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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

# Mu and Delta Opioid Receptors Are Coexpressed and Functionally Interact in the Enteric Nervous System of the Mouse Colon

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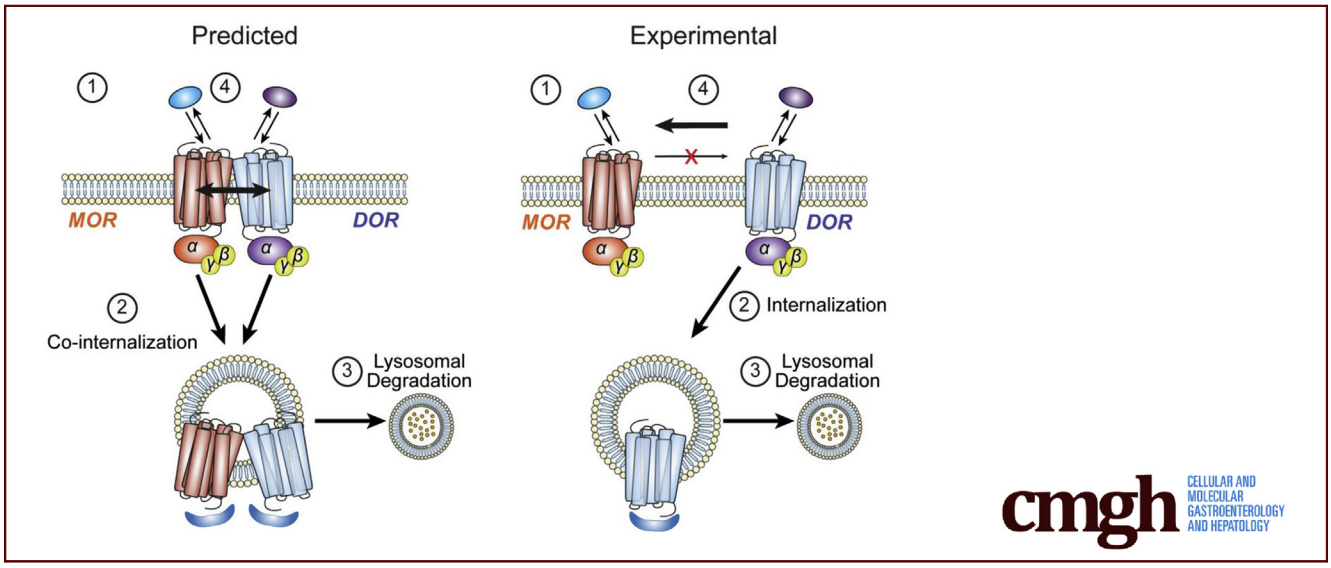


## SUMMARY

G protein-coupled receptors physically and functionally interact, leading to unique signaling. We report that delta and mu opioid receptors are coexpressed and functionally interact in myenteric neurons. Our findings have implications for opioid-based therapies for motility disorders and pain.

**BACKGROUND & AIMS:** Functional interactions between the mu opioid receptor (MOR) and delta opioid receptor (DOR) represent a potential target for novel analgesics and may drive the effects of the clinically approved drug eluxadoline for the treatment of diarrhea-predominant irritable bowel syndrome. Although the enteric nervous system (ENS) is a likely site of action, the coexpression and potential interaction between MOR and DOR in the ENS are largely undefined. In the present study, we have characterized the distribution of MOR in the mouse ENS and examined MOR-DOR interactions by using pharmacologic and cell biology techniques.

**METHODS:** MOR and DOR expression was defined by using MORmCherry and MORmCherry–DOR-eGFP knockin mice. MOR-DOR interactions were assessed by using DOR-eGFP internalization assays and by pharmacologic analysis of neurogenic contractions of the colon.

**RESULTS:** Although MOR was expressed by approximately half of all myenteric neurons, MOR-positive submucosal neurons were rarely observed. There was extensive overlap between MOR and DOR in both excitatory and inhibitory pathways involved in the coordination of intestinal motility. MOR and DOR can functionally interact, as shown through heterologous desensitization of MOR-dependent responses by DOR agonists. Functional evidence suggests that MOR and DOR may not exist as heteromers in the ENS. Pharmacologic studies show no evidence of cooperativity between MOR and DOR. DOR internalizes independently of MOR in myenteric neurons, and MOR-evoked contractions are unaffected by the sequestration of DOR.

**CONCLUSIONS:** Collectively, these findings demonstrate that although MOR and DOR are coexpressed in the ENS and functionally interact, they are unlikely to exist as heteromers under physiological conditions. *(Cell Mol Gastroenterol Hepatol 2020;9:465–483; https://doi.org/10.1016/j.jcmgh.2019.11.006)*

*Keywords:* G Protein-Coupled Receptor; Opioid Receptor; Heteromer; Heterologous Desensitization.

Opiates that target the mu 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] Opioid-induced constipation results from the direct activation of MOR expressed by enteric neurons and can be so severe that it leads to patient noncompliance 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 pharmacologic and physicochemical properties of MOR ligands, in particular biased agonism.[2–4] However, recent investigations of these biased agonists indicate that side effects, including constipation and respiratory depression, may still be retained.[5–8] Thus, there is a need for greater understanding of how opioid receptor regulation and signaling underlie the control of physiological processes, including GI motility.

The guinea pig ileum has been used as the gold standard tissue for assaying opioid receptor function in the enteric nervous system (ENS),[9] and we have previously characterized the neurochemical coding and identity of MOR-positive neurons in this model.[10] However, the development of tolerance to MOR agonists by this tissue is inconsistent with clinically observed constipation.[11] The mouse is commonly used to assess efficacy and potential side effects of novel opioid receptor ligands.[2,12,13] In marked contrast to the guinea pig ileum, MOR signaling is resistant to the development of tolerance in the mouse colon, leading to sustained constipation.[14] This supports the use of this tissue as a preclinical model of MOR regulation in the human colon. However, a detailed characterization of MOR distribution in the mouse GI tract is lacking. In the present study, we have used tissues from transgenic mice expressing MOR fused to a C-terminal red fluorescent protein tag (MORmCherry[15]) to comprehensively characterize the distribution of MOR in the mouse ENS.

At a cellular level, G protein-coupled receptors (GPCRs) such as MOR can interact with other membrane proteins. One way through which GPCRs may functionally interact is through the sharing or recruitment of the same intracellular proteins.[16] Distinct GPCRs can also directly interact with other receptors to form a unique signaling unit known as a heteromer. The MOR and delta opioid receptor (DOR) heteromer (MOR-DOR) is a well-characterized example and has been identified as a potential target for pain therapy,[17] although this model has recently been challenged.[18] The first evidence that MOR and DOR are coexpressed by individual neurons was provided by electrophysiological analysis of agonist-induced hyperpolarization of myenteric neurons of the guinea pig ileum.[19] However, subsequent studies showed that DOR is not functionally expressed in this tissue,[9] raising questions about the selectivity of the agonists available at the time. The extensive overlap between MOR and DOR in the mouse ENS may be indicative of a native functional interaction between these 2 receptors.[20] The activation of either receptor in the ENS dampens both motility and secretion through the inhibition of myenteric and submucosal neurons, respectively.[21] MOR-DOR heteromer formation in the ENS has been proposed on the basis of labeling using a MOR-DOR selective antibody, and targeting of interactions between MOR and DOR in the GI tract may be clinically beneficial.[22] The mixed MOR agonist/DOR antagonist eluxadoline (Viberzi) is clinically approved for the management of diarrhea-predominant irritable bowel syndrome[23] and is proposed to partially exert its effects through MOR-DOR.[13,22] To date, a systematic analysis of interactions between MOR and DOR and their respective distributions has not been performed in the colon, and whether MOR and DOR can functionally interact in this system is unclear. A detailed characterization of MOR and DOR in the ENS will broaden the fundamental understanding of opioid receptor function and regulation in the ENS, provide a better mechanistic understanding to assist the development of opiate therapeutics for digestive 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 by using transgenic mice expressing either MORmCherry[15] or both MORmCherry and DOR tagged with a C-terminal enhanced green fluorescent protein (MORmCherry-DOR-eGFP[15]). The ability of MOR and DOR to functionally interact in the ENS and the potential mechanisms involved were determined by using receptor endocytosis assays and by pharmacologic examination of neurogenic contractions of the colon.[24]

## Results

### Cellular Distribution of MORmCherry

MORmCherry labeling was most evident in myenteric neurons of the ileum and distal colon. There was no detectable MORmCherry expression in smooth muscle of the muscularis externa. No immunolabeling for mCherry was detected in tissues from C57Bl/6J wild-type mice. Closer examination revealed that MORmCherry was localized to punctate structures in the soma of a subset of myenteric neurons, with no evidence for labeling of proximal neurites or nerve fibers within the muscularis externa.

[a]Authors share co-first authorship.

***Abbreviations used in this paper:*** CalR, calretinin; ChAT, choline acetyltransferase; DAMGO, [D-Ala$^2$,*N*-MePhe$^4$, Gly-ol]-enkephalin; DMSO, dimethyl sulfoxide; DOR, delta opioid receptor; EFS, electrical field stimulation; ENS, enteric nervous system; GFP, green fluorescent protein; GI, gastrointestinal; GPCR, G protein-coupled receptor; IPAN, intrinsic primary afferent; IR, immunoreactive; MOR, mu 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; RT, room temperature.

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This subcellular distribution contrasts with that described in myenteric neurons using immunofluorescence, where a significant proportion of MOR immunoreactivity (-IR) is associated with the cell surface.[10,25] To further confirm this observation, MOR-GFP was transiently expressed in cultured myenteric neurons. A proportion of MOR-GFP was effectively trafficked and localized to the cell surface of the soma and neurites. Treatment with the MOR agonist [D-Ala$^2$,*N*-MePhe$^4$, Gly-ol]-enkephalin (DAMGO) (1 $\mu$mol/L, 30 minutes) resulted in internalization of MOR-GFP from the cell surface to endosome-like structures (Figure 1*A*). Equivalent observations were made in cultured dorsal root ganglion neurons after treatment with loperamide (Figure 1*B*). Thus, we conclude that the subcellular distribution of MORmCherry in enteric neurons 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. For this reason, all subsequent characterization using MORmCherry knockin mice was restricted to the assessment of the anatomic, but not subcellular, distribution of this receptor.

### *MORmCherry Is Expressed by Neurons Controlling Excitatory and Inhibitory Neuromuscular Transmission*

The distribution of MORmCherry in the myenteric region was examined in whole mount preparations of the ileum and distal colon. The relative distribution of MORmCherry in distinct neurochemically defined subsets of myenteric neurons is summarized in Table 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 choline acetyltransferase (ChAT)-positive neurons in both the ileum and colon (Figure 2*A*). MORmCherry was also detected in neuronal nitric oxide synthase (nNOS)-expressing neurons, with a lower percentage overlap in the colon (Figure 2*B*). No MORmCherry expression was evident in larger diameter multipolar neurons that were also positive for calretinin (CalR) (Figure 2*C*), ChAT, or neurofilament M (NFM). These neurons exhibit the morphologic and neurochemical characteristics of putative intrinsic primary afferent neurons (IPANs), although these have not been definitively identified in the mouse colon.

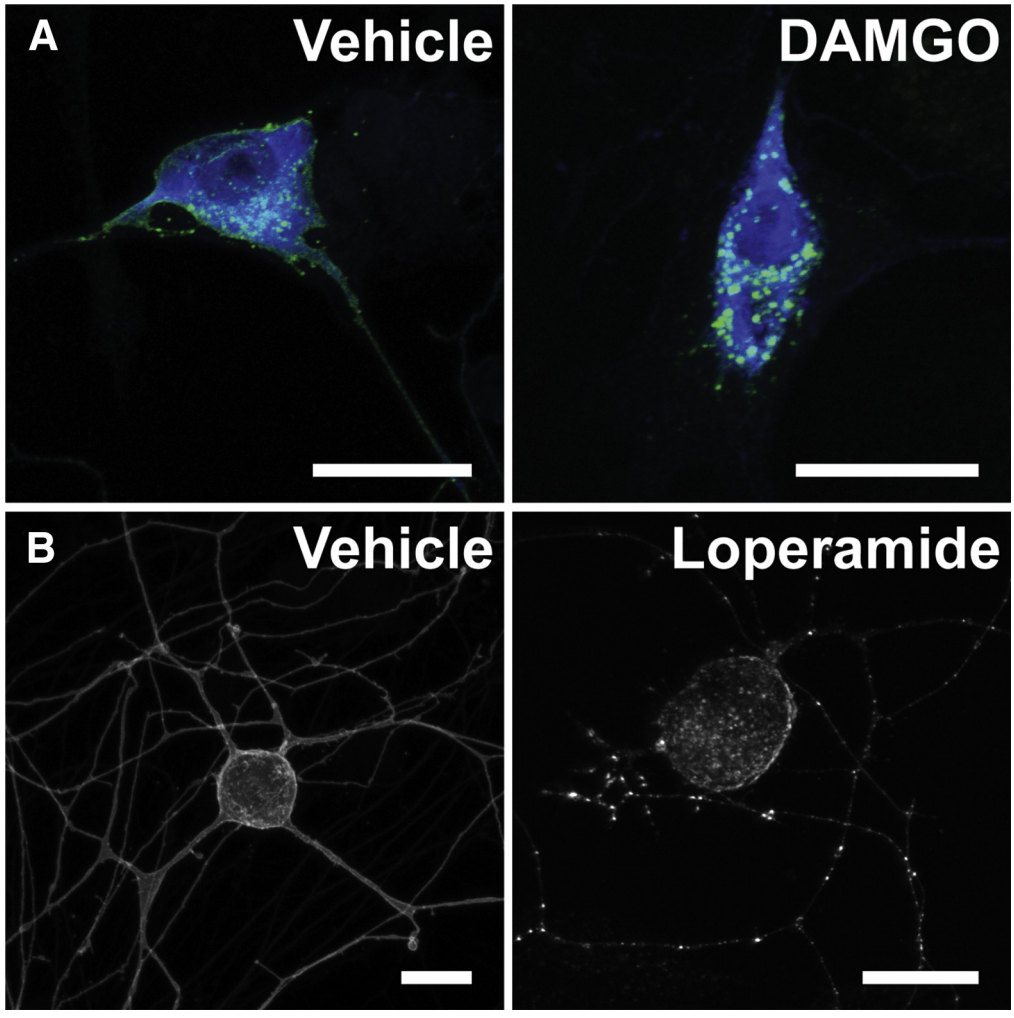



**Figure 1. MOR-GFP is expressed at the cell surface of myenteric and sensory neurons.** (*A*) MOR-GFP was localized to the cell surface and to intracellular structures in transiently transfected myenteric neurons in culture. Treatment with the prototypical MOR agonist DAMGO (1 $\mu$mol/L, 30 minutes) was associated with loss of cell surface MOR-GFP and corresponding increase in labeling of endosome-like structures, consistent with MOR endocytosis. Labeling for the neuron-specific marker PGP9.5 is presented in *blue*. (*B*) Equivalent redistribution of MOR-GFP was observed in the soma and neurites of cultured dorsal root ganglion neurons after treatment with loperamide (1 $\mu$mol/L, 30 minutes). Scale: 20 $\mu$m.

**Table 1.** Quantification of MORmCherry Expressing Neurons in the Myenteric Plexus (n = 7–21 Mice per Group)

| Region | Hu | CalR | ChAT | NOS | DOR-eGFP |
|---|---|---|---|---|---|
| Ileum | 1550/1550 (100%) | 542/1526 (34.8% ± 3.1%)[a] | 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 | 542/931 (57.8% ± 4.6%)[b]; 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 MORmCherry-positive population (ie, in the ileum, of 1526 cells expressing MORmCherry, 542 expressed CalR).
[b]Number of MORmCherry-positive cells in the marker-positive population (ie, in the ileum, of 931 cells expressing CalR, 542 also expressed MORmCherry).

MORmCherry was not detected in glial fibrillary acidic protein–positive enteric glial cells and extraganglionic cells of the myenteric region, suggesting that MOR is not expressed by key non-neuronal cell types involved in the control of intestinal motility including smooth muscle cells, interstitial cells of Cajal, platelet-derived growth factor receptor $\alpha$ positive fibroblast-like cells, and muscularis macrophages.

## Limited MORmCherry Expression by Submucosal Neurons

Although extensive MORmCherry expression was observed in myenteric neurons of the ileum, no labeling was detected in submucosal neurons within the same tissue preparation (n = 0/415 neurons, 6 mice; Figure 3*A*). MORmCherry was detected in a very limited number of submucosal neurons of the distal colon, where it was generally coexpressed with nNOS (not quantified; Figure 3*B* and *C*). These findings suggest that MOR expression in the mouse intestine is largely restricted to myenteric neurons.

## MORmCherry and DOR-eGFP Are Coexpressed in a Subset of Myenteric Neurons

Functional interaction between MOR and DOR requires the coexpression of the individual protomers by the same cell. MOR and DOR overlap was examined by using MORmCherry–DOR-eGFP double knockin mice.[15,18] A summary of the neuronal populations in which MOR and DOR are coexpressed is presented in Table 2. MORmCherry and DOR-eGFP were codistributed in a subset of Hu-IR myenteric neurons in the ileum (31%) and distal colon (21%; Figure 4*A*). MORmCherry and DOR-eGFP were coexpressed in a subset of nNOS-positive (Figures 4*B* and 5*A*), ChAT-positive (Figures 4*C* and 5*B*), and small diameter CalR-positive neurons (Figures 4*D* and 5*C*) in both regions. Thus, there is potential for functional interaction between these receptors to occur in the ENS.

## 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-IR in tissue from DOR-eGFP mice (Figure 6). Labeling for the *Oprm1*$^{-/-}$ validated monoclonal antibody UMB3[26] was detected at the cell surface and cytosol of a large proportion of myenteric neurons and with nerve fibers associated with the circular muscle layer. MOR-IR often overlapped with DOR-eGFP labeling, consistent with the extensive coexpression demonstrated by using MORmCherry–DOR-eGFP knockin mice. However, the quality of immunolabeling was inconsistent under the staining conditions used and was not quantified for this reason.

## DOR and MOR Functionally Interact in a Heterologous Manner in Myenteric Neurons

The extensive coexpression of MOR and DOR by myenteric neurons suggests that these receptors may functionally interact in the ENS. Such functional interaction may be detected as heterologous desensitization whereby activation of a protomer with a selective agonist desensitizes the response of the other protomer to its corresponding agonist.[16] This interaction was examined in the colon by first exposing preparations to an agonist for one receptor (1 $\mu$mol/L, 5 minutes) and then measuring subsequent responses to either the same agonist or to an agonist for the other receptor (homologous and heterologous desensitization, respectively).

The selective MOR agonists DAMGO and morphine produced robust, concentration-dependent contractions of the colon (Figure 7*A*, *D*, and *E*). Prior exposure to either agonist did not alter the magnitude of subsequent contractions to the same agonist (Figure 7*B*, *D*, and *E*), consistent with the resistance of MOR to desensitization in myenteric neurons.[27] In contrast, preincubation with the DOR agonist SNC80, a high efficacy DOR agonist,[27] abolished subsequent DAMGO-evoked (10 nmol/L–10 $\mu$mol/L; $P < .05$ vs vehicle,

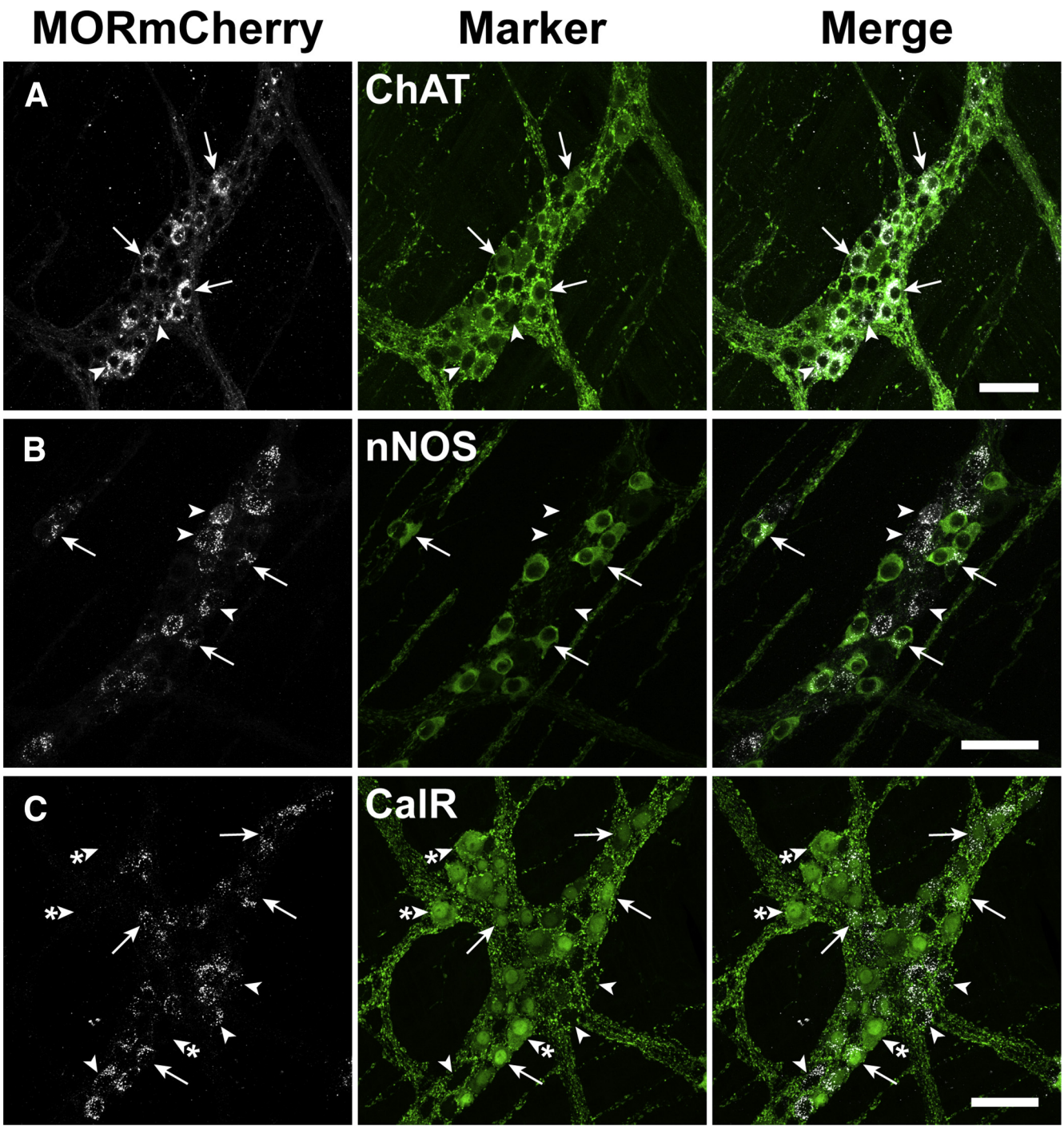



**Figure 2. MORmCherry expression in the myenteric plexus of distal colon.** MORmCherry was distributed in a subset of all Hu-positive neurons (not shown). There was overlap with (*A*) ChAT-positive cholinergic neurons, (*B*) nNOS expressing inhibitory neurons, and (*C*) CalR expressing excitatory neuronal populations. MORmCherry was not detected in large diameter CalR-positive neurons, indicating lack of expression by intrinsic primary afferent neurons (*arrowheads with asterisks*). *Arrows*, coexpression with marker; *arrowheads*, no-coexpression with marker. Scale: 50 $\mu$m.

n = 5–6; Figure 7*C* and *D*) and morphine-evoked contractions (100 nmol/L–10 $\mu$mol/L; $P < .01$ vs vehicle, n = 6–7; Figure 7*E*). ARM390, a partial agonist with lower intrinsic efficacy than SNC80,[27] did not significantly affect DAMGO-evoked (Figure 7*C* and *D*) or morphine-evoked contractions (1 nmol/L–10 $\mu$mol/L, n = 5–6; $P > .05$; Figure 7*E*). Thus, although MOR in the ENS is resistant to homologous desensitization, responses to MOR agonists can be desensitized in a heterologous manner by DOR, consistent with functional interaction between these 2 receptors.

Both SNC80 and ARM390 evoke tonic contractions of colon strips.[27] Prior treatment with SNC80 completely

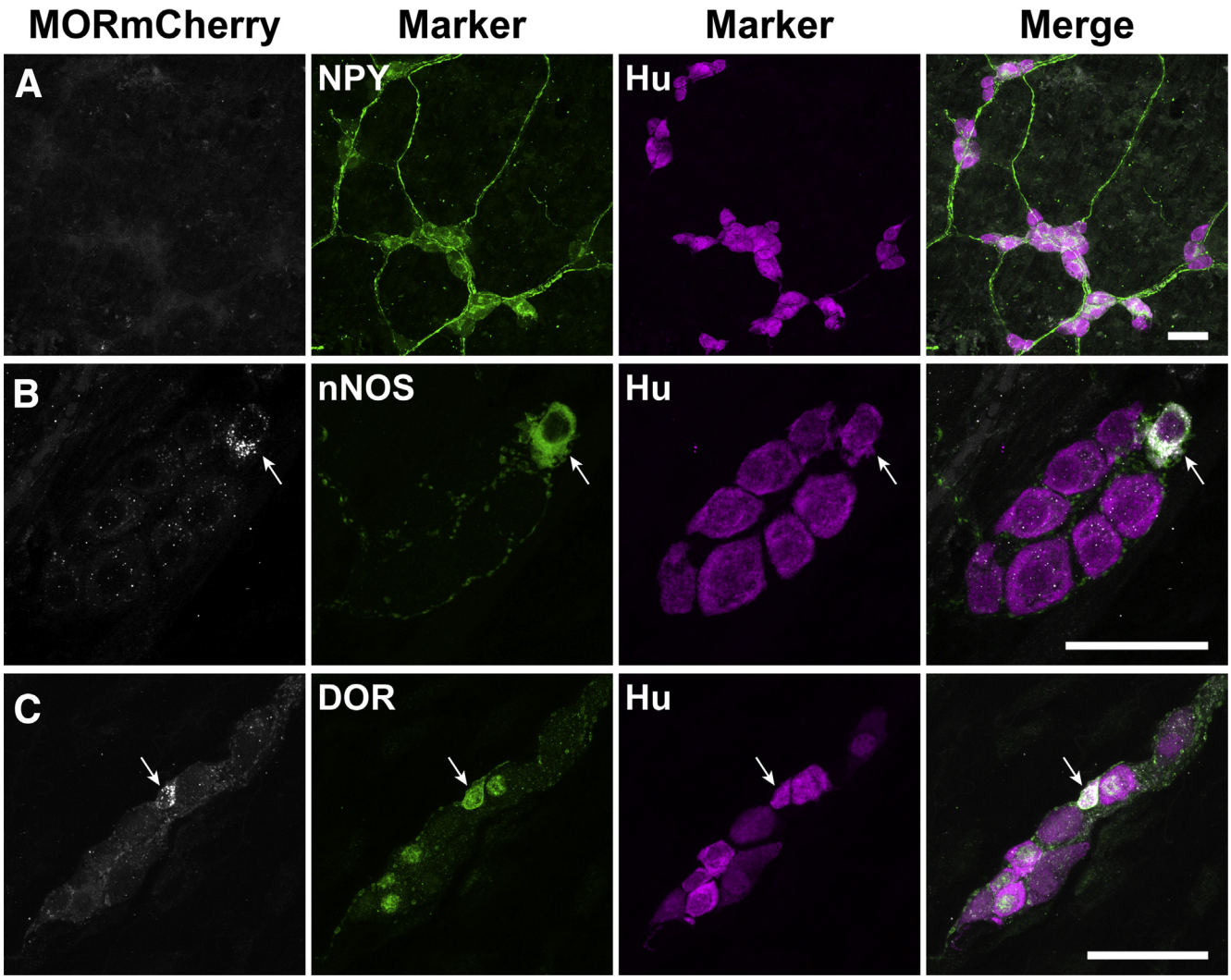



**Figure 3. MORmCherry expression in the submucosal plexus.** (*A*) MORmCherry labeling in the ileum was not detected in neurons of the submucosal plexus, as identified by NPY and Hu staining. (*B*) MORmCherry was expressed by small number of submucosal neurons of distal colon. These neurons were generally nNOS positive. (*C*) MOR and DOR were coexpressed in very limited number of submucosal neurons. Scale: 50 $\mu$m. *Arrows* represent coexpression of markers.

desensitized subsequent contractile responses to the same agonist[27] (Figure 7*F*). In contrast, ARM390 had no effect on SNC80-mediated contractions (Figure 7*F*). Prior exposure to DAMGO significantly augmented the initial SNC80-evoked contraction at 1 nmol/L compared with the vehicle (n = 8–12; $P < .05$), indicative of sensitization.

**Table 2.** Quantification of MORmCherry/DOR-eGFP Expressing Neurons in the Myenteric Plexus (n = 5–6 Mice per Group)

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

[a]Number of marker-positive neurons in the MORmCherry/DOR-eGFP–positive population (ie, in the ileum, of 790 cells expressing MOR/DOR, 310 expressed CalR).
[b]Number of MORmCherry/DOR-eGFP–positive cells in the marker-positive population (ie, in the ileum, of 765 cells expressing CalR, 310 also expressed MOR).

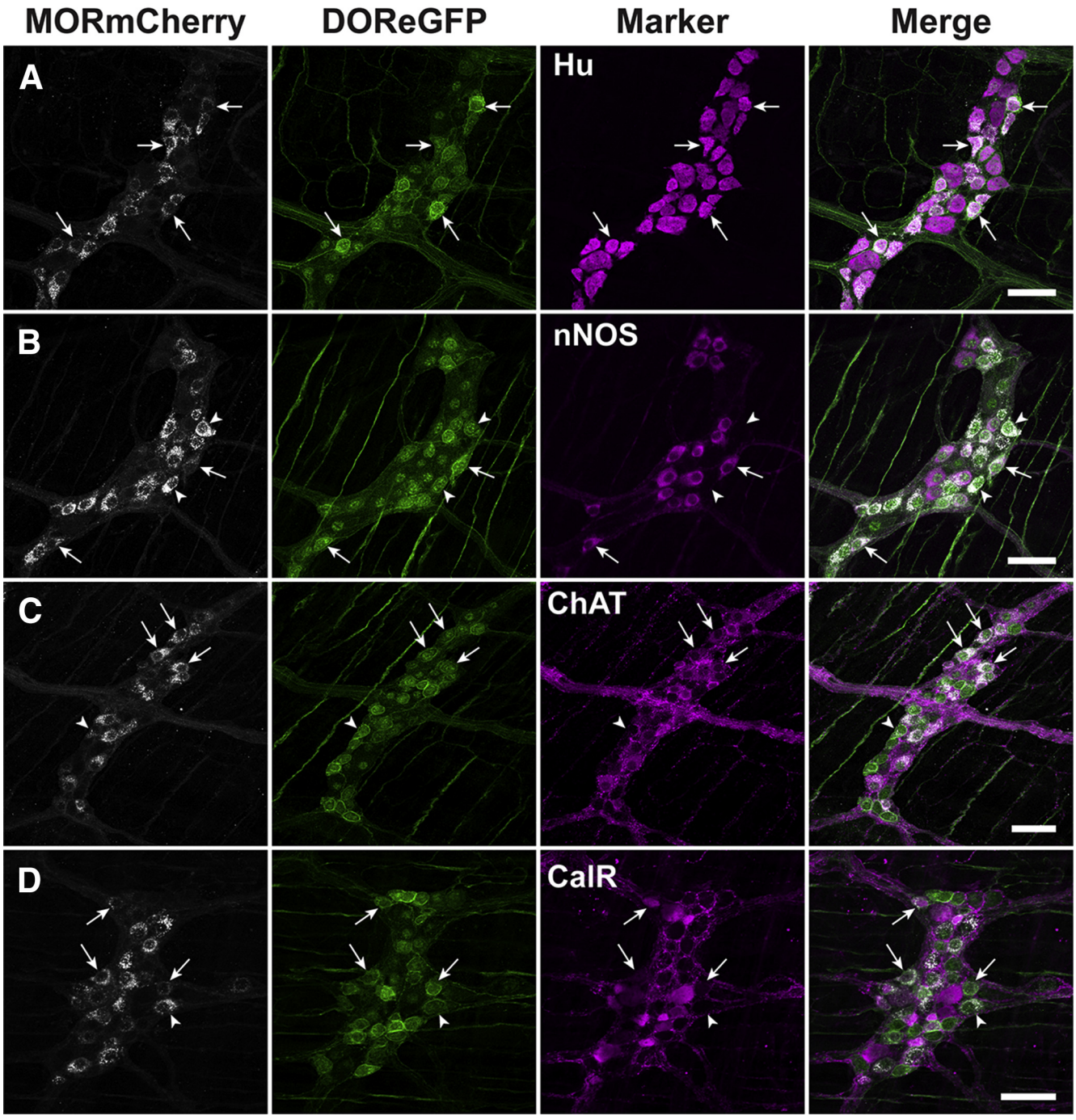



**Figure 4. Examples of overlap between MOR and DOR in different myenteric neuronal populations of distal colon.** (*A*) All MOR-DOR expressing cells were positive for pan-neuronal marker Hu (*arrows*). (*B*) Both nNOS positive (*arrows*) and negative (*arrowheads*) neurons expressed MOR-DOR. Similarly, MOR and DOR were coexpressed in subset of both ChAT (*C*) and CalR (*D*) positive neuronal populations. Scale: 50 $\mu$m.

However, there was no significant change to SNC80 responses at any other concentration (10 nmol/L–10 $\mu$mol/L; Figure 7*F*). Thus, DOR is desensitized in a homologous manner in myenteric neurons. These data further support the functional coexpression of MOR and DOR in enteric pathways.

### *MOR and DOR Do Not Function as Heteromers in the ENS*

We assessed whether MOR and DOR form heteromers in the ENS by using both pharmacologic and cell biology approaches. The agonist CYM51010, selective for the MOR-DOR heteromer, was initially used to probe for this

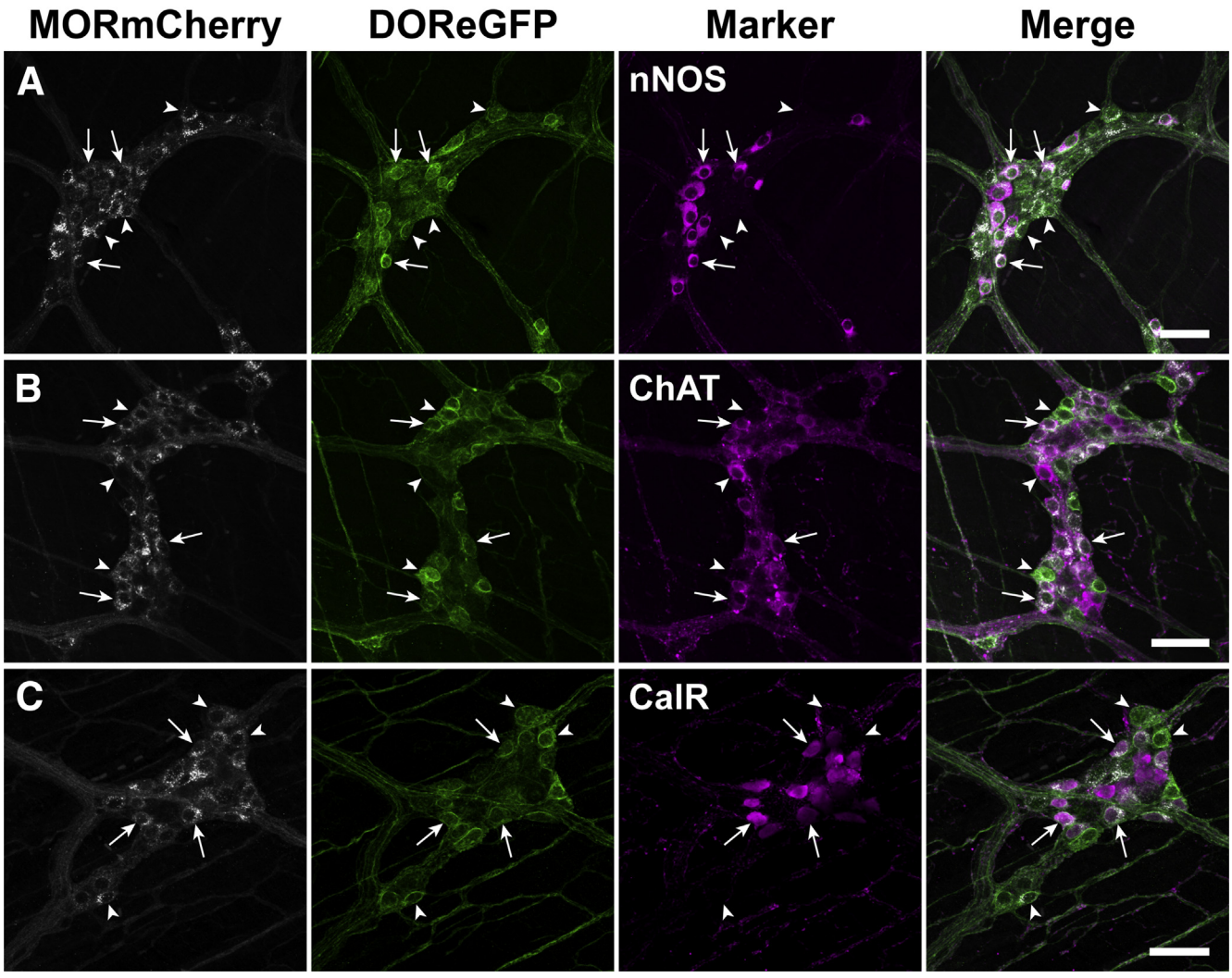



**Figure 5. Examples of overlap between MOR and DOR in different myenteric neuronal populations of the ileum.** (*A*) Both nNOS positive (*arrows*) and negative (*arrowheads*) neurons coexpressed MOR and DOR. Similarly, MOR and DOR were coexpressed in subset of both ChAT (*B*) and CalR (*C*) positive neuronal populations. Scale: 50 $\mu$m.

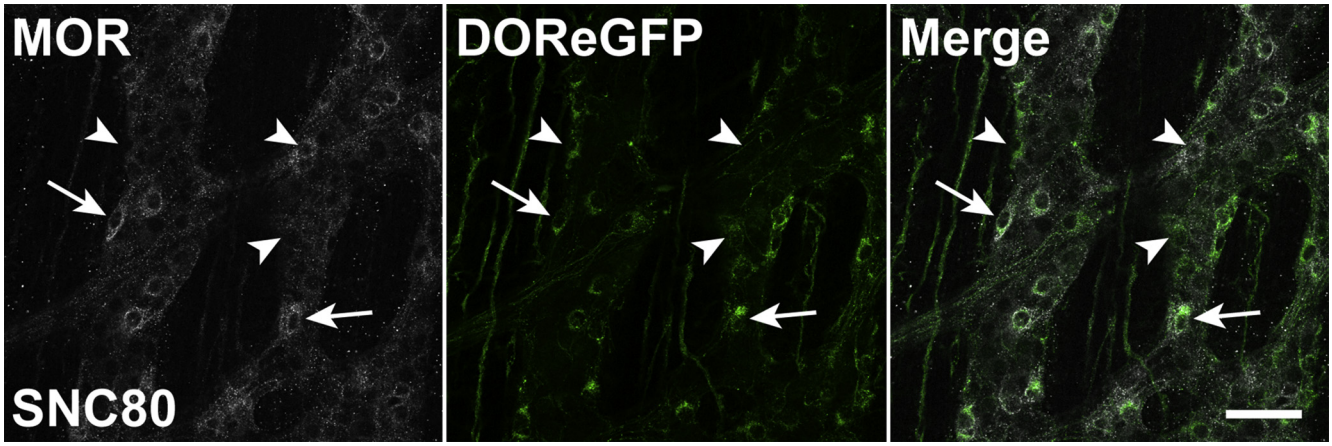
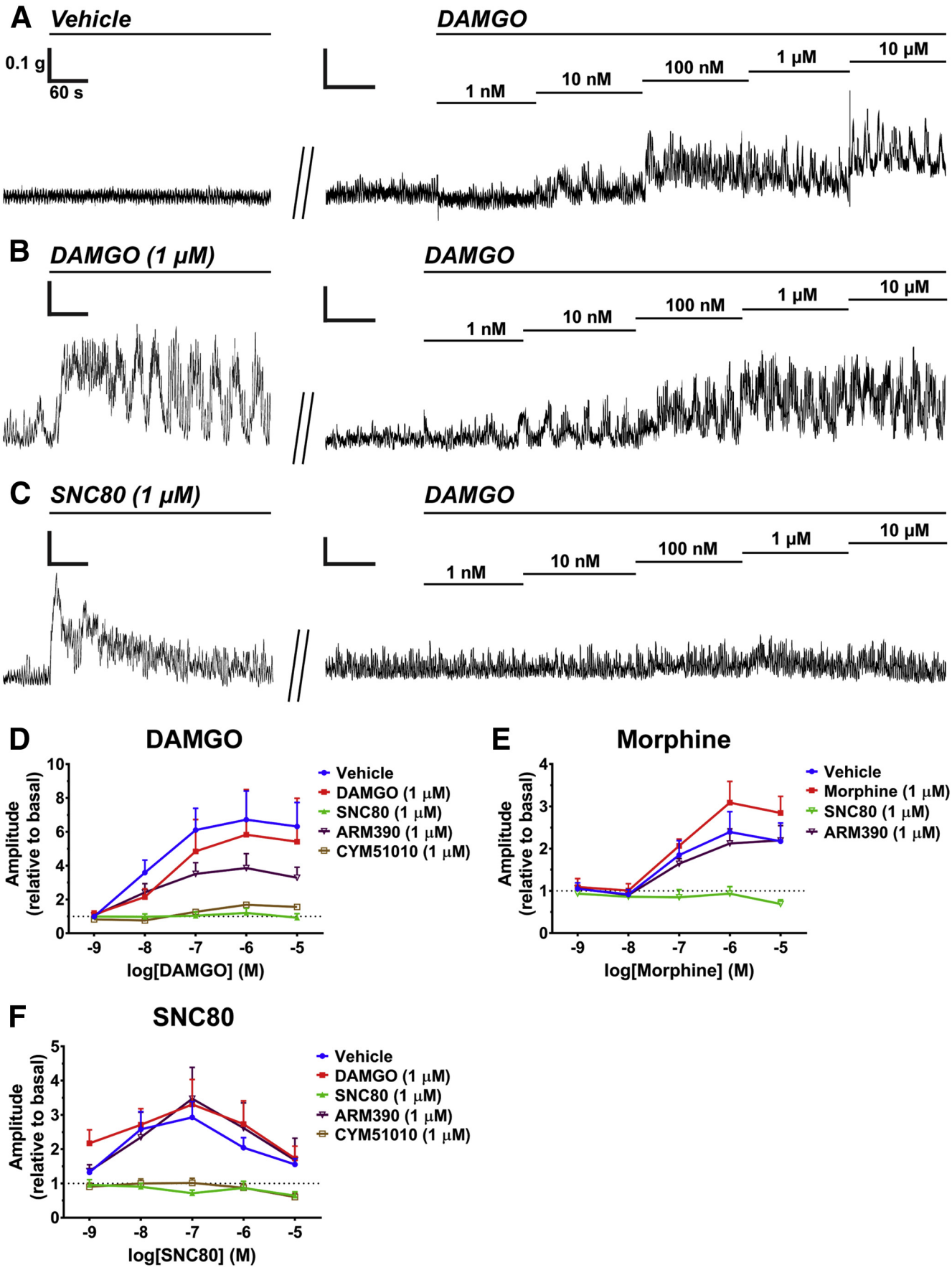



**Figure 6. Extensive overlap between MOR-IR and DOR-eGFP in myenteric plexus.** A subset of myenteric neurons expressed both MOR-IR and DOR-eGFP (*arrows*). Whole mounts were treated with SNC80 (100 nmol/L, 30 minutes) to concentrate DOR-eGFP in endosomes to facilitate detection. Scale bar: 20 $\mu$m. *Arrows,* coexpression of markers; *arrowheads,* no coexpression of markers.


A
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DAMGO
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B
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D
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F
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interaction.[28] The direct contractile effects of CYM51010 on the colon were blocked by the DOR antagonist naltrindole (NLT) but not by the MOR antagonist CTOP (Figure 8*A*–*D*). CYM51010 (1 $\mu$mol/L) blocked subsequent contractions to both SNC80 and DAMGO (Figure 7*D* and *F*), consistent with the desensitizing effects of efficacious DOR agonists. CYM51010 promoted robust internalization of DOR-eGFP in both myenteric and submucosal neurons, which was blocked by NLT (Figure 8*E*–*I*). Pharmacologic assessment of receptor coupling using CHO-hDOR cells also supported a DOR-dependent mechanism of action (Figure 8*J*), preventing further use of this compound as a specific tool to probe for heteromer function.

Formation of MOR-DOR heteromers is also associated with allosteric interactions between individual receptors, which are revealed in the presence of MOR or DOR selective antagonists.[29] MOR and DOR agonists inhibit EFS-evoked neurogenic contractions through actions on excitatory neurons.[27] 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 at 100 nmol/L (Figure 9*A*). In contrast, NLT (1 $\mu$mol/L) inhibited DAMGO-mediated responses, consistent with decreased receptor selectivity at higher concentrations. The inhibitory actions of SNC80 ($pEC_{50}$ = 7.06 ± 0.24, $E_{max}$ = 84.6% ± 5.9%, n = 6) were insensitive to the MOR selective antagonist CTOP (1 $\mu$mol/L; Figure 9*B*). These data indicate a lack of cooperativity between MOR and DOR in the ENS.

Cointernalization of protomers is also expected to occur if they exist as a heteromeric unit.[30] MOR and DOR cointernalization was examined in enteric neurons of the colon of DOR-eGFP mice (Figure 9*C* and *D*).[20,24] DOR-eGFP was mainly confined to the plasma membrane of myenteric neurons under control conditions (73.8% ± 2.6% cell surface DOR-eGFP, mean ± 95% confidence interval, n = 73 neurons). Treatment with SNC80 (1 $\mu$mol/L) resulted in significant DOR-eGFP endocytosis into endosomes (41.9% ± 2.9%, n = 58 neurons). In contrast, DOR-eGFP was retained at the cell surface after treatment with DAMGO (1 $\mu$mol/L; 77.8% ± 1.5%, n = 115). The subcellular distribution of MOR-IR was qualitatively assessed. Treatment with DAMGO (1 $\mu$mol/L, 30 minutes) resulted in labeling of endosome-like structures, whereas MOR-IR remained at the cell surface after SNC80 treatment (Figure 9*C*). To confirm that these 2 receptors internalize independently in enteric neurons, we examined whether prior activation 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%, intraperitoneally) or SNC80 (10 mg/kg, intraperitoneally; 3 hours), which effectively promotes DOR internalization.[27] SNC80-evoked contractions were desensitized in the SNC80-pretreated group, confirming effective removal of functional DOR from the cell surface (Figure 9*E*; 10 nmol/L–1 $\mu$mol/L, n = 5–7; $P < .05$).[27] In marked contrast, DAMGO-evoked contractions were unaffected by SNC80 pretreatment (Figure 9*F*), indicating that MOR function is retained after internalization of DOR. Together these findings are consistent with independent regulation of the 2 receptors in myenteric neurons.

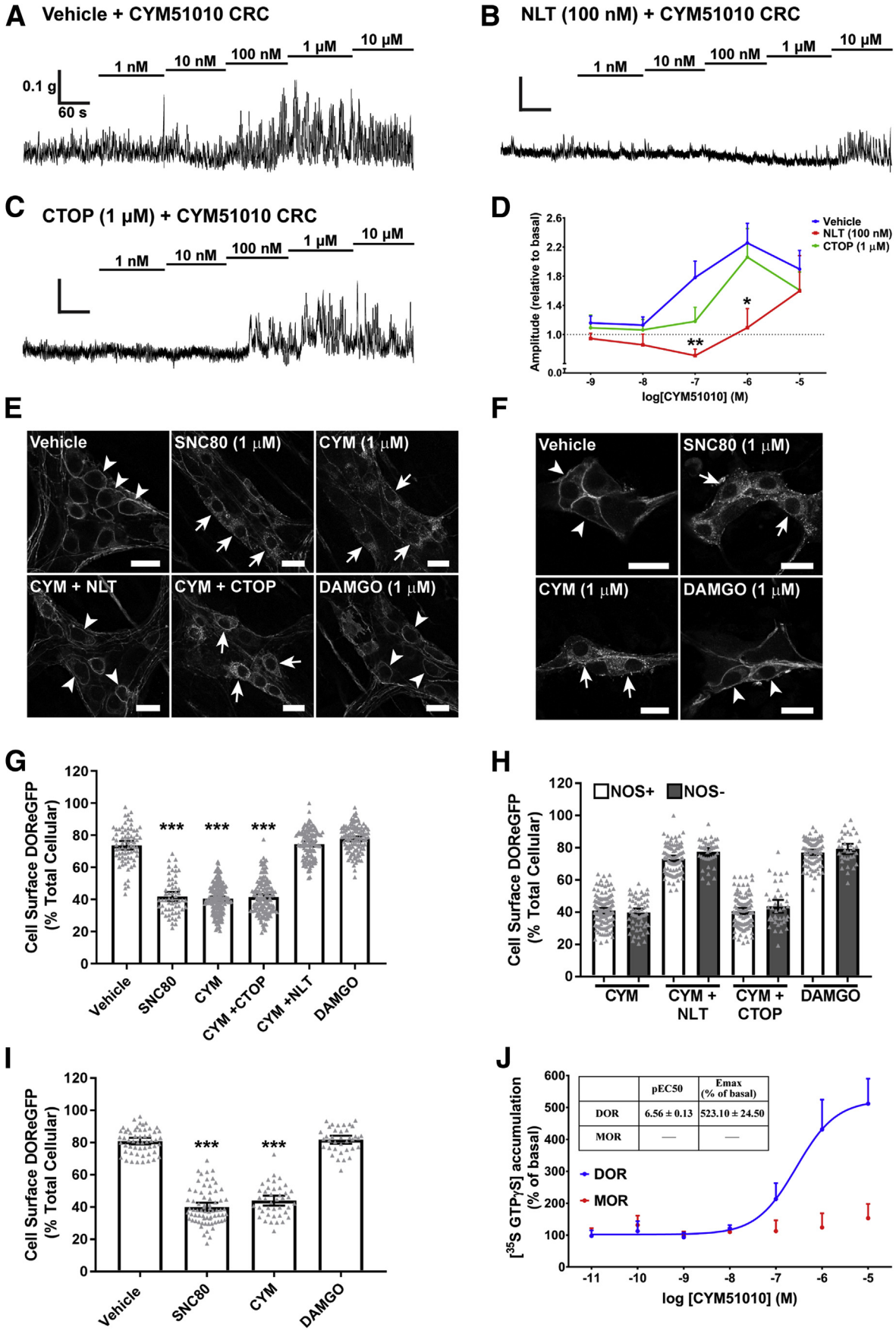
## Discussion

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 neurons of the myenteric plexus, (2) that MOR and DOR can functionally interact via heterologous desensitization, and (3) that this functional interaction is unlikely to be due to heteromerization.

### *Distribution of MOR in the ENS*

In marked contrast to the guinea pig intestine[10] and despite its relevance for animal models to assess opioid-induced side effects, 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, we examined the distribution of MOR by using transgenic mice expressing MORmCherry under the control of the endogenous promoter *Oprm1*.[15] 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.[21] MOR agonists inhibit electrically stimulated contractions of the mouse ileum and colon,[14] which correspond to the expression of MORmCherry by ChAT-positive excitatory neurons. MOR agonists increase basal tone and reduce neurogenic relaxations,[31] and this correlates with localization of MORmCherry to nNOS-positive inhibitory neurons. MORmCherry was predominantly expressed by the cholinergic neuronal population of the ileum and colon. This contrasts with previous studies of the guinea pig intestine where MOR-IR was mainly in the nitrergic neuronal

**Figure 7.** (***See previous page***). **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* and *E*) DAMGO- and morphine-evoked contractions were significantly reduced by strongly 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 weakly internalizing DOR agonist ARM390. Data are presented as mean ± standard error of the mean, n = 5–12 mice per group. Statistical comparisons were made at individual concentrations by Kruskal-Wallis test, followed by Dunn's post hoc and are presented in the main text.


A
Vehicle + CYM51010 CRC
1 nM
10 nM
100 nM
1 μM
10 μM
0.1 g
60 s
B
NLT (100 nM) + CYM51010 CRC
C
CTOP (1 μM) + CYM51010 CRC
D
Amplitude (relative to basal)
log[CYM51010] (M)
Vehicle
NLT (100 nM)
CTOP (1 μM)
E
Vehicle
SNC80 (1 μM)
CYM (1 μM)
CYM + NLT
CYM + CTOP
DAMGO (1 μM)
F
Vehicle
SNC80 (1 μM)
CYM (1 μM)
DAMGO (1 μM)
G
Cell Surface DOReGFP (% Total Cellular)
Vehicle
SNC80
CYM
CYM +CTOP
CYM +NLT
DAMGO
H
NOS+
NOS-
Cell Surface DOReGFP (% Total Cellular)
CYM
CYM + NLT
CYM + CTOP
DAMGO
I
Cell Surface DOReGFP (% Total Cellular)
Vehicle
SNC80
CYM
DAMGO
J
[35S GTPγS] accumulation (% of basal)
pEC50
Emax (% of basal)
DOR
6.56 ± 0.13
523.10 ± 24.50
MOR
log [CYM51010] (M)

population of these regions.[10] Putative IPANs, which could be identified by CalR- and ChAT-IR, their size, and by their distinctive Dogiel type II morphology (revealed by NFM or CalR labeling), were negative for MORmCherry. This matches our previous description of MOR immunolabeling in the guinea pig intestine.[10] In direct contrast to our study, Smith et al[32] recorded MOR-dependent responses from dissociated neurons with AH electrophysiological properties (ie, putative IPANs) from the mouse ileum and colon. However, putative interneurons and motoneurons, which functionally express MOR,[21] did not respond to morphine. These 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.[33] We report that a slightly higher percentage of total neurons express MORmCherry in the ileum relative to the distal colon. We have reported a similar distribution in our previous quantitative analysis of MOR-IR in the guinea pig GI tract.[10]

The activation of MOR on submucosal neurons reduces secretion and may contribute to the constipating effects of opioids.[21] Morphine inhibited neurogenic chloride secretion in the mouse colon, consistent with functional expression of MOR by submucosal neurons.[34] Previous descriptive studies have reported extensive immunolabeling of submucosal neurons using MOR antibodies.[10] However, the predicted functional distribution differs to the neuronal population identified by immunolabeling.[10] 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 that needs to be investigated further.

## Coexpression of MOR and DOR by Myenteric Neurons

Overlap between MOR and DOR positive neuronal populations has been described.[20] Recent evidence suggests that the MOR-DOR heteromer expressed by myenteric neurons mediates the actions of eluxadoline.[22] Despite the potential clinical importance of the MOR-DOR heteromer in the ENS, a fundamental understanding of receptor interactions at the cellular and physiological level is lacking. The distribution and neurochemical coding of neurons that express both MOR and DOR were examined by using MORmCherry–DOR-eGFP mice. This mouse line was used previously to map the distribution of these receptors with high specificity in the central nervous system and in pain pathways.[15,18] Our findings demonstrate that there is extensive overlap between MOR and DOR in the myenteric plexus and supports our previous study using DOR-eGFP mice in combination with validated MOR antibodies and a labeled MOR ligand.[20] However, the earlier study did not examine the neurochemical coding of neurons that coexpressed both receptors. Approximately one-fourth of total myenteric neurons in the ileum (30%) and colon (22%) expressed both MORmCherry and DOR-eGFP. 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.[21] The predicted cooperativity between MOR and DOR may also enhance the modulatory effects of endogenous opioids. MOR and DOR were coexpressed by cholinergic and nitrergic neurons, suggesting a potential role for functional interactions between the receptors in the modulation of excitatory and inhibitory motor pathways, respectively.

## MOR and DOR Functionally Interact via Heterologous Desensitization

The extensive coexpression of MOR and DOR suggests that these 2 receptors may interact in the ENS. GPCRs may interact at the cellular level through alternative mechanisms including heterologous desensitization.[16] Very few studies have specifically examined heterologous GPCR desensitization in the ENS. Activation of the neurokinin 1 receptor ($NK_1R$) desensitized neurokinin 3 receptor ($NK_3R$)-

**Figure 8.** (***See previous page***). **CYM51010 is a selective DOR agonist in the ENS.** (*A*) CYM51010 evoked concentration-dependent contractions (*A*) that were effectively blocked by NLT (*B*) but not by CTOP (*C*). (*D*) Concentration-response curves of contractions evoked by CYM51010. Data are presented as mean ± standard error of the mean, 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 (**$P < .01$ and *$P < .05$ compared with vehicle-treated group). (*E*) DOR-eGFP was internalized from the cell surface to endosomes of myenteric neurons of the colon after treatment with SNC80 (data from Figure 9*D*) and CYM51010. CYM51010-evoked internalization was effectively blocked by NLT but not by CTOP. No redistribution of DOR-eGFP was detected on stimulation with MOR agonist DAMGO (taken from Figure 9*D*). (*F*) Both SNC80 and CYM51010, but not DAMGO, promoted DOR-eGFP internalization in submucosal neurons of the ileum, a site in which no MORmCherry expression was detected. Scale: 20 μm. *Arrowheads,* DOR-eGFP retained at plasma membrane; *arrows,* DOR-eGFP internalized. (*G*) Quantitative analysis of DOR-eGFP distribution in myenteric neurons. (*H*) Equivalent DOR-eGFP endocytosis in NOS positive and negative populations in response to CYM51010 (1 μmol/L). Selective inhibition of DOR (NLT) did not result in a difference in CYM51010-evoked DOR-eGFP internalization between the 2 populations. Similarly, there was no difference in DOR-eGFP internalization in NOS positive and negative populations after treatment with either CYM51010 in the presence of CTOP or MOR agonist DAMGO. (*I*) Quantitative analysis of DOR-eGFP distribution in submucosal neurons. Endocytosis data are presented as mean values ± standard error of the mean and were analyzed by one-way analysis of variance with Dunnett's post hoc test. Individual data points are represented as *triangles*. ***$P < .001$ compared with vehicle treatment. (*J*) CYM51010 produced a concentration-dependent increase in G protein activation only in DOR expressing CHO cells. Data are presented as mean ± standard error of the mean from 4 independent experiments.

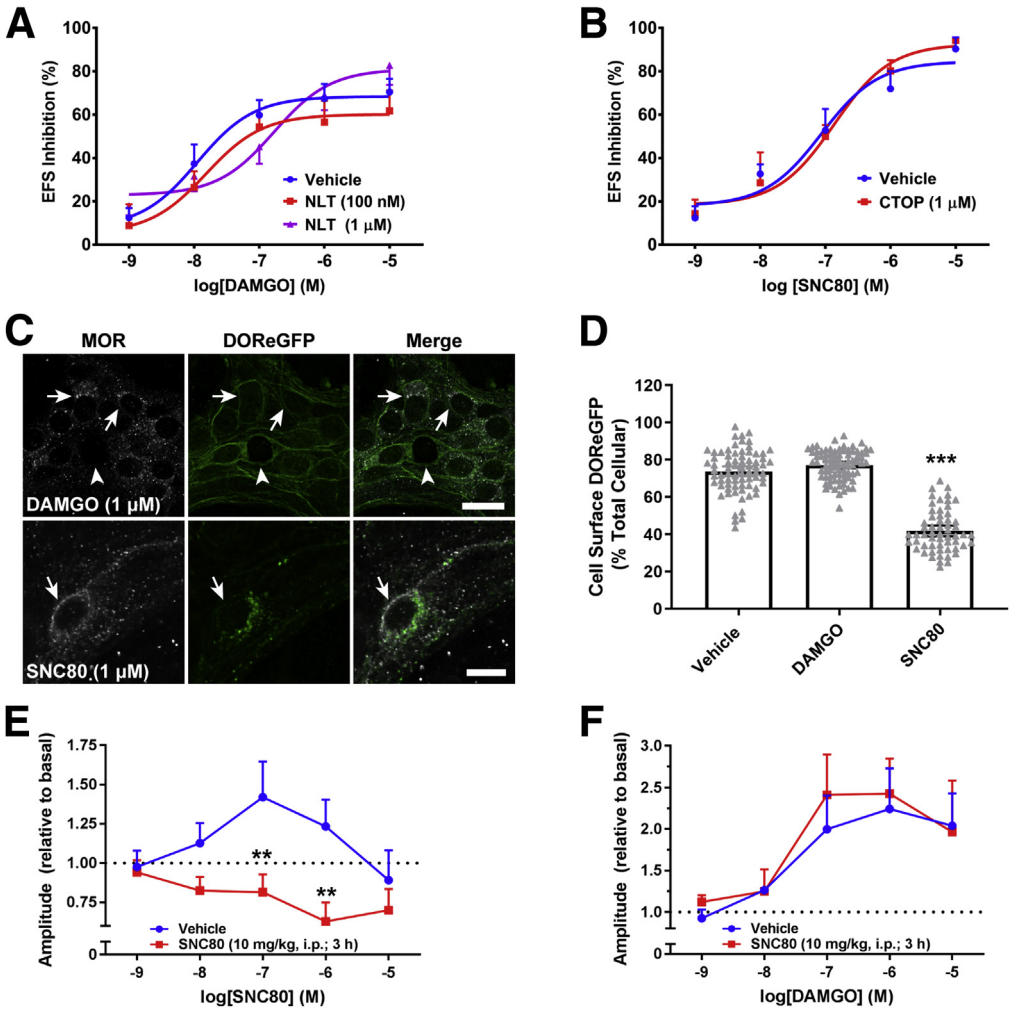



**Figure 9. DOR and MOR do not display functional cooperativity and traffic independently in enteric neurons.** (*A*) DAMGO-mediated effects were unaffected by DOR antagonist NLT at low concentration (100 nmol/L) but were reduced when used at higher concentration (1 μmol/L). (*B*) Responses to SNC80 were unaffected by MOR antagonist CTOP. Data are expressed as mean ± standard error of the mean, n = 5–9 mice per group. (*C*) DOR-eGFP was internalized from the cell surface to endosomes of myenteric neurons of the colon after treatment with SNC80 but not DAMGO. No redistribution of MOR-IR was detected on stimulation with SNC80, whereas DAMGO promoted robust endocytosis. (*D*) Quantitative analysis of DOR-eGFP distribution in (*C*). Scale: 20 μm. ***$P < .001$, one-way analysis of variance with Dunnett's post hoc test. *Arrows,* coexpression of markers; *arrowheads,* no coexpression of markers. (*E*) Internalization of DOR by SNC80 (3 hours, in vivo) effectively inhibited subsequent responses to SNC80, consistent with development of acute tolerance. (*F*) DAMGO-evoked contractions are unaffected by DOR sequestration. Data are presented as mean ± standard error of the mean, n = 5–7 mice per treatment group. Statistical analyses were conducted at each concentration by using Student unpaired *t* test. **$P <$ .01 compared with the vehicle-treated group.

mediated secretory responses in the guinea pig distal colon.[35] Prior $NK_1R$ activation also reduced agonist-mediated $NK_3R$ internalization in cultured myenteric neurons.[36] 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. However, this was unidirectional because MOR agonists did not desensitize responses to DOR agonists. A possible explanation may be because MOR agonists do not promote the recruitment of regulatory proteins to the receptor in colonic myenteric neurons, as supported by the inability of MOR to undergo homologous desensitization.[14] Alternatively, the inability of MOR to undergo homologous desensitization may be due to a large receptor reserve.[37] MOR-evoked colonic contractions are susceptible to desensitization in β arrestin 2 knockout tissues.[14] SNC80 robustly recruits β arrestin,[38] and it is possible that cross-desensitization of MOR occurs through sequestration of β arrestin 2.[36] The partial agonist ARM390 had a relatively minimal effect on MOR-mediated responses. SNC80 and ARM390 display marked differences in their ability to recruit β arrestin and to internalize DOR,[38] which supports this mechanism of interaction. Small molecule endocytic inhibitors have been developed and are commonly used to study the involvement of GPCR internalization and β arrestin recruitment to cell signaling.

However, we have recently demonstrated that these inhibitors block neurogenic processes in the colon and are unsuitable for functional studies.[39] Therefore, the underlying mechanism of heterologous desensitization of MOR by DOR agonists was not examined. DOR-mediated desensitization of MOR 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.[24] This may affect the activity of endogenous MOR-acting ligands including enkephalins or endorphins.

### Lack of Evidence for Heteromerization of MOR and DOR in the ENS

The existence of GPCR heteromers under native conditions and their role in physiological processes remain controversial primarily because of the difficulty in examining their distribution and function in tissues and in vivo. Furthermore, the formation of MOR-DOR heteromers in native tissue may be both cell-dependent and system-dependent.[18,40] Demonstration of a unique pharmacologic profile is one of the key requirements for recognition of heteromer formation in native systems.[41,42] Several observations outlined in this study indicate that MOR and DOR are unlikely to function as a heteromer in the ENS.

The inhibitory actions of either MOR or DOR agonists were unaffected by antagonism of the other receptor, indicating a lack of cooperativity between these receptors in the ENS. A similar approach was used to characterize MOR-DOR heteromer expression by ventral tegmental area neurons. In this study, MOR-induced hyperpolarization was enhanced in the presence of selective DOR antagonists.[40] However, this may be an overinterpretation because there was a population of myenteric neurons that expressed only MOR or DOR. Therefore, we used additional approaches to examine whether MOR-DOR heteromers exist in the colon. Another common way to probe GPCR heteromerization is to examine whether the receptors co-internalize.[18,30] The MOR-selective agonist DAMGO, which robustly internalizes MOR,[10] did not internalize DOR-eGFP in myenteric neurons. Conversely, MOR-IR was retained at the cell surface in SNC80-treated preparations. These observations indicate that MOR and DOR internalization occurs independently in the ENS. The same approach was used to describe the independent internalization of MOR and DOR in somatosensory neurons.[18] 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.[30] Prior internalization of DOR by SNC80 did not significantly suppress subsequent DAMGO-evoked contractions, which would be expected to occur if DOR and MOR were co-internalized. DOR-mediated responses were effectively desensitized because all subsequent responses to SNC80 were reduced.[27] These functional observations are consistent with retention of MOR-IR at the cell surface of SNC80-treated neurons. Collectively, the experimental evidence that we present in this study indicates that MOR-DOR heteromers are either unlikely to exist in the ENS or play a very minor role in the control of neuromuscular transmission (Figure 10). Furthermore, in contrast to a recent study,[22] our data suggest that eluxadoline most likely exerts its effects on the colon through a heteromer-independent mechanism.

In summary, we have defined the neurochemistry of MOR-positive myenteric neurons in the mouse intestine. We demonstrate that MOR and DOR are coexpressed by a subset of myenteric neurons, where they may potentially interact to influence GI function. 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 pharmacologic 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 novel opportunity to limit the negative GI effects of opioid analgesics.

## Materials and Methods

### Animals

C57Bl/6J and DOR-eGFP knockin mice[43] (6–8 weeks, male) were purpose bred by the Monash Animal Research Platform. Mice were housed under a 12-hour light/dark cycle in a temperature controlled (24°C) environment, with free access to food and water. MORmCherry and MORmCherry-DOR-eGFP knockin mice (male and female) were maintained at 21°C ± 2°C and housed under tightly controlled conditions as described.[15] All procedures involving C57Bl/6J and DOR-eGFP mice were approved by the Monash Institute of Pharmaceutical Sciences animal ethics committee. Studies using MORmCherry and MORmCherry/DOR-eGFP mice were performed in agreement with the European legislation (directive 2010/63/EU acting on protection of laboratory animals) and received agreement from the French ministry (APAFIS 20 1503041113547 (APAFIS#300.02)).

### Reagents

Carbamoylcholine (carbachol), CTOP, CYM51010, DAMGO, NLT, and nicardipine hydrochloride were obtained from Sigma-Aldrich (St Louis, MO). SNC80 was obtained from Cayman Chemical Company (Ann Arbor, MI), ARM390 from Tocris Bioscience (Bristol, UK), morphine hydrochloride from MacFarlan Smith (Edinburgh, UK), and tetrodotoxin citrate from Alomone (Jerusalem, Israel).

### Immunolabeling

MORmCherry and MORmCherry/DOR-eGFP knockin mice were killed with ketamine/xylazine (11/10 mg/kg, intraperitoneally). The ileum and colon were excised and placed in ice-cold phosphate-buffered saline (PBS). Tissues for sectioning and whole mounts were prepared as described.[20,24] Target proteins were detected by indirect immunofluorescence using primary antibodies outlined in Table 3. Briefly, sections and whole mounts were incubated in blocking buffer (5% normal horse serum, 0.1% Triton X-100 in PBS containing 0.1% sodium azide; 1 hour, room

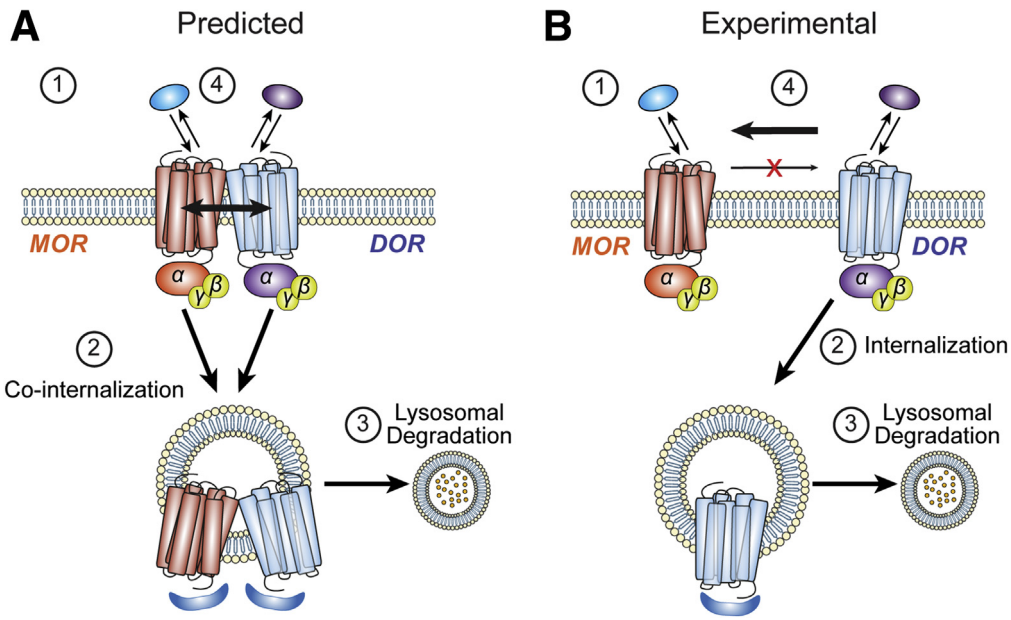



**Figure 10. Overview of and comparison between the predicted interactions between MOR-DOR heteromers and experimental evidence presented in this study.** (*A*) In the predicted model in which MOR and DOR exist as heteromers: (1) MOR and DOR are coexpressed, (2) MOR-DOR co-internalize and (3) co-degrade, and (4) MOR and DOR functionally interact in a bidirectional manner, leading to a unique pharmacologic profile. (*B*) Experimental evidence demonstrates that (1) MOR and DOR are coexpressed, but (2) do not co-internalize or (3) co-degrade, and (4) MOR and DOR can functionally interact through heterologous desensitization.

temperature [RT]) and then labeled with primary antibodies (diluted in blocking buffer, 4°C, overnight). Primary antibodies were detected by using donkey secondary antibodies conjugated to Alexa Fluor 405, 488, 568, or 647 dyes (1:500; 1 hour, RT; Thermo Fisher Scientific, Waltham, MA). Tissue sections were also labeled with the nuclear marker 4',6-diamidino-2-phenylindole (DAPI) (1:1,000, 5 minutes). Preparations were mounted in ProLong Diamond anti-fade mountant (Thermo Fisher Scientific).

### *Imaging and Analysis for Expression Studies*

Preparations were imaged by using a Leica (Wetzlar, Germany) TCS SP8 confocal microscope system. Five confocal images including myenteric or submucosal ganglia were captured per preparation as z-stacks (×40 objective, 0.75 zoom, NA 1.3, 16-bit depth, 1024 × 1024-pixel resolution). One tissue preparation of the distal ileum and the distal colon was examined per immunolabeling combination for each mouse. The area per image was 150,451 $\mu m^2$, with

**Table 3.** Primary Antibodies Used in This Study

| Antigen/neurochemical marker | Host | Dilution | Code and manufacturer |
|---|---|---|---|
| Calretinin (CalR) | Goat | 1:1000 | AB1550 (Merck) RRID:AB_90764 |
| Choline acetyltransferase (ChAT) | Goat | 1:200 | AB144P (Merck) RRID:AB_2079751 |
| Glial fibrillary acidic protein (GFAP) | Chicken | 1:1000 | ab4674 (Abcam) RRID:AB_304558 |
| GFP | Chicken | 1:500 | ab13970 (Abcam) RRID:AB_300798 |
| HuC/D | Human | 1:25,000 | Anti-Hu[45] |
| mCherry (DsRed1) | Rabbit | 1:1000 | 632496 (Clontech) RRID:AB_10013483 |
| MOR | Rabbit | 1:500 | UMB3; ab134054 (Abcam)[26] |
| Neurofilament M (NFM) | Chicken | 1:1000 | ab134458 (Abcam) |
| Neuronal nitric oxide synthase (nNOS) | Sheep | 1:1000 | GTX89962 (GeneTex) RRID:AB_10725945 |
| Neuropeptide Y (NPY) | Sheep | 1:1000 | AB1583 (Merck) RRID:AB_2236176 |

a total area of 752,254 $\mu m^2$ captured per whole mount preparation. The overlap of MORmCherry or MORmCherry/DOR-eGFP with neurochemically defined neuronal subpopulations was measured as described.[10,20] All neurons per ganglion were analyzed by using the FIJI distribution of ImageJ (V1.52n; Wayne Rasband, NIH). Counts were performed by 2 individuals to reduce potential experimenter bias and were presented as a percentage of positive neurons relative to different populations and were also expressed as neuronal counts.

## DOR-eGFP Internalization Assay

Whole mounts of the ileum and distal colon of DOR-eGFP knockin mice were prepared and allowed to recover before use (Krebs containing 1 $\mu$mol/L tetrodotoxin and 10 $\mu$mol/L nicardipine, 37°C, 1 hour, bubbled with 5% $CO_2$, 95% $O_2$). Preparations were treated with antagonists (37°C, 20 minutes) or vehicle (0.1% dimethyl sulfoxide [DMSO]) and then washed with ice-cold Krebs (3 × 5-minute washes). These were then exposed to agonist (1 $\mu$mol/L SNC80, 1 $\mu$mol/L CYM51010, or 1 $\mu$mol/L DAMGO; 4°C, 1 hour, in the presence of antagonist or vehicle), washed (3 × 5-minute washes, ice-cold Krebs), and recovered to allow DOR-eGFP endocytosis (agonist-free Krebs with or without antagonist, 37°C, 30 minutes). Tissues were fixed (4% PFA, overnight, 4°C). Fixative was cleared (3 × 10-minute washes, PBS), and circular muscle-myenteric plexus whole mounts were prepared. Preparations were labeled for eGFP, nNOS, and Hu immunoreactivities. Some preparations were also labeled for qualitative assessment of MOR distribution.

## Imaging and Analysis of DOR-eGFP Internalization

Five confocal images including myenteric or submucosal ganglia were captured per preparation (×40 objective, ≥2.0 zoom, 16-bit depth, 1024 × 1024-pixel resolution). The subcellular distribution of DOR-eGFP was determined as described.[27] Briefly, the subcellular distribution of DOR-eGFP within the neuronal soma was determined by using nNOS and Hu immunoreactivities to define cellular morphology. Images were converted to binary (ie, positive or negative pixels) by using the nucleus to define the threshold for positive staining. At least 34 neurons from preparations from 3–5 mice were analyzed per treatment group. Cell surface-associated DOR-eGFP was expressed as a relative percentage of total cellular DOR-eGFP 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[20,44] and nucleofected with 600 ng human MOR-GFP by using a Lonza 4D-Nucleofector system.[44] Cells were cultured (4 days in vitro), treated (100 nmol/L DAMGO, 1 $\mu$mol/L loperamide, or vehicle, 30 minutes at 37°C), fixed (4% PFA, 20 minutes on ice), immunostained (GFP, Hu, glial fibrillary acidic protein), 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 modified Eagle medium containing 10% fetal bovine serum and 0.3 mg/mL Hygromycin (37°C, 5% $CO_2$, 95% $O_2$). Cell membranes were prepared for GTP$\gamma$S $^{35}$S assay. Briefly, cells were grown to confluence and washed with warm PBS (pH 7.4). Cells were detached with warm Versene and pelleted by centrifugation (350*g*, 3 minutes, RT). The pellet was resuspended in ice-cold homogenization buffer (20 mmol/L HEPES, 10 mmol/L $MgCl_2$, 100 mmol/L NaCl, 1 mmol/L EGTA, pH 7.4) and homogenized for three 10-second intervals at maximum setting, with 30-second cooling periods on ice between each burst. The homogenates were centrifuged (600*g*, 10 minutes, 4°C), the pellet was discarded, and the supernatant was re-centrifuged (20,000*g*, 4°C, 1 hour). The final pellet was resuspended in 20 mmol/L HEPES, 10 mmol/L $MgCl_2$, and 100 mmol/L NaCl, pH 7.4 using a syringe. Protein concentration was determined by using bicinchoninic acid quantification method with bovine serum albumin as the standard. Aliquots were stored at −80°C until required for GTP$\gamma$S $^{35}$S assay.

## GTP$\gamma$S $^{35}$S Binding Assays

GTP$\gamma$S $^{35}$S binding experiments were performed by using cell-membrane homogenates as described. Membrane homogenates (10 $\mu$g) were equilibrated in a 200-$\mu$L volume of GTP$\gamma$S $^{35}$S assay buffer (20 mmol/L HEPES, 10 mmol/L $MgCl_2$, 100 mmol/L NaCl, 30 $\mu$g/mL saponin, and 0.1% bovine serum albumin, pH7.4) containing varying concentrations of CYM51010 and 10 $\mu$mol/L or 30 $\mu$mol/L guanosine diphosphate (MOR and DOR, respectively; 30 minutes, RT). After this time, 50 $\mu$L [$^{35}$S] (0.3 nmol/L) was added, and incubation was continued for an additional 60 minutes (RT). Incubation was terminated by rapid filtration with a Packard plate harvester onto 96-well GF/C filter plates, followed by 3 washes with ice-cold Tris buffer (50 mmol/L Tris-HCl, 10 mmol/L $MgCl_2$, 100 mmol/L NaCl, pH 7.6). After drying for 3 hours at 55°C, the GF/C filter plates were sealed with melt-on scintillator sheets. Bound [$^{35}$S] was solubilized in 40 $\mu$L Microscint-20, and radioactivity was measured in a MicroBeta counter (Perkin-Elmer Life Sciences, Waltham, MA). Data were analyzed by using GraphPad Prism (San Diego) v8.0.1. Agonist concentration-response curves from GTP$\gamma$S $^{35}$S experiments were fitted to the three-parameter logistic equation to derive estimates for agonist potencies ($pEC_{50}$) and maximal agonist responses ($E_{max}$).

## Tissue Contraction Assays

Distal colons from C57Bl/6J mice were excised and prepared for tissue contraction assays of the circular muscle layer as described in detail.[24,27] Colons were placed in 10 mL water-jacketed organ baths containing Krebs solution (in mmol/L; 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 by 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 were equilibrated for 30 minutes before use. Drugs were applied at a volume of 10 $\mu$L into organ baths. After the completion of each experiment, 10 $\mu$mol/L 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 $s^{-1}$, 60 V), which was applied through platinum electrodes incorporated into the tissue holder.[24] Tissues were incubated with DMSO (0.1%), NLT (100 nmol/L), or CTOP (1 $\mu$mol/L) for the entire experiment. Once reproducible baseline responses were maintained ($\geq$3 sets, 5-minute intervals), tissues were treated cumulatively with agonists (1 nmol/L–10 $\mu$mol/L, 5 minutes). Tissues were electrically stimulated (3 sets, 5-minute intervals) after each drug addition and then washed (5 minutes). The amplitudes of EFS-evoked contractions were compared with baseline responses (ie, 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.[24] Tissues were treated with DMSO (0.1%), NLT (100 nmol/L), or CTOP (1 $\mu$mol/L) for 15 minutes, followed by cumulative exposure to increasing concentrations of CYM51010 (1 nmol/L–10 $\mu$mol/L, 2 minutes). 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 DMSO (0.1%), a selective DOR agonist (SNC80 or ARM390), a selective MOR agonist (DAMGO or morphine), or CYM51010 (all 1 $\mu$mol/L, 5 minutes). Tissues were washed (3 washes, 5-minute intervals), and increasing concentrations of SNC80, DAMGO, or morphine (1 nmol/L–10 $\mu$mol/L, 2-minute 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%, intraperitoneally; 3 hours) or SNC80 (10 mg/kg, intraperitoneally; 3 hours). At this dose and time point, SNC80 promotes significant internalization of DOR-eGFP in the soma, proximal neurites, and nerve fibers of myenteric neurons.[27] Tissue strips were prepared as described above. After equilibration, either DAMGO or SNC80 (1 nmol/L–10 $\mu$mol/L, 2 minutes) was cumulatively added to the bath, and maximal contraction amplitudes were measured and analyzed as described above.

### Statistical Analyses

Data were expressed as the mean ± standard error of the mean, and graphs were constructed in GraphPad Prism v8.0.1. All groups for image analysis were compared by one-way analysis of variance, followed by Dunnett's multiple comparison test. For the contraction assays, specific statistical analyses used for each experiment are indicated in the respective figure legends. $P < .05$ was defined as significantly different to the null hypothesis of no difference between means at the 95% confidence level.

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J.J.D. designed and conducted experiments and assisted with manuscript preparation. S.E.C. supervised the study, performed preliminary experiments, and assisted with manuscript preparation. A.S., P.R., R.A.C., and V.P. performed experiments. C.V., A.C., N.A.V., and M.C. provided critical assessment of the manuscript. D.M. provided tissue from MOR-DOR knockin mice, performed experiments, and provided critical assessment of the manuscript. D.P.P. supervised the study, designed and conducted experiments, and wrote the manuscript.

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