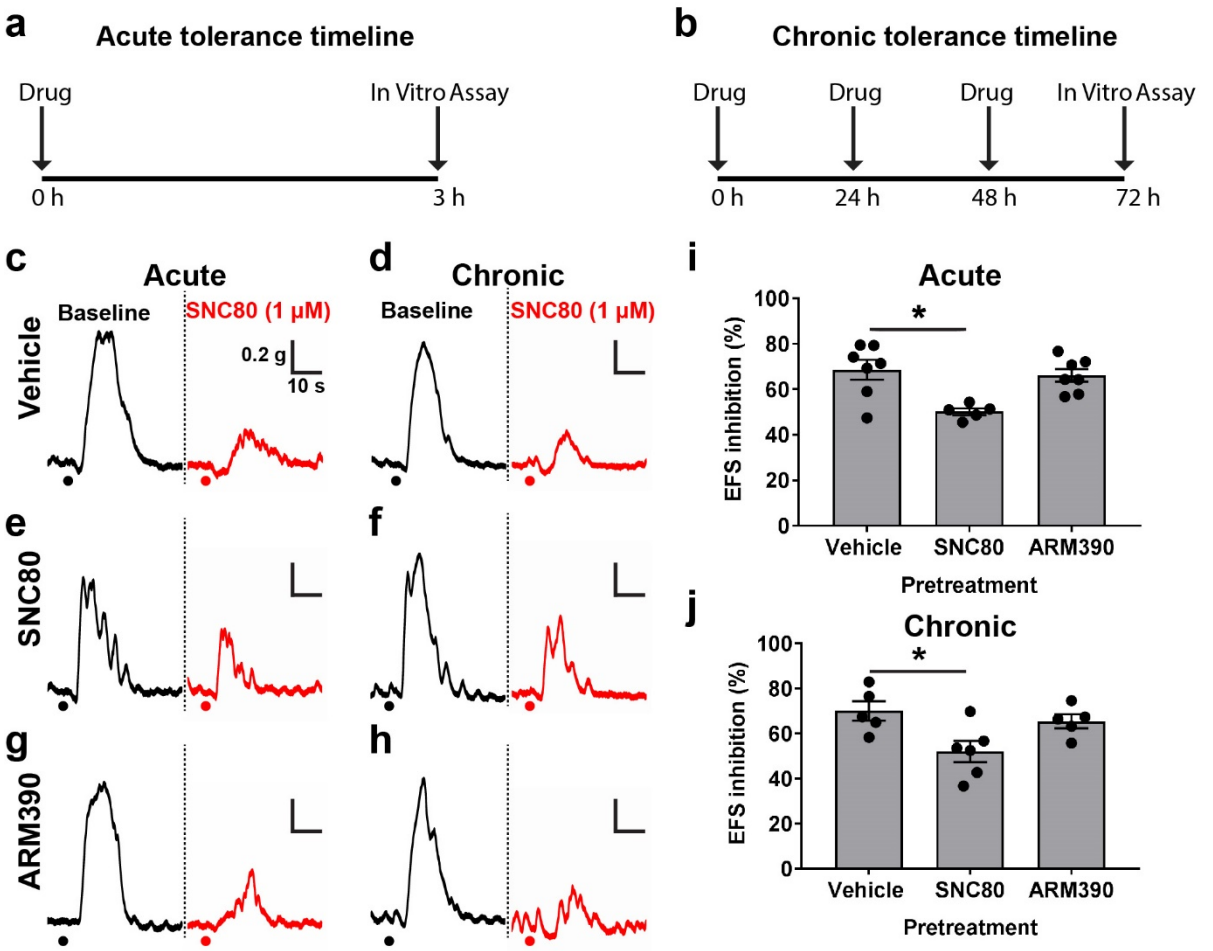



**Fig. 3** Myenteric excitatory motor pathways develop acute and chronic tolerance to SNC80. (**a, b**) Timeline of the acute and chronic tolerance experiments. Bath addition of the DOR agonist SNC80 (1 µM) diminished EFS-evoked contractions in colons from (**c**) acute and (**d**) chronic vehicle-treated mice. Agonist-mediated inhibition of electrically-stimulated contractions was reduced in the (**e**) acute and (**f**) chronic SNC80-pretreated group, indicative of tolerance. SNC80 suppressed EFS-evoked contractions of colons after (**g**) acute and (**h**) chronic exposure to ARM390, indicating that DOR function was retained. Quantitative analysis of the SNC80-evoked reduction in EFS-evoked contractions from the (**i**) acute and (**j**) chronic agonist pre-treated groups. Dots represent where EFS was applied. Data points are expressed as mean ± s.e.m., n=5-13 mice per treatment group. Statistical analyses were conducted using one-way ANOVA followed by Tukey's post-hoc test (*$p<0.05$).

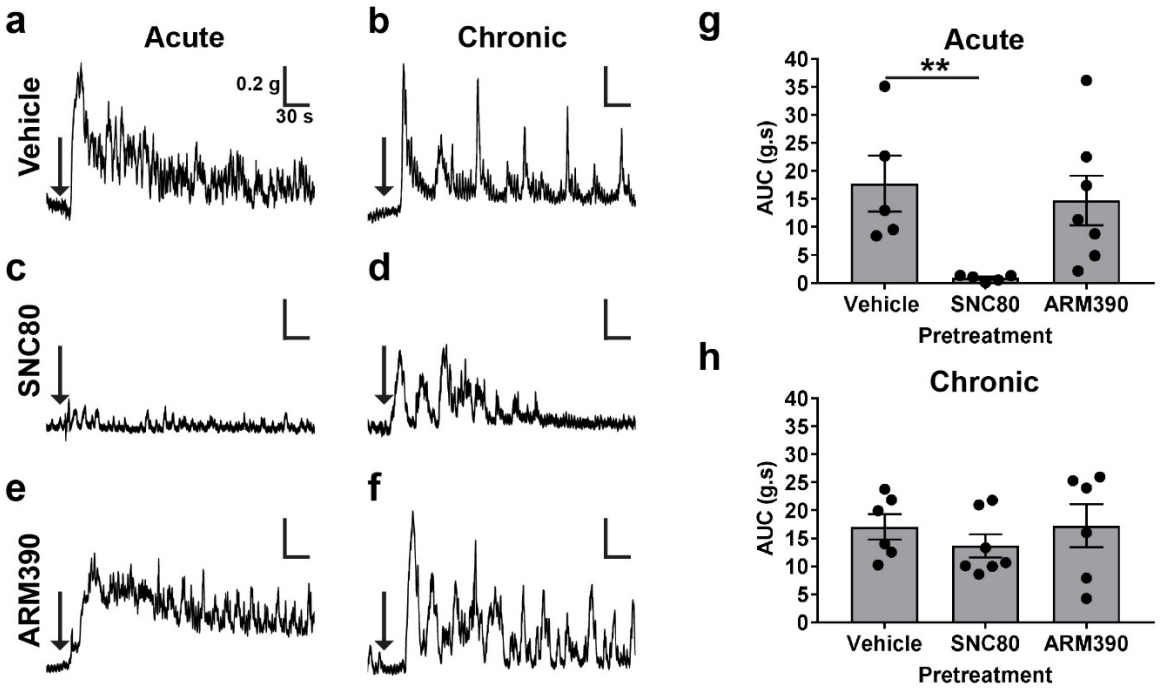



**Fig. 4** Myenteric inhibitory motor pathways develop acute, but not chronic, tolerance to SNC80. The DOR agonist SNC80 (1 μM) evoked a robust contraction in colons following acute (**a**) and chronic (**b**) treatment with vehicle. These effects were abolished in the acute (**c**), but not the chronic (**d**) SNC80 pretreated-group. Application of SNC80 also evoked a robust contraction in the acute (**e**) and chronic (**f**) ARM390-treated group. Quantitative analysis of SNC80-evoked contractions following acute (**g**) or chronic (**h**) drug treatment. Arrows represent the bath addition of SNC80 (1 μM). Data points represent the mean ± s.e.m., n=5-12 mice per treatment group. Acute tolerance data were analyzed by one-way ANOVA followed by Tukey's post-hoc test. Statistical comparisons for the chronic tolerance data were performed using Kruskal Wallis's test followed by Dunn's multiple comparison test (**p<0.01).

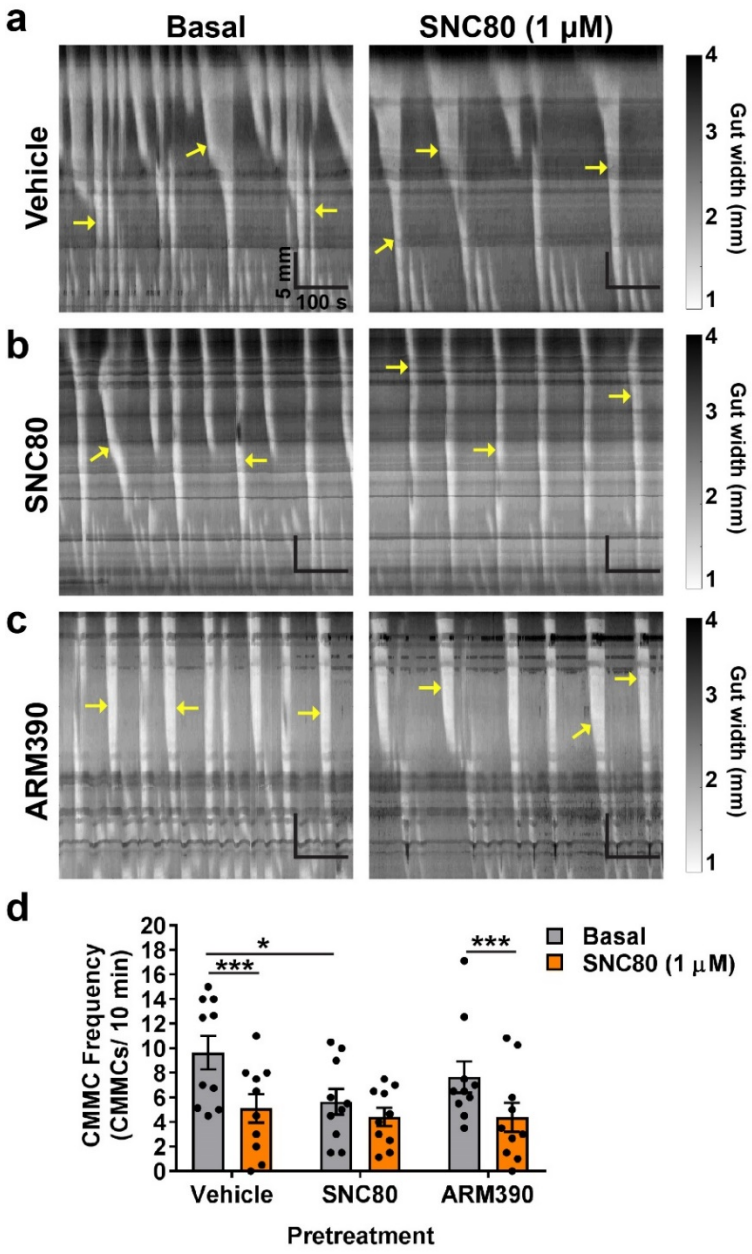



**Fig. 5** Acute administration of SNC80 has a prolonged inhibitory effect on CMMC frequency. (**a**) The number of CMMCs generated in the acute vehicle treated group was diminished after the bath addition of SNC80 (1 µM). (**b**) CMMC frequency was significantly reduced under basal conditions in the acute SNC80-treated group compared to the vehicle-treated group. Subsequent bath application of SNC80 had no further effect on CMMC frequency. (**c**) The number of CMMCs generated in colons from the ARM390-treated group under basal conditions was similar to that in the vehicle-treated group. Application of SNC80 significantly reduced CMMC frequency, indicating that DOR retained function under these conditions. (**d**) Quantitative analysis of the changes in CMMC frequency for each treatment under basal conditions and following bath addition of SNC80. Representative CMMCs are indicated by yellow arrows. Data are expressed as mean ± s.e.m., n=10 mice per treatment group. Statistical analyses were conducted using two-way repeated measures ANOVA. Dunnett's multiple comparison test was used to compare means across treatment groups. Sidak's multiple comparison test was used to compare means within treatment groups (*$p<0.05$ and ***$p<0.001$).

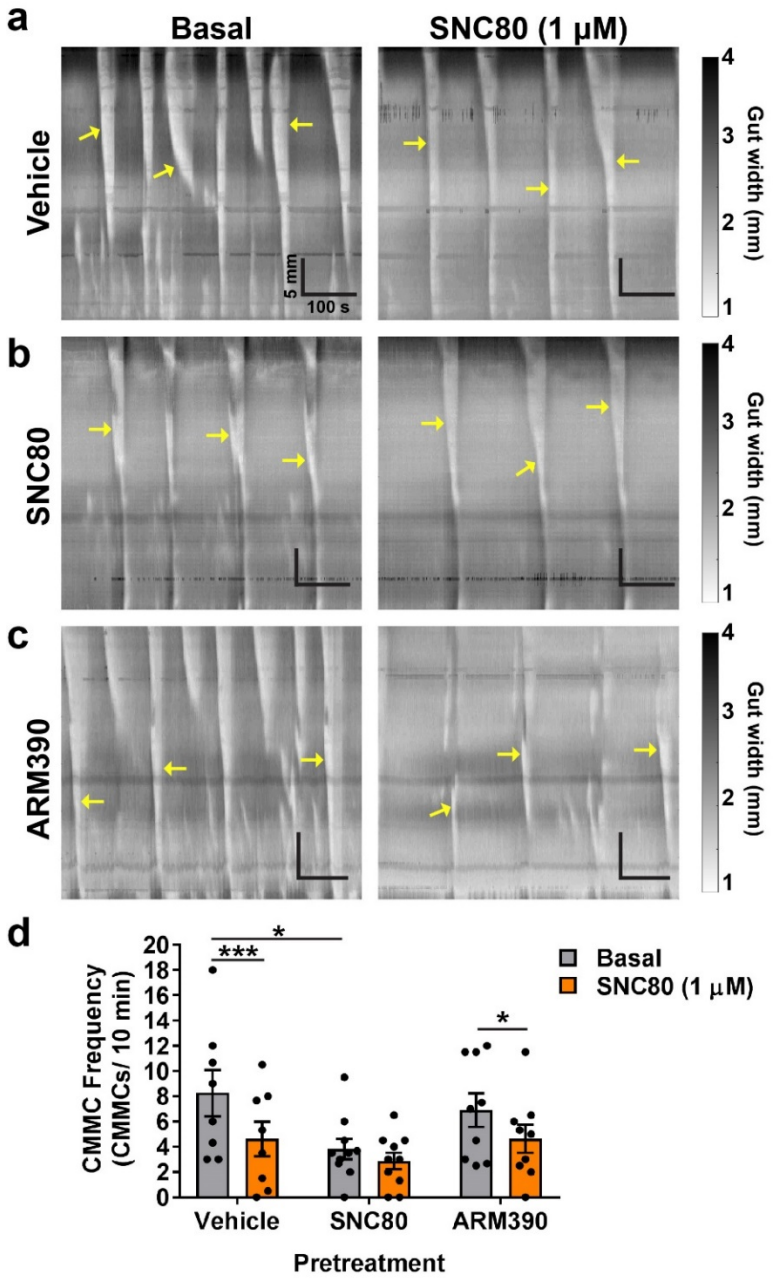



**Fig. 6** The effects of chronic DOR activation on CMMC frequency are agonist-dependent. (**a**) The *in vitro* addition of SNC80 (1 µM) significantly reduced CMMC frequency in the chronic vehicle-treated group. (**b**) The number of CMMCs generated in the chronic SNC80-treated group were significantly lower under basal conditions relative to vehicle. Bath addition of SNC80 had no further effect on CMMC frequency. (**c**) CMMC frequency was similar under basal conditions in the ARM390-treated group compared to the vehicle. Subsequent bath addition of SNC80 significantly reduced CMMC frequency (**d**). Quantitative analysis of the changes in CMMC frequency for each treatment under basal conditions and following bath addition of SNC80. Yellow arrows indicate representative CMMCs. Data are expressed as mean ± s.e.m., n=8-10 mice per treatment group. Statistical analyses were performed using two-way repeated measures ANOVA. Dunnett's multiple comparison test was used to compare means across treatment groups. Sidak's multiple comparison test was conducted to compare means within treatment groups.

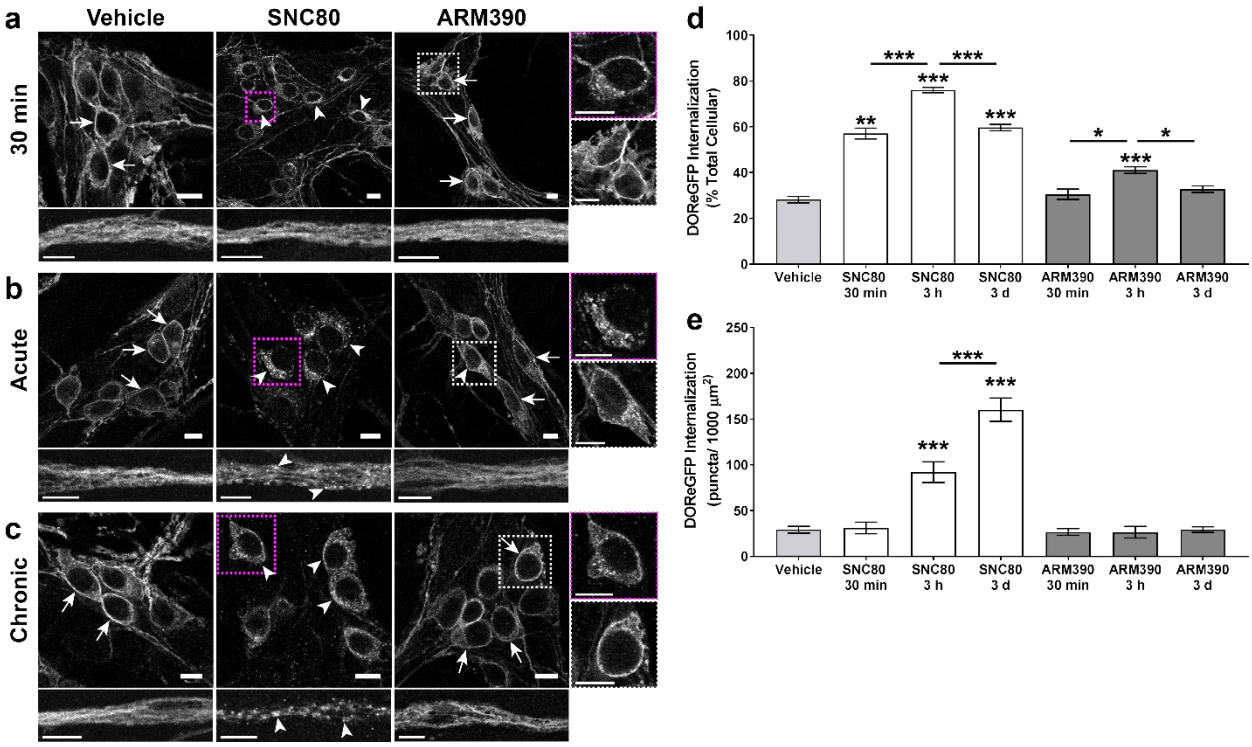



**Fig. 7** DOReGFP trafficking is an agonist and time-dependent process. **(a)** *In vitro* treatment with SNC80 (1 µM), but not ARM390 (1 µM), for 30 min resulted in significant internalization of DOReGFP in the soma of myenteric neurons (arrowheads: internalization, arrows: no internalization). No change in DOReGFP distribution in circular muscle nerve fibers was detected. **(b)** Acute *in vivo* administration of both SNC80 and ARM390 (10 mg/kg, 3h) resulted in significant redistribution of DOReGFP to punctate endosome-like structures. SNC80, but not ARM390, exposure promoted endocytosis in circular muscle nerve fibers (arrowheads). **(c)** Chronic *in vivo* administration of SNC80, but not ARM390 (both 10 mg/kg, 3 d), was associated with robust DOReGFP internalization in both the soma and nerve fibers of myenteric neurons. **(d)** Graph summarizing the subcellular distribution of DOReGFP in myenteric neurons under the different treatment conditions tested. (**e)** Pixel densitometry data measuring DOReGFP subcellular distribution in circular muscle nerve fibers under the different treatment conditions. Scale: 10 µm. Data are expressed as mean ± s.e.m., n=3-5 mice per treatment group. Statistical comparisons performed using Kruskal Wallis's test followed by Dunn's multiple comparison test (**p<0.01, ***p<0.001).

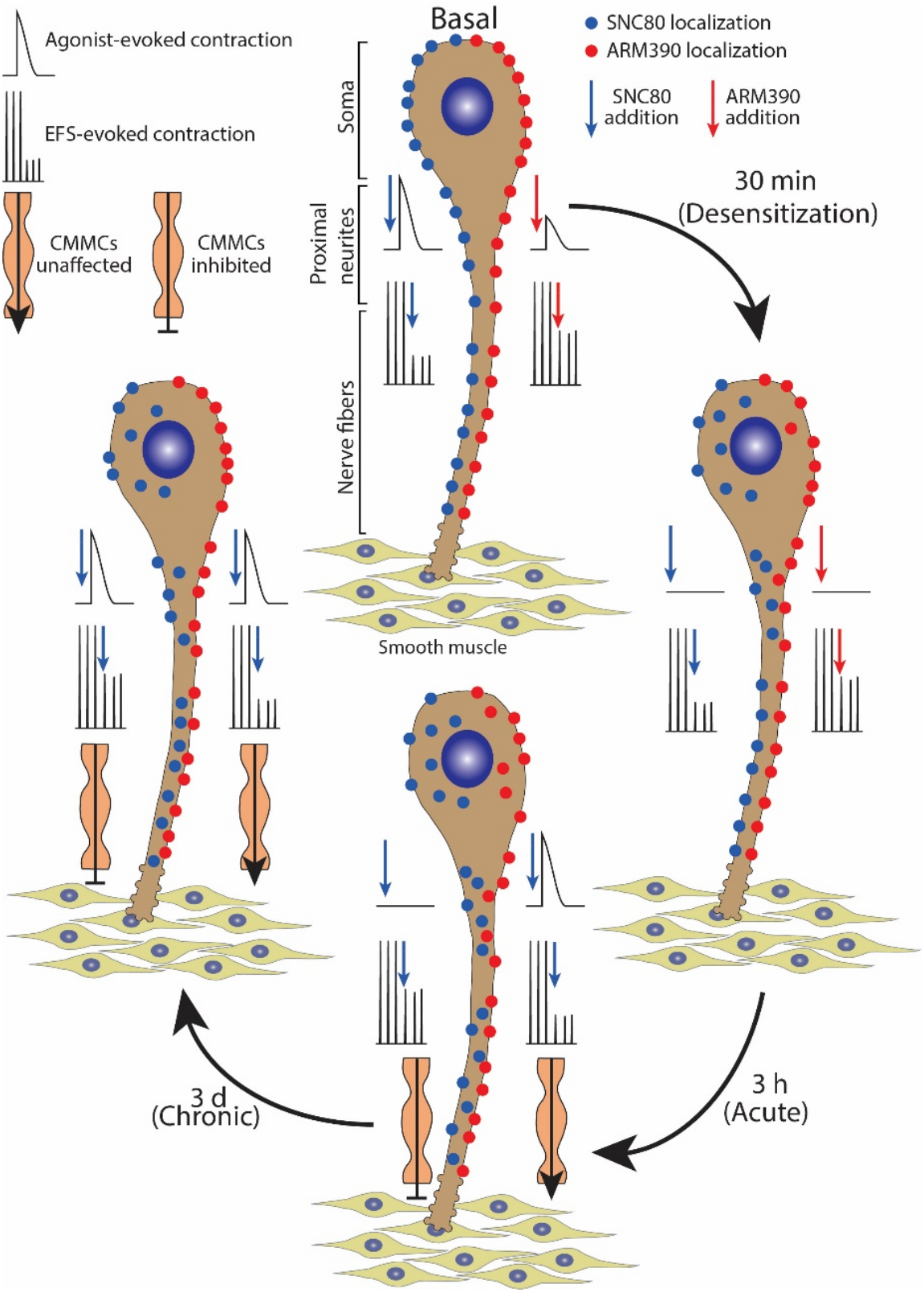



**Fig 8** Schematic summarizing the link between DOReGFP trafficking and the regulation of DOR agonist-mediated effects on neuromuscular transmission in the colon. Under basal conditions, DOReGFP was mainly localized to the plasma membrane and the addition of a DOR agonist produced a robust functional response. After 30 min, SNC80 promoted internalization of DOReGFP in the soma

and proximal neurites, while ARM390 had no effect. Functional responses to both ARM390 and SNC80 were desensitized in inhibitory motor pathways, whereas they retained their efficacy in excitatory pathways. This demonstrates that DOR endocytosis is not a prerequisite for the desensitization of agonist-mediated responses. After an acute exposure (3 h) to SNC80, DOReGFP was significantly internalized in all compartments of the neuron. In contrast, a significant proportion of total DOReGFP remained at the plasma membrane following acute ARM390 treatment. The internalization profile correlated with the susceptibility to tolerance development in assays using muscle strips. Agonist-mediated internalization also correlated with prolonged inhibitory effects on motility. DOReGFP was significantly internalized in both the soma and nerve fibers following chronic treatment (3 d) with SNC80. However, there was a significant increase in DOReGFP associated with the plasma membrane of the soma compared to the respective acute treatment group. In addition, DOReGFP internalization was more pronounced in the nerve fibers compared to the acute SNC80-treatment group. DOReGFP was retained at the cell surface following chronic treatment with ARM390. DOR internalization was correlated with both the development of tolerance in assays using muscle strips and the sustained inhibition of colonic motility.

**Table 1.** Quantitative analysis of myenteric neuronal populations following chronic treatment with DOR agonists.

| | Hu/ GFP (%) | GFP/ Hu (%) | NOS/ GFP (%) | GFP/ NOS (%) | Hu/ NOS (%) |
|---|---|---|---|---|---|
| Vehicle | 43.8 ± 3.9 | 100 ± 0 | 73.6 ± 1.7 | 70.9 ± 6.0 | 41.8 ± 2.3 |
| SNC80 (10 mg/kg, i.p.;3 d) | 49.2 ± 1.9 | 100 ± 0 | 70.9 ± 6.0 | 63.6 ± 1.3 | 40.7 ± 0.7 |
| ARM390 (10 mg/kg, p.o.; 3 d) | 45.9 ± 1.3 | 100 ± 0 | 75.2 ± 2.3 | 67.4 ± 0.9 | 41.2 ± 1.0 |

Numbers: Vehicle: 1091 neurons examined from 3 mice; SNC80: 2107 neurons examined from 5 mice; ARM390: 1703 neurons examined from 5 mice.

Hu/ GFP represents the percentage of all Hu-positive neurons that is also DOReGFP-positive.

GFP/ Hu represents the percentage of DOReGFP-positive neurons that is also Hu-positive.

NOS/ GFP represents the percentage of NOS-positive neurons that is also DOReGFP positive.

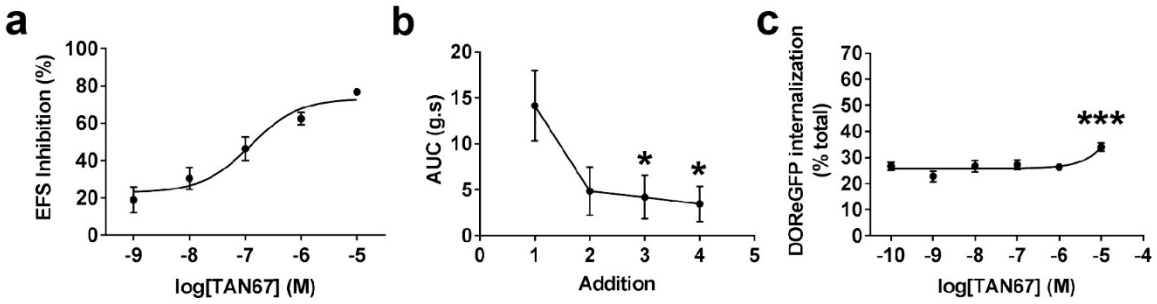
GFP/ NOS represents the percentage of GFP-positive neurons that is also NOS positive.

Hu/ NOS represents the percentage of Hu-positive neurons that is also NOS positive.

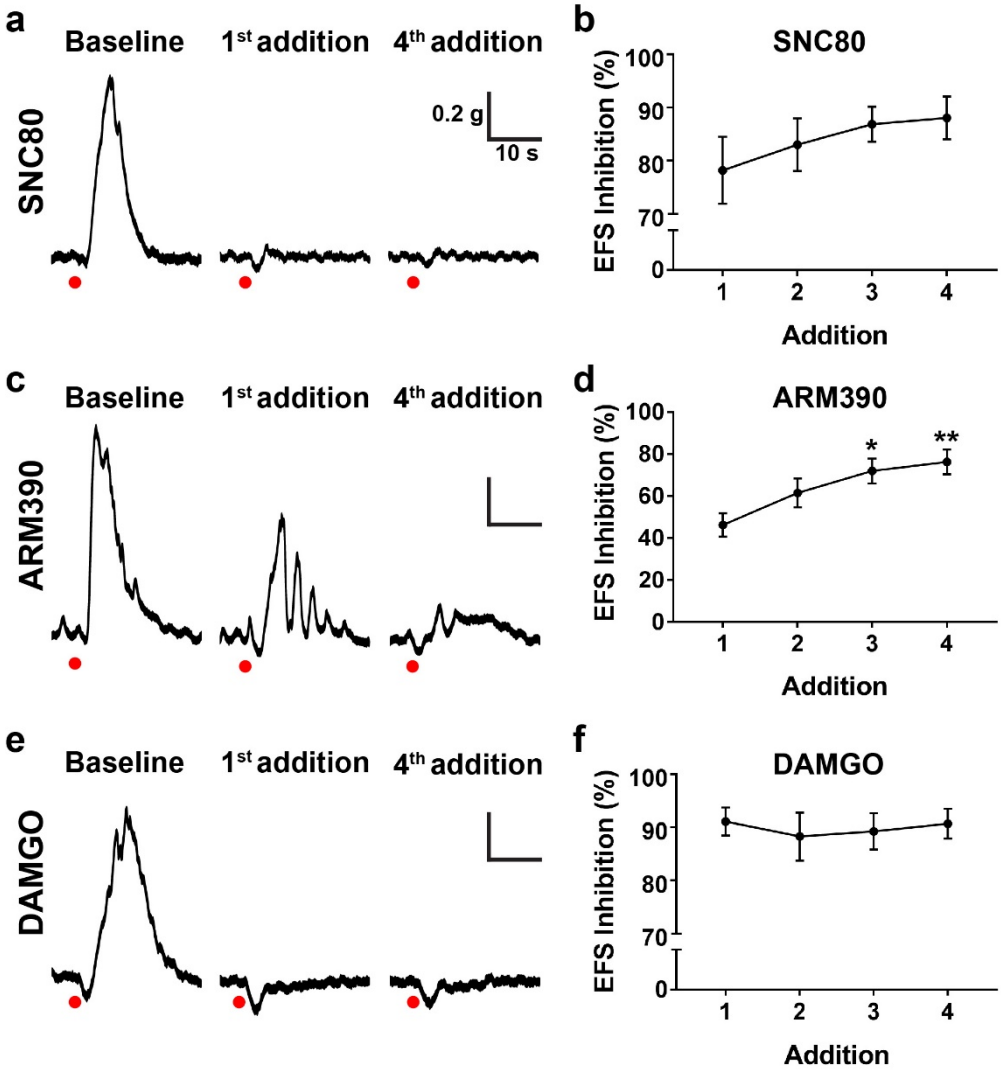
Data were compared across treatment conditions using a one-way ANOVA followed by Tukey's multiple comparison test.

**Supplementary Table 1.** Summary of tolerance generation for different opioid receptor agonists under the assay conditions used in this study.

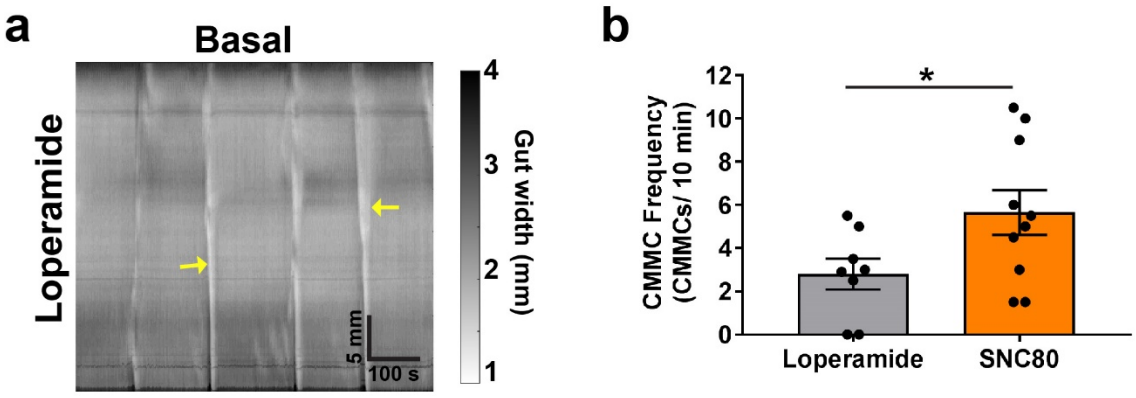
| | Agonist treatment | 30 min | Acute | Chronic |
|---|---|---|---|---|
| Excitatory motor pathways *(Inhibition of EFS-evoked contraction assay)* | SNC80 | No recovery | Partial tolerance | Partial tolerance |
| | ARM390 | No recovery | No tolerance | No tolerance |
| | DAMGO | Recovered/No Desensitization | | |
| Inhibitory motor pathways *(Direct agonist-evoked contraction assay)* | SNC80 | Desensitized | Tolerance | No tolerance |
| | ARM390 | Desensitized | No tolerance | No tolerance |
| Whole colon motility (*Inhibition of CMMCs*) | SNC80 | | Prolonged | Prolonged |
| | ARM390 | | No tolerance | No tolerance |
| | Loperamide | | Prolonged | |




**Supplementary Fig. 1** TAN67 acts as a partial DOR agonist in the colon. (**a**) TAN67 inhibited EFS-evoked contractions in a concentration-dependent manner. (**b**) TAN67-evoked contractions were desensitized following repeated exposures to agonist. (**c**) TAN67 (1 nM- 10 μM) only weakly internalized DOReGFP in myenteric neurons (n=21-52 neurons from 3-5 mice). Data are expressed as mean ± s.e.m.; N=5-6 mice for tissue contraction experiments. Statistical comparison performed by one-way repeated measures ANOVA followed by Dunnett's post-hoc test (*$p<0.05$ and ***$p<0.01$).




**Supplementary Fig. 2** Prolonged inhibitory effects of DOR and MOR agonists on electrically-stimulated contractions of the colon. (**a, b**) EFS-evoked contractions remained suppressed after each subsequent exposure to SNC80. (**c, d**) The efficacy at which ARM390 inhibited electrically-stimulated contractions significantly increased at the third and fourth addition. (**e, f**) DAMGO maintained its ability to diminish EFS-evoked contractions at each subsequent exposure. Arrows indicate where EFS was applied. Data points are expressed as mean ± s.e.m., n=5-7 mice per treatment. Statistical analyses for the SNC80 and DAMGO data were conducted using a one-way repeated measures ANOVA followed by Dunnett's post-hoc test. Statistical analyses for the ARM390 data were performed using Friedman's test followed by Dunn's post-hoc analysis (*$p<0.05$ and **$p<0.01$ compared to 1st addition).




**Supplementary Fig. 3** An acute treatment (3 h) with the MOR agonist loperamide reduced CMMC frequency. (**a, b**) Relatively fewer CMMCs were generated in the loperamide-treated group compared to the equivalent acute SNC80-treated group under basal conditions. The data set presented for the SNC80-pretreated group was taken from **Fig. 5d**. Yellow arrows indicate representative CMMCs. Data are expressed as mean ± s.e.m., n=8-10 mice per treatment group. Statistical analysis was conducted using the Student's t-test (*$p<0.05$).

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# Chapter 4
# *Clathrin and GRK2/3 inhibitors block delta opioid receptor internalization in myenteric neurons and inhibit neuromuscular transmission in the colon.*

Clathrin and GRK2/3 inhibitors block delta opioid receptor internalization in myenteric neurons and inhibit neuromuscular transmission in the colon.

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Running Title: *Endocytic inhibitors suppress enteric neuron function*

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**Key Words**: G protein-coupled receptor (GPCR), opiate opioid, clathrin, dynamin, receptor endocytosis, receptor internalization, neuroscience, neurotransmitter receptor

**Abstract:** Endocytosis is a major mechanism through which cellular signaling by G protein-coupled receptors (GPCRs) is terminated. However, recent studies demonstrate that GPCRs are internalized in an active state and continue to signal from within endosomes, resulting in effects on cellular function that are distinct to those arising at the cell surface. Endocytosis inhibitors are commonly used to define the importance of GPCR internalization for physiological and pathophysiological processes. Here we provide the first detailed examination of the effects of these inhibitors on neurogenic contractions of gastrointestinal smooth muscle; a key preliminary step to evaluate the importance of GPCR endocytosis for gut function. Inhibitors of clathrin-mediated endocytosis (PitStop2: PS2) or GRK2/3-dependent phosphorylation (Takeda compound 101: Cmpd101), significantly reduced GPCR internalization. However, they also attenuated cholinergic contractions through different mechanisms. PS2 abolished contractile responses by colonic muscle to SNC80 and morphine, which strongly and weakly internalize delta and mu opioid receptors, respectively. PS2 did not affect the increased myogenic contractile activity following removal of an inhibitory neural influence (tetrodotoxin) but suppressed electrically-evoked neurogenic contractions. $Ca^{2+}$ signaling by myenteric neurons in response to exogenous ATP was unaffected by PS2, suggesting inhibitory actions on neurotransmitter release, rather than neurotransmission. In contrast, Cmpd101 attenuated contractions to the cholinergic agonist carbachol, indicating direct effects on smooth muscle. We conclude that although PS2 and Cmpd101 are effective blockers of GPCR endocytosis in enteric neurons, these inhibitors are unsuitable for the study of neurally-mediated gut function due to their inhibitory effects on neuromuscular transmission and smooth muscle contractility.

G protein-coupled receptors (GPCRs) enable cells to detect and respond appropriately to changes in their extracellular environment. GPCRs represent a significant therapeutic target, with 30%

of currently approved drugs targeting this family of receptors (1). Traditionally, GPCRs have been described as proteins that transduce signals from the cell surface. These signals are effectively terminated by two major mechanisms: receptor desensitization and endocytosis. The demonstration that activated GPCRs can continue to signal from within endosomes, resulting in cellular responses that are spatially and temporally distinct to those derived from the cell surface represents a major conceptual advancement to the field of GPCR biology. In this model, internalized activated GPCRs form endosomal signaling complexes known as 'signalosomes' that elicit signals distinct from those initiated at the plasma membrane. This '*positional dynamism*' defines both the nature and duration of the ensuing cellular signaling events downstream of receptor activation (2,3). Intracellular GPCRs can drive unique signaling events and may play a potential role in pathophysiology or represent a distinct therapeutic target. For example, '*location bias*' has been described for endogenous opioid peptides and their synthetic derivatives, which can activate the mu and delta opioid receptors (MOR and DOR, respectively) at the cell surface to promote internalization of activated receptors into endosomes. In contrast, clinically important opioid drugs. such as morphine, can cross the plasma membrane to directly activate MOR in the Golgi network. What remains to be defined are the implications that potential activation of three spatially distinct signaling pathways downstream of the same GPCR could have on neuronal functions, such as the differential control of neuronal excitability (4). Similar differences between the relative accessibility of endogenous ligands and drugs to preformed receptors at the Golgi apparatus have been shown for the β1 adrenergic receptor (5). Location bias has also been described for mGluR5, which is localized to both the cell surface and to intracellular membranes in dorsal horn neurons of the spinal cord. Neuropathic pain induced by nerve injury was associated with increased mGluR5 at the nuclear membrane of these neurons. Inhibition of nuclear mGluR5, but not cell surface receptor, was effective at attenuating pain behavior and related cellular signaling (6). These studies demonstrate that pharmacological targeting of GPCR signaling at specific subcellular locales may prove to be an effective and novel therapeutic approach.

Recent studies have defined the importance of endosomal signaling to physiological and pathophysiological processes, including acute pain. This has significant implications for therapy, and the potential for selectively targeting endosomal GPCR (eGPCR) signaling for the control of pain has recently been highlighted. In these studies, small molecule inhibitors of endocytosis and delivery of lipidated antagonists to endosomes was effective in suppressing acute mechanical algesia in mice (7-9), with the conclusion that endosomal signaling by pronociceptive GPCRs drives prolonged pain behavior. Whether eGPCR signaling similarly contributes to the enteric neuronal hyperexcitability and associated gastrointestinal (GI) dysfunction that occurs following inflammation or infection of the intestine (10) is presently unknown.

Classically, the primary function of endocytosis is to effectively terminate downstream signaling through sequestration of the GPCR away from the cell surface. Activated GPCRs are phosphorylated by G protein-coupled receptor kinases (GRKs) to promote beta arrestin (βArr) recruitment and subsequent internalization of receptors through an AP2-, clathrin- and dynamin-dependent mechanism. It is now appreciated that internalization of GPCRs is required for the full signaling repertoire and that some specific signals depend on receptors forming additional signaling platforms in intracellular compartments. Key proteins essential for effective endocytosis can be targeted genetically or through use of small molecule inhibitors. The processes that these proteins control include GRK-dependent phosphorylation, βArr interaction with clathrin adaptor protein AP2, dynamin GTPase activity, and clathrin coated pit formation. The importance of endocytosis for the control of enteric neuron function is unknown beyond its role in receptor desensitization and resensitization. Endocytic inhibitors have been used to examine mechanisms of agonist-evoked GPCR endocytosis and GPCR-mediated signaling in enteric neurons (11-14). However, at present there has been very limited examination of the role that internalized GPCRs may play in gut function, or of eGPCR signaling in enteric neurons in general. Agonist-evoked internalization of the neurokinin 1 receptor ($NK_1R$) in myenteric neurons leads to endosomal signaling

through MAPK, which is sustained if endosomal retention of the receptor is favored (12,15). Although these studies demonstrated that the endosomal endopeptidase endothelin converting enzyme 1 (ECE-1) controls the duration of this signaling and subsequent $NK_1R$ recycling and resensitization at the cell surface, the role of receptor endocytosis in physiologically important neurogenic processes in the GI tract, such as the coordination of motility, remains undetermined.

In the present study we have examined the utility of small molecule inhibitors of clathrin and GRK2/3 to determine the importance of endocytosis for the actions of GPCRs on intestinal motility, the primary function of myenteric neurons. We have focused on MOR and DOR as representative GPCRs due to existing knowledge about the trafficking and function of these receptors in the enteric nervous system (ENS) (16-19). MOR and DOR are established and emerging targets for the treatment of moderate to severe pain, respectively, and clinically important opioids, such as morphine, can promote severe intractable constipation through MOR activation on enteric neurons (19). MOR and DOR share a common mechanism of action in the ENS. Both are $G_i$-coupled receptors, inhibit electrically-stimulated contractions, and their respective agonists evoke contractions when directly applied to intestinal tissue (16,20-22). MOR and DOR expression in the colon is almost exclusively restricted to enteric neurons (17,18,23) and the effects of both MOR and DOR agonists on colonic motility are mediated through a neurogenic mechanism. Finally, agonists that robustly or weakly promote MOR or DOR endocytosis in myenteric neurons have been identified (17,18,24,25). Thus, examination of opioid receptors provides an ideal opportunity to determine the functional importance of GPCR endocytosis in the control of neuromuscular transmission in the GI tract. As a first and fundamental step towards defining the importance of GPCR internalization in enteric neurotransmission, the suitability of established endocytosis inhibitors for use in standard assays of GI motility must be determined. This study has examined the effectiveness of small molecule inhibitors of clathrin and GRK2/3 to inhibit agonist-evoked DOR internalization, and their potential impact on the neurogenic control of intestinal smooth muscle contractions. We demonstrate that although these compounds can inhibit GPCR endocytosis in enteric neurons, significant effects on neuromuscular transmission and direct actions on smooth muscle can confound interpretation of the functional role that GPCR endocytosis plays in GI motility.

**Results.**

PS2 is a tool compound that is routinely used to examine the importance of clathrin and the process of endocytosis for cell surface protein function. PS2 complexes with the terminal domain of clathrin, preventing interaction with endocytic ligands containing clathrin box motifs (26). Recently, PS2 has enabled determination of the relative contribution of intracellular signaling for physiological and pathophysiological effects of GPCR activation (7,27). Cmpd101 is a GRK2/3 inhibitor that has been used to inhibit mu opioid receptor internalization and desensitization in model cell lines and neurons (28,29). In the present report we determined the potential utility of these small molecule inhibitors for the study of the neurogenic actions of GPCRs on GI functions.

***DOReGFP endocytosis is significantly inhibited by clathrin and GRK2/3 inhibition.*** To assess the effectiveness of PS2 and Cmpd101 in inhibiting native GPCR endocytosis in myenteric neurons we examined SNC80-evoked redistribution of DOR using colon tissue from knockin mice expressing DOR tagged with a C-terminal eGFP (DOReGFP). Under basal conditions DOReGFP was localized to the cell surface of a subset of myenteric neurons (% cell surface associated: mean ± s.e.m 78.6% ± 1.1, 95% confidence interval: 76.5-80.8; n=70 neurons; **Fig. 1A, B**). Treatment with PS2 (15 μM) or Cmpd101 (10 μM) alone did not significantly alter the subcellular distribution of DOReGFP under basal conditions (*PS2*: 80.7% ± 1.3, 78.1-83.2; n=35; *Cmpd101*: 82.2% ± 0.7, 80.7-83.7, n=62). Exposure to the DOR agonist SNC80 (1 μM, 30 min, $EC_{80}$ concentration) promoted significant redistribution of DOReGFP from the cell surface to endosome-like structures (49.5% ± 1.02, 47.5-51.6; n=106 neurons; $p<0.001$). PS2 pretreatment significantly diminished DOReGFP internalization to SNC80 (79.8% ± 0.8, 78.2-81.4; n= 127; $p<0.001$). The inactive control for PS2 (PS2i, 15 μM: 44.7% ± 1.0, 42.7-46.8; n= 66; $p=0.12$) did not

significantly affect SNC80-induced DOReGFP endocytosis. Inhibition of GRK2/3 with Cmpd101 also significantly attenuated SNC80-evoked DOReGFP endocytosis (69.5% ± 1.7, 66.3-72.8; n=101, p<0.001).

***Inhibitory neurotransmission is unaffected by endocytic inhibitors.*** The resting tone of the colon is maintained by nitrergic inhibitory motor neurons. Disinhibition of this input results in a sustained contraction of the external smooth muscle layers (30). The basal tone of the colon was unaffected by the addition of either PS2 or Cmpd101, indicating that the inhibitory input remained intact (**Fig. 2A-C**). Removal of this inhibition by the neurotoxin tetrodotoxin (TTX) resulted in a tonic contraction (0.12 ± 0.04 g, n=6; **Fig. 2A, D**), which was unaffected by PS2 (0.24 ± 0.09 g, n=6; **Fig. 2B, D**) and Cmpd101 (0.23 ± 0.13 g, n=6; **Fig. 2C, D**). This confirms that these compounds have no detectable influence on inhibitory neuromuscular transmission.

***PS2 and Cmpd101 reduce cholinergic contractions through distinct mechanisms.*** Transmural electrical field stimulation (EFS) produces a neurogenic contraction which is mediated primarily by the release of acetylcholine (ACh) and activation of muscarinic receptors on GI smooth muscle cells. Inhibition of electrically-evoked intestinal contractions is commonly used to characterize opioid receptor agonists. We examined the effect of PS2 and Cmpd101 on the amplitude of EFS contractions to assess their suitability for use in this assay. Both PS2 (39.7 ± 3.6 % of baseline EFS, n=8) and Cmpd101 (46.9 ± 8.7 %, n=6) significantly suppressed EFS-evoked contractions compared to vehicle (88.7 ± 8.8 %, n=6; p<0.001 and p<0.01, respectively) (**Fig. 3A, C-E**). PS2i, the inactive control for PS2, did not affect EFS-evoked contractions (91.9 ± 8.5 %, n=6; **Fig. 3B & E**). In contrast to Cmpd101, PS2 produced a graded reduction in contraction amplitude with each successive electrical stimulus (1$^{st}$ stimulation: 64.1 ± 4.8 %, 2$^{nd}$: 37.1 ± 4.3 %, p<0.001 and 3$^{rd}$: 26.0 ± 4.9 %, p<0.001; **Fig. 3E**). This is consistent with depletion of existing stores of ACh and a reduced capacity to effectively replace vesicular stores. These observations indicate that both PS2 and Cmpd101 are unsuitable for examination of GPCR function in assays involving assessment of electrically-evoked contractions.

The direct actions of endocytic inhibitors on smooth muscle were measured using carbachol, which produced robust concentration-dependent contractions ($pEC_{50}$= 5.96 ± 0.37, $E_{max}$= 1.77 ± 0.35 g, n=5; **Fig. 4A, D**). These responses were unaffected by PS2 ($pEC_{50}$= 5.67 ± 0.18, $E_{max}$= 1.52 ± 0.16 g, n=6; **Fig. 4B, D**), but were significantly decreased by Cmpd101 ($pEC_{50}$= 5.37 ± 0.35, $E_{max}$= 0.76 ± 0.20 g, n=5, p=0.02; **Fig. 4C, D**). Together these findings suggest that PS2 and Cmpd101 exert anticholinergic effects through distinct mechanisms; while PS2 reduces cholinergic contractions via a neurogenic mechanism, Cmpd101 exerts its inhibitory effects, in part, through actions on muscarinic signaling in smooth muscle cells.

***PS2 does not affect the activation of myenteric neurons.*** The inhibition of EFS-evoked contractions by PS2 could be due to a general inhibitory or deleterious effect on myenteric neuron function, or through decreased synaptic vesicle formation and vesicular release of excitatory neurotransmitters (31). To determine the mechanism of action, the potential inhibitory effects of PS2 on myenteric neurons were examined using $Ca^{2+}$ imaging of myenteric wholemount preparations. Preparations were challenged with ATP, which activates both GPCRs (P2Y) and ion channels (P2X) (32). The effects of PS2 and PS2i on ATP-evoked (100 µM) increases in intracellular $Ca^{2+}$ levels in neurons and glia were examined. PS2 or PS2i did not significantly alter the peak amplitude of ATP responses by neurons (N= 333) and glia (N= 517) relative to vehicle controls (**Fig 5A-C**, n=3-4 mice per group). The PGP9.5-positive nerve fibers associated with the circular muscle layer also responded to ATP in the presence of PS2 (**Fig 5D**). These data demonstrate that PS2-dependent inhibition of neurogenic contractions is unlikely to be through adverse effects on neuronal activation or neuronal viability.

***PS2 reduces GPCR-mediated responses via an internalization-independent mechanism.*** PS2 has been used to investigate GPCR internalization and signaling in recombinant cell systems and in natively expressing cells and tissues (7,13,27). We have demonstrated that PS2 alone does not

influence contractions to TTX. Like TTX, opioid receptor agonists produce a neurogenic smooth muscle contraction which is mediated via the disinhibition of the resting tone (16,33). Thus, we hypothesized that contractile responses to both internalizing and non-internalizing opioid receptor agonists would be similarly unaffected by PS2. To test this prediction, the functional effects of PS2 on GPCR-mediated responses of the colon were examined using SNC80 and morphine, which were chosen due to their markedly different abilities to promote opioid receptor endocytosis. SNC80 strongly internalizes DOR (16,18), whereas acute exposure to morphine activates MOR signaling in myenteric neurons without promoting receptor endocytosis (11,24,34). SNC80 elicited sustained contractions in a concentration-dependent manner. These responses were biphasic with a maximum response at 100 nM (0.09 ± 0.03 g, n=11). PS2 effectively abolished these responses (**Fig. 6B, Fig. S1**). Morphine (1 μM) also evoked a rapid and sustained contraction (0.14 ± 0.04 g, n=6) which was significantly reduced by PS2 (0.03 ± 0.04 g, n=6, $p<0.05$; **Fig. 6A, B**). The effects of PS2 on opioid-dependent suppression of neurogenic contractions and of Cmpd101 on opioid-evoked contractions were not examined due to the effects of these inhibitors on EFS- and carbachol-stimulated contractions, respectively. These data indicate that the PS2-dependent inhibition of GPCR-mediated colonic contractions is via an internalization-independent mechanism.

## Discussion

***Use of endocytic inhibitors for physiological studies of gastrointestinal function.*** Our data shows that the inhibitor of clathrin-mediated endocytosis PS2 effectively prevents agonist-evoked GPCR endocytosis in enteric neurons. However, PS2 blocked contractions evoked by agonists that either strongly or weakly internalize opioid receptors (SNC80 and morphine, respectively), indicating that this effect was independent of receptor endocytosis. Moreover, our functional studies support a model in which the effects of PS2 are mediated through actions on neurotransmitter release from the ENS, rather than through block of target GPCR endocytosis or inhibition of smooth muscle activity (**Fig. 7**). PS2 has been used to define the importance of endosomal signaling in model cells and neurons (7-9,27) and has also been administered intrathecally to block nociceptive signaling from second order neurons of the spinal cord (7). Although direct examination of compartmentalized signaling in enteric neurons can be similarly determined using PS2, the present study demonstrates that currently available tools cannot be used to determine the functional importance of GPCR endocytosis and endosomal signaling to GI motility. Approaches such as endosomally-targeted antagonists (7-9) or evaluation of receptor internalizing vs non-internalizing agonists will be better suited for this type of study.

Cmpd101 is an inhibitor of GRKs 2 and 3 (35), which are key enzymes required for phosphorylation of activated GPCRs and for ensuing βArr recruitment, receptor endocytosis and desensitization. Cmpd101 blocks MOR desensitization and endocytosis in model cell lines and locus coeruleus neurons (28,29,36), consistent with the GRK2/3-mediated multi-site phosphorylation of this receptor (36,37). In contrast, the C-terminus of DOR is not highly phosphorylated (38) and the requirement for GRK2 activity for DOR trafficking appears to be dependent on the cellular background and potentially on the neuronal subtype examined (39,40). While we did not detect a significant difference in SNC80-evoked internalization profiles across nNOS positive and negative neurons upon treatment with SNC80 (DiCello & Poole, *unpublished data*), we show that Cmpd101 was an effective inhibitor of SNC80-evoked DOReGFP endocytosis in myenteric neurons. However, Cmpd101 suppressed cholinergic contractions, rendering it unsuitable for use in standard assays of GI function.

***PS2 suppresses neuromuscular transmission.*** Although PS2 attenuates excitatory neuromuscular transmission, it is possible that this is mediated through altered synaptic vesicle formation, rather than through reduced transmitter release or block of neuronal depolarization. Inhibitory neurotransmission in the ENS is primarily mediated through the gaseous transmitter NO. TTX contracts GI smooth muscle through suppression of nitrergic signaling, which effectively removes an underlying inhibitory influence to reveal myogenic activity.

Our data suggest that release of transmitter stores, such as those for ACh, is a clathrin-dependent process, whereas NO generation and release is mediated through a clathrin-independent mechanism. This is consistent with the effect of clathrin depletion on the quantal size and relative amount of readily releasable ACh-containing vesicles (41), the loading of ACh into clathrin-sculpted synaptic vesicles (42), and the clathrin-dependent uptake of choline into endosomes (42,43). In contrast, NO release is through diffusion across membranes and is therefore unlikely to be impacted by clathrin inhibition (44). This model is supported by key findings of the present study: 1) PS2 diminished EFS-evoked cholinergic contractions in a graded manner, which is consistent with depletion of existing stores and a reduced capacity to effectively replace vesicular stores; 2) nitrergic transmission and TTX-evoked contractions were unaffected by PS2; 3) Direct activation of GI smooth muscle by carbachol was unaffected by PS2; 4) myenteric neurons responded normally to exogenous application of ATP in the presence of PS2. Mechanistically, these data support a clathrin-dependent effect on vesicle formation, rather than a direct suppression of neuromuscular transmission or effect on GI smooth muscle.

***PS2 influences opioid receptor signaling independently of endocytosis.*** The effects of DOR agonists on neurogenic contractions of the colon are correlated with the receptor internalizing properties of the agonist. SNC80, which strongly internalizes DOR, evokes robust contractions, effectively inhibits electrically-stimulated contractions, and rapidly desensitizes DOR (16). In contrast, the related compound ARM390, which only weakly internalizes DORcGFP at higher concentrations, exhibits a reduced capability to evoke contractions, inhibit EFS-contractions, and desensitize DOR (45). The distinct antinociceptive capacities of these two ligands and the subsequent development of analgesic tolerance has been attributed to their respective internalizing properties (46). However, ARM390 is a partial agonist in the ENS and reduced effector coupling capacity is the most likely explanation for the agonist-dependent differences observed in the colon (16). The block of morphine-evoked contractions by PS2 supports this hypothesis, as morphine does not promote MOR endocytosis in myenteric neurons (24,34,47). The equivalent block of contractions to both SNC80 and morphine indicates that PS2 is likely to have effects on downstream signaling from opioid receptors that are endocytosis-independent and not directly related to effects of PS2 on neurotransmission. This does not exclude effects of PS2 on lateral movement and clustering of MOR within the plasma membrane, which can also significantly influence downstream signaling (27).

***Cmpd101 suppresses cholinergic smooth muscle contractions.*** Cmpd101 inhibited electrically-evoked neurogenic contractions, suggesting an action on neuron depolarization, neurotransmitter release, or muscarinic receptor signaling in smooth muscle effector cells. Direct examination of contractions to carbachol demonstrated a significant reduction in efficacy, but not potency, in the presence of Cmpd101. Thus, Cmpd101 has a direct effect on GPCR-mediated smooth muscle activity. The specific effects of Cmpd101 on signaling downstream of GPCRs in GI smooth muscle and enteric neurons is presently undefined. However, our proposed mechanism of action is consistent with a previous report that Cmpd101 inhibits GPCR- and electrically-evoked prostatic smooth muscle contractions (48).

**Conclusions.** Commonly used and commercially available endocytosis inhibitors are effective tools for the study of GPCR signaling in isolated neurons (27). However, the present study demonstrates that they are likely to prove unsuitable for studies of the neurogenic control of complex physiological processes in the GI tract. Clathrin inhibition is associated with altered neurotransmitter release and uptake in the CNS (31,42), and we provide evidence that PS2 negatively impacts neuromuscular transmission in the gut. This presents a potentially significant challenge to their use for studying GI function, as many processes are mediated through agonist actions at the level of the ENS. Moreover, our data indicate that there are inhibitory effects of PS2 on GPCR signaling that are likely to be endocytosis-independent. Thus, caution should be taken when interpreting physiological studies derived using these inhibitors.

**Experimental Procedures**

***Animals.*** C57Bl/6J and DOReGFP knockin mice (49) (6-8 weeks, male) were purpose bred by the Monash Animal Research Platform. Wnt1-GCaMP3 mice (4-6 weeks, male and female) were generated as described (50). Mice were housed under a 12h light/dark cycle, temperature-controlled conditions (24ºC), and with free access to food and water. All procedures involving mice were approved by the Monash Institute of Pharmaceutical Sciences and The University of Melbourne animal ethics committees.

***Reagents.*** Reagents used were from the following suppliers: PitStop2 (PS2) and PitStop2 inactive control (PS2i) (Abcam); Cmpd101 and SNC80 (Tocris); morphine hydrochloride (MacFarlan Smith); tetrodotoxin citrate (Alomone); nicardipine hydrochloride, carbamoyl choline (carbachol), and ATP (Sigma Aldrich).

***Endocytosis assays.*** The effectiveness of endocytosis inhibitors was determined using SNC80-induced DOReGFP endocytosis as a standard assay (16,18). Briefly, DOReGFP knockin mice were euthanized by cervical dislocation and the distal colon was harvested and placed in modified Krebs buffer (containing 10 μM nicardipine and 1 μM TTX). Wholemount preparations were recovered (Krebs + TTX + nicardipine, 37°C, 1 h, bubbled with 95% $O_2$/ 5% $CO_2$). Preparations were incubated with inhibitors or vehicle (37°C, 20 min), then washed with ice-cold Krebs (3 x 5 min washes). These were then incubated with SNC80 (1 μM) with or without endocytosis inhibitors (4°C, 1 h), washed (3 x 5 min washes, ice-cold Krebs), then recovered (agonist-free Krebs, 37°C, 30 min). Tissues were fixed (4% paraformaldehyde, overnight, 4°C). Fixative was cleared (3 x 10 min washes, PBS) and circular muscle-myenteric plexus wholemounts were prepared. These were blocked (5% normal horse serum, 0.1% Triton X-100 in PBS containing 0.1% sodium azide, 1 h, RT), then labeled by indirect immunofluorescence for eGFP (rabbit anti-GFP, 1:1,000, ThermoFisher, #AA11122), nNOS (goat anti-nNOS, 1:1,000, Genetex, #89962), and the pan-neuronal marker Hu (human anti-Hu, 1:25,000, (51)) (48 h at 4°C, diluted in blocking buffer). Primary antibody binding was detected using donkey secondaries conjugated to Alexa -488 (anti-rabbit, ThermoFisher, 1:500), -568 (anti-goat, ThermoFisher, 1:500) and -647 (anti-human, Jackson ImmunoResearch, 1:500) (1 h at room temperature, diluted in PBS). Tissues were washed (3 x 10 min, PBS), then mounted using ProLong Diamond antifade mountant (ThermoFisher).

***Microscopy and image analysis***. Images were captured using an SP8 TCS confocal system. Five images including myenteric ganglia were captured per preparation (40x objective, NA 1.3, >2.0 zoom, 16-bit depth, 1024 x 1024-pixel resolution). The subcellular distribution of DOReGFP within the neuronal soma was determined using nNOS and Hu immunoreactivities to define cellular morphology. Images were converted to binary (i.e. positive or negative pixels) using the nucleus to define the threshold for positive staining. A total of at least 35 neurons from 3-5 preparations were analyzed per treatment group. The subcellular distribution of DOReGFP was expressed as the relative percentage of total cellular DOReGFP that was associated with the plasma membrane. Single optical sections containing the neuronal soma and nucleus were included in analysis. A threshold based on the absence of nuclear DOReGFP was set for each image. Images were converted to binary with pixels set to either '0' (negative) or '255' (positive). Individual neurons were outlined based on Hu and nNOS immunoreactivities, and total positive pixels were determined. A second region of interest was drawn immediately beneath the plasma membrane allowing measurement of intracellular pixels. Finally, nuclear labeling was quantified and subtracted from total and intracellular values. The relative percentages of membrane [=Total − (Intracellular + Nuclear positive pixels)] and cytosolic (=Intracellular - Nuclear positive pixels) labeling were then determined.

***Calcium imaging of wholemount preparations.*** The effects of PS2 and PS2i on neuronal function were assessed by calcium imaging of circular muscle-myenteric plexus wholemount preparations of the colon from Wnt1-GCaMP3 mice, which express the genetically-encoded calcium biosensor GCaMP3 only in neural crest-derived cells (i.e. enteric neurons and glia; (50)). Tissues were imaged in calcium assay buffer (10 mM HEPES, 0.5% BSA, 10 mM D-glucose, 2.2 mM $CaCl_2.H_2O$, $MgCl_2.6H_2O$, 2.6 mM KCl, 150 mM NaCl) using a Leica SP8-MP multiphoton system (HC PLAN

APO 0.95 NA/25x water immersion objective). Images were captured at 16-bit depth, pinhole of 8-10 airy units, 1024 x 1024-pixel resolution at a frame rate of 1 image every 0.86 s). Preparations were incubated with PS2 or PS2i (both 15 μM) for 20 min prior to and during imaging. Tissues were challenged with ATP (100 μM) and the responses were recorded over a 2 min period. Wholemounts were then fixed and stained for PGP9.5 and GFAP immunoreactivities to identify all neurons and nerve fibers, and enteric glial cells, respectively. Nuclei were labeled with DAPI. Immunofluorescence was imaged (20x objective, 1.3 NA). The calcium imaging and immunolabeled images were overlaid using key landmarks with the bUnwarp plugin in ImageJ (v1.52i) (52). Myenteric neurons were identified by PGP9.5 labeling and enteric glial cells were identified based on GFAP-immunoreactivity and DAPI staining. The calcium responses were extracted using ImageJ and normalized to baseline (F/F0). Positive responses were defined based on an elevation above baseline of greater than 10%.

***Tissue contraction assays.*** Muscle strips were prepared from the distal colon as decribed (16). Briefly, tissues were placed in 10 mL water-jacketed organ baths containing Krebs solution (in mMol $L^{-1}$; NaCl 118; KCl 4.70, $NaH_2PO_42H_2O$ 1; $NaHCO_3$ 25; $MgCl_26H_2O$ 1.2; D-Glucose 11; $CaCl_22H_2O$ 2.5) and maintained at 37°C and bubbled with 95% $O_2$/ 5% $CO_2$. Isometric contractions of the circular muscle were measured by a Grass FT03 force displacement transducer (Grass Instruments, Quincy, MA, USA). Data were acquired with a PowerLab 4/SP system and viewed using LabChart software (v.5; AD Instruments Pty. Ltd., Castle Hill, NSW). Strips were placed under a resting tension of 0.5-1 g and were equilibrated for 30 min prior to use. Drugs were applied at a volume of 10-15 μL. Following the completion of each experiment, tissues were contracted with carbachol (10 μM) to evaluate viability. Tissues that were unresponsive to carbachol were omitted from analysis.

Electrically-stimulated contractions. Neurogenic contractions were evoked by transmural EFS (0.5 msec duration, 3 pulses $s^{-1}$, 60V), which was applied through platinum electrodes incorporated into the tissue holders (16). Once reproducible baseline contractile responses to EFS were maintained (minimum of 3 sets, 5 min intervals), tissues were washed and preincubated (10 min) with either vehicle (0.1% DMSO), PS2 (15 μM), or Cmpd101 (10μM). EFS (3 sets, 5 min intervals) were subsequently applied to the muscle strips. The amplitudes of EFS-evoked contractions were compared to baseline responses (i.e. in the absence of inhibitors). Data were expressed as % of baseline EFS-evoked contractions.

Drug-evoked contractions. After the equilibration period, EFS was applied (2 sets, 2 min intervals) to determine tissue viability. Muscle strips were washed and preincubated (10 min) with either vehicle or inhibitors as described above. TTX was added to the bath to determine the effects on inhibitory neurotransmission. To measure the direct effects of the inhibitors on smooth muscle contractility, the non-selective muscarinic agonist carbachol, which evokes contractions mainly through actions on smooth muscle (53) was added cumulatively to the bath (1 nM-10 μM). Data were fitted to three-parameter nonlinear regression curves using Prism (v8.01, GraphPad Software Inc, CA, USA) and $EC_{50}$ and $E_{max}$ values were determined. In another set of experiments, tissues were exposed to either SNC80 (1 nM- 10 μM; cumulative additions every 2 min) or morphine (1 μM). For each experiment, the maximum contraction amplitude was measured and corrected to baseline activity (2 min prior to addition of endocytic inhibitors). Data were expressed as grams of tension (g) generated.

**Statistical analysis.** Data were expressed as the mean ± s.e.m. and graphs were constructed in Prism v8.0.1. All groups for image analysis were compared by one-way ANOVA. For contraction assays, specific statistical analyses used for each experiment are stated in the respective figure legends. $P<0.05$ was defined as significantly different to the null hypothesis of no difference between means at the 95% confidence level.

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**FOOTNOTES**

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The abbreviations used are: ACh, acetylcholine; βArr, beta arrestin; Cmpd101, Takeda compound 101; DOR, delta opioid receptor; DOReGFP, DOR tagged with a C-terminal eGFP; EFS, electrical field stimulation; eGPCR, endosomal GPCR; ECE-1, endothelin converting enzyme 1; ENS, enteric nervous system; GI, gastrointestinal; GPCRs, G protein-coupled receptors; GRKs, G protein-coupled receptor kinases; MOR, mu opioid receptor; $NK_1R$, neurokinin 1 receptor; PS2, PitStop2; PS2i, inactive control for PS2; TTX, tetrodotoxin.

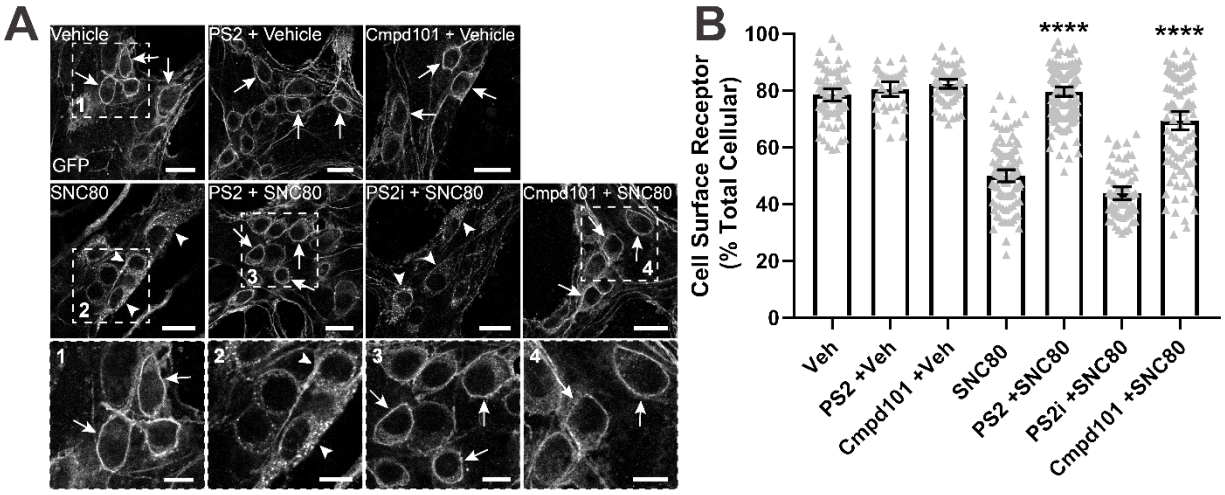


**Figure 1. PS2 and Cmpd101 are effective inhibitors of DOR endocytosis in myenteric neurons**. (**A**) Under basal conditions DOReGFP was localized to the cell surface of myenteric neurons (*inset 1*, arrows). SNC80 (1 μM, 30 min) stimulated internalization of DOReGFP from the cell surface to endosomes (*inset 2*, arrowheads), which was effectively blocked by PS2 (*inset 3*, arrows) and by Cmpd101 (*inset 4*, arrows). The inhibitors alone had no effect on the cellular distribution of DOReGFP. Scale: 20 μm and 10 μm (inset images). (**B**) Quantitative analysis of the percentage of total DOReGFP at the cell surface under each treatment condition (mean ± 95% confidence interval). **** $p < 0.0001$ (one-way ANOVA with Tukey post-hoc test). Comparisons shown for inhibitors relative to SNC80 treatment.

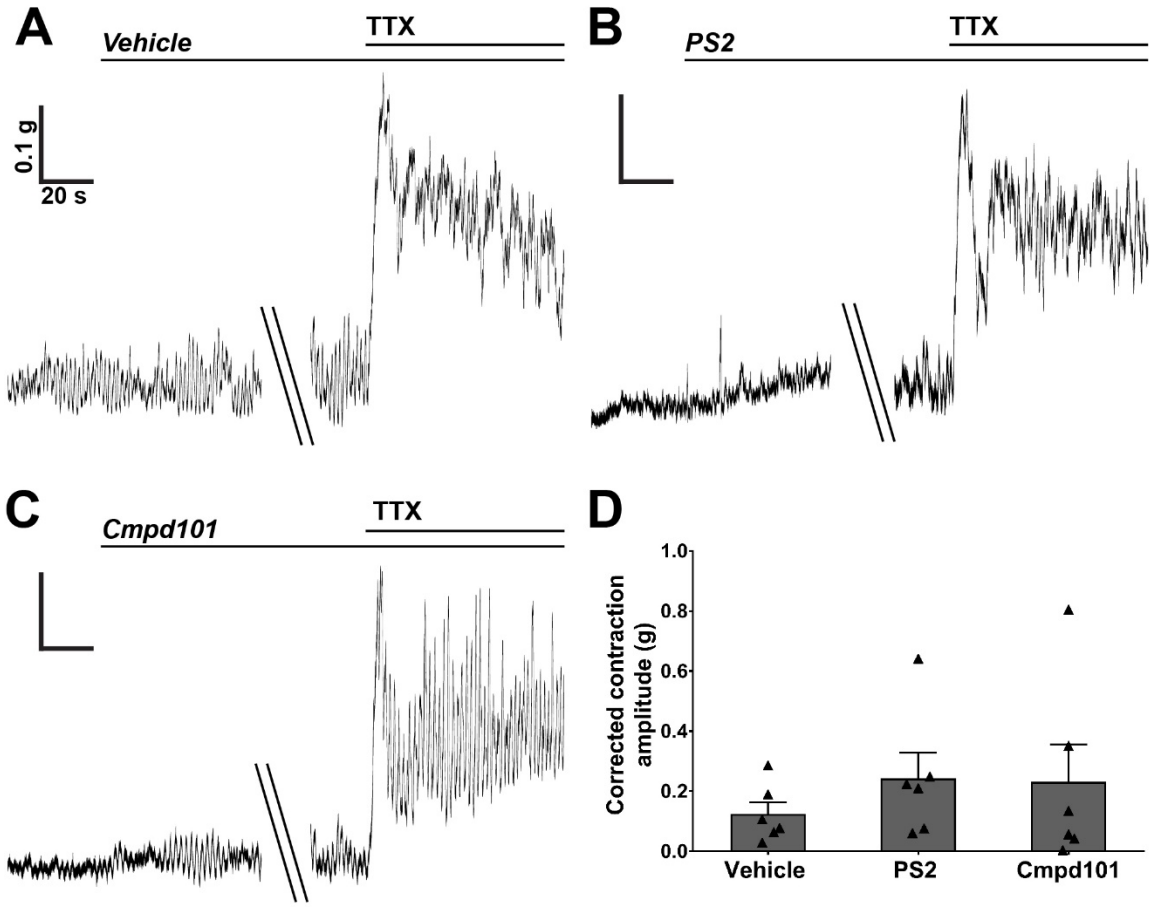


**Figure 2. TTX-evoked contractions are unaffected by endocytic inhibitors.** (**A**) The addition of the neuronal blocker TTX (1 μM) produced a tonic contraction of the colon, consistent with the block of an inhibitory tone. (**B**) Pre-incubation with either PS2 (15 μM) or (**C**) Cmpd101 (10 μM) had no effect on TTX-evoked contraction. (**D**) Quantitative analysis of TTX-mediated responses. Data are expressed as mean ± s.e.m, n= 6 mice per group. Statistical analysis conducted by Kruskal-Wallis' test.

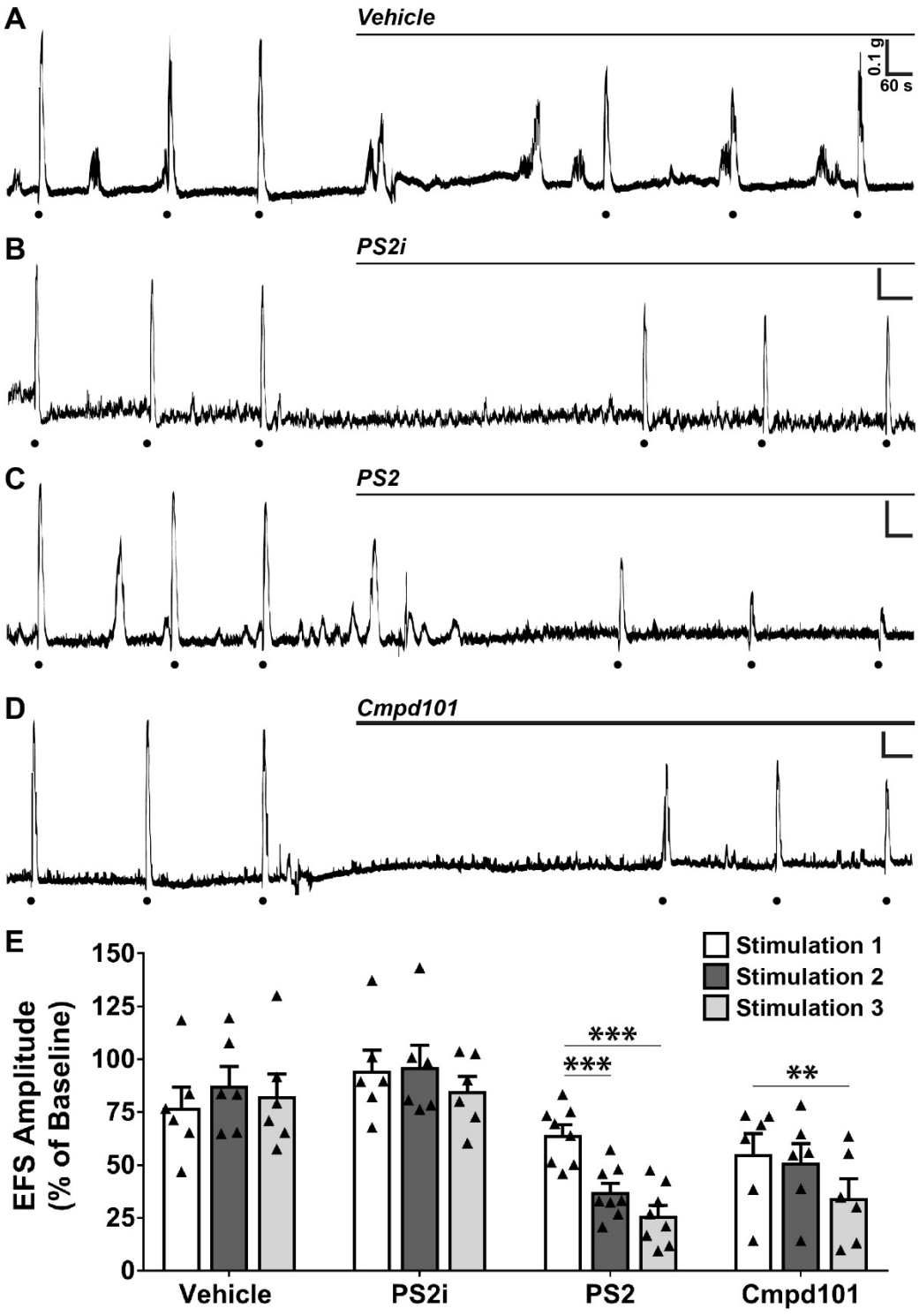


**Figure 3. Endocytic inhibitors reduce electrically-stimulated contractions of the colon.** (**A**) The amplitude of EFS-evoked contractions was unaffected by the addition of vehicle. Black circles represent where EFS was applied. (**B**) Exposure to PS2i (15 μM) had no effect on contractions, whereas PS2 (15 μM) inhibited subsequent EFS-evoked contractions in a graded manner (**C**). (**D**) GRK2/3 inhibition by Cmpd101 (10 μM) suppressed EFS-evoked contractions. (**E**) Quantitative analysis of contractions to sequential electrical stimulations following exposure to the different treatments. Data are expressed as mean ± s.e.m, n= 6-8 mice per group, with individual responses represented by triangles. Individual EFS amplitudes were compared using a repeated measures two-way ANOVA followed by Tukey's post-hoc test (**p<0.01 and ***p<0.001).

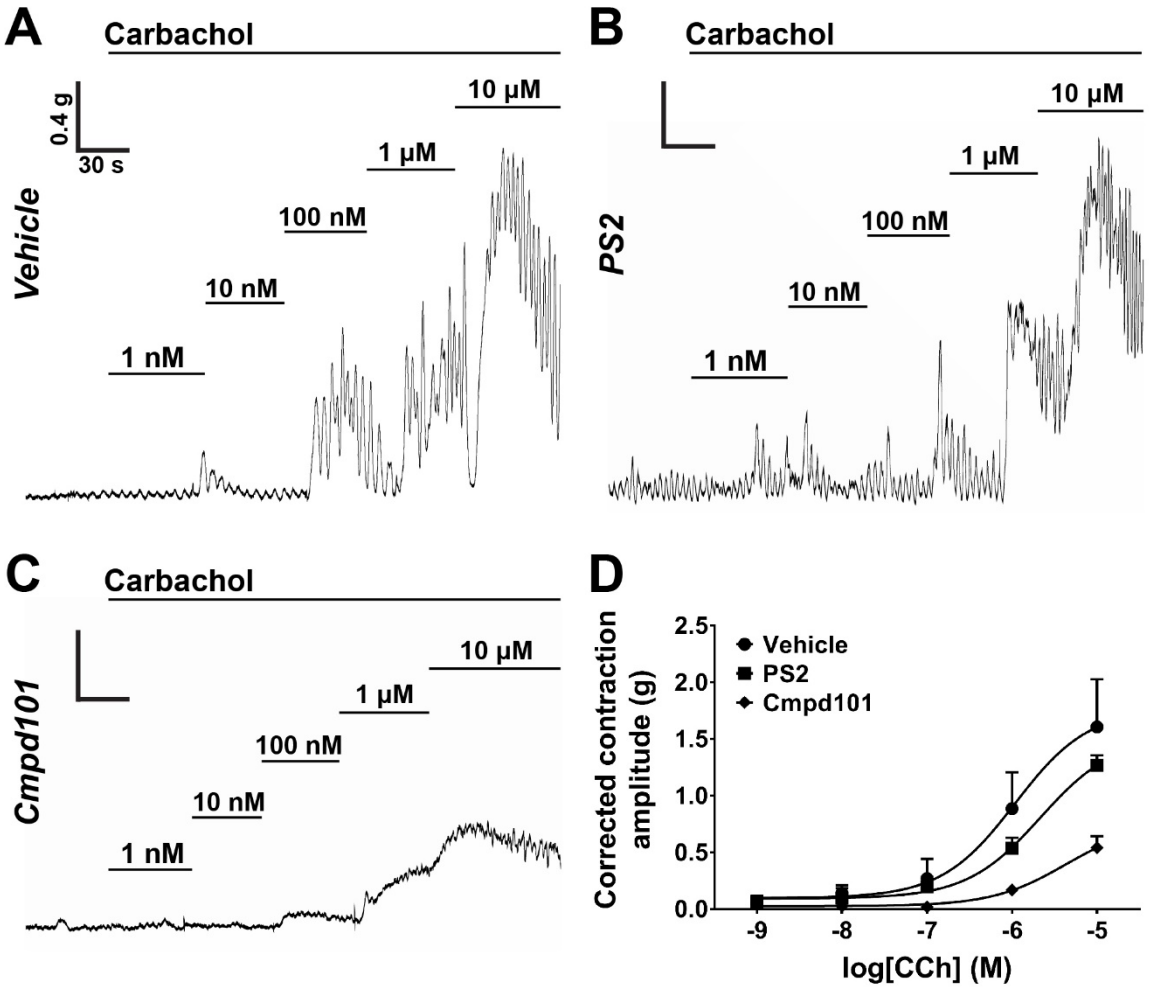


**Figure 4. PS2 and Cmpd101 exert anticholinergic effects through different mechanisms.** (**A**) Carbachol produced robust contractions of colonic smooth muscle in a concentration-dependent manner. These contractions are mediated through direct actions on the smooth muscle. (**B**) PS2 (15 µM) did not affect carbachol-mediated responses. (**C**) Cmpd101 decreased the efficacy of carbachol-evoked contractions, consistent with a direct action on smooth muscle. (**D**) Concentration response curves of carbachol-induced contractions. Data are expressed as mean ± s.e.m, n= 5-6 mice per group.

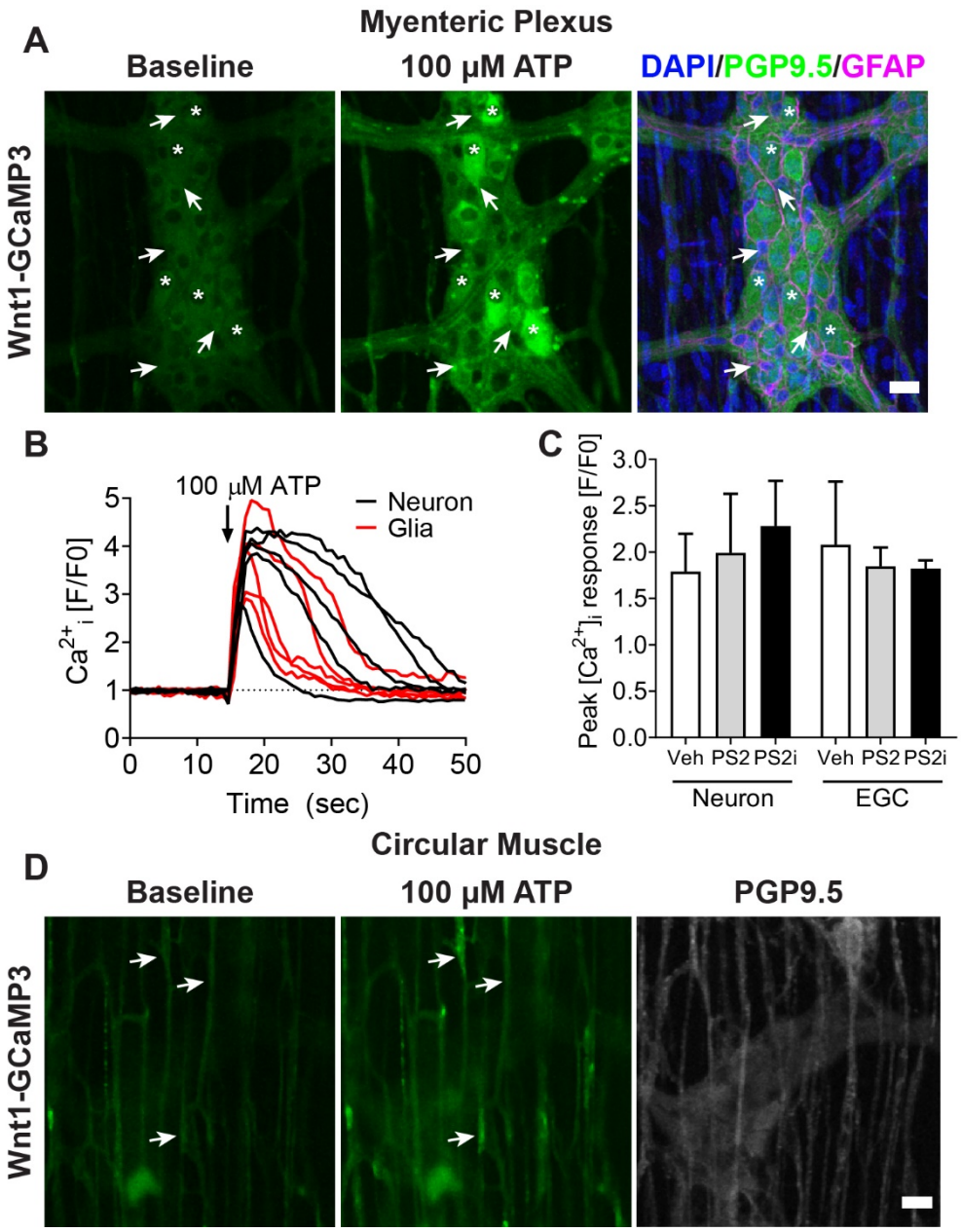


**Figure 5**. **PS2 does not affect agonist-evoked activation of myenteric neurons and glia**. (**A**) Example images demonstrating activation of myenteric neurons (asterisks; PGP9.5-positive) and glia (arrows; GFAP-positive) by ATP (100 µM). (**B**) Example traces of ATP-dependent $Ca^{2+}$ responses from neurons (black) and glia (red) derived from the preparation shown in (**A**). (**C**) Summary of the peak amplitude of responses by neurons and glia (EGC) to ATP in the presence of vehicle (DMSO), PS2, or PS2i (mean ± s.e.m., preparations from n=3-4 mice per group). (**D**) Examples of ATP-evoked $Ca^{2+}$ responses by nerve fibers of the circular muscle layer in the presence of PS2. Scale: 30 µm. Treatment groups were compared by one-way ANOVA with Tukey's post-hoc test.

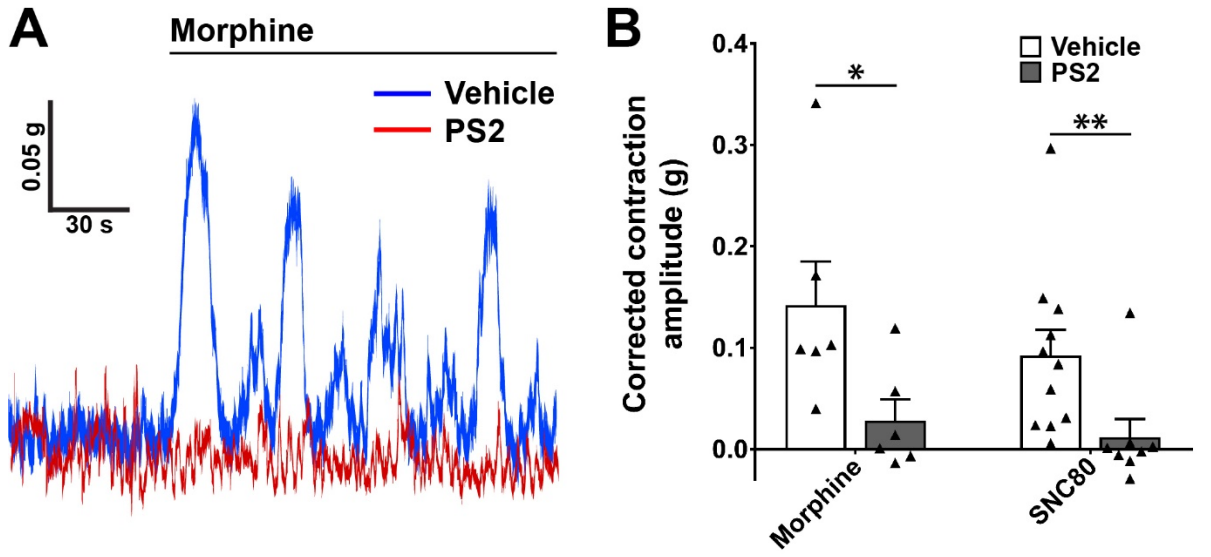


**Figure 6. PS2 inhibits GPCR-mediated responses by the colon via an endocytosis-independent mechanism.** (**A**) Morphine (1 μM), which does not promote MOR internalization, evoked a robust contraction of the colon. This was effectively blocked by PS2 (15 μM). (**B**) Quantitative analysis of the effect of PS2 on morphine- and SNC80 (100 nM)-evoked contractions. Data are presented as individual values and expressed as mean ± s.e.m, n= 6-11 mice per group. Data for the SNC80-treated groups were taken from **Fig. S1**. Morphine-treated groups were compared by unpaired Student's t-test. SNC80-treated groups were compared by Mann-Whitney's test (*p<0.05, **p<0.01).

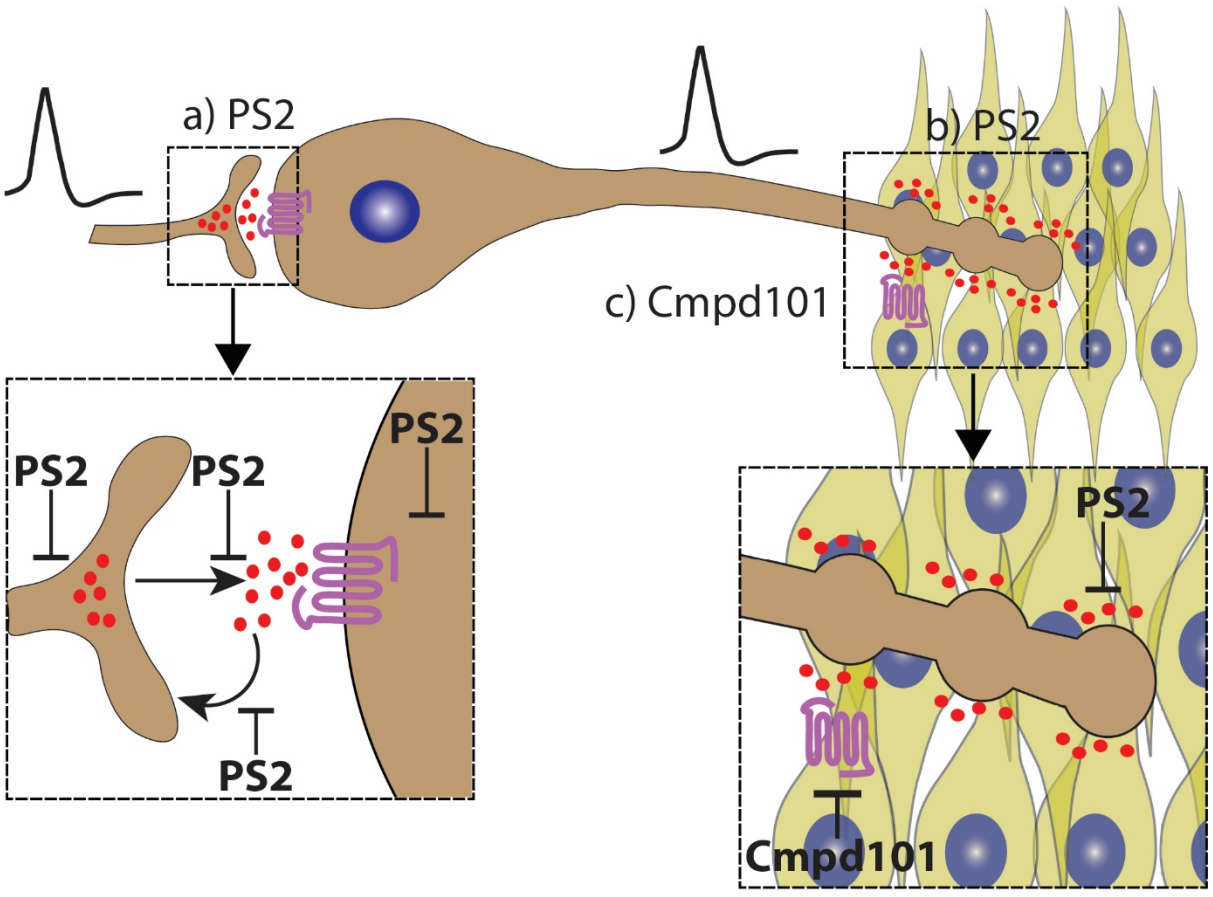


**Figure 7. Proposed mechanisms of action of endocytic inhibitors on neuromuscular transmission in the ENS. a)** PS2 suppresses synaptic transmission through effects on vesicle formation and release, reuptake of neurotransmitters and negative modulation of GPCR signaling. PS2 influences vesicle size and number and suppresses excitatory neurotransmitter release at synapses and neurotransmitter uptake into presynaptic terminals. PS2 also inhibits downstream signaling from postsynaptic GPCRs. **b)** PS2 blocks release of transmitter from nerve fibers associated with the circular muscle layer. **c)** Cmpd101 does not block neurotransmitter release but inhibits cholinergic contractions through a direct influence on signaling by GPCRs on smooth muscle cells.

*Supporting information included:*

Figure S1

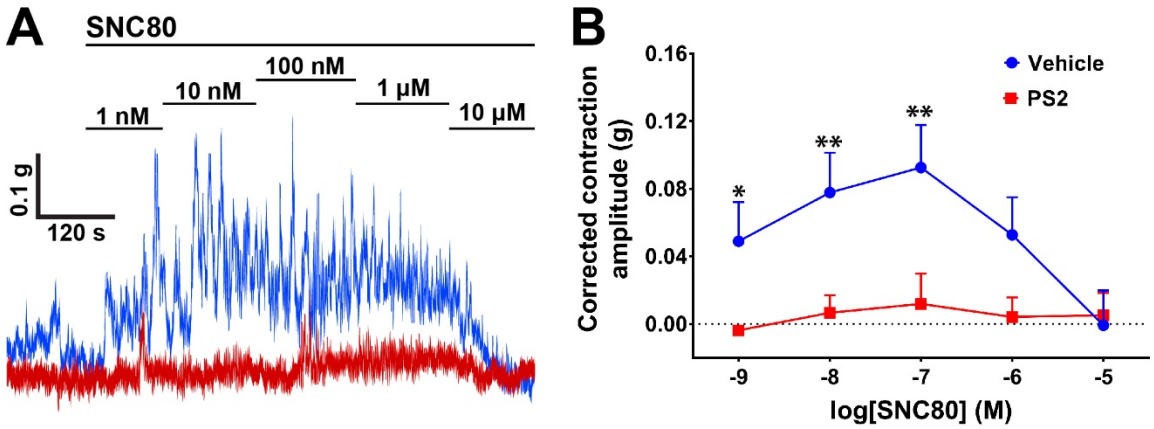


**Figure S1**. (**A**) PS2 inhibits contractile responses to SNC80. SNC80 evoked concentration-dependent contractions of the circular muscle of the colon (blue), which was effectively abolished in the presence of PS2 (15 µM). (**B**) Concentration-response relationship demonstrating block of SNC80 responses by PS2. Data points represent the mean ± s.e.m. of n= 8-11 experiments. Values at each concentration were compared across groups by Mann-Whitney test (*p<0.05, **p<0.01)

# Chapter 5
# *Evidence for functional interaction between the Mu and Delta opioid receptors in the mouse enteric nervous system.*

**Evidence for functional interaction between the Mu and Delta opioid receptors in the mouse enteric nervous system.**

**Short title: MOR and DOR interaction in the ENS**

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**Abbreviations:** CalR, calretinin; ChAT, choline acetyltransferase; DAMGO, [D-Ala$^2$,*N*-MePhe$^4$, Gly-ol]-enkephalin; DOR, δ opioid receptor; ENS, enteric nervous system; GFP green fluorescent protein; GI, gastrointestinal; GPCR, G protein-coupled receptor; GRK; G protein receptor kinase; IBS-D, diarrhea-predominant form of irritable bowel syndrome; IPAN, intrinsic primary afferent; IR, immunoreactive; MOR, μ opioid receptor; NFM, neurofilament M; $NK_1R$, neurokinin 1 receptor; $NK_3R$, neurokinin 3 receptor; NLT, naltrindole hydrochloride; nNOS, neuronal nitric oxide synthase; OBD; opioid-induced bowel dysfunction; PKA, protein kinase A; PKC, protein kinase C

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**Author Contributions**: JJD designed and conducted experiments and assisted with manuscript preparation. SEC supervised the study, performed preliminary experiments, and assisted with manuscript preparation. AS, RAC and VP performed experiments. NAV, MC, AC and CV provided critical assessment of the manuscript. DM provided tissue from knockin mice and provided critical assessment of the manuscript. DPP supervised the study, designed and conducted experiments and wrote the manuscript.

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**Synopsis.** This study has characterized the distribution of the therapeutically important mu opioid receptor in the mouse enteric nervous system. Although there is extensive coexpression of MOR with the delta opioid receptor, there is limited functional evidence to support the existence of MOR-DOR heteromers. However, MOR and DOR may functionally interact in enteric neurons, as demonstrated by heterologous desensitization.


## Abstract

**Background & Aims.** The μ and δ opioid receptors (MOR and DOR, respectively) may exist as heteromers. MOR and DOR heteromers may drive effects of the clinically-approved IBS-D drug eluxadoline and represent a potential target for novel analgesics. At present, sites of co-expression and potential interaction between MOR and DOR in the enteric nervous system (ENS) are undefined. In the present study we have characterized the distribution of MOR in the ENS of the mouse and examined MOR-DOR interactions using pharmacological and cell biology techniques. **Methods.** MOR and DOR expression were examined using MORmCherry and MORmCherry-DOReGFP knockin mice. MOR-DOR interactions were assessed using DOReGFP internalization assays, and pharmacological analysis of neurogenic contractions of the colon. **Results**. MOR is expressed by approximately half of all myenteric neurons, but MOR-positive submucosal neurons were rarely observed. We demonstrate extensive overlap between MOR and DOR in both excitatory and inhibitory pathways involved in the coordination of intestinal motility. Functional evidence indicates that MOR and DOR do not exist as heteromers in the ENS. DOR internalizes independently of MOR in myenteric neurons. Pharmacological studies demonstrate no evidence of cooperativity between MOR and DOR. Furthermore, the MOR-DOR biased agonist CYM51010 exerts its effects on neurogenic contractions of the colon mainly through DOR. MOR and DOR can functionally interact, as shown through unidirectional heterologous desensitization of MOR-dependent responses by DOR. Collectively, these findings demonstrate that although MOR and DOR are coexpressed and functionally interact, they do not exist as heteromers in the ENS under physiological conditions.


## Introduction

Opiates which target the μ opioid receptor (MOR) are the leading treatment for moderate to severe pain. Although their analgesic efficacy is unparalleled, their prolonged use is commonly associated with adverse and limiting side-effects including dependence, analgesic tolerance, respiratory depression and opioid-induced bowel dysfunction (OBD). OBD is a collection of on-target gastrointestinal (GI) side-effects with the most frequent being intractable constipation.[1] This constipation can be so severe that it leads to patient non-compliance with opioid therapy, ultimately resulting in ineffective pain relief. Drug discovery strategies to develop safer and more efficacious opioid analgesics are currently focused on exploiting different pharmacological and physicochemical properties of MOR-ligands.[2-4] However, subsequent investigations of these compounds indicate that side-effects including constipation and respiratory depression may be retained.[5-8] Thus, there is a need for greater understanding of how opioid receptor regulation and signaling underlies the control of physiological processes, including GI motility.

There are several ways that G protein-coupled receptors (GPCRs) can interact at the cellular level. GPCRs may directly or indirectly interact to form a distinct signaling unit known as a heteromer. The MOR and δ opioid receptor (DOR) heteromer (MOR-DOR) is a well-characterized example and has been identified as a potential target for pain therapy. The existence of MOR-DOR heteromers in nociceptive pathways is supported by pharmacological and receptor trafficking studies that identify unique activities compared to the individual receptors alone.[9] The occupation of one receptor within a heteromer enhances the binding affinity and signaling of the other in both heterologous cell lines and membrane preparations from the spinal cord, consistent with an allosteric interaction between MOR and DOR.[10-13] Furthermore, the addition of morphine or a high efficacy DOR agonist promotes the trafficking of MOR into late endosomes for degradation, rather than recycling back to the cell surface for resensitization.[14, 15] MOR degradation also reduces functional receptor at the cell surface and is implicated in the development of morphine tolerance.[14] DOR antagonism may augment the analgesic efficacy of clinically relevant MOR agonists while reducing analgesic tolerance.[10, 16] This mechanism may underlie the actions of the mixed MOR agonist- DOR antagonist eluxadoline (Viberzi®),[17, 18] which is clinically approved for the treatment of diarrhea-predominant irritable bowel syndrome (IBS-D) .[19]

Although there are distinct therapeutic advantages to targeting MOR-DOR, the existence of these heteromers *in vivo* remains controversial.[20] Characterization of MOR-DOR has mainly been performed on heterologous cell lines and membrane preparations, and issues have been raised concerning the specificity of reagents routinely used to probe receptor expression and interaction *in vivo*.[10, 20-25] Recent evidence using highly specific detection methods demonstrates minimal overlap between MOR and DOR in nociceptive pathways.[20] Moreover, MOR and DOR were internalized independently in the small population of neurons that expresses both receptors, and MOR-mediated responses were unaffected by prior DOR activation. MOR-mediated analgesia is unaffected by DOR antagonism, indicating a lack of cooperativity between these receptors.[22, 24] Thus, the current literature suggests that both receptors function independently in pain pathways.

The enteric nervous system (ENS) provides an ideal native system in which to probe for functional interactions between MOR and DOR. The activation of either receptor in the ENS dampens both motility and secretion through the inhibition of myenteric and submucosal neurons, respectively. The first evidence for MOR and DOR coexpression in a native system was provided by electrophysiological analysis of agonist-induced hyperpolarization of myenteric neurons of the guinea pig intestine.[26] However, DOR is not functionally present in the guinea pig ileum,[27, 28] which questions the selectivity of the agonists available at the time of this study. There is extensive overlap between MOR and DOR in the mouse ENS,[29] and evidence for heteromer formation using a MOR-DOR selective antibody.[17] Antagonism of DOR in membrane preparations of the mouse ileum augments MOR-mediated signaling, indicative of positive cooperativity between these receptors.[17] Thus, both expression and functional analysis supports formation of MOR-DOR heteromers in the ENS. Concerns about the specificity of antibodies used to probe for GPCRs in native tissues have been raised.[30] Moreover, MOR-DOR distribution and signaling has not been examined in the colon, which is the region most impacted by OBD and affected in IBS-D. The neurochemical coding of enteric

neurons that co-express MOR and DOR in the mouse, a species commonly used in mechanistic and preclinical studies,[5, 31-33] is unknown. Although MOR-DOR is a proposed target for the approved IBS-D drug eluxadoline, fundamental understanding of this interaction at the cellular and physiological level remains limited. A detailed characterization of MOR-DOR in the ENS will better inform development of opiate therapeutics for intestinal disorders and identify potential GI side-effects associated with use of MOR-DOR targeted analgesics.

In the present study, we used a multidisciplinary approach to examine whether MOR and DOR functionally interact in a native system. The neurochemical coding of enteric neurons expressing MOR or both MOR and DOR was determined with high specificity using transgenic mice expressing either MOR with a C-terminal red fluorescent protein (MORmCherry) or both MORmCherry and DOR tagged with a C-terminal enhanced green fluorescent protein (MORmCherry/DOReGFP).[21] The ability of MOR and DOR to functionally interact was determined using receptor endocytosis assays and by pharmacological examination of neurogenic contractions.[31]

## Results

**MORmCherry Expression.** MORmCherry labeling was most evident in myenteric neurons in sections of the antrum, ileum and distal colon (**Fig. S1**). There was no detectable MORmCherry expression in smooth muscle of the *muscularis externa*. There was limited labeling of cells of the lamina propria and diffuse staining of the epithelium. MORmCherry was localized to punctate structures within the soma of a subset of myenteric neurons, with no evidence for labeling of proximal neurites or nerve fibers associated with the circular or longitudinal layers of the *muscularis externa*. This subcellular distribution of MOR is inconsistent with that described in myenteric neurons using immunofluorescence, where a significant proportion of MOR immunoreactivity is associated with the cell surface. [34, 35] We examined MOR labeling of myenteric neurons in colonic wholemounts using a validated antibody (UMB3).[36] MOR immunoreactivity was associated with the cell surface and intracellular structures and labeling was also detected in nerve fibers in the circular muscle layer (**Fig. S2**), consistent with our previous observations in the guinea pig ENS.[34] To further confirm this observation, MOR-GFP was transiently expressed in cultured myenteric neurons. MOR-GFP was effectively trafficked and localized to the cell surface of the soma and neurites and was also associated with intracellular structures. Treatment with the MOR agonist DAMGO (1 μM, 30 min) resulted in internalization of MOR-GFP from the cell surface to endosome-like structures (**Fig. S3A**). Equivalent observations were made in cultured dorsal root ganglion neurons (**Fig. S3B**). Thus, we conclude that the intracellular distribution of MORmCherry is most likely due to a trafficking defect associated with expression of the fusion protein, rather than a true reflection of MOR distribution in myenteric neurons. All subsequent characterization using MORmCherry knockin mice was restricted to distribution studies for this reason.

**MORmCherry is expressed by neurons controlling excitatory and inhibitory neuromuscular transmission**. The distribution of MORmCherry in the myenteric region was examined in wholemount preparations of the ileum and distal colon. The relative expression of MORmCherry in distinct neurochemically-defined subsets of myenteric neurons is summarized in **Table 1** and **Fig. 1**. MORmCherry was localized to a subset of all Hu-immunoreactive (-IR)

neurons, with similar proportions of all neurons labeled between the ileum and colon. MORmCherry was mainly localized to ChAT-positive neurons in both the ileum and colon. MORmCherry was also detected in nNOS-expressing neurons, with a relatively lower percentage overlap in the colon. MORmCherry was not detected in larger diameter neurons that were also positive for calretinin, ChAT, or NFM. These neurons exhibit the morphological and neurochemical characteristics of intrinsic primary afferent neurons (IPANS). Labeling was also absent in proximal neurites or in nerve fibers associated with the *muscularis externa* presumably due to the sequestration of MORmCherry in neuronal somata. There was no evidence for MORmCherry expression by GFAP-positive enteric glial cells. MOR was similarly absent from extraganglionic cells of the myenteric region, suggesting that MOR is not expressed by key cell types involved in the control of intestinal motility including interstitial cells of Cajal, PDGFR$\alpha^+$ fibroblast-like cells, and muscularis macrophages.

**MORmCherry is not expressed by submucosal neurons.** MORmCherry labeling of Hu-positive submucosal neurons was not observed in the ileum (**Fig. 2**). Labeling was present in myenteric ganglia within the same preparation, confirming both genotype and effective binding and detection of the dsRed1 antibody. MORmCherry was detected in a very limited number of submucosal neurons of the distal colon, where it was generally coexpressed with nNOS (not quantified; **Fig. 2**). These findings suggest that MOR expression in the mouse intestine is highly restricted to myenteric neurons.

**MORmCherry and DOReGFP are co-expressed in a subset of myenteric neurons.** A fundamental requirement for the formation of MOR-DOR heteromers is the co-expression of the individual protomers by the same cell. We examined overlap between MOR and DOR using MORmCherry-DOReGFP knockin mice.[21] A detailed summary of the neuronal populations in which MOR and DOR coexpression was detected is presented in **Table 2** and representative images are shown in **Fig. 3**. MORmCherry and DOReGFP were colocalized in a subset of all Hu-IR neurons in the ileum (31%) and distal colon (21%). MORmCherry and DOReGFP were coexpressed in a subset of ChAT-positive neurons in both the ileum and colon. MORmCherry and DOReGFP were also colocalized in nNOS-expressing neurons. These data highlight that MOR and DOR are coexpressed by a significant proportion of all myenteric neurons and that identifies the myenteric plexus as a site where heteromerization may potentially occur. Although DOReGFP was localized to submucosal neurons of the ileum, the absence of MORmCherry indicates that this is a site where putative MOR-DOR heteromers cannot form.

**MOR immunoreactivity is detected in myenteric neurons and is coexpressed with DOR**. To demonstrate localization of MOR at the cell surface, expression in nerve fibers innervating colonic circular muscle and overlap with DOR, we performed a qualitative assessment of the distribution of MOR immunoreactivity (**Fig. S2**). Labeling for the *Oprm1*$^{-/-}$ validated monoclonal antibody UMB-3[36] was associated with the cell surface and cytosol of a large proportion of myenteric neurons, and with nerve fibers associated with the circular muscle layer. MOR immunoreactivity often overlapped with DOReGFP labeling, consistent with the extensive coexpression demonstrated using MORmCherry-DOReGFP knockin mice.

**MORmCherry/DOReGFP Coexpression**

**Table 1.** Quantification of MORmCherry expressing neurons in the myenteric plexus (n=7-21 mice per group).

| Region | Hu | CalR | ChAT | NOS | DOReGFP |
|---|---|---|---|---|---|
| Ileum | 1550/1550 (100 %) | [a]542/1526 (34.8 % ±3.1) | 544/1118 (49.8 % ± 2.9) | 676/1844 (34.9 % ± 1.6) | 1857/3039 (60.9% ± 2.4) |
| | 1550/2624 (59.1 % ± 2.7); n=10 | [b]542/931 (57.8 % ± 4.6); n=9 | 544/842 (65.9 % ± 3.3); n=7 | 676/831 (80.1 % ± 2.2); n= 12 | 1857/2362 (78.9% ± 2.5); n=19 |
| Colon | 2217/2217 (100 %) | 1293/2928 (45.1 % ± 2.7) | 988/1899 (56.0 % ± 3.6) | 408/1552 (23.2 % ± 3.1) | 2151/4431 (46.1 % ± 2.0) |
| | 2217/4550 (48.7 % ± 2.4); n=12 | 1293/2512 (51 % ± 2.3); n=11 | 988/1655 (61.3 % ± 1.9); n= 8 | 408/1105 (33.5 % ± 4.9); n=9 | 2152/3673 (55.1 % ±2.6); n=21 |

[a]Number of marker-positive neurons in the MORmCherry-positive population (i.e.; in ileum, of 1526 cells expressing MORmCherry, 542 expressed CalR).

[b]Number of MORmCherry-positive cells in the marker-positive population (i.e.; in ileum, of 931 cells expressing CalR, 542 also expressed MORmCherry).

**Table 2.** Quantification of MORmCherry/DOReGFP expressing neurons in the myenteric plexus (n=5-6 mice per group).

| Region | Hu | CalR | ChAT | NOS |
|---|---|---|---|---|
| Ileum | 497/497 (100 %) | [a]310/790 (40.4 % ± 3.8) | 203/471 (44.0 % ± 5.3) | 194/481 (39.9 % ± 3.1) |
| | 497/1657 (30.9 % ± 4.1); n=6 | [b]310/765 (41.3 % ± 3.9); n=8 | 203/539 (38.1 % ± 5.1); n=5 | 194/350 (55.9 % ± 2.3); n=6 |
| Colon | 425/425 (100 %) | 349/691 (56.7 % ± 4.6) | 130/278 (49.1 % ±2.7) | 178/370 (39.9 % ± 9.4) |
| | 425/1896 (21.2 % ± 2.8); n=6 | 349/940 (37 % ± 3.0); n=6 | 130/560 (25.0 % ± 3.2); n=5 | 178/525 (26.0% ± 6.2); n=5 |

[a]Number of marker-positive neurons in the MORmCherry/DOReGFP -positive population (i.e.; in ileum, of 790 cells expressing MOR/DOR, 310 expressed CalR).

[b]Number of MORmCherry/DOReGFP -positive cells in the marker-positive population (i.e.; in ileum, of 765 cells expressing CalR, 310 also expressed MOR).

**MOR and DOR internalize independently in enteric neurons.** The co-internalization of individual protomers is a standard assay to demonstrate heteromer formation, with the assumption that both interacting partners will be endocytosed upon activation.[15, 37] The requirement for MOR activity for effective agonist-evoked DOR endocytosis was examined in enteric neurons of the ileum and colon of DOReGFP knockin mice.[29, 31]

DOReGFP was mainly confined to the plasma membrane of myenteric neurons under control conditions (73.8 ± 1.3% cell surface DOReGFP, mean, n=73 neurons; **Fig. 4A, C**). Treatment with SNC80 (1 μM) resulted in significant DOReGFP endocytosis into endosomes (42.9 ± 2.3 %, n=24). In marked contrast, DOReGFP was retained at the cell surface following treatment with DAMGO (1 μM; 77.8 ± 0.8 %, n=115). CYM51010 (1 μM), a biased agonist for the MOR-DOR heteromer,[22] stimulated DOReGFP endocytosis in all neurons examined (40.6 ± 0.7 %, n=182). This internalization was effectively blocked by the DOR antagonist NLT (74.5 ± 0.8 %, n=125), but not by the MOR antagonist CTOP (41.5 ± 0.9 %, n=143). Equivalent DOReGFP endocytosis was detected in nNOS positive and negative neuronal populations (**Fig. S4**).

Agonist-evoked DOReGFP internalization was similarly examined in submucosal neurons of the ileum. These neurons express DOR, but not MOR (present study),[29] thus providing an ideal opportunity to examine specific activation of DOR. DOReGFP was mainly localized to the cell surface under basal conditions (81 ± 1.0 %, n=55; **Fig. 4B, D**). Treatment with SNC80 resulted in marked internalization of DOReGFP in all submucosal neurons examined (40.1 ± 1.3 %, n=70), whereas DAMGO did not significantly alter the subcellular distribution of DOReGFP (81.9 ± 1.2 %, n=37). CYM51010 promoted endocytosis of

DOReGFP in submucosal neurons (44.1 ± 1.5 %, n=41), confirming that this agonist can directly activate DOR.

The subcellular distribution of MOR immunoreactivity was qualitatively assessed. MOR labeling was detected at the cell surface of a large proportion of myenteric neurons of the colon where it was often co-distributed with DOReGFP (**Fig. S2**). Treatment with DAMGO (1 μM, 30 min) resulted in labeling of endosome-like structures with the MOR-antibody and retention of DOReGFP at the cell surface. Treatment with SNC80 (30 min) resulted in complete internalization of DOReGFP, while MOR immunoreactivity remained at the cell surface. Thus, MOR and DOR undergo independent agonist-evoked internalization in myenteric neurons. Although MOR and DOR are coexpressed, the independent manner in which they are internalized upon activation indicates that they are unlikely to exist and function as heteromers in the ENS.

**MOR-mediated inhibition of neuromuscular transmission is mechanistically independent of DOR.** Formation of MOR-DOR heteromers is associated with allosteric interactions between individual receptors. This results in enhanced receptor-mediated signaling of one protomer following the occupation of the partner receptor and is proposed to underlie enhanced MOR-dependent analgesia in the presence of a DOR antagonist.[10, 11] Allosteric interaction between MOR and DOR in myenteric neurons was examined pharmacologically. DAMGO inhibited EFS-evoked contractions in a concentration-dependent manner ($pEC_{50}$=8.0 ± 0.3, $E_{max}$=68.4 ± 4.4 %, n=9). The actions of DAMGO were unaffected by NLT (100 nM; **Fig. 5A**). In contrast, a higher concentration of NLT (1 μM) inhibited DAMGO-mediated responses ($pEC_{50}$=6.8 ± 0.3, $E_{max}$=81.1 ± 6.5 %, n=5; $p<0.05$) consistent with lower selectivity at higher concentrations.[38] The inhibitory actions of SNC80 ($pEC_{50}$=7.06 ± 0.24, $E_{max}$=84.58 ± 5.93 %, n=6) were insensitive to CTOP (1 μM; **Fig. 5B**). These data indicate that there is no cooperativity between MOR and DOR in the ENS, consistent with actions at individual receptors, rather than MOR-DOR heteromers.

**CYM51010-evoked colonic contractions are DOR- and MOR-dependent.** MOR and DOR agonists can evoke myogenic smooth muscle contractions through removal of an underlying inhibitory neural influence.[31] CYM51010 treatment resulted in a rapid and sustained concentration-dependent contraction of colonic circular muscle which was maximal at 1 μM (**Fig. 6A, D**). NLT (100 nM) significantly inhibited the CYM51010 response (100 nM-1 μM, n=7-9; $p<0.01$; **Fig. 6B, D**), whereas CTOP (1 μM) had no effect (1 nM- 10 μM, n=9-10; $p>0.05$; **Fig. 6C, D**). These results correlated with the ability of CYM51010 to enhance [$^{35}$S] GTPγS binding in membrane preparations from CHO cells expressing DOR, but not MOR (**Fig. 6E**). Thus, the actions of CYM51010 on inhibitory neural pathways in the ENS are primarily mediated through direct activation of DOR.

The effects of CYM51010 on excitatory pathways were determined. CYM51010 inhibited EFS-evoked contractions of the colon in a concentration-dependent manner ($pEC_{50}$=7.60 ± 0.14, $E_{max}$=86.42 ± 2.72%, n=13; **Fig. S5A, E**). NLT (100 nM) treatment resulted in a significant parallel rightwards shift of the CYM51010 concentration-response curve ($pEC_{50}$=6.81 ± 0.15, $E_{max}$=87.70 ± 4.41 %, n=6; $p<0.01$; **Fig. S5B, E**). CTOP (1 μM) also shifted the CYM51010 concentration-response curve rightwards ($pEC_{50}$=6.80 ± 0.18,

$E_{max}$=89.19 ± 5.26 %, n=6; p<0.01; **Fig. S5C, E**). Simultaneous treatment with both antagonists had an additive inhibitory effect on CYM51010 responses, confirming antagonism of the individual receptor subtypes ($pEC_{50}$=5.89 ± 0.16, $E_{max}$=89.38 ± 7.22 %, n=7; p<0.001; **Fig. S5D, E**). These results support an action by CYM51010 at either DOR or MOR expressed in excitatory pathways.

**MOR-mediated responses are unaffected by DOR sequestration**. The co-degradation hypothesis proposes that high efficacy DOR agonists also promote MOR endocytosis and trafficking to lysosomes, rather than to recycling pathways.[14] This results in a sustained reduction of functional MOR and DOR on the cell surface and may be indicative of heteromer formation. We examined whether prior activation and internalization of DOR was associated with a corresponding reduction in MOR agonist evoked colonic contractions. Mice were administered a single dose of either saline (0.9%, i.p.) or SNC80 (10 mg/kg, i.p; 3 h), and subsequent tissue responses to either SNC80 or DAMGO were examined *in vitro*. SNC80-evoked contractions were desensitized in the SNC80-pretreated group, confirming effective removal of functional DOR from the cell surface (**Fig. 7B**; 10 nM-1 μM, n= 5-7; p<0.05). In marked contrast, DAMGO-evoked contractions were unaffected by SNC80 pre-treatment (**Fig. 7A**). Thus, MOR function is retained following removal of DOR from the cell surface, consistent with independent regulation of the two receptors in myenteric neurons.

**Cross-talk between DOR and MOR occurs in a unidirectional manner.** Interactions between GPCRs can also occur through heteromer-independent mechanisms, including via heterologous desensitization.[39, 40] This possibility was specifically examined by first exposing tissues to an agonist for one receptor (1 μM, 5 min) then measuring resulting changes in the responses to either the same agonist (homologous) or to an agonist for the other receptor (heterologous). Agonists with varying receptor internalizing properties were used to determine the involvement of endocytosis and associated processes.

The selective MOR agonists DAMGO and morphine produced robust, concentration-dependent contractions of the colon (**Fig. 8A, D, E**). Prior exposure to the same agonist did not alter the magnitude of agonist-evoked contractions (**Fig. 8B, D, E**), consistent with the resistance of MOR to desensitization in myenteric neurons.[32, 33] In contrast, pre-incubation with SNC80, which stimulates robust DOR internalization, abolished subsequent DAMGO- (10 nM-10 μM; p<0.05 and 0.01 vs. vehicle, n= 5-6; **Fig. 8C, D**) and morphine-evoked contractions (100 nM- 10 μM; p<0.01 vs vehicle, n= 6-7; **Fig. 8E**). ARM390, which only weakly internalizes DOR, did not significantly attenuate DAMGO-evoked responses relative to the vehicle control (**Fig. 8C, D**). Furthermore, ARM390 had no effect on morphine-evoked contractions (1 nM-10 μM, n=5-6; p>0.05; **Fig. 8E**). Thus, although MOR in the ENS is resistant to homologous desensitization, responses to MOR agonists can be desensitized in a heterologous manner by DOR. These data support functional interaction between these two receptors. The selective DOR agonist SNC80 evoked tonic contractions of colon strips.[31] Prior treatment with SNC80 completely desensitized subsequent contractile responses to SNC80, consistent with desensitization of DOR (**Fig. 8F**). In contrast, the partial agonist ARM390 had no effect on SNC80-mediated contractions (**Fig. 8F**). Prior exposure to DAMGO significantly augmented the initial SNC80-evoked contraction at 1 nM compared to the vehicle (n= 8-12; p<0.05), indicative of sensitization. However, there was no significant change to SNC80 responses at

any other concentration (10 nM- 10 μM, **Fig. 8F**). Thus, DOR is desensitized in a homologous-manner in enteric neurons. CYM51010-dependent desensitization of MOR- and DOR was similarly examined. CYM51010 (1 μM) blocked both SNC80- and DAMGO- evoked contractions (**Fig. 8D, F**), consistent with the desensitizing effects of SNC80 and with our pharmacological evidence for a DOR-dependent mechanism of action. These data support the functional coexpression of MOR and DOR in pathways involved in inhibitory neurotransmission and are consistent with overlap between these receptors in the nitrergic neuronal population. Although MOR and DOR can functionally interact, the unidirectional nature of this interaction indicates that this is unlikely to be through formation of heteromers.

## Discussion

In the present study, we examined whether MOR and DOR are coexpressed and can functionally interact in the ENS. We demonstrate that 1) there is extensive coexpression of MOR and DOR in the myenteric plexus 2) that MOR and DOR are unlikely to exist as heteromers in this system and 3) that MOR and DOR can functionally interact in a unidirectional manner.

**The organization of MOR in the ENS.** Opiates directly activate MOR on enteric neurons leading to the inhibition of GI motility and secretion.[41] MOR in the ENS is also the therapeutic target of peripherally-restricted agonists and antagonists, such as loperamide and Alvimopan, for the treatment of acute diarrhea and OBD, respectively.

Comparison with MOR expression in other studies. Expression of MOR by a range of different cell types in the GI tract has been reported, although the distributions described are not consistent between studies. MORmCherry was exclusively expressed by Hu-positive myenteric neurons, with no MORmCherry detected in GFAP-positive enteric glial cells. This contrasts with Bauman *et al.* (2017) who examined an EGC cell line but is consistent with Bhave *et al.* (2017). We did not observe labeling of smooth muscle cells within tissues, which is supported by our functional evidence that the effects of MOR activation on smooth muscle activity are of neurogenic origin. Previous evidence for expression was mainly obtained using isolated smooth muscle cells and may reflect phenotypic changes that occur under culture conditions.[44] Moreover, there was no labeling of ICC or PDGFRα$^+$ cells in the myenteric region. Expression of MOR by ICC has been reported.[45, 46] However, Ho *et al.* (2003) demonstrated that MOR-IR was very closely associated with ICC but did not directly label these cells. MOR may also be expressed by gut-associated T cells and myeloid cells.[48] We did not detect any labeling of CD45$^+$ leukocytes in our preparations including an absence of MORmCherry-positive cells within submucosal vessels or the mucosa. Furthermore, there was no evidence for MORmCherry expression by muscularis macrophages (CD68$^+$) or by cells with equivalent distribution patterns close to myenteric ganglia, within the submucosal layer, or associated with submucosal vessels.[49] Finally, there was potentially weak, diffuse labeling of intestinal epithelia. [50, 51] However, these cells did not exhibit intense punctate labeling as seen in myenteric neurons and there were no positive cells evident in basal epithelia layers. Thus, the distribution of MORmCherry in the gastrointestinal tract of healthy mice is not congruent with expression by non-neuronal cells as described in the existing literature.

MOR in the myenteric plexus. The general effects of MOR agonists on GI function are conserved across species, and the mouse is commonly used as to assess efficacy and potential side-effects of novel opioid receptor-ligands.[2, 3, 18] Although the guinea pig ileum has been used as the gold standard tissue for assaying opioid receptor function in the ENS,[28, 52, 53] the development of MOR tolerance by this tissue is inconsistent with clinically observed constipation, which is sustained with treatment due to the absence of tolerance.[52] In contrast, MOR signaling is sustained in the mouse colon,[33] supporting the use of this tissue as a preclinical model of MOR regulation in the human colon.

In marked contrast to the guinea pig intestine,[34, 47] there is very limited information available regarding the neurochemistry of MOR-positive neurons in the mouse ENS. To avoid specificity concerns that are commonly associated with GPCR-targeted antibodies and potential issues with low level detection,[20, 24, 30] we examined the distribution of MOR using transgenic mice expressing MORmCherry under the control of the endogenous promoter *Oprm1*.[21] The distribution pattern that we describe using these mice is consistent with the established function of MOR in the ENS. Activation of MOR leads to hyperpolarization of enteric neurons through the opening of $K^+$ channels. Agonists may also act presynaptically to inhibit neurotransmitter release via closure of $Ca^{2+}$ channels. Collectively, these mechanisms suppress action potential firing and neurotransmission, and underlie the inhibitory actions of opiates on secretomotor function and motility of the GI tract.[41, 54] MOR agonists inhibit electrically-stimulated contractions of the mouse ileum and colon,[32, 33] which is consistent with the expression of MORmCherry by ChAT-positive excitatory neurons. MOR agonists increase basal tone and reduce neurogenic relaxations and this correlates with localization of MORmCherry to nNOS-positive inhibitory neurons.[55] MORmCherry was predominantly expressed by the cholinergic population of the ileum and colon. This contrasts with previous studies of the guinea pig intestine using validated MOR antibodies, where MOR expression was mainly in the nitrergic neuronal population of these regions.[34] There was a clear difference in the distribution of MORmCherry in the nitrergic population of the ileum (81% of NOS+ neurons were MOR+) compared to the colon (37 %). MOR was mainly expressed in the nitrergic population throughout the guinea pig GI tract, suggesting that there are species-dependent differences in MOR distribution. IPANs, which could be identified by calretinin- and ChAT-immunoreactivity, size and by their distinctive Dogiel type II morphology (revealed by NFM or calretinin labeling), were negative for MORmCherry. This is consistent with our previous description of MOR immunolabeling in the guinea pig intestine.[34] In direct contrast to our study, Smith *et al*. (2012 and 2014) recorded MOR-dependent responses from dissociated neurons with AH electrophysiological properties (i.e. IPANs)[58] from the mouse ileum and colon. However, myenteric neurons that lacked a prominent afterhyperpolarizing potential (i.e. interneurons and motoneurons), which are established to functionally express MOR (present study),[41, 55] did not respond to morphine. These fundamental differences may reflect altered MOR distribution and expression or phenotypic changes to neuronal populations that may occur under culture conditions. Differences in the relative proportion of MOR-IR neurons between intestinal regions have been described, with sparse expression in the ileum relative to the colorectum.[59] We report that a slightly higher percentage of total neurons express MORmCherry in the ileum relative to distal colon. This is consistent with our previous quantitative analysis in the guinea pig GI tract.[34]

MOR in the submucosal plexus. The activation of MOR on submucosal neurons reduces secretion and may contribute to the constipating effects of opioids.[41] Morphine inhibited neurogenic chloride secretion in the mouse colon, consistent with functional expression of MOR by submucosal neurons.[60] Previous descriptive studies have reported extensive immunolabeling of submucosal neurons using MOR antibodies.[34, 45, 61] However, the predicted functional distribution differs to the neuronal population identified by immunolabeling.[34, 60] We report that MORmCherry was expressed in a very limited population of submucosal neurons of the colon and was not detected in the ileum. Thus, there appears to be a disconnect between MOR distribution and function in submucosal secretomotor pathways.

**MOR and DOR are co-expressed in the ENS.** Functional coexpression and overlap between MOR and DOR positive neuronal populations has been described.[26, 29, 61] Recent evidence suggests that the MOR-DOR heteromer mediates the actions of Eluxadoline,[17]a clinically-approved mixed MOR agonist/ DOR antagonist for the management of diarrhea-predominant irritable bowel syndrome (IBS-D).[19] Despite the potential clinical importance of the MOR-DOR heteromer in the ENS, fundamental understanding of this interaction at the cellular and physiological level is lacking. An absolute requirement for heteromer formation is the co-expression and colocalization of both receptors.[9] The distribution and neurochemical coding of neurons which express both MOR and DOR was examined using MORmCherry/DOReGFP mice. This mouse line was used previously to map the distribution of these receptors with high specificity in the CNS and pain pathways.[20, 21] Our findings demonstrate that there is extensive overlap between MOR and DOR in the myenteric plexus and supports our previous study using DOReGFP mice in combination with validated MOR antibodies and a labeled MOR ligand.[29] However, the earlier study did not examine the neurochemical coding of neurons which co-expressed both receptors. Approximately a quarter of total myenteric neurons in the ileum (30 %) and colon (22 %) expressed both MORmCherry and DOReGFP. Thus, there is significant potential for these receptors to functionally interact at the cellular level in myenteric neurons. Furthermore, both receptors are activated by endogenous enkephalins, which are inhibitory neurotransmitters in the ENS and dampen neuronal excitability.[41, 62] The predicted cooperativity between MOR and DOR may also enhance the modulatory effects of endogenous opioids.[12] MOR and DOR were co-expressed by cholinergic and nitrergic neurons, suggesting a potential role for heteromeric receptors in the modulation of excitatory and inhibitory motor pathways, respectively.

**MOR and DOR functionally interact through a heteromer-independent mechanism.** The existence of GPCR heteromers under native conditions and their role in physiological processes remains controversial due primarily to the difficulty in examining their distribution and function in tissues and *in vivo*. However, Wang *et al*. (2018) demonstrated using the MORmCherry/DOReGFP knockin mouse line, validated antibodies, and *in situ* hybridization that these receptors are expressed by distinct neuronal populations in pain pathways. Furthermore, MOR and DOR are not co-internalized in the small subset of neurons that express both receptors. In contrast to neurons of the CNS and somatosensory pathways, our distribution data indicates that the ENS is a more suitable system in which to study potential MOR-DOR interactions. Collectively, the experimental evidence that we present in this study indicate that

MOR-DOR heteromers are either unlikely to exist in the ENS or play a very minor role in the control of neuromuscular transmission (**Fig. 9**).

**Receptor trafficking studies.** CYM51010 has previously been characterized as a biased agonist for the MOR-DOR heteromer in heterologous cell lines and *in vivo*.[22] CYM51010 inhibited EFS-evoked contractions which were blocked by either a MOR- or DOR-selective antagonist. However, CYM51010-evoked contractions were only effectively blocked by the DOR-selective antagonist NLT. The reasons underlying the different selectivity for MOR in the two assays and pathways examined are unclear. Endocytosis was only blocked by NLT, confirming a DOR-dependent mechanism of action for CYM51010. Trafficking of the MOR-DOR heteromer in heterologous cell lines is antagonized by selective antagonists for either MOR or DOR.[14, 15, 37] The inability of CTOP to inhibit CYM51010-mediated DOReGFP internalization supports an exclusively DOR-dependent mechanism of action. Moreover, DOReGFP internalization was evident in submucosal neurons of the ileum, which do not express detectable MORmCherry. These findings are supported by our GTPγ[S] studies in recombinant CHO cells expressing MOR or DOR, which indicate effective coupling of CYM51010 to DOR only. It should be noted that CYM51010 activates GIRK channels in dorsal horn neurons of $DOR^{-/-}$ mice,[20] indicating that other modes of action may mediate its effects.

**Allosteric cooperativity between MOR and DOR.** The MOR-DOR heteromer exhibits distinct signaling and trafficking properties compared to the individual protomers when examined in heterologous cell lines and membrane preparations. Demonstration of a unique pharmacological profile is one of the key requirements for recognition of heteromer formation in native systems.[63] Several observations outlined in this study indicate that MOR and DOR do not function as a heteromer in the ENS. The MOR-selective agonist DAMGO, which robustly internalizes MOR in myenteric neurons,[34] did not internalize DOReGFP in myenteric neurons. Conversely, MOR immunoreactivity was retained at the cell surface in SNC80-treated preparations. These observations indicate that MOR and DOR internalization occurs independently. The co-degradation hypothesis states that the MOR-DOR heteromer is targeted for lysosomal degradation and this effectively reduces the amount of functional receptor at the cell surface.[14] Prior internalization of DOR by SNC80 did not significantly suppress subsequent DAMGO-evoked contractions, which would be expected to occur if DOR and MOR are co-internalized. DOR-mediated responses were effectively desensitized since all subsequent responses to SNC80 were reduced. These functional observations are consistent with retention of MOR-IR at the cell surface of SNC80-treated neurons. These data indicate that MOR and DOR are internalized independently, which is inconsistent with their existence as heteromers.

Expression of MOR-DOR heteromers by ventral tegmental area neurons was demonstrated through enhanced MOR-induced hyperpolarization in the presence of selective DOR antagonists. [13] In the present study, inhibitory effects of either MOR or DOR agonists were unaffected by antagonism of the other receptor, indicating a lack of cooperativity between MOR and DOR in the ENS. Eluxadoline has been reported to activate MOR-DOR heteromers in the ENS.[17] However, our data suggest that Eluxadoline most likely exerts its effects on the colon through a heteromer-independent mechanism. Eluxadoline reduced castor oil-induced diarrhea in $DOR^{-/-}$ mice consistent with a MOR-mediated mechanism of action. Antagonism of

DOR can enhance the velocity of propagating contractile waves during peristalsis.[64] Eluxadoline is a mixed MOR agonist-DOR antagonist and may therefore act through the two distinct receptors to form an interaction at the physiological level, rather than through activation of heteromers. The current findings also highlight that tools currently used to probe for the MOR-DOR heteromer in tissue may lack sufficient specificity to accurately assess their functional expression.

**MOR and DOR functionally interact in a heterologous manner in the ENS.** Although our data demonstrate that MOR and DOR are unlikely to form heteromers in the ENS, GPCRs may also interact at the cellular level through alternative mechanisms including heterologous desensitization.[40] To our knowledge, very few studies have specifically examined heterologous GPCR desensitization in the ENS. Activation of the neurokinin 1 receptor ($NK_1R$) desensitized neurokinin 3 receptor ($NK_3R$)-mediated secretory responses in the guinea pig distal colon.[65] Prior $NK_1R$ activation also reduced agonist-mediated $NK_3R$ internalization in cultured myenteric neurons. [66] These studies provide physiological evidence for heterologous GPCR desensitization in the ENS. In the present study, the high efficacy DOR agonist SNC80 desensitized MOR-mediated contractions of the colon. MOR-evoked colonic contractions are susceptible to desensitization in $\beta Arr2^{-/-}$ tissues.[32, 67] SNC80 robustly recruits β arrestins,[68] and it is possible that cross-desensitization of MOR occurs through sequestration of βArr2.[66] The partial agonist ARM390 had relatively minimal effect on MOR-mediated responses. SNC80 and ARM390 display marked differences in their ability to recruit βArrs and to internalize DOR,[68] which supports this mechanism of interaction. The unidirectional cross-talk between MOR and DOR may have important physiological implications for motility. The activation of DOR by endogenous opioids is enhanced during both colonic inflammation and states of high intraluminal pressure.[31] This may affect the activity of endogenous MOR-acting ligands including enkephalins or endorphins. Furthermore, CYM51010 desensitized DAMGO-evoked contractions which confirms a similar mechanism of action to high efficacy DOR agonists.

Heterologous desensitization may also occur through related intracellular mechanisms including the sharing of the same G protein pool,[69] activation of second messenger kinases such as PKC or PKA,[70] phosphorylation by GRKs[71] or the recruitment of arrestins to the inactive receptor.[72] Specific examination of these pathways in tissues is limited by the ubiquitous expression of partner proteins and kinases, such as PKC.[73] Furthermore, small molecule inhibitors of endocytosis and GRK can negatively impact neurogenic and smooth muscle contractions which limits their use in functional studies (Dicello *et al* unpublished).

In conclusion, we demonstrate that MOR and DOR are coexpressed by a subset of myenteric neurons in the mouse intestine. Although our data indicate that it is unlikely that MOR and DOR form heteromers, the functional interaction between these receptors that we have identified may represent a unique pharmacological target for therapy. The inhibition of the effects of morphine on the ENS by prior exposure to a high efficacy DOR agonist may provide a unique opportunity to limit the negative gastrointestinal effects of opioid analgesics.

## Methods

**Animals**. C57Bl/6J and DOReGFP knockin mice[74] (6-8 weeks, male) were purpose bred by the Monash Animal Research Platform. Mice were housed under a 12h light/dark cycle, controlled

temperature (24ºC), with free access to food and water. MORmCherry and MORmCherry/DOReGFP mice (male and female) were maintained at x and housed under tightly controlled conditions as described.[21] All procedures involving mice were approved by the Monash Institute of Pharmaceutical Sciences and Université de Strasbourg animal ethics committees.

**Reagents.** Carbamoyl choline (carbachol), CTOP, CYM51010, [D-Ala$^2$,*N*-MePhe$^4$, Gly-ol]-enkephalin (DAMGO), naltrindole hydrochloride (NLT), and nicardipine hydrochloride (Sigma-Aldrich). SNC80 (Cayman Chemical), ARM390 (Tocris Bioscience), morphine hydrochloride (MacFarlan Smith), tetrodotoxin citrate (TTX, Alomone).

**Immunolabeling.** MORmCherry and MORmCherry/DOReGFP knockin mice were euthanized with ketamine/ xylazine (11/10 mg/kg, i.p.). The ileum and colon were excised and placed in ice cold PBS. Tissues for sectioning were fixed in 4% paraformaldehyde overnight, washed (3x PBS washes), cryoprotected in 30 % sucrose (PBS, 0.1 % sodium azide, overnight) and embedded in optimal cutting temperature compound. Frozen sections (16 µm) were then prepared for immunolabeling. Wholemount preparations of myenteric and submucosal plexuses were prepared as described.[29, 31] Sections and wholemounts were incubated in blocking buffer (5% bovine serum albumin, 0.1% Triton X-100 in PBS containing 0.1% sodium azide; 1 h, RT) and then labeled with primary antibodies outlined in **Table 3** (diluted in blocking buffer, 4°C, overnight). Primary antibodies were detected using donkey secondary antibodies conjugated to Alexa Fluor® 405, 488, 568 or 647 dyes (1:500; 1 h, RT; Thermofisher). Tissue sections were also labeled with the nuclear marker 4',6-diamidino-2-phenylindole (DAPI; 1:1,000, 5 min). Preparations were mounted in ProLong Diamond anti-fade mountant (Thermofisher).

**Imaging and analysis for expression studies.** Preparations were imaged using a Leica-SP8-TCS confocal microscope system. Five confocal images including myenteric or submucosal ganglia were captured per preparation as z-stacks (40x objective, NA 1.3, 16-bit depth, 1024 x 1024-pixel resolution). Overlap of MORmCherry or MORmCherry/DOReGFP with neurochemically defined neuronal subpopulations was measured as described.[31, 34] Counts were presented as a percentage of positive neurons relative to different populations and were also expressed as neuronal counts.

**DOReGFP internalization assay.** Wholemounts of the ileum and distal colon of DOReGFP knockin mice were prepared and allowed to recover before use (Krebs containing 1 µM TTX and 10 µM nicardipine, 37°C, 1 h, bubbled with 5% $CO_2$, 95% $O_2$). Preparations were treated with antagonists (37°C, 20 min) or vehicle (0.1 % DMSO), then washed with ice-cold Krebs (3 x 5 min washes). These were then exposed to agonist (1 µM SNC80, 1 µM CYM51010 or 1 µM DAMGO; 4°C, 1h, in the presence of antagonist or vehicle), washed (3 x 5 min washes, ice-cold Krebs), and recovered to allow DOReGFP endocytosis (agonist-free Krebs with or without antagonist, 37°C, 30 min). Tissues were fixed (4% PFA, overnight, 4°C). Fixative was cleared (3 x 10 min washes, PBS) and circular muscle-myenteric plexus wholemounts were prepared. Preparations were labeled for eGFP, nNOS, and Hu immunoreactivities. Some preparations were also labeled for qualitative assessment of MOR distribution.

**Table 3**. **Primary antibodies used in this study.**

| Antigen/neurochemical marker | Host | Dilution | Code and Manufacturer |
|---|---|---|---|
| Calretinin (CalR) | Goat | 1:1,000 | AB1550 (Merck) |
| CD45 | Rat | 1:500 | 30-F11 (BioLegend) |
| Choline Acetyltransferase (ChAT) | Goat | 1:200 | AB144P (Merck) |
| Glial fibrillary acidic protein (GFAP) | Chicken | 1:1,000 | ab4674 (Abcam) |
| GFP | Chicken | 1:500 | ab13970 (Abcam) |
| HuC/D | Human | 1:25,000 | Anti-Hu (Luchinetti 1998) |
| mCherry (DsRed1) | Rabbit | 1:1,000 | 632496 (Clontech) |
| MOR | Rabbit | 1:500 | ab134054 (Abcam) |
| Neurofilament M (NFM) | Chicken | 1:1,000 | ab134458 (Abcam) |
| Neuronal nitric oxide synthase (nNOS) | Sheep | 1:1,000 | GTX89962 (Genetex) |

**Imaging and analysis of DOReGFP internalization.** Five confocal images including myenteric or submucosal ganglia were captured per preparation (40x objective, ≥2.0 zoom, 16-bit depth, 1024 x 1024-pixel resolution). The subcellular distribution of DOReGFP within the neuronal soma was determined using nNOS and Hu immunoreactivities to define cellular morphology. Images were converted to binary (i.e. positive or negative pixels) using the nucleus to define the threshold for positive staining. At least 30 neurons from preparations from 3-5 mice were analyzed per treatment group. Cell surface-associated DOReGFP was expressed as a relative percentage of total cellular DOReGFP labeling.

**Myenteric and dorsal root ganglion neuron culture and transfection.** Myenteric neurons of the colon and dorsal root ganglion neurons were isolated by mechanical and enzymatic digestion [29, 76] and nucleofected with 600 ng human MOR-GFP using an Amaxa Nucleofector system.[76] Cells were cultured (4 d *in vitro*), treated (DAMGO 100 nM, 30 min or vehicle), fixed (4% PFA, 20 min on ice), immunostained (GFP, Hu, GFAP) and imaged by confocal microscopy.

**Cell Lines and Membrane Preparation.** Flp-In Chinese hamster ovary (CHO) cells stably expressing either human DOR or MOR were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 0.3 mg/ml of Hygromycin (37°C, 5 % $CO_2$, 95 % $O_2$). Cell membranes were prepared for GTPγS $^{35}S$ assay. Briefly, cells were grown to confluence and washed with warm phosphate-buffered saline (pH 7.4). Cells were detached

with warm Versene and pelleted by centrifugation (350*g,* 3 min, room temperature (RT)). The pellet was resuspended in ice-cold homogenization buffer (20 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM EGTA, pH 7.4) and homogenized for three 10-s intervals at maximum setting, with 30-s cooling periods on ice between each burst. The homogenates were centrifuged (600*g*, 10 min, 4ºC), the pellet was discarded, and the supernatant was re-centrifuged (20,000*g*, 4ºC, 1 h). The final pellet was resuspended in 20 mM HEPES, 10 mM $MgCl_2$ and 100 mM NaCl, pH 7.4 using a syringe. Protein concentration was determined using bicinchoninic acid quantification method with bovine serum albumin (BSA) as the standard. Aliquots were stored at -80ºC until required for GTPγS $^{35}$S assay.

**GTPγS $^{35}$S Binding Assays.** GTPγS $^{35}$S binding experiments were performed using cell-membrane homogenates as described. Membrane homogenates (10 μg) were equilibrated in a 200-μl volume of GTPγS $^{35}$S assay buffer (20 mM HEPES, 10 mM $MgCl_2$ and 100 mM NaCl, 30μg/ml Saponin and 0.1% BSA, pH7.4) containing varying concentrations of CYM51010 and 10 μM or 30 μM GDP (MOR and DOR, respectively; 30 min, RT). After this time, 50 μl of [$^{35}$S] (0.3 nM) was added and incubation was continued for an additional 60 min (RT). Incubation was terminated by rapid filtration with a Packard plate harvester onto 96-well GF/C filter plates followed by three washes with ice-cold Tris buffer (50 mM Tris-HCl, 10mM $MgCl_2$, 100 mM NaCl, pH7.6). After drying for 3 h at 55ºC, the GF/C filter plates were sealed with melt-on scintillator sheets. Bound [$^{35}$S] was solubilized in 40μL Microscint-20 and radioactivity was measured in a MicroBeta counter (Perkin-Elmer Life Sciences).

**Tissue contraction assays.** Tissues were placed in 10 mL water-jacketed organ baths containing Krebs solution (in mM; NaCl 118; KCl 4.70, $NaH_2PO_4.2H_2O$ 1; $NaHCO_3$ 25; $MgCl_2.6H_2O$ 1.2; D-Glucose 11; $CaCl_2.2H_2O$ 2.5) and maintained at 37°C and bubbled with 95% $O_2$/5% $CO_2$. Isometric contractions of the circular muscle were measured by a Grass FT03 force displacement transducer (Grass Instruments, Quincy, MA). Data were acquired with a PowerLab 4/SP system and viewed using LabChart software (v.5; AD Instruments Pty. Ltd., Castle Hill, NSW). Tissues were placed under a resting tension of 0.5-1 g and were equilibrated for 30 min prior to use. Drugs were applied at a volume of 10 μL into organ baths. Following the completion of each experiment, 10 μM carbachol was added to evaluate tissue viability. Tissues that were unresponsive to carbachol were omitted from analysis.

**Electrically-evoked contractions**. Neurogenic contractions were evoked by transmural electrical field stimulation (EFS; 0.5 msec duration, 3 pulses $s^{-1}$, 60V), which was applied through platinum electrodes incorporated into the tissue holder.[31] Tissues were incubated with either DMSO (0.1%), NLT (100 nM), CTOP (1 μM) for the entire experiment. Once reproducible baseline responses were maintained (≥3 sets, 5 min intervals), tissues were treated cumulatively with agonists (1 nM-10 μM, 5 min). Tissues were electrically stimulated (3 sets, 5 min intervals) following each drug addition, then washed (5 min). The amplitudes of EFS-evoked contractions were compared to baseline responses (i.e. in the absence of agonist). Data were expressed as % inhibition of the average baseline EFS-evoked contraction.

**Measurement of CYM51010-evoked contractions.** MOR and DOR agonists produce a tonic, neurogenic contraction of colonic circular muscle.[31] Tissues were treated with either DMSO (0.1%), NLT (100 nM), or CTOP (1 μM) for 15 min followed by cumulative exposure to increasing concentrations of CYM51010 (1 nM-10 μM, 2 min). The amplitude of the maximum contraction to CYM51010 was measured and expressed relative to basal activity.

**Heterologous desensitization of MOR- and DOR-dependent contractions**. Tissues were exposed to either DMSO (0.1 %), a selective DOR agonist (SNC80 or ARM390), a selective MOR agonist (DAMGO or morphine) or CYM51010 (all 1 μM, 5 min). Tissues were washed (3 washes, 5 min intervals), and increasing concentrations of either SNC80, DAMGO or morphine (1 nM-10 μM, 2 min intervals) were added cumulatively to the bath. Peak contraction amplitudes were measured as described above.

**Effect of DOR endocytosis on MOR- and DOR-dependent contractions.** Mice were administered a single dose of either vehicle (saline; 0.9 %, i.p; 3 h) or SNC80 (10 mg/kg, i.p; 3 h). At this dose and time point, SNC80 promotes significant internalization of DOReGFP in the soma, proximal neurites and nerve fibers of myenteric neurons (DiCello et al *manuscript submitted*). Tissue strips were prepared as described above. Following equilibration, either DAMGO or SNC80 (1 nM-10 μM, 2 min) were cumulatively added to the bath, and maximal contraction amplitudes were measured and analyzed as described above.

**Statistical Analyses.** Data were expressed as the mean ± s.e.m. and graphs were constructed in GraphPad Prism v8.0.1. All groups for image analysis were compared by one-way ANOVA followed by Tukey's multiple comparison test. For the contraction assays, specific statistical analyses used for each experiment are indicated in the respective figure legends. $P<0.05$ was defined as significantly different to the null hypothesis of no difference between means at the 95% confidence level.

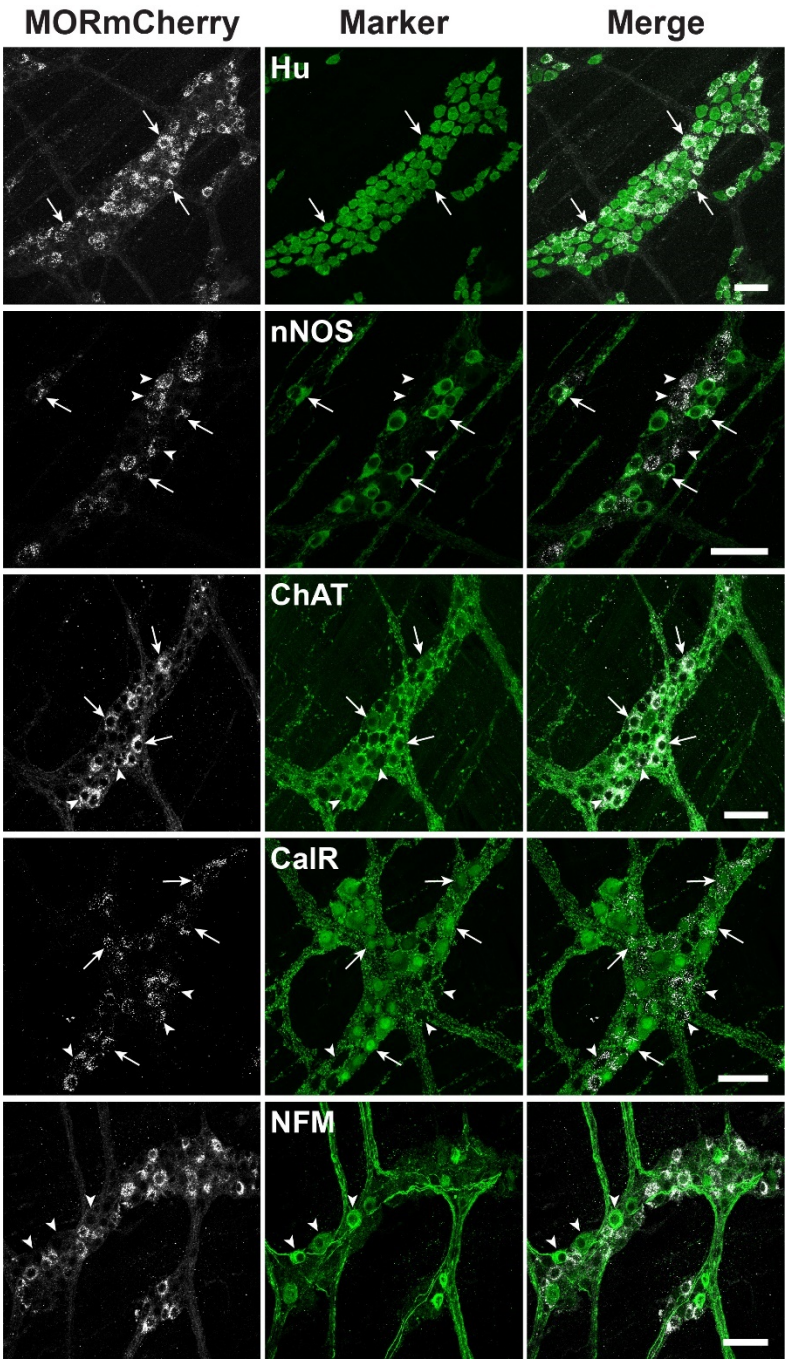



**Fig 1.** MORmCherry expression in the myenteric plexus of the distal colon. MORmCherry was distributed in a subset of all Hu-positive neurons. There was overlap with nNOS expressing inhibitory neurons, and with ChAT and calretinin expressing excitatory neuronal populations. MORmCherry was not detected in large diameter calretinin- or NFM-positive neurons indicating a lack of expression by intrinsic primary afferent neurons. Arrows: coexpression with marker; Arrowheads: no coexpression with marker. Scale: 50 µm.

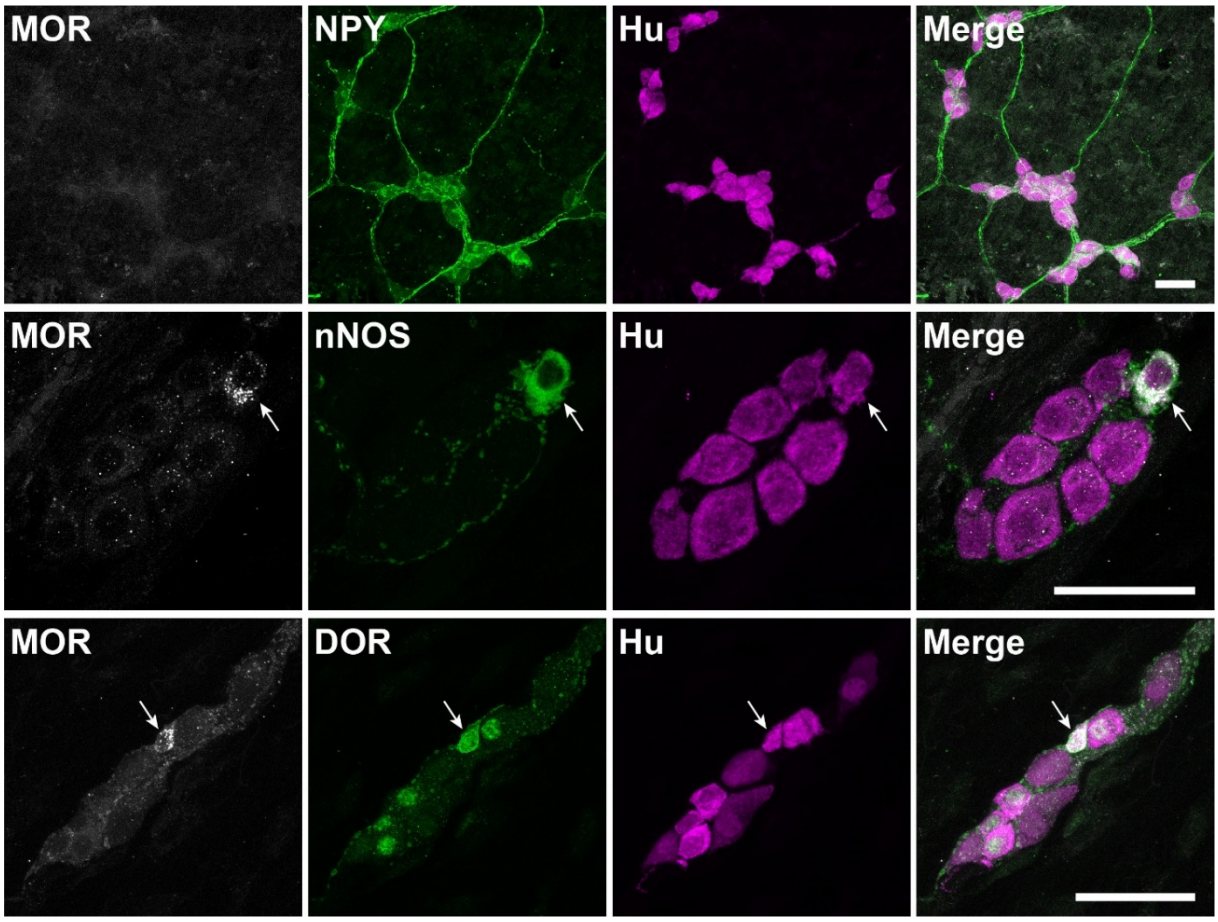



**Fig 2.** MORmCherry expression in the submucosal plexus. **Top**: MORmCherry labeling was not detected in neurons of the submucosal plexus of the ileum, as identified by NPY and Hu staining. **Middle:** MORmCherry was expressed by a small number of submucosal neurons of the distal colon. These neurons were generally nNOS positive. **Bottom:** MOR and DOR were coexpressed in a very limited number of submucosal neurons of the distal colon. Scale: 50 µm.

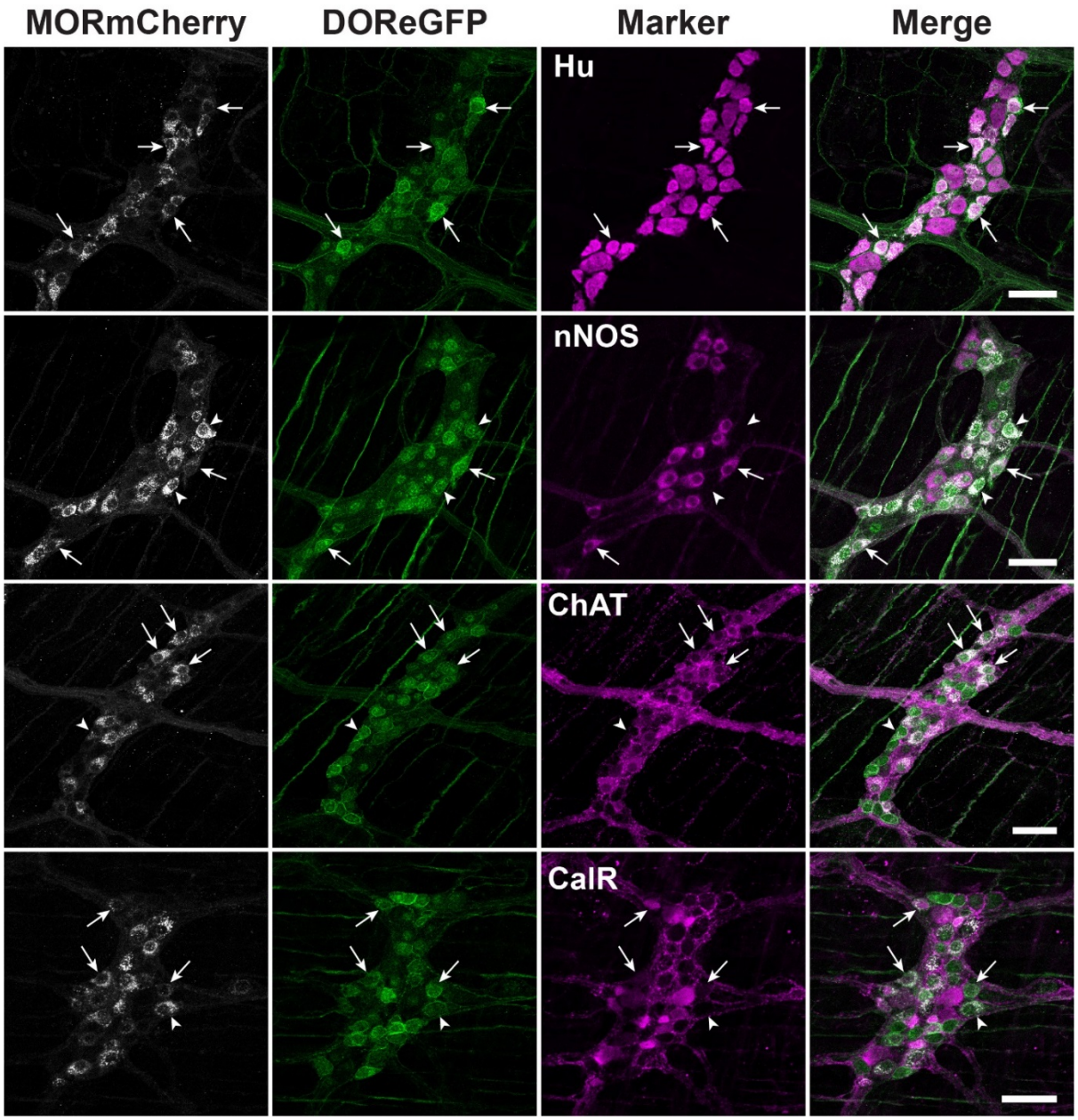



**Fig 3.** Examples of overlap between MOR and DOR in different myenteric neuronal populations of the distal colon. All MOR-DOR expressing cells were positive for the pan-neuronal marker Hu (arrows). Both nNOS positive (arrows) and negative (arrowheads) neurons expressed MOR-DOR. Similarly, MOR and DOR were coexpressed in both ChAT and CalR positive and negative population. Scale: 50 μm.

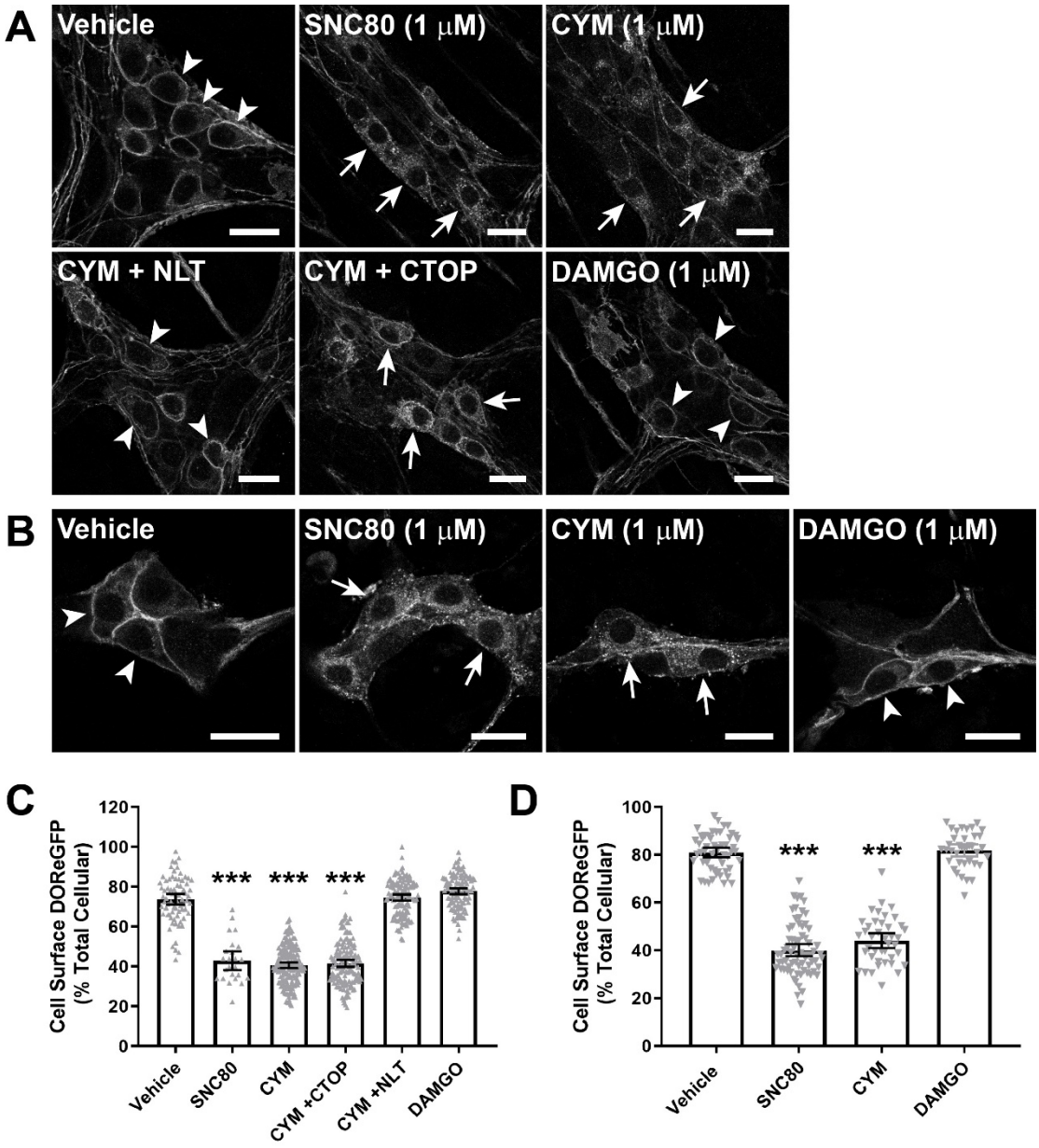



**Fig 4.** DOReGFP internalization occurs independently of MOR. **A**. DOReGFP was internalized from the cell surface to endosomes of myenteric neurons of the colon following treatment with SNC80 and CYM51010. CYM51010-evoked internalization was effectively blocked by NLT, but not by CTOP, indicating a DOR-dependent mechanism of action. No redistribution of DOReGFP was detected upon stimulation with the MOR agonist DAMGO. **B**. Both SNC80 and CYM51010, but not DAMGO, promoted DOReGFP internalization in submucosal neurons of the ileum, a site in which no MORmCherry expression was detected. Quantitative analysis DOReGFP distribution in (**C**) myenteric and (**D**) submucosal neurons following different treatments. Arrowheads: DOReGFP retained at the plasma membrane; Arrows: DOReGFP internalization. Scale: 20 μm, *** P<0.001, One-way ANOVA with Tukey's post-test.

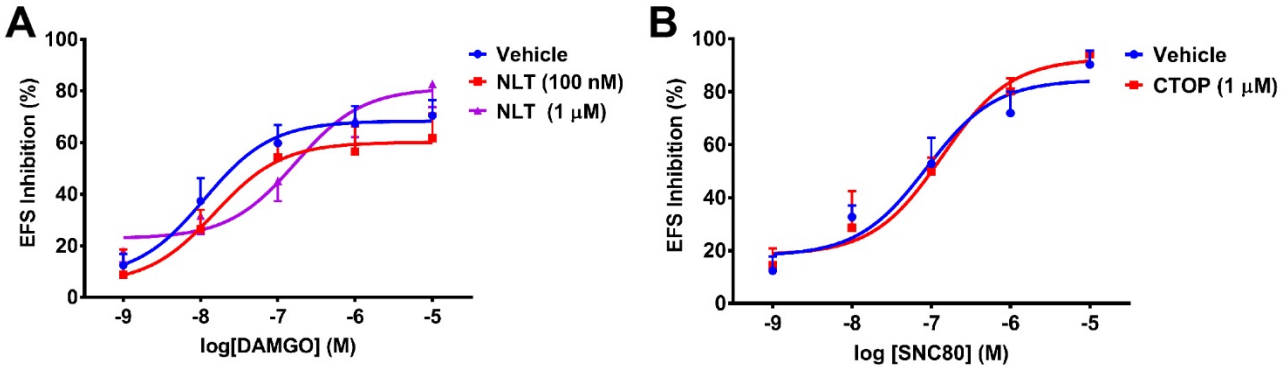



**Fig 5.** No evidence for cooperativity between MOR and DOR in excitatory motor pathways. **A**. DAMGO-mediated effects were unaltered by a low concentration of naltrindole (NLT). However, a higher concentration of NLT (1 µM) inhibited responses to DAMGO. **B**. Responses to SNC80 were unaffected by CTOP. Data are expressed as mean ± s.e.m, n= 5-9 mice per group.

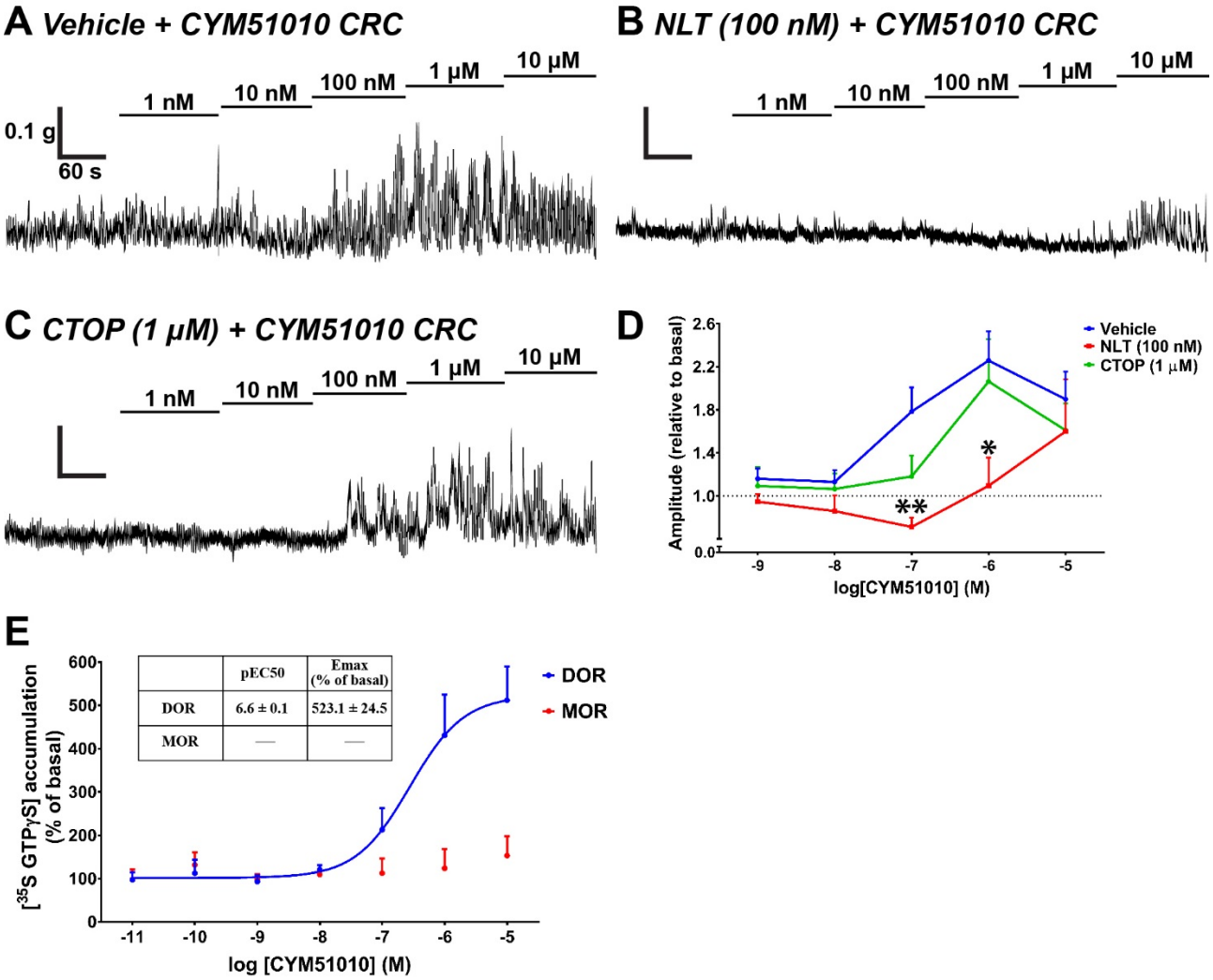



**Fig 6.** CYM51010-evoked contractions are mediated through DOR**. A**. CYM51010 evoked concentration-dependent contractions that were effectively blocked by naltrindole (NLT; **B**). **C.** In contrast, MOR inhibition by CTOP did not significantly alter the magnitude of contractile responses to CYM51010. **D.** Concentration-response curves of contractions evoked by CYM51010. Data are presented as mean ± s.e.m, n= 7-10 mice per group. Treatment groups were compared at each individual concentration by Kruskal Wallis' test followed by Dunn's post-hoc analysis (**p<0.01 and *p<0.05 compared to vehicle-treated group). **E.** CYM51010 produced a concentration-dependent increase in G protein activation only in DOR expressing CHO cells. Data are presented as mean ± s.e.m from 4 independent experiments.

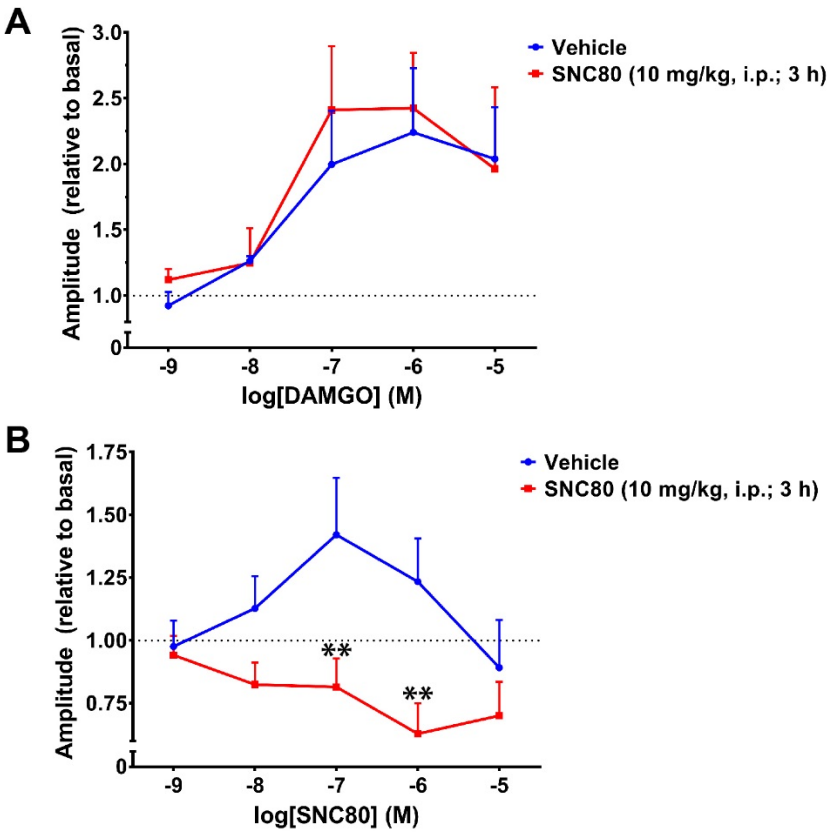



**Fig 7.** DOR internalization does not block MOR-dependent contractions of the colon. **A**. Internalization of DOR by SNC80 (3h, *in vivo*) does not inhibit DAMGO-evoked contractions **B.** Sustained activation of DOR effectively inhibited subsequent responses to SNC80, consistent with the development of acute tolerance. Data are presented as mean ± s.e.m, n= 5-7 mice per treatment group. Statistical analyses were conducted at each concentration using Student's unpaired t-test (**p<0.01 compared to the vehicle-treated group).

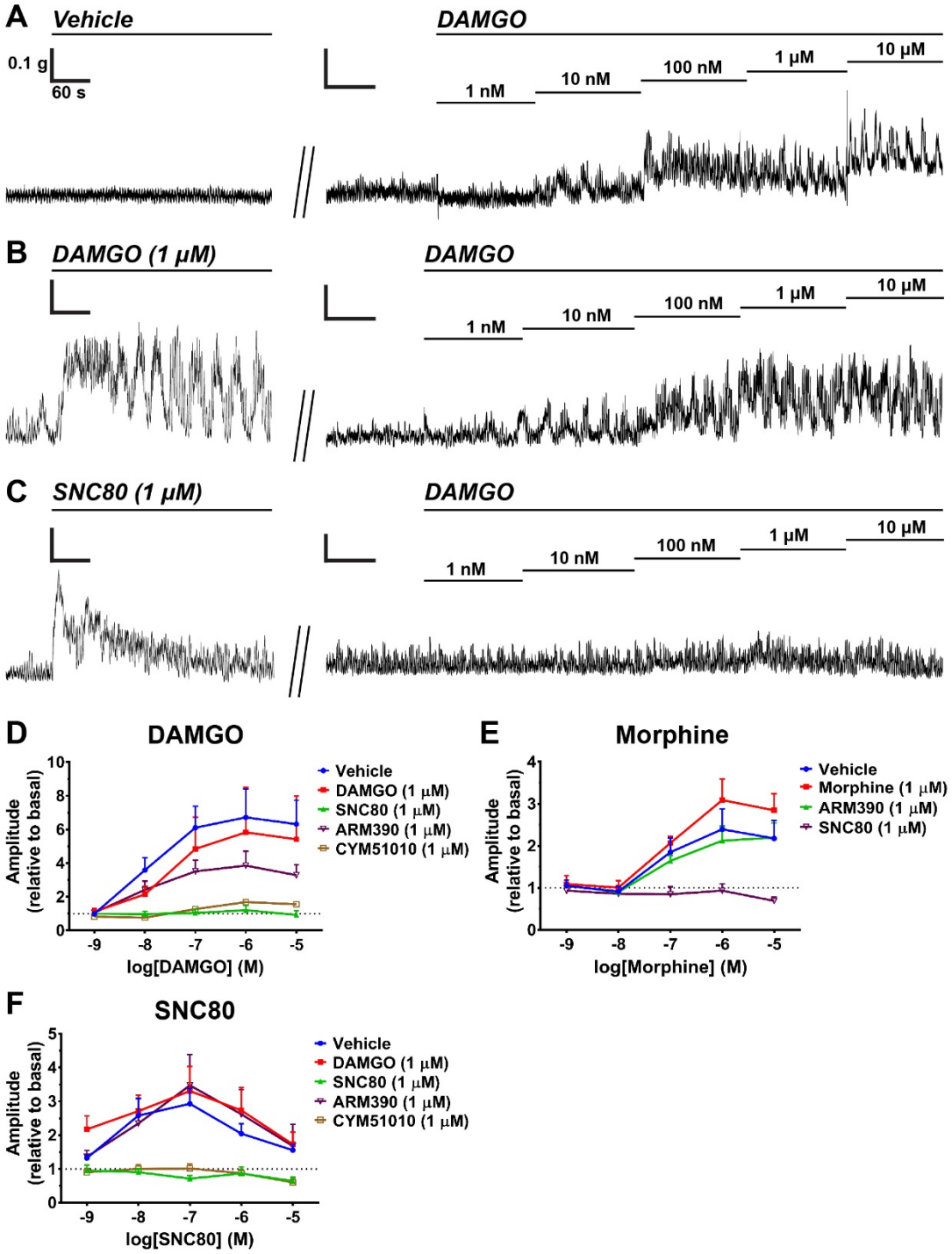
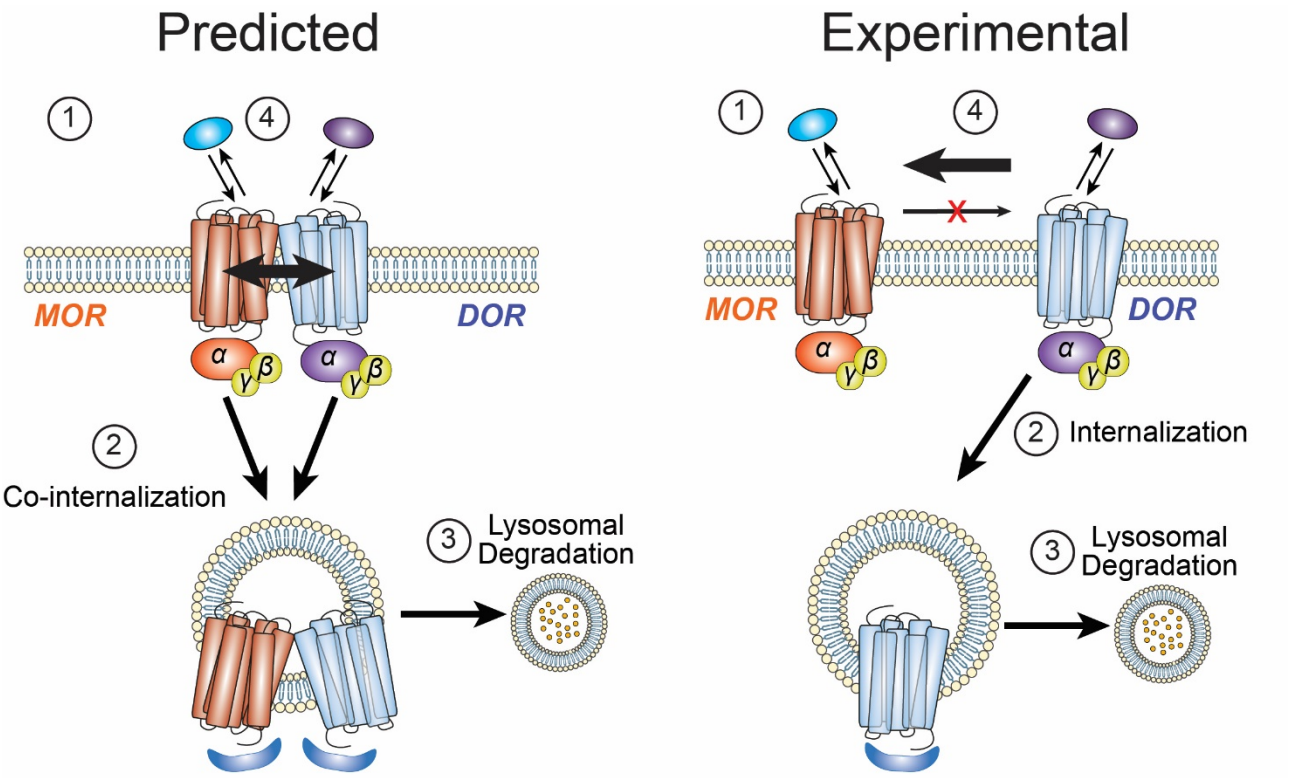
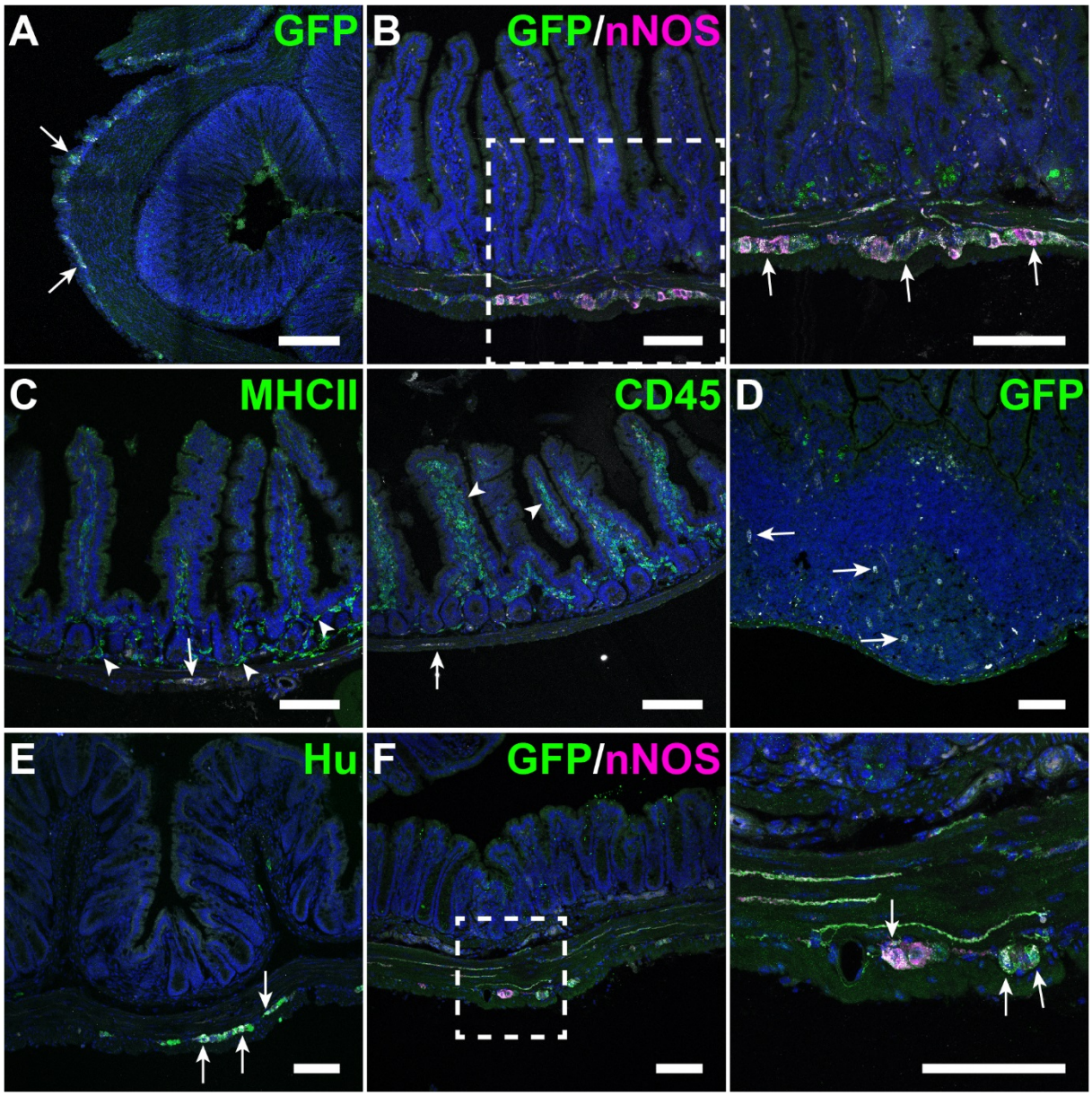



**Fig 8.** MOR and DOR functionally interact in enteric circuitry controlling inhibitory neuromuscular transmission. **A.** DAMGO evoked concentration-dependent contractions**,** which were unaffected by prior exposure to DAMGO (**B). C.** In contrast, treatment with SNC80 prevented all subsequent contractions to DAMGO. **D, E.** DAMGO- and morphine-evoked contractions were significantly reduced by strong internalizing DOR agonists, but not by MOR agonists. **F.** SNC80-dependent contractions were blocked by SNC80 and CYM51010, but not by MOR agonists or the weak internalizing DOR agonist ARM390. Data are presented as mean ± s.e.m, n= 5-12 mice per group.

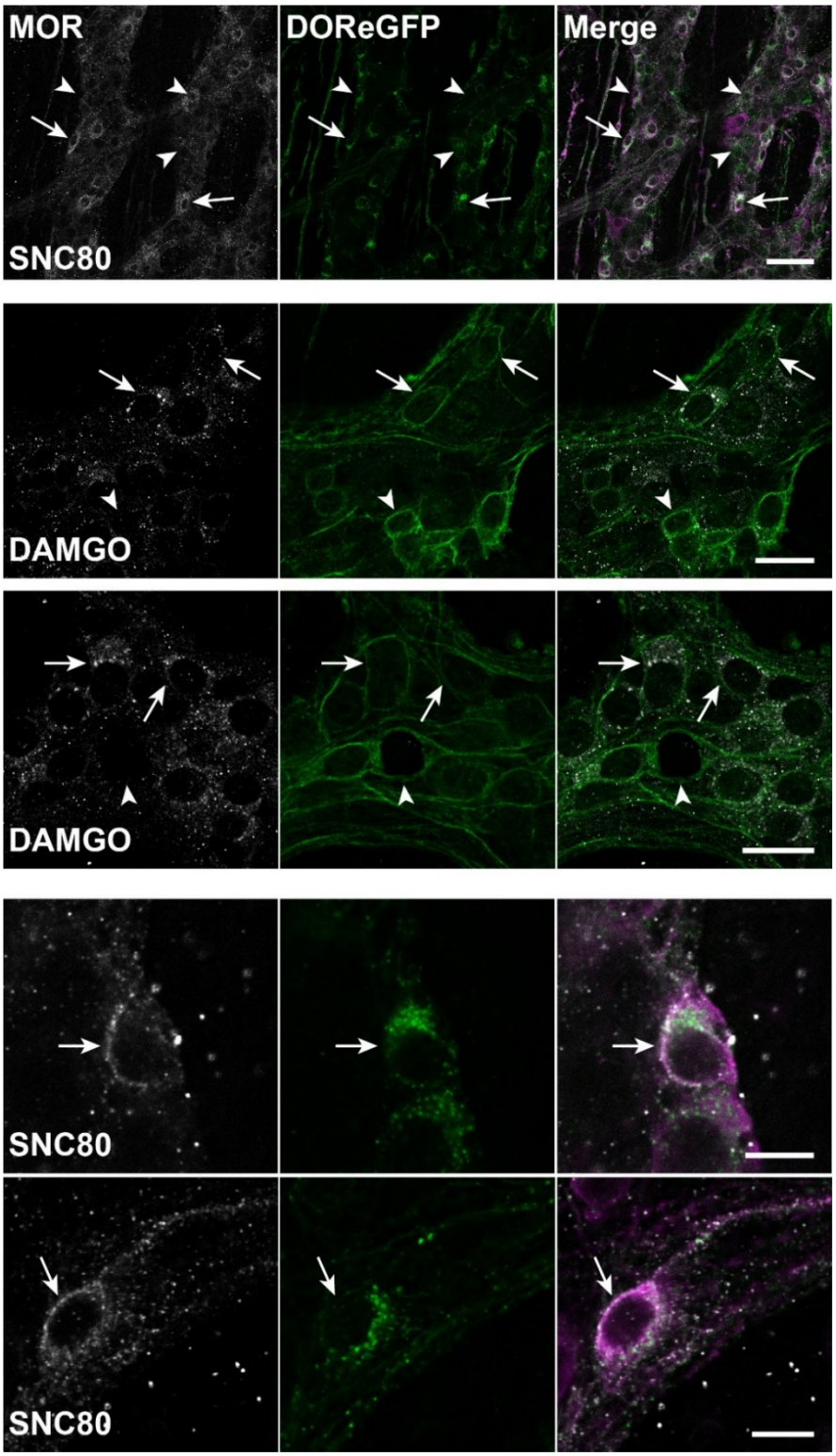



| Property Examined | Independent | Functional Interaction | Heteromer |
|---|---|---|---|
| Coexpression by same neuron | + | + | + |
| Independent endocytosis | + | - | - |
| No co-degradation of MOR | + | - | - |
| No cooperativity between MOR and DOR | + | - | - |
| Unidirectional Heterologous Desensitization | + | + | - |

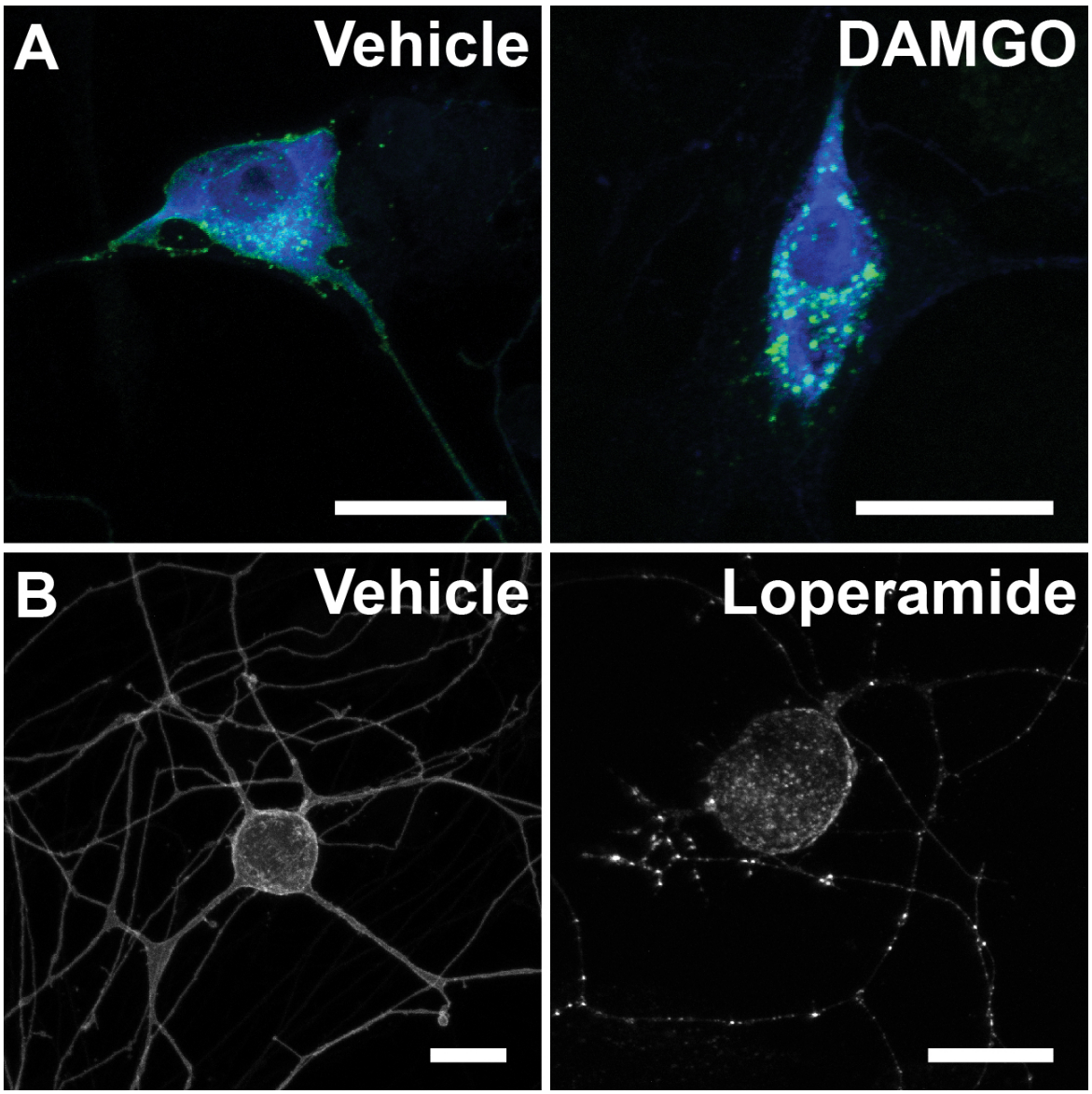
**Fig 9.** Overview of, and comparison between, the predicted interactions between MOR-DOR heteromers and the experimental evidence presented in this study. **A.** In the predicted model in which MOR and DOR exist as heteromers: 1. MOR and DOR are coexpressed; 2. MOR-DOR co-internalize and 3. co-degrade and 4. MOR and DOR functionally interact in a bidirectional manner leading to a unique pharmacological profile. **B.** Experimental evidence demonstrates that 1. MOR and DOR are coexpressed, but 2. do not cointernalize or 3. co-degrade; 4. MOR and DOR functionally interact in a unidirectional manner and this interaction is not associated with a unique pharmacological profile. **C**. Table summarizing evidence supporting three models of MOR-DOR expression in the ENS: independent expression, functional interaction, and heteromer formation.



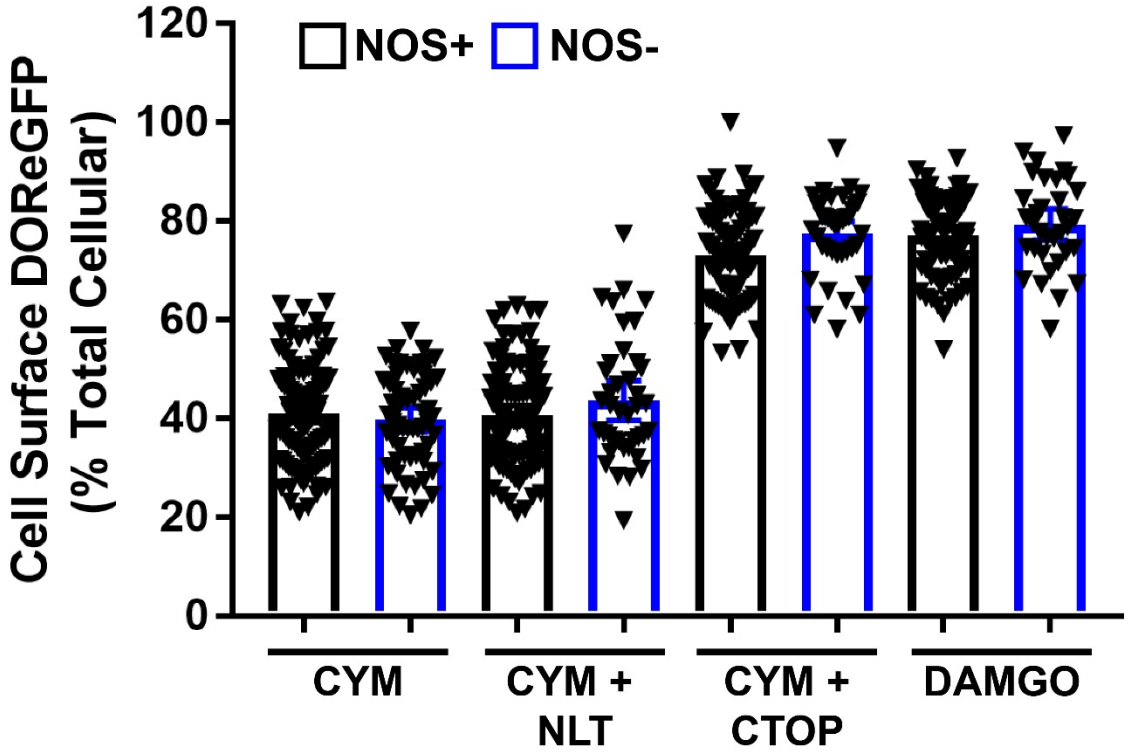

**Supplemental Fig 1.** Distribution of MORmCherry (grey) and overlap with DOReGFP in different regions of the gastrointestinal tract. **A.** MORmCherry expression in the antrum was restricted to myenteric neurons. A subset of these neurons coexpressed DOReGFP (arrows). **B.** A similar distribution and overlap with DOReGFP was observed in the ileum. **C.** MORmCherry (arrows) was not detected in MHCII- or CD45-positive cells of the ileal mucosa (arrow heads), but was detected in cells within lymphoid tissue (**D). E.** MORmCherry was expressed by Hu-positive myenteric neurons of the distal colon (arrows). **F.** There was overlap with DOReGFP- and nNOS-immunoreactive neurons (subset with arrows). Scale: 100 µm. (Antrum= 250 µm).



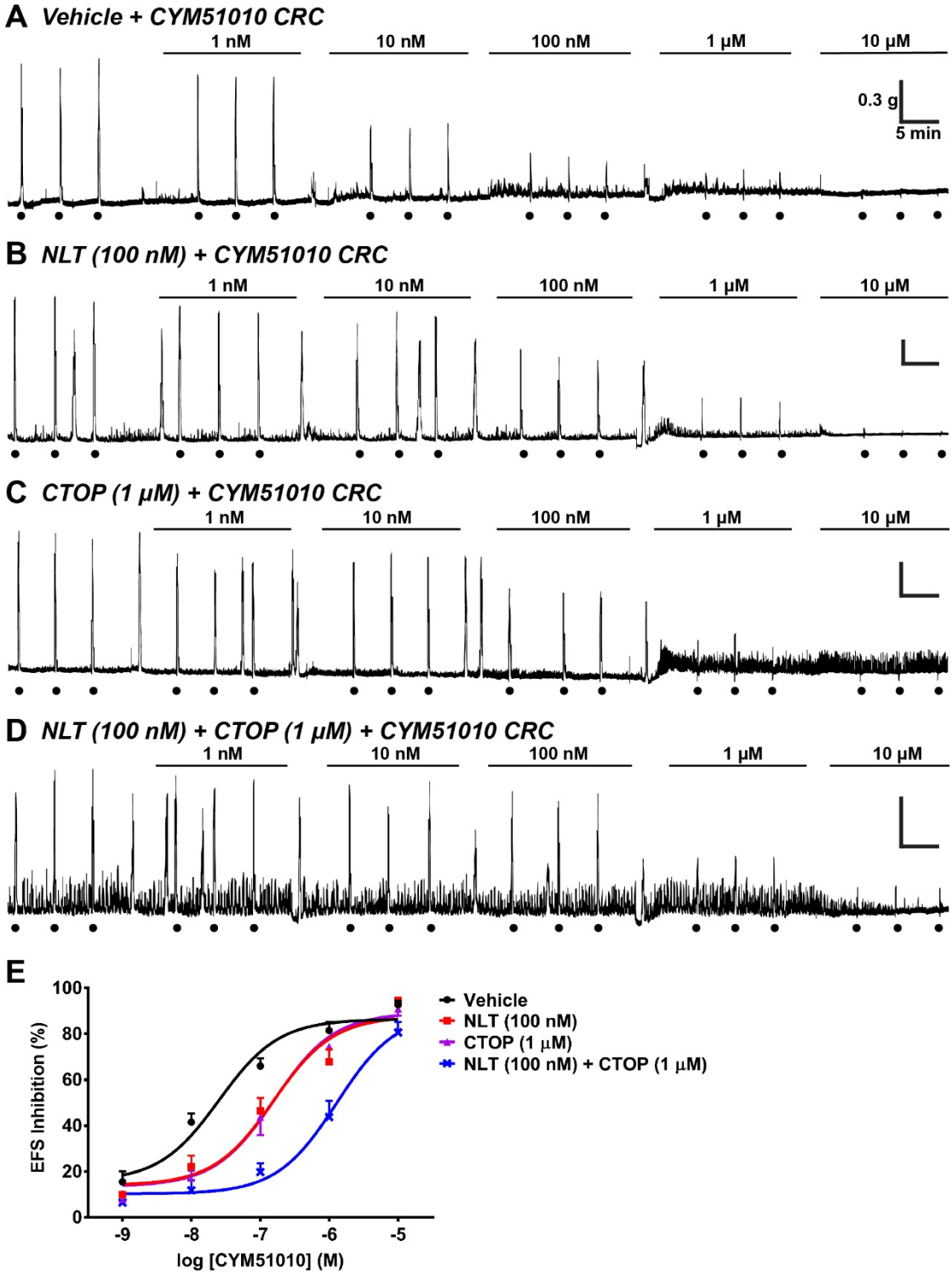

**Supplemental Fig 2**. Independent trafficking of MOR and DOR in myenteric neurons. There was extensive overlap between DOReGFP and MOR-immunoreactivity. MOR-IR was retained at the cell surface following treatment with SNC80 (1 μM, 30 min), whereas DOReGFP was internalized. Treatment with DAMGO (1 μM, 30 min) promoted internalization of MOR-IR. DOReGFP remained at the cell surface in the same neurons. Arrows: Coexpression of marker; Arrowheads: No coexpression of marker. Scale: 20 μm.




**Supplemental Fig 3**. MOR-GFP is expressed at the cell surface of myenteric and sensory neurons. **A.** MOR-GFP was localized to the cell surface and to intracellular structures in transiently-transfected myenteric neurons in culture. Treatment with the prototypical MOR agonist DAMGO (1 μM, 30 min) was associated with a loss of cell surface MOR-GFP and a corresponding increase in labeling of endosomes-like structures, consistent with MOR endocytosis. **B.** Equivalent observations were made in cultured dorsal root ganglion neurons following treatment with loperamide (1 μM, 30 min). Scale: 20 μm.




**Supplemental Fig 4.** Equivalent DOReGFP endocytosis in NOS positive and negative populations in response to the MOR-DOR heteromer-biased agonist CYM51010 (1 μM). Selective inhibition of DOR (NLT) did not result in a difference in CYM51010-evoked DOReGFP internalization between the two populations. Similarly, there was no difference in DOReGFP internalization in NOS+ and NOS- positive populations following treatment with either CYM51010 in the presence of CTOP or the MOR agonist DAMGO. Data are presented as mean values ± s.e.m. and were analysed by one-way ANOVA with Tukey's post-hoc test. Individual data points are represented as triangles.




**Supplemental Fig 5.** CYM51010 is a non-selective agonist for MOR and DOR in excitatory motor pathways**. A.** CYM51010 inhibited electrically-stimulated contractions in a concentration-dependent manner. Responses to CYM51010 were effectively blocked by (**B**) naltrindole (NLT) and (**C**) CTOP. **D.** Preincubation with both CTOP and NLT further inhibited CYM51010-mediated responses. **E**. Concentration-response curves of CYM51010-mediated effects on EFS-evoked contractions. Data are expressed as mean ± s.e.m, n= 6-13 mice per group.

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# Chapter 6
# *General Discussion*

## 6.1 Functional expression of DOR in the mouse colon and its contribution to motility

Despite several studies demonstrating both anatomical and functional expression of DOR by myenteric neurons (Bauer et al., 1991; Foxx-Orenstein et al., 1998; Menzies et al., 1999; Poole et al., 2011), there is still controversy regarding the relative contribution of peripheral DOR to GI motility. In **Chapter 2**, we established that DOR agonists inhibit neurogenic contractions of the colon and elevate GI smooth muscle tone, confirming functional expression of DOR in the ENS. These data correlate with the expression of DOR by both cholinergic and nitrergic motoneurons, respectively (Poole et al., 2011). Contraction assays using muscle strips are considered to be an indirect output of motility but are effective for screening drug targets in the GIT. However, data obtained from these assays do not always correlate with effects of agents on the complex motility patterns observed in the intact colon. For example, although exposure to either a NOS inhibitor or the neuronal blocker TTX produces a similar tonic contraction of colonic muscle strips (**Chapter 2**), these two compounds have distinct effects on propagating motor patterns. In the rodent model, NOS inhibitors enhance the frequency of propagating motor patterns, whereas exposure to TTX ablates motility (Brierley et al., 2001; Costa et al., 2017). This highlights that factors in addition to effects on contractility of muscle strips need to be considered when developing assays to predict drug-mediated effects on motility. Furthermore, peristalsis requires the complex interplay of the neural reflex circuitry and effectors within an intact system (Costa et al., 2017; **Figure 1.1**). Colonic motility is commonly assayed *in vitro* using spatiotemporal mapping of the intact colon, or *in vivo* by measuring faecal output or bead expulsion. Since SNC80 can slow colonic transit through a central mechanism (Broccardo et al., 1998) and peripherally-restricted DOR agonists are lacking, we assessed the contribution of peripheral DOR to GI motility using the spatiotemporal mapping technique.

CMMCs are peristaltic contractions which are commonly measured in spatiotemporal mapping studies (Costa et al., 2015). The propulsion of contents through the colon is likely to be mediated by a combination of CMMCs and the activation of the neuromechanical loop (described in ***1.1.4 Peristalsis***; Spencer et al., 2016). In **Chapter 3**, we reported that the high efficacy DOR agonist SNC80 inhibited CMMCs in the mouse colon. In addition to our finding that DOR is functionally expressed by myenteric neurons, we have also demonstrated that DOR regulates complex motor patterns. A similar finding was reported in the intact guinea pig colon, where the selective DOR agonist DPDPE reduced the propulsive velocity of an artificial pellet (Foxx-Orenstein et al., 1998). However, the inhibition of colonic transit by SNC80 *in vivo* was unaffected by the peripherally-restricted opioid receptor antagonist naloxone methiodide, indicative of a central mechanism of action (Broccardo et al., 1998). Taken together, this suggests that *in vivo* studies cannot directly examine the role of myenteric DOR in motility due to a confounding central action of an exogenous agonist. Furthermore, we reported difficulty in blocking the direct effects of SNC80 on neurogenic contractions. This may explain why peripherally-restricted opioids antagonists did not inhibit SNC80-mediated responses *in vivo*. The development of a peripherally-restricted high efficacy DOR agonist may help clarify the contribution of DOR expressed by myenteric neurons to colonic transit, which, to my knowledge, is not currently commercially available.

Our results also indicate that DOR-mediated inhibition of motility is a highly agonist-specific process. In direct contrast to SNC80, ARM390 had no effect on the occurrence of CMMCs. We propose that this is because ARM390 acts as a partial DOR agonist in the ENS. ARM390 was less efficacious than SNC80 at inhibiting neurogenic contractions and elevating the basal tone of colonic muscle strips, consistent with partial agonism. ARM390 is a full agonist in assays of G protein activation in spinal cord membrane preparations, adenylyl cyclase inhibition in recombinant cell systems where DOR is overexpressed, and analgesic efficacy (Marie et al., 2003; Pradhan et al., 2009). Thus, the pharmacological properties of ARM390 display 'system-bias'. System-dependent signalling at MOR

has been reported in the ENS. Morphine-mediated inhibition of colonic propulsion was reduced in βArr2-/- mice (Raehal et al., 2005), whereas the central analgesic action of morphine was enhanced (Bohn et al., 1999). Furthermore, responses to morphine in the colon are retained following prolonged treatment, whereas tolerance develops to morphine in central neurons (Arttamangkul et al., 2008; Ross et al., 2008). The potential for system-specific actions indicates the need to directly examine signalling in the ENS.

Our data indicates that weak internalizers of DOR have lower propensity than SNC80 to reduce colonic motility. The weak internalizing agonist TAN67 displayed similar pharmacological properties to ARM390 in our contraction assays. These data may explain the inability of the weak internalizing DOR agonist JNJ-20788560 to inhibit colonic transit in mice (Codd et al., 2009). Moreover, patients administered either ADL5747 or ADL5859 did not develop constipation or other GI disturbances in clinical trials. Both of these agonists are weak internalizers of DOR in central neurons (Nozaki et al., 2014). No studies to date have examined the effect of a full DOR agonist on human colonic motility. An alternative explanation is that DOR agonists may lack general efficacy in the human colon. Therefore, it will be critical to determine whether DOR agonists have motility-associated side effects in humans. Following this, future studies may focus on determining whether other weak internalizers of DOR such as KNT-127 or the ADL compounds have similar pharmacological properties to both ARM390 and TAN67 in the ENS. This may have important implications for the development of DOR therapeutics for CNS disorders without motility-associated side effects. Weak internalizers of DOR represent a better therapeutic option for a range of CNS disorders compared to the strong internalizing agonist SNC80. They have already been shown to have retained efficacies for reducing inflammatory and migraine pain following repeated administrations, whereas analgesic tolerance rapidly develops to SNC80. Equivalent actions were reported for anti-depressive and anxiolytic effects of DOR agonists (Nozaki et al., 2012; Nozaki et al., 2014; Pradhan et al., 2009; Rowan et al., 2014). In addition, the administration of SNC80, but not ARM390, results in opioid-induced hyperalgesia in rats (Rowan et al., 2014). In marked contrast to SNC80, the administration of either ARM390 or ADL5859 did not induce seizures in mice (Chung et al., 2015). Collectively, our data indicates that ARM390, and potentially other weak internalizers of DOR, may have a lower propensity for producing motility-associated side-effects which may contribute to a safer side-effect profile. Again, it will be critical in determining whether full DOR agonists influence colonic motility in humans.

We also established that DOR function is enhanced during colitis. This was associated with an increase in the density of DOR-expressing nerve fibers innervating the circular smooth muscle. This type of sensitization is consistent with reports that the ability of DOR agonists to inhibit intestinal transit is augmented during colitis (Pol, Ferrer, & Puig, 1994). Moreover, we demonstrated that DOR is internalized in myenteric neurons during inflammation which confirms that there is increased release of endogenous opioids. Boué *et al*. (2014) provided evidence that the enhanced release of enkephalins from T cells during colitis is important for suppressing visceral pain. Pain associated with colonic distension during inflammation was enhanced following the administration of naloxone methiodide, supporting an analgesic effect of peripheral endogenous opioids. However, the contribution of this endogenous opioid release and the sensitization of DOR function to complex GI motor patterns are unknown. Colitis is associated with neuronal hyperexcitability and endogenous opioids may suppress this excitability, similar to the actions of opioids on colonic nociceptors during inflammation (Guerrero-Alba et al., 2017). Future work in this area may include determining whether endogenous DOR activation during colitis influences colonic motility. Hoffman *et al*. (2011) demonstrated that exposure of the inflamed colon to pharmacological agents ($Cs^+$ and ZD7288) which act to restore the AHP and subsequently reduce neuronal excitability improved dysmotility. The enkephalinase inhibitor racecadotril may be a

useful pharmacological tool to examine whether enhancing DOR activation restores the generation of colonic motor patterns during colitis.

## 6.2 Comparison between DOR and MOR-mediated effects on motility

Although we have established that SNC80 inhibits CMMCs, additional factors may be important for determining whether DOR may be a suitable therapeutic target for dysmotility. We examined whether SNC80 disrupted peristaltic contractions to a similar degree as the constipating MOR agonist loperamide. Loperamide is only administered for acute diarrhea symptoms as prolonged treatment often leads to constipation. We demonstrated that both acute and chronic *in vivo* administration of SNC80 only partially reduced CMMCs, whereas the administration of loperamide almost abolished these motor patterns. Our data are concordant with other studies which reported that MOR agonists effectively disrupt peristaltic contractions of the isolated colon. Altarifi *et al.* (2017) determined that the movement of an artificial pellet through an intact colon was effectively blocked by either morphine or TRV130. This is consistent with reported findings *in vivo*, whereby constipation developed to both agonists. A separate study also demonstrated that acute morphine exposure reduced CMMC frequency in a concentration-dependent manner, with final concentrations above 10 nM abolishing propagating motility (Beckett, Staikopoulos, & Hutchinson, 2018). Since these motor patterns are retained following exposure to SNC80, we expect content to be propelled *in vivo*. Examining the influence of SNC80 on the propagation of an artificial pellet through the intact colon may determine whether DOR agonists effectively inhibit transit. This also reflects a limitation of the spatiotemporal mapping assay in that it does not provide direct evidence of content movement. Again, the development of a peripherally-restricted DOR agonist may also help correlate our spatiotemporal mapping findings with colonic transit.

MOR and DOR agonists supress neurogenic responses of both excitatory and inhibitory pathways in muscle strips. However, MOR agonists were more efficacious than SNC80 at reducing CMMCs. In **Chapter 5**, we comprehensively characterized with high specificity the neurochemical coding of MOR-expressing enteric neurons in the mouse. A high proportion of excitatory motoneurons expressed MOR. This correlated with the ability of MOR ligands to either completely or nearly abolish propulsive motor patterns (**Chapter 3**; Altarifi et al., 2017). Fida *et al.* (1997) used a pharmacological approach to examine the contribution of excitatory and inhibitory pathways to the frequency and amplitudes of CMMCs in the mouse colon. CMMCs were completely abolished following exposure to the muscarinic antagonist hyoscine, whereas the NOS inhibitor NOLA increased the frequency of CMMCs. This suggests that excitatory motor pathways are largely responsible for generating CMMCs, whereas inhibitory motor pathways maintain their consistency. In marked contrast to MOR, a high proportion of the nitrergic population expressed DOR compared to the cholinergic population (**Chapter 5**; Poole et al., 2011). Therefore, the relatively small proportion of cholinergic neurons expressing DOR may explain the ability of the colon to still generate CMMCs following exposure to SNC80. This demonstrates the importance of characterizing the neurochemistry of enteric neurons that express distinct GPCRs.

## 6.3 Long term effects of DOR activation to motility

The development of tolerance to desired drug-mediated responses is a common problem associated with opioid receptors (Williams et al., 2013). Tolerance is also a system-dependent phenomenon which may lead to unwanted side-effects. For example, tolerance readily develops to morphine-mediated analgesia, whereas the inhibitory effects of morphine on colonic motility are retained leading to OIC (Akbarali et al., 2014; Williams et al., 2013).

In **Chapter 3** we examined whether DOR agonists retain their inhibitory effects on motility with repeated exposures. The finding that SNC80 retains its inhibitory effects on propagating contractions following both acute (3 h) and daily administration indicates potential use for motility disorders including IBS-D. Importantly, the ability of the colon to still generate CMMCs following prolonged exposure to SNC80 suggests that colonic transit may be slowed rather than completely inhibited. This may be particularly important because constipation is a major cause of patient non-compliance to opiate treatments. To our knowledge, this is the first study to directly examine long-term effects of GPCR agonists on the generation of CMMCs. Therefore, we have provided a new methodological approach to determine the development of tolerance to GPCR-mediated effects on colonic motility.

Another key finding is that tolerance to DOR-mediated responses is a system-dependent process. Tolerance develops to SNC80 in central neurons (Pradhan et al., 2009; Pradhan et al., 2010), whereas we found that this is not the case in the ENS. This confirms that the regulation of GPCR responses in colonic myenteric neurons may differ to central neurons (Ross et al., 2008). The development of tolerance in the CNS is attributed to reduced receptor availability at the cell surface following SNC80 treatment (Pradhan et al., 2009). However, our data suggests that the prolonged effects of DOR activation to motility are independent of receptor availability. DOR internalization was prominent in both the soma and nerve fibers innervating the smooth muscle following both acute and chronic exposure to SNC80. It is possible that DOR may continue to signal in endosomes or in other organelles, such as the Golgi (Carbone et al., 2019; Stoeber et al., 2018). As discussed in ***Section 6.5***, the development of specific endosomal inhibitors may shed light on the role that signalling by intracellular DOR plays in SNC80-mediated responses in the colon.

## 6.4 Targeting endogenous DOR signalling in the ENS

Enkephalinergic nerve fibers are in close apposition to DOR-positive neurons in the myenteric plexus (**Chapter 2**; Poole et al. 2011). In **Chapter 2**, both chemical (veratridine) and electrical stimulation resulted in DOR internalization, indicative of endogenous activation of DOR-expressing neurons by enkephalins. These types of stimuli are considered unfocused because of their generalized effect on myenteric neurons. Veratridine is a voltage-gated sodium channel opener which causes neuron hyperexcitability and spasmodic contractions (Hoffman et al., 2011), and electrical stimulation promotes the release of neurotransmitters which evokes a neurogenic relaxation followed by a contraction. These assays are useful for the screening of endogenous GPCR activation within the myenteric plexus. For example, MOR is internalized in myenteric neurons following electrical stimulation (Minnis et al., 2003). A limitation of these assays is that they provide little information about the activation of GPCR-expressing neurons during a well-defined GI reflex, such as motility. To determine whether DOR is activated during motility reflexes, we mechanically evoked colonic propagating contractions and then examined DOR endocytosis in myenteric neurons. DOR was retained at the plasma membrane under basal conditions, whereas redistribution to intracellular compartments occurred following elevation of intraluminal pressure. This is the first detailed report of GPCR trafficking in myenteric neurons following a well-characterized physiological event. Our internalization data also correlated with reports that DOR antagonism accelerates the propagation of a pellet in a small segment of the guinea pig colon (Foxx-Orenstein et al., 1998). This demonstrates that DOR is activated and may be involved in the dynamic interplay of myenteric neurons which is critical for propulsive motility.

Our finding that DOR-expressing myenteric neurons are activated during motility was the first step in demonstrating that this system may be targeted by allosteric modulation. Data which were generated during my PhD, but not included in this thesis, indicated that a positive allosteric modulator (PAM) of DOR (BMS-986187; Burford et al., 2015) reduced the frequency of CMMCs during high, but

not basal, intraluminal pressures. This correlated with the internalization of DOR in myenteric neurons during elevated intraluminal pressure states. Moreover, the *in vivo* administration of BMS-986187 reduced bead expulsion in stressed, but not healthy mice and delayed the onset of castor oil-induced diarrhea. Stress-induced defecation is a preclinical model of the symptoms of IBS-D and was used to examine the anti-diarrheal effects of the FDA approved drug eluxadoline (Wade et al., 2012). Therefore, our data from **Chapter 2** which established endogenous activation of DOR and our unpublished work examining positive allosteric modulation of DOR highlights the important modulatory role of enkephalinergic signalling to motility. This also suggests that a negative allosteric modulator (NAM) of DOR, which supresses receptor signalling without displaying efficacy, may reduce enkephalinergic signalling and thereby enhance motility. In addition, a MOR NAM may reduce MOR signalling in enteric neurons and subsequently control OIC. To date, opioid receptor NAMs have not been developed. Our data also suggests that racecadotril may be mucosally-restricted since it has no effects on motility. However, this is just an assumption and no studies have directly examined this. In addition, racecadotril may not necessarily act at the colon because studies which directly examined its anti-secretory activity were limited to the small intestine of laboratory animals and humans (Primi et al., 1999).

## 6.5 Contribution of GPCR endocytosis to motility

In **Chapter 2** and **Chapter 3**, we reported the novel finding that DOR endocytosis in myenteric neurons is polarized, with rapid internalization in the soma and neurites, and delayed onset of internalization in the nerve fibers. This is also the first study to examine the kinetics of GPCR endocytosis within these different neuronal compartments. Previous studies which examined GPCR trafficking in myenteric neurons were restricted to the soma and neurites. $NK_1R$ internalization in the soma of myenteric neurons was associated with inflammation (Poole et al., 2015). Similarly, DOR was internalized in the soma of myenteric neurons during active colitis. However, we correlated the enhanced function of DOR with the retention of receptor at the plasma membrane of nerve fibers innervating the muscle. The associated functional changes to $NK_1R$ trafficking during colitis may depend on receptor availability on the nerve fibers. We have also provided the first detailed characterization of *real time* GPCR trafficking in enteric neurons. This methodological approach will also be useful for determining the spatial and temporal regulation of GPCR internalization during disease states including colitis.

It is now widely accepted that many GPCRs continue to signal from within endosomes. Only a limited number of studies have examined endosomal signalling in myenteric neurons (Cottrell et al., 2009; Pelayo et al., 2011). We established that the internalization of DOR in both nerve fibers innervating the circular smooth muscle and the soma correlated with the prolonged inhibitory actions of DOR agonists on CMMCs. There are other possible factors which may account for our data. The effectiveness of ARM390 to promote DOR coupling to G proteins at the cell surface may potentially contribute to its low efficacy in reducing the frequency of CMMCs. Furthermore, distinct agonists may display ligand-biased signalling. For example, ARM390-mediated analgesia is reduced in βArr2-/- mice (Pradhan et al., 2016). This suggests ARM390s efficacy in recruiting βArr2 to DOR, rather than its receptor internalizing properties, is important for its pharmacological response. The direct inhibition of endocytosis may provide a better approach to examine whether there is a link between endocytosis and colonic motility.

In **Chapter 4**, we confirmed that the small molecule endocytic inhibitors PS2 and Cmpd101 are useful for examining GPCR trafficking in myenteric neurons (Poole et al., 2015). However, these inhibitors alone either inhibit neurogenic contractions (PS2) or muscle contractility (Cmpd101). Therefore, they are unsuitable for examining the contribution of endocytosis to motility. Our findings are also important for the GPCR field in that these commonly used inhibitors may also affect neurotransmission in other systems. The use of lipidated GPCR antagonists may provide a better option

to examine the role of endosomal signalling on GI reflexes. Lipidated antagonists anchor at the plasma membrane and have proven to be effective for the delivery of drugs to endosomes (Jensen et al., 2017). They can be used to define the contribution of endosomal signalling to physiological processes (Jensen et al., 2017; Jimenez-Vargas et al., 2018; Yarwood et al., 2017). For example, a lipidated $NK_1R$ antagonist was used to establish the importance of endosomal signalling in second order spinal neurons to pain transmission (Jensen et al., 2017). Although $NK_1R$ is expressed by myenteric neurons and signals from within endosomes (Pelayo et al., 2011), colonic motility is unaffected by selective $NK_1R$ agonists (Holzer, Schluet, & Maggi, 1995). This indicates that the lipidated $NK_1R$ antagonist will be unsuitable for determining the contribution of GPCR endocytosis to motility. To my knowledge, lipidated opioid receptor antagonists have not been developed. Nanoparticles, which selectively deliver drugs to endosomes, may also be a future option to study the importance of endocytosis to GI motility (Zhou et al., 2011). Future work in this area may also include screening GPCR activation at the cellular level in a real system. Genetically-encoded biosensors which detect GPCR activation in real time were recently developed and used to establish that both MOR and DOR can continue to signal from within endosomes. Synthetic opioid receptor agonists may also penetrate through the membrane and activate their respective receptors located on the Golgi network of striatal neurons (Stoeber et al., 2018). These biosensors may also provide detail of the signalling of opioid receptors in enteric neurons.

## 6.6 Opioid receptor interactions in the ENS

Considerable attention has focused on the ability of GPCRs to form heteromers (Gomes et al., 2016). Heteromers may provide a novel therapeutic target which offers distinct pharmacological properties to the individual receptors. However, proving the concept of heteromers is a controversial topic since elucidating their expression and function *in vivo* is difficult. The International Union of Basic and Clinical Pharmacology have developed set criteria for the acceptance of GPCR heteromers (Pin et al., 2007; **Table 1.2**). Only a few studies have examined whether class A GPCRs functionally interact in the ENS. The MOR-DOR heteromer in the ENS has gained attention because eluxadoline is proposed to exert partial effects through this interaction. Fujita *et al.* (2014) investigated the protein levels of MOR-DOR in the ENS using a monoclonal antibody selective for the heteromer (Gupta et al., 2010). However, immunostaining was still present in tissue from DOR-/- mice. This study also used the antibody to characterise the mechanism of action of eluxadoline. The inhibitory effects of eluxadoline on colonic transit were partially blocked by the MOR-DOR antibody. With our expertise in opioid receptor pharmacology and GPCR biology in the ENS, we examined whether criterion 2 (**Table 1.2**) requirements are met for this proposed interaction. This states that *there must be evidence of a new or different pharmacological property of the heteromer in native systems*. In **Chapter 5**, we established that MOR and DOR are coexpressed by a high proportion of myenteric neurons, indicating the possibility of an intracellular interaction. However, we demonstrated that MOR and DOR internalize independently and exhibit no functional co-cooperativity in myenteric neurons, indicative of the absence of a novel pharmacological fingerprint. We also probed for MOR-DOR using CYM51010 which is a proposed biased agonist for the heteromer (Gomes et al., 2013). However, we established that this drug exerts most of its actions solely through DOR in the ENS, and stimulates G protein activity in CHO cells transfected with hDOR, but not hMOR. Based on our pharmacological data which provides strong evidence that MOR and DOR do not form heteromers in the ENS, we propose that eluxadoline exerts its actions through a heteromer-independent mechanism. Criterion 3 was not assessed because we do not have access to DOR-/- or MOR-/- mice. Our data suggests that functional assays using these mice are redundant because we did not observe any novel pharmacological properties in wildtype tissue.

Despite evidence suggesting that MOR and DOR do not form heteromers, we determined that strong internalizers of DOR desensitized MOR-evoked contractions of the colon in a unidirectional manner. This is the first study to directly examine heterologous desensitization of opioid receptors in

the ENS, and the contribution of this process to functional responses of myenteric neurons. Our finding that responses to morphine were effectively abolished by preincubation with strong internalizers of DOR may have important clinical implications for the treatment of OIC. For example, we demonstrated that morphine-mediated effects on neurogenic contractions are suppressed by a low concentration of SNC80. This may be particularly important because current pharmacological treatments for OIC include peripherally-restricted opioid receptor antagonists. These drugs display adverse side-effects including GI pain and diarrhea (Holzer, 2009). A limitation to our finding is that the underlying mechanism of the unidirectional heterologous desensitization remains unknown. Pradhan *et al.* (2016) determined that the analgesic efficacy of DOR agonists is reduced in both βArr1-/- and βArr2-/- mice. These mice may also be used to determine whether βArr1 or 2 are implicated in the desensitization of MOR-mediated responses by SNC80. In addition, Williams *et al.* (2018) used a phosphodeficient MOR mouse to determine that phosphorylation is critical for the desensitization and development of morphine tolerance in locus coeruleus neurons. A separate study by Kliewer *et al.* (2019) determined that the analgesic efficacies of fentanyl and morphine are enhanced in transgenic mice expressing phosphodeficient MOR. These studies demonstrate the importance of MOR phosphorylation to the overall response to agonists. These mice may also be used to identify whether the desensitization of MOR responses by SNC80 is a phosphorylation-dependent process. Another limitation to our findings is that we assayed heterologous desensitization using muscle strips. Future work should focus on examining whether this also translates to motility. This may be assayed by exposing the colon to a low concentration of SNC80 and determining whether this reverses MOR-mediated inhibition of CMMCs.

In conclusion, our findings have provided a better understanding of both opioid receptor and GPCR biology in the ENS. We have confirmed that DOR is functionally expressed by myenteric neurons and is endogenously activated during motility. This also demonstrates that DOR can be used as a model GPCR for examining allosteric modulation in the ENS. This conceptual advancement in the GPCR field has not been directly examined in the ENS, although unpublished reports suggest that this approach is feasible and potentially of therapeutic use. Furthermore, drug discovery efforts to develop MOR ligands without motility-associated side effects have failed potentially due to the lack of our basic understanding of GPCR signalling in the ENS. Conceptual advancements in the GPCR field, including endosomal signalling and heteromerization, were also examined in this thesis. Although we did not define the role of endosomal signalling to motility, our findings provide important evidence that commonly used small molecule endocytic inhibitors suppress neurotransmission or tissue contractility. We also demonstrated using the same pharmacological approaches which defined MOR-DOR in recombinant cell systems that this functional interaction probably doesn't occur in the ENS. Therefore, the proposed mechanism of action of eluxadoline on GI motility is probably via MOR. This body of work contributes greatly to our understanding of GPCR signalling in myenteric neurons at the neurophysiological level and provides evidence that DOR may be a safer therapeutic target for dysmotility than current clinical opiates.

# Chapter 7
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