Localization and Regulation of Fluorescently Labeled Delta Opioid Receptor, Expressed in Enteric Neurons of Mice

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BACKGROUND & AIMS: Opioids and opiates inhibit gastrointestinal functions via μ, δ, and κ receptors. Although agonists of the δ opioid receptor (DOR) suppress motility and secretion, little is known about the localization and regulation of DOR in the gastrointestinal tract. **METHODS:** We studied mice in which the gene that encodes the enhanced green fluorescent protein (eGFP) was inserted into *Oprd1*, which encodes DOR, to express an approximately 80-kilodalton product (DOReGFP). We used these mice to localize DOR and to determine how agonists regulate the subcellular distribution of DOR. **RESULTS:** DOReGFP was expressed in all regions but was confined to enteric neurons and fibers within the muscularis externa. In the submucosal plexus, DOReGFP was detected in neuropeptide Y–positive secretomotor and vasodilator neurons of the small intestine, but rarely was observed in the large bowel. In the myenteric plexus of the small intestine, DOReGFP was present in similar proportions of excitatory motoneurons and interneurons that expressed choline acetyltransferase and substance P, and in inhibitory motoneurons and interneurons that contained nitric oxide synthase. DOReGFP was present mostly in nitrergic myenteric neurons of colon. DOReGFP and μ opioid receptors often were co-expressed. DOReGFP-expressing neurons were associated with enkephalin-containing varicosities, and enkephalin-induced clathrin- and dynamin-mediated endocytosis and lysosomal trafficking of DOReGFP. DOReGFP replenishment at the plasma membrane was slow, requiring de novo synthesis, rather than recycling. **CONCLUSIONS: DOR localizes specifically to submucosal and myenteric neurons, which might account for the ability of DOR agonists to inhibit gastrointestinal secretion and motility. Sustained down-regulation of DOReGFP at the plasma membrane of activated neurons could induce long-lasting tolerance to DOR agonists.**

Keywords: Trafficking; Opiate Drug; Constipation; Morphine.

Opioids and opiates inhibit gastrointestinal functions via G-protein–coupled μ, δ, and κ opioid receptors (MOR, DOR, and KOR).[1,2] Opiates that activate MOR, DOR, and KOR inhibit intestinal peristalsis in the guinea pig,[3,4] and opioid-receptor antagonists disrupt peristaltic contractions in guinea pig, mouse, and rat, suggesting that endogenous opioids modulate this reflex.[3–6] Opioid-receptor agonists also inhibit secretion from the rat jejunum.[7] The mechanisms underlying these actions are of interest because they mediate constipation, which is the major limiting side effects of opiate analgesics.

Anatomic and pharmacologic studies provide insight into the location and function of opioid receptors in the gut. However, it is unclear whether opioids and opiates exert their effects by activating receptors on enteric neurons or other cell types within the gut, or act centrally. Although the location and activation of MOR in enteric neurons have been studied extensively because of its importance in morphine-induced constipation,[8] less is known about the distribution and activation of DOR and KOR in the gastrointestinal tract. Electrophysiological studies have indicated that opioids regulate enteric neurons by DOR- and MOR-dependent mechanisms.[9] However, it is not known whether these receptors are co-expressed by enteric neurons, where they may respond to the same agonists and could interact.

Agonists promote endocytosis of many G-protein–coupled receptors in neurons, including opioid receptors, and redistribution of receptors to endosomes is a hallmark of activation. Endocytosis attenuates G-protein–dependent signaling by depleting receptors from the cell surface, but also activates G-protein–independent signaling by recruiting receptors and adaptor proteins, such as β-arrestins, to endosomes.[10] Endosomal sorting targets receptors to the plasma membrane, where recycling mediates resensitization, or to lysosomes, where degradation down-regulates signaling. Activated DOR traffics to lysosomes,[11,12] but also can recycle.[13] Nothing is known about the mechanism and pathway of DOR trafficking in enteric neurons.

We localized DOR in enteric neurons and determined the mechanism and pathway of agonist-stimulated trafficking of DOR. To enable specific detection and permit

Abbreviations used in this paper: ChAT, choline acetyltransferase; Cy3-dermorphin, Cy3-[Lys[7], Cys-[8]]dermorphin; DOR, δ opioid receptor; eGFP, enhanced green fluorescent protein; IPANs, intrinsic primary afferent neurons; IR, immunoreactive/immunoreactivity; KOR, κ opioid receptor; MOR, μ opioid receptor; NOS, nitric oxide synthase; SOM, somatostatin; SP, substance P; SSTR2A, somatostatin receptor 2A.

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direct observation of receptor trafficking in real time, we studied mice in which enhanced green fluorescent protein (eGFP) was knocked into the DOR gene *Oprd1*.[14] We detected DOReGFP in specific populations of submucosal and myenteric neurons, some of which co-expressed MOR. Agonists stimulated clathrin- and dynamin-mediated endocytosis of DOR, which trafficked via early endosomes to lysosomes, and repletion of cell surface receptors required de novo synthesis.

Materials and Methods

See the Supplementary Materials and Methods section for complete methodologic details, and Supplementary Table 1 for sources and dilutions of antibodies.

Animals

Mice expressing DOR with C-terminal eGFP have been characterized.[12,14–16] Mice (male and female, 20–30 g) were anesthetized with tribromoethanol (Avertin, Sigma, St. Louis, MO; 250 mg/kg, intraperitoneally [IP]) and killed by bilateral thoracotomy. The University of California San Francisco Institutional Animal Care and Use Committee approved all procedures.

Western Blotting

Tissue extracts (60 μg protein) were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and DOReGFP was detected by Western blotting for GFP.

Immunofluorescence

Sections and whole-mounts of enteric ganglia were incubated with primary antibodies, washed, and incubated with fluorescent secondary antibodies (1 h, room temperature). To facilitate detection of neuropeptides in the soma, mice were treated with colchicine (2.5 mg/kg, IP) 6 hours before tissue collection. To facilitate detection of DOReGFP-positive submucosal neurons, mice were treated with the DOR agonist SNC80 (10 mg/kg, subcutaneously [SC]) 30 minutes before tissue collection, which concentrated the receptor in endosomes where it was readily detected. This strategy was not necessary for detecting DOReGFP in myenteric neurons.

DOReGFP Trafficking

To examine DOR trafficking in vivo, mice were treated with SNC80 (10 mg/kg, SC), and whole-mounts of myenteric and submucosal plexuses were prepared at various times. To examine trafficking in organotypic cultures, ileal segments were opened, pinned mucosa-down onto silicone-lined dishes, and equilibrated (1 h, 37°C) in Krebs' buffer containing nicardipine (1 μmol/L) and tetrodotoxin (1 μmol/L). Tissues were stimulated with SNC80 or Met-enkephalin (10, 100 nmol/L) for 1 hour at 4°C, washed, incubated at 37°C for varying times to allow DOR trafficking to occur, and were fixed and processed for immunofluorescence. To induce endogenous opioid release, tissues were stimulated with KCl (50 mmol/L; 2-min wash, 30-min recovery) in buffer containing nicardipine, bestatin, captopril, leupeptin, phosphoramidon, and thiorphan (10 μmol/L). To examine trafficking in isolated neurons, myenteric neurons were dispersed enzymatically from the ileum or distal colon, and were cultured for 7–10 days.[17] Cultured neurons were stimulated with DOR agonists, fixed, and processed for immunofluorescence.

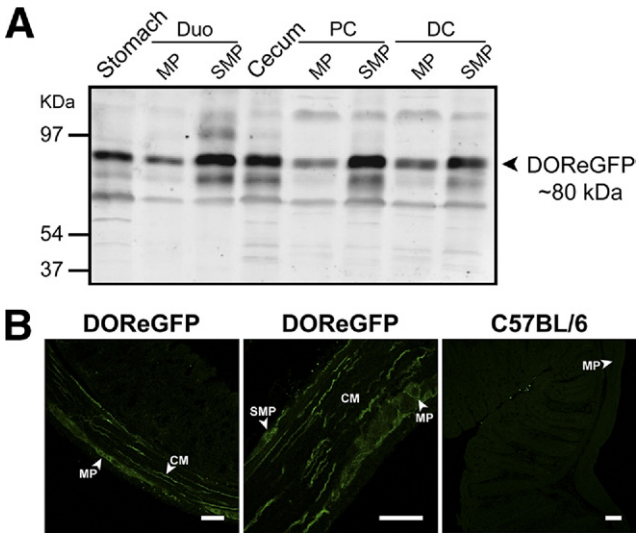

Figure 1. Expression and localization of DOR in the gastrointestinal tract. (*A*) DOReGFP was detected as an approximately 80-kilodalton protein in Western blots of stomach, duodenum (Duo), cecum, proximal colon (PC), and distal colon (DC), including muscularis externa/myenteric plexus (MP) and submucosa/submucosal plexus (SMP). (*B*) DOReGFP-IR was localized to neurons of myenteric and submucosal plexuses and to nerve fibers within longitudinal and circular (CM) smooth muscle layers (*left* and *middle panels*) of colon. There was no detectable GFP-IR distal colon of wild-type mice (*right panel*), showing specificity. *Scale bar*, 50 μm.

Imaging

Specimens were examined using a Zeiss LSM510 META confocal microscope (Carl-Zeiss, Thornwood, NY). Expression of GFP relative to the total neuronal population and functional neuronal subtypes was determined by co-labeling with neurochemical markers.[18,19]

Statistical Analysis

Data are expressed as the mean ± standard error of the mean and were analyzed using the Student *t* test or 1-way analysis of variance with the Newman–Keuls or Bonferroni post hoc test. A *P* value of less than .05 was considered significant.

Results

DOR Is Expressed in Enteric Neurons

DOReGFP immunoreactivity (IR) was detected as a prominent band of approximately 80 kilodaltons in all regions (Figure 1*A*), consistent with the combined sizes of DOR and eGFP.[12] Other immunoreactive proteins may correspond to oligomers or degradation products. No signal was detected in tissues from wild-type mice (not shown). We localized DOReGFP in sections by indirect immunofluorescence using GFP antibody to amplify the signal. DOReGFP-IR was detected in the esophagus, corpus, antrum, gall bladder, duodenum, ileum, cecum, proximal colon, and distal colon, where it was localized to enteric ganglia and nerve fibers (Figure 1*B*). Within ganglia, DOReGFP-IR colocalized with the neuronal marker PGP9.5 in a subset of myenteric and submucosal neurons, but it did not colocalize with glial fibrillary acidic protein, which identifies enteric glial cells (not shown). Nerve fibers

containing DOReGFP-IR projected along the axes of the circular and longitudinal muscle layers of the muscularis externa (Figure 1*B*), and contained nitric oxide synthase (NOS)-IR and vasoactive intestinal polypeptide-IR (not shown), indicating origination from myenteric inhibitory motoneurons.[19] DOReGFP-IR did not colocalize with calcitonin gene-related peptide-IR in nerve fibers innervating the mucosa (not shown), indicating that DOReGFP is not expressed in the terminals of intrinsic primary afferent neurons (IPANs).[19,20] DOReGFP-IR was not detected in gastrointestinal or vascular smooth muscle, interstitial cells of Cajal, or enterocytes. GFP antibody did not stain tissues from wild-type mice, confirming specificity (Figure 1*B*).

DOR Is Expressed by Secretomotor/Vasodilator Submucosal Neurons

Supplementary Table 2 reports the proportions of submucosal neurons co-expressing DOReGFP-IR and neurochemical markers. DOReGFP-IR colocalized with PGP9.5-IR in submucosal neurons, but there were marked differences in the proportions of neurons expressing DOReGFP-IR in the small and large intestines. In the duodenum and ileum, DOReGFP-IR was detected in most submucosal neurons, whereas few neurons expressed DOReGFP-IR in the cecum and colon. We estimate by extrapolation from neurochemical coding studies[18] that approximately 50% of all submucosal neurons of the ileum express DOReGFP. In the ileum, 90% of neurons expressing DOReGFP-IR co-expressed neuropeptide Y–IR (identifies noncholinergic secretomotor and vasodilator neurons[18]) (Supplementary Figure 1*A*). Of neurons expressing DOReGFP-IR, 8% co-expressed choline acetyl transferase (ChAT)-IR (identifies cholinergic secretomotor neurons) and 11% co-expressed somatostatin (SOM)-IR (localized to cholinergic submucosal neurons of the ileum) (Supplementary Figure 1*A*), consistent with the minimal colocalization of ChAT and DOReGFP. In the distal colon, there was a smaller degree of overlap between DOReGFP-IR and neuropeptide Y–IR (39%) than in the ileum, and DOReGFP-IR and ChAT-IR rarely were colocalized (10%) (Supplementary Figure 1*B*). A substantial proportion of submucosal neurons in the large intestine that expressed DOReGFP-IR co-expressed NOS-IR (cecum, 96%; proximal colon, 33%; distal colon, 79%; Figure 2*C*). Although most submucosal neurons of the cecum expressed NOS-IR at a low level, those neurons expressing DOReGFP-IR had intense signals for NOS-IR (Supplementary Figure 1*C*). The relative mean fluorescence intensity of NOS-IR in DOReGFP-negative neurons was 0.50 ± 0.03 (n = 188 neurons) and in DOReGFP-positive neurons was 1.00 ± 0.03 (n = 35 neurons). These neurons were unipolar.

DOR Is Expressed by Nitrergic and Cholinergic Myenteric Neurons

Supplemental Table 3 reports the proportions of myenteric neurons co-expressing DOReGFP-IR and neurochemical markers. Intense DOReGFP-IR was detected at the plasma membrane of the soma and axons of a subset of myenteric neurons, with little intracellular signal (Figure 2). In all regions (antrum to distal colon), approximately 50% of all PGP9.5-IR myenteric neurons co-expressed DOReGFP-IR, with the exception of the proximal colon where only approximately 38% of myenteric neurons were DOReGFP positive (Figure 2*A*). There were marked differences in the neuronal subtypes that expressed DOReGFP in the small and large intestine. The proportion of DOReGFP-IR neurons co-expressing NOS-IR (identifies inhibitory motoneurons and interneurons) was 44%–53% in the small intestine (Figure 2*B*) and 66%–95% in the large intestine (Supplementary Figure 2*A*). The proportion of DOReGFP-IR neurons co-expressing ChAT-IR (identifies cholinergic excitatory motoneurons, interneurons, and IPANs) was 41%–44% in the small intestine (Figure 2*C*) and only 9%–30% in the large intestine (Supplementary Figure 2*B*). In the small and large intestines, DOReGFP-IR was not detected in large ChAT-IR neurons, which probably are IPANs. DOReGFP-IR was not detected in neurons with Dogiel type II morphology that expressed neurofilament M–IR (identifies IPANs and other neurons in the mouse myenteric plexus[19]) in any of the regions examined (Figure 2*D*, Supplementary Figure 2*C*). DOReGFP-IR did not colocalize with the IPAN marker calcitonin gene-related peptide–IR (not shown).[19,20]

DOR and SOM Receptor Somatostatin Receptor 2A Are Expressed by Distinct Nitrergic Myenteric Neurons

Functional studies of peristalsis suggest that DOR is co-expressed with SOM receptor somatostatin receptor 2A (SSTR2A) by inhibitory motoneurons containing NOS and vasoactive intestinal polypeptide in mouse colon.[5] SSTR2A-IR colocalizes with NOS-IR and vasoactive intestinal polypeptide–IR in myenteric neurons, suggesting overlap with DOReGFP-IR.[21,22] To examine this possibility, we simultaneously localized DOReGFP, SSTR2A, and NOS in myenteric plexus whole-mounts of large intestine. There was no detectable colocalization of DOReGFP-IR and SSTR2A-IR (Figure 2*E*). Although DOReGFP-IR and SSTR2A-IR were colocalized extensively with NOS-IR, triple labeling indicated that the 2 receptors were present in distinct populations of NOS-IR neurons. Neurons co-expressing SSTR2A-IR and NOS-IR were larger (diameter, 27.98 ± 0.74 μm; 56 neurons) than neurons co-expressing DOReGFP-IR and NOS-IR (diameter, 20.80 ± 0.36 μm; 187 neurons; $P < .0001$). Neurons expressing NOS-IR mostly co-expressed DOReGFP-IR (76% distal colon), with the remaining neurons either co-expressing SSTR2A-IR (17%) or only NOS-IR (7%). SOM-IR was detected in few myenteric neurons, which were characterized by intense labeling of Golgi-like structures and by filamentous morphology (Supplementary Figure 2*D*). A small proportion (3%–4%) of DOReGFP-IR neurons co-expressed SOM-IR in the ileum and distal colon, where SOM is present in a subset of descending interneurons.[5,19] The minimal overlap between DOR and SSTR2A or SOM is at variance with predictions of pharmacologic studies.

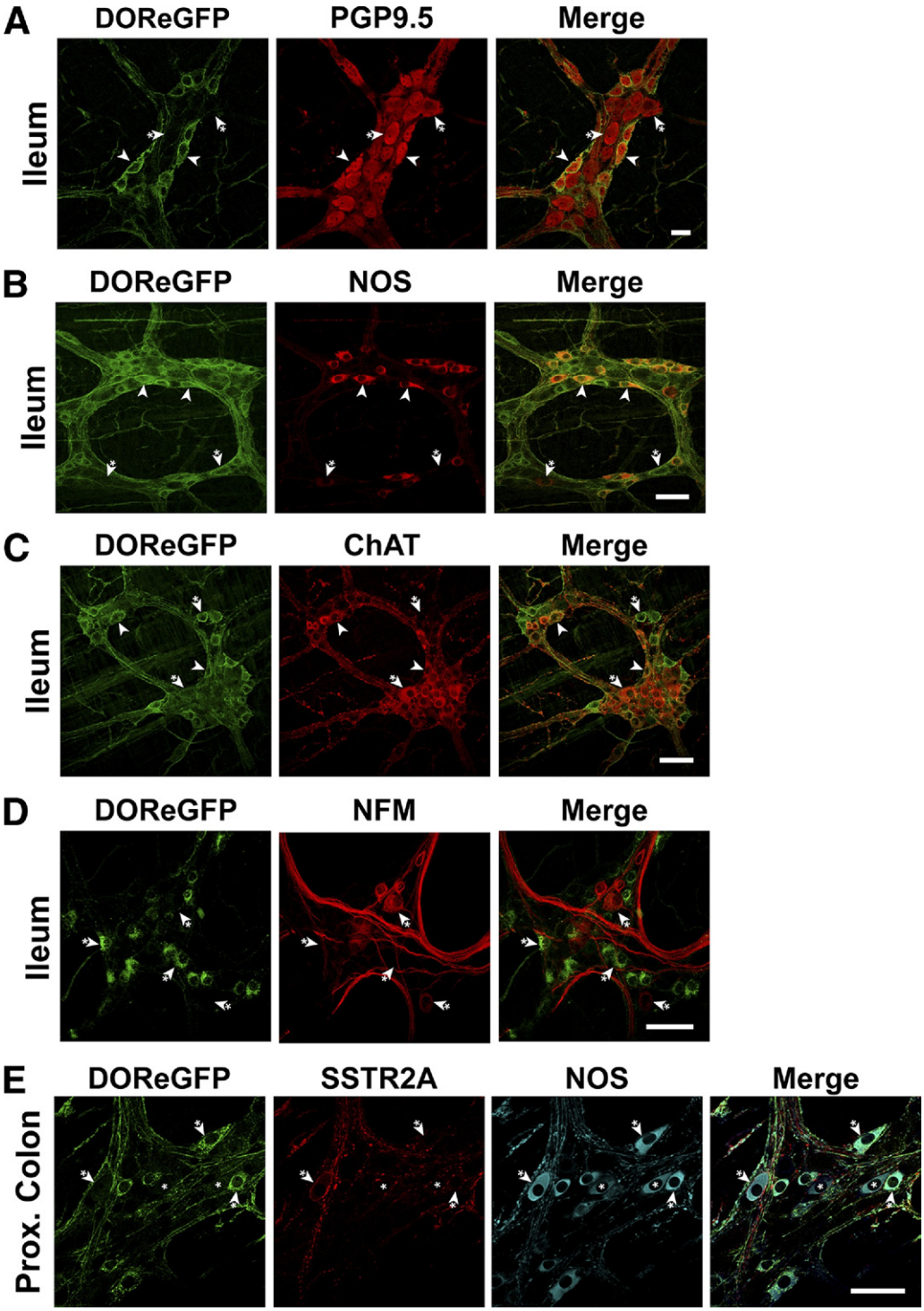

Figure 2. Localization of DOR to nitrergic and cholinergic myenteric neurons of ileum and colon. (*A*) DOReGFP-IR was detected in approximately 50% of all PGP9.5-IR myenteric neurons (*arrowheads* indicate colocalization, *arrowheads with asterisk* indicate lack of colocalization). (*B* and *C*) DOReGFP-IR extensively colocalized with (*B*) NOS-IR and (*C*) ChAT-IR in small neurons (*arrowheads*), but was absent from large ChAT-IR neurons (*arrowheads with asterisks*). (*D*) DOReGFP-IR was not colocalized with neurofilament M (NFM)-IR. (*E*) DOReGFP-IR and SSTR2A-IR were localized to distinct NOS-IR neurons in proximal colon (*arrowheads with asterisks*). Neurons positive only for NOS-IR are indicated by an asterisk. *Scale bars*, 50 μm.

DOR Is Co-expressed With Substance P but Not the Neurokinin 1 Receptor in Myenteric Neurons

Tachykinins are excitatory peristaltic neurotransmitters.[5] Of DOReGFP-IR myenteric neurons, 33% co-expressed substance P (SP)-IR in the ileum and 25% co-expressed SP-IR in the distal colon of colchicine-treated mice (Supplementary Figure 2*E*). In the distal colon, only 1% of DOReGFP-IR neurons co-expressed neurokinin 1 receptor–IR (Supplementary Figure 2*F*). Neurokinin 1 re-

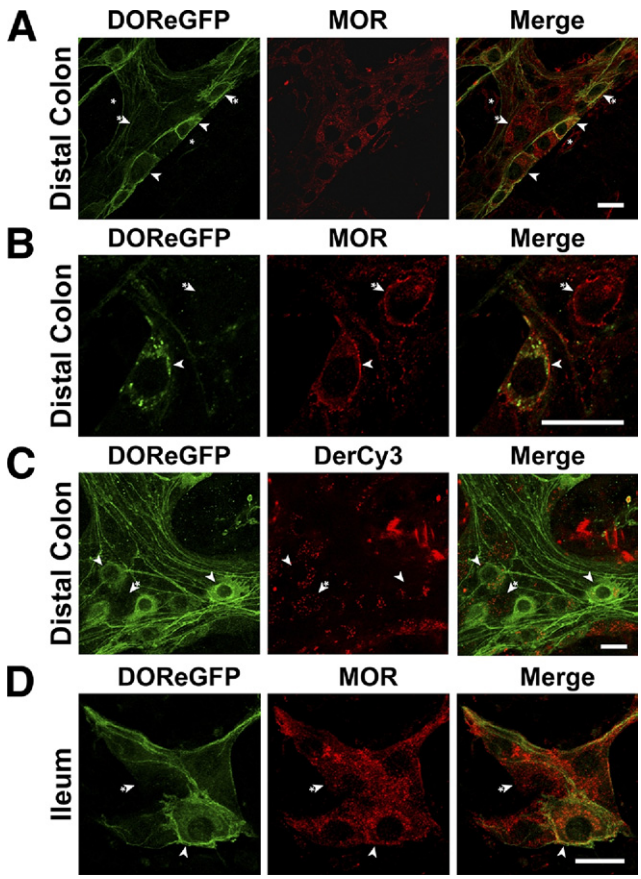

Figure 3. Colocalization of DOR and MOR in a subpopulation of enteric neurons. (*A*) DOReGFP-IR colocalized in myenteric neurons of distal colon with MOR-IR (*arrowheads*), but MOR-IR also was detected in large neurons that did not express DOReGFP-IR (*arrowheads with asterisks*) and in interstitial cells of Cajal (*asterisks*). (*B*) MOR-IR was present at the cell surface of some myenteric neurons, including those positive for DOReGFP-IR (*arrowhead*). (*C*) Cy3-dermorphin (DerCy3) trafficked to endosomes of myenteric neurons of distal colon, including neurons expressing DOReGFP-IR (*arrowheads*). (*D*) DOReGFP-IR colocalized with MOR-IR in some submucosal neurons of the ileum (*arrowheads*), but MOR-IR also was detected in neurons that did not express DOReGFP-IR (*arrowheads with asterisks*). *Scale bars*, 20 μm.

ceptor–IR neurons mainly were multipolar and larger than DOReGFP-IR neurons. The colocalization of DOR with SP in ascending interneurons and excitatory circular muscle motoneurons[19] suggests that DOR is appropriately located to inhibit SP release and indirectly modulate neurokinin 1–receptor activation, but DOR could not directly regulate neurokinin 1 receptor.

DOR and MOR Are Co-expressed by Enteric Neurons

To examine DOR/MOR colocalization, we localized DOReGFP-IR and detected MOR by using a characterized MOR-specific antibody[8] that does not stain tissue from MOR-deficient mice[16] and by studying uptake of the MOR-selective agonist Cy3-[Lys[7], Cys[8]]dermorphin (Cy3-dermorphin). In myenteric ganglia of the distal colon, MOR-IR was localized to a subset of neurons and to myenteric interstitial cells of Cajal (Figure 3*A*). Approximately 77%–85% of all DOReGFP-IR myenteric neurons co-expressed MOR-IR, and 57%–61% of MOR-IR neurons co-expressed DOReGFP (Figure 3*A* and *B*). However, MOR-IR also was detected in large neurons, probably IPANs, that did not express DOReGFP-IR. Cy3-dermorphin (100 nmol/L) bound and trafficked to endosomes of myenteric neurons, many of which also expressed DOReGFP-IR (Figure 3*C*), although the extent of colocalization was not quantified. Naloxone (1 μmol/L) abolished binding of Cy3-dermorphin, confirming specific interaction with MOR (not shown). MOR-IR was detected in submucosal neurons of ileum and distal colon, many of which also expressed DOReGFP-IR (Figure 3*D*), although colocalization was not quantified. There were marked differences in the subcellular localization of DOR and MOR in some neurons where these receptors were co-expressed. In all neurons, DOReGFP was localized predominantly to the plasma membrane. However, MOR-IR was either mostly intracellular (Figure 3*A*) or mostly at the plasma membrane (Figure 3*B*).

Enkephalinergic Varicosities Innervate DOR Myenteric Neurons

We examined the proximity between DOR and the endogenous agonist enkephalin and determined whether endogenous agonists could activate DOR. Enkephalin IR was detected in varicosities of fibers surrounding the soma and neurites of DOReGFP-IR neurons, as well as neurons that did not express DOReGFP-IR, in the myenteric (Figure 4*A*) and submucosal (not shown) plexuses of ileum and colon. Enkephalinergic varicosities also were associated intimately with nerve fibers containing DOReGFP-IR in the circular muscle and deep muscular plexus (Figure 4*A*, right panel). Enkephalin-IR and DOReGFP-IR did not colocalize in the soma of myenteric (Figure 4*A*) or submucosal (not shown) neurons. To examine whether endogenous opioids activate DOR, we depolarized organotypic preparations of distal colon using KCl, which induces neuropeptide release,[17] and examined the subcellular distribution of DOReGFP-IR. KCl induced endocytosis of DOReGFP-IR (Figure 4*B* and *C*), indicating receptor activation. The DOR-selective antagonist naltrindole (100 nmol/L) abolished KCl-induced endocytosis.

Agonists Induce Clathrin- and Dynamin-Dependent DOR Endocytosis

We examined agonist-induced DOR trafficking in myenteric neurons in vivo, in organotypic cultures, and in isolated neurons.

Mice were treated with SNC80 (10 mg/kg SC) or vehicle (control), and DOReGFP-IR was localized in myenteric and submucosal plexus whole-mounts. DOReGFP-IR was confined to the plasma membrane of myenteric and submucosal neurons from vehicle-treated mice (Figure 4*D*, myenteric plexus). Within 10–30 minutes after SNC80

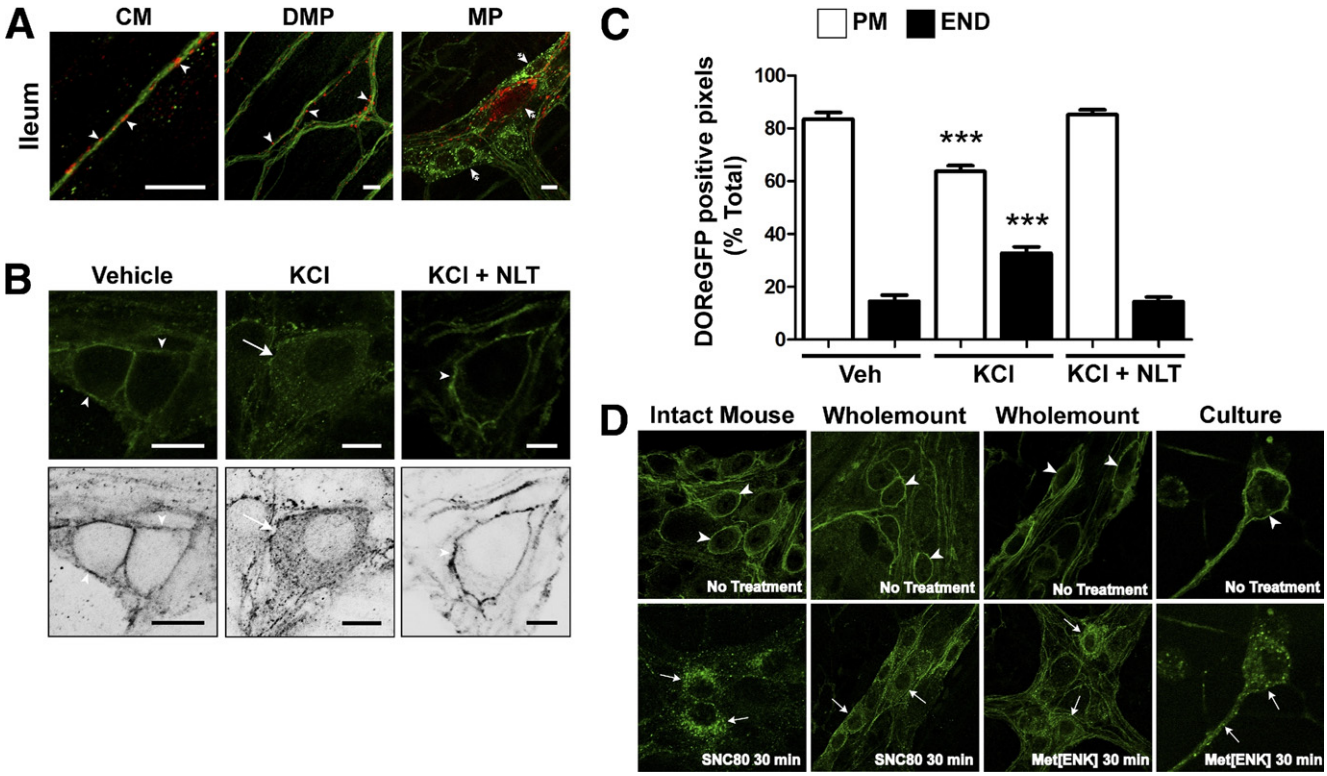

Figure 4. Localization of DOR and enkephalin (ENK), and opioid-induced activation of DOR in the myenteric plexus. (*A*) ENK-IR (*red*) varicosities were associated intimately with DOReGFP-IR (*green*) in nerve fibers in circular muscle (CM) and deep muscular plexus (DMP) (*arrowheads*). DOReGFP-IR was not colocalized with ENK-IR in myenteric neurons (MP, *arrowheads with asterisks*, *right panel*). (*B*) KCl but not vehicle stimulated DOReGFP-IR endocytosis in organotypic myenteric plexus whole-mounts from distal colon. Naltrindole (NLT, *right panel*) abolished DOR endocytosis. *Lower panels* are inverted images. *Scale bars*, 10 μm for *panels A* and *B*. (*C*) Quantitative analysis confirmed KCl-induced depletion of DOReGFP-IR from plasma membrane (PM) and concomitant increase in DOReGFP-IR in endosomes (END), and that naltrindole abolished endocytosis. ***$P < .0001$ to vehicle (Veh). (*D*) DOReGFP-IR endocytosis in myenteric neurons of distal colon after SNC80 injection into the intact animal (*left panels*), treatment of organotypic whole-mounts with SNC80 or Met-enkephalin (Met[ENK]) (*middle panels*), or incubation of cultured neurons with Met-enkephalin (*right panels*). *Right panel* shows eGFP fluorescence in the same cultured neuron.

treatment, DOReGFP-IR was detected in endosomes in the soma and axons of myenteric (Figure 4*D*) and submucosal (not shown) neurons.

To examine the precise timing and concentration dependence of DOR trafficking, we incubated organotypic whole-mounts of distal colon with SNC80 (10, 100 nmol/L), Met-enkephalin (100 nmol/L), or vehicle (0–120 min, 37°C). In vehicle-treated preparations, DOReGFP-IR was confined to the plasma membrane (Figure 4*D*). SNC80 induced receptor clustering at the plasma membrane within 10 minutes and maximal internalization within 30–60 minutes (Figure 4*D*). Quantitative analysis indicated that 90.4% ± 0.7% (89 neurons) of total DOReGFP-IR was at the plasma membrane in unstimulated neurons, and that 42.7% ± 5.1% (22 neurons) of total DOReGFP-IR was at the plasma membrane 60 minutes after SNC80 treatment (100 nmol/L, Supplementary Figure 3*A*). Met-enkephalin also stimulated DOReGFP internalization (Figure 4*D*). Both SNC80 and Met-enkephalin induced DOR-eGFP endocytosis in the presence of tetrodotoxin (100 nmol/L), suggesting a direct action of these agonists on the DOReGFP receptor. Naltrindole abolished endocytosis (not shown), confirming selectivity.

By studying myenteric neurons in culture, we evaluated DOReGFP trafficking in the soma and neurites of the same neurons in real time. DOReGFP-IR was detected in 46% of PGP9.5-positive myenteric neurons in culture (26 of 57 neurons), indicating retained expression (Supplementary Figure 3*B*). In unstimulated neurons, DOReGFP was distributed uniformly at the plasma membrane of the soma and neurites (Figure 4*D*, Supplementary Figure 3*B*). SNC80 and Met-enkephalin (100 nmol/L) induced DOReGFP internalization, detected by live imaging of DOReGFP and by immunofluorescence detection of GFP. Within 10 minutes, DOReGFP was clustered at the plasma membrane of the soma and neurites, and after 30 minutes DOReGFP was redistributed to endosomes and depleted from the plasma membrane (Figure 4*D*, Supplementary Figure 3*B*). Naltrindole prevented SNC80-stimulated (Supplementary Figure 3*C*) and Met-enkephalin–stimulated (not shown) trafficking, confirming specific activation of DOR.

To examine the mechanism of DOR endocytosis, we treated organotypic cultures with hypertonic sucrose (0.45 mol/L, which blocks clathrin-mediated endocytosis[17]), or Dynasore (80 μmol/L, which inhibits dynamin

guanosine triphosphatase[23]). Sucrose and Dynasore inhibited SNC80-evoked endocytosis of DOReGFP-IR in whole-mounts (Figure 5). Sucrose or Dynasore alone were without effect. These findings were confirmed using live imaging of cultured neurons (not shown).

DOR Traffics via Early Endosomes to Lysosomes and Does Not Recycle

To examine the subcellular pathway of DOR trafficking, we simultaneously localized DOReGFP with early endosomal antigen 1 (identifies early endosomes), and LAMP1 or LysoTracker (identify lysosomes). In cultured myenteric neurons, Met-enkephalin stimulated redistribution of DOReGFP-IR to early endosomal antigen 1–positive early endosomes within 10–30 minutes in the soma (Figure 6*A*) and in neurites (Supplementary Figure 3*D*). After 60–120 minutes, DOR-GFP had traversed the endosomal system and was detected in lysosomes of the soma (Figure 6*B*). Although we did not detect lysosomes in the neurites using LysoTracker or LAMP1 antibodies (not shown), after 60 minutes DOReGFP was detected in vesicles in neurites that were distinct from early endosomes (Supplementary Figure 3*D*). DOReGFP-IR also colocalized with LAMP1 in the soma of myenteric neurons of whole-mounts 60–120 minutes after administration of SNC80 to the intact mouse, and DOReGFP was still detected in lysosomes after 16 hours (Supplementary Figure 3*E*). Because DOR traffics to lysosomes and there are no prominent intracellular stores, DOR replenishment at the plasma membrane probably requires receptor synthesis. To examine this process, cultured neurons were incubated briefly with Met-enkephalin (100 nmol/L, 10 min) or vehicle, washed, and then recovered in agonist-free medium. Met-enkephalin, but not vehicle, induced translocation of DOReGFP to endosomes and lysosomes within 0.5–2 hours. DOReGFP-IR was replenished at the plasma membrane within 6–16 hours (Figure 6*C*). Cycloheximide (70 μmol/L) did not affect DOReGFP-IR endocytosis, but prevented recovery of receptor at the plasma membrane (Figure 6*D* and *E*), indicating a requirement for new receptor synthesis.

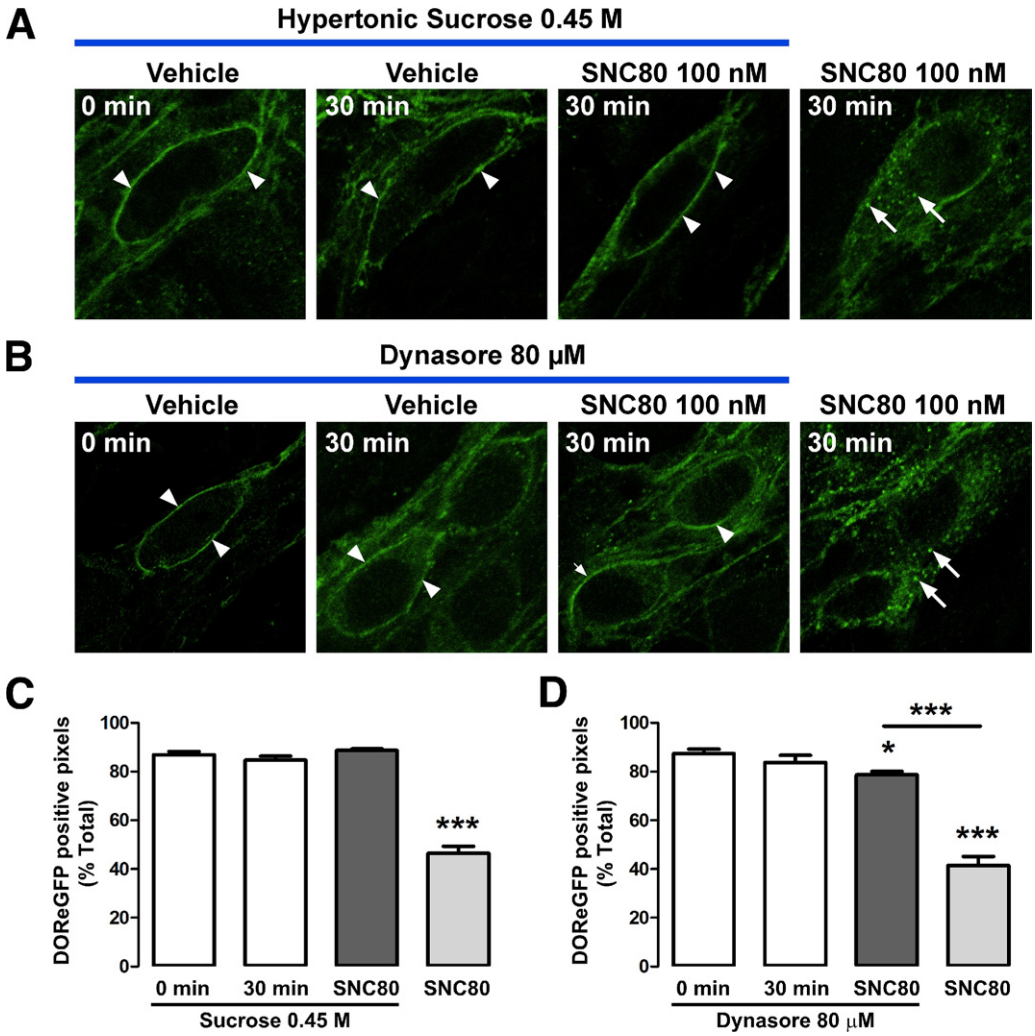

Figure 5. Clathrin- and dynamin-dependent endocytosis of DOR in myenteric neurons. (*A*) Hypertonic sucrose or (*B*) Dynasore inhibited SNC80-induced endocytosis of DOReGFP-IR in myenteric neurons in organotypic cultures from distal colon. Quantitative analysis confirmed inhibitory effects of (*C*) sucrose and (*D*) Dynasore. *$P < .05$; ***$P < .0001$ compared to control. n = 30–39 neurons from 3 or more mice per data point.

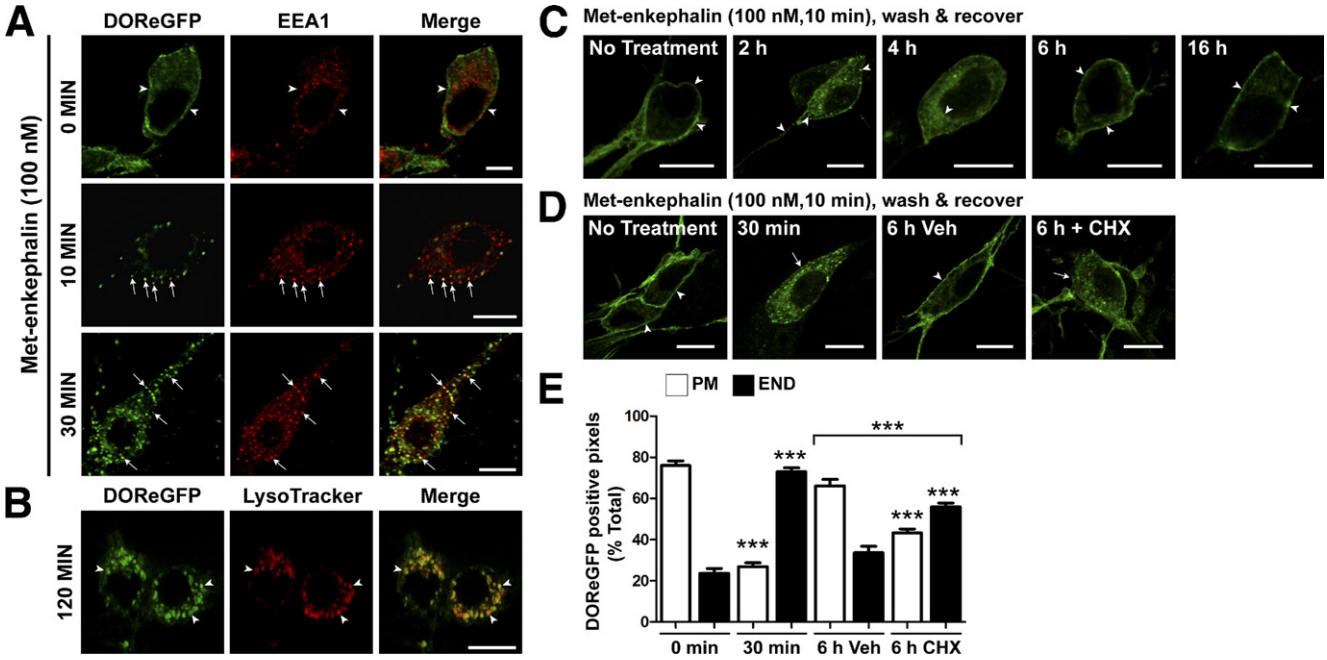

Figure 6. Intracellular trafficking of DOR in myenteric neurons. (*A*) Met-enkephalin induced DOReGFP-IR trafficking to early endosomal antigen 1 (EEA1)-positive early endosomes (*arrows*) of cultured myenteric neurons after 10–30 minutes. *Scale bars*, 10 μm. (*B*) After 120 minutes, DOReGFP-IR was detected in LysoTracker-positive lysosomes in the soma. *Scale bar*, 20 μm. (*C*) Transient stimulation of cultured neurons with Met-enkephalin stimulated DOReGFP-IR endocytosis, which was replenished at the plasma membrane only after 6–16 hours recovery. (*D*) Cycloheximide (CHX) inhibited recovery of cell surface DOReGFP at 6 hours, as confirmed by quantitative analysis (*E*). *Scale bars*, 10 μm for *panels C* and *D*. PM, plasma membrane–associated DOReGFP; END: endosome-associated DOReGFP. ***$P < .0001$, n = 20–78 neurons from 3 or more mice per data point.

Discussion

We report a detailed examination of the expression and regulation of DOR in the enteric nervous system. DOReGFP knock-in mice, previously used to study DOR in sensory and central neurons,[12,14–16] enabled specific detection, avoiding concerns about antibody selectivity. DOR is expressed by secretomotor submucosal neurons, and inhibitory nitrergic and excitatory cholinergic/tachykinergic myenteric motoneurons. Agonists induce clathrin- and dynamin-mediated endocytosis of DOR in the soma and axons, although DOR traffics to lysosomes only in the soma, suggesting differences in the fate and regulation of the receptor in the soma and axons. Restoration of surface DOR is slow, requiring de novo synthesis. Agonists may activate DOR on myenteric neurons to suppress peristalsis,[3–5] and the prolonged intracellular retention of activated DOR may induce sustained unresponsiveness of activated neurons.

Location and Function of DOR in Secretomotor Neurons of the Submucosal Plexus

We report that most ileal submucosal neurons express DOR, in particular neuropeptide Y–positive, noncholinergic, secretomotor neurons.[18] In contrast, DOR rarely is expressed in colonic submucosal neurons, where it is confined to nitrergic neurons. These findings support the localization of DOR to submucosal neurons of rat and pig.[24] Although activation of opioid receptors inhibits electrolyte and fluid secretion,[2] the mechanism of inhibition is not clear. Intracerebroventricular administration of DOR agonist inhibits cholera toxin–stimulated jejunal secretion in rats via stimulation of sympathetic postganglionic fibers and activation of α2 adrenoceptors on submucosal neurons.[7] Our results suggest that such indirect mechanisms could operate in the large intestine, where the small number of DOR-expressing neurons may limit their influence on secretomotor activity. However, the extensive expression of DOR in secretomotor neurons of the small intestine indicates that agonists can inhibit secretion by direct action on submucosal neurons. Electrophysiological studies indicate that DOR agonists inhibit activity of submucosal neurons of guinea pig ileum and cecum,[25,26] thereby suppressing secretion.[2] Enkephalinergic fibers innervating submucosal ganglia of the guinea pig small intestine originate in the myenteric plexus, with little evidence for expression by submucosal neurons.[27] Our inability to detect enkephalin in submucosal neurons supports these findings and suggests interaction between the myenteric and submucosal plexuses in any DOR-dependent regulation of secretion.

Location and Function of DOR in Excitatory and Inhibitory Motoneurons and Interneurons of the Myenteric Plexus

DOR agonists may inhibit motility by effects on central and enteric neurons. Peripheral SNC80 inhibits gastrointestinal propulsion in the mouse, and centrally penetrant but not peripherally restricted naloxone derivatives suppress this effect, suggesting a central mecha-

nism.[28] The observation that DOR knockdown in the rat brain inhibits the antipropulsive actions in the colon of peripheral SNC80 supports a central mechanism.[29] These reports are at variance with our demonstration that peripheral SNC80 directly activates (ie, internalizes) DOR in myenteric neurons. DOR agonists hyperpolarize these neurons, causing dysmotility and suppression of propulsion.[2] Intraventricular DOR agonists do not affect intestinal propulsion in rats,[30] and DOR agonists affect contractility and peristalsis of isolated rat and mouse colon,[31] supporting a direct, peripherally mediated mechanism.

We detected DOR in excitatory (ChAT/SP-positive) and inhibitory (NOS/SOM-positive) myenteric motoneurons, and in nerve fibers innervating deep muscular plexus and circular muscle, supporting observations in rat and pig.[24] DOR was associated intimately with enkephalin-containing varicosities, and endogenous opioids activated (ie, internalized) DOR in myenteric neurons, suggesting that opioids suppress peristalsis by activating DOR on excitatory and inhibitory myenteric neurons. Opioid release from the rat colon declines during the inhibitory phase and increases during the excitatory phase of peristalsis.[6] Met-enkephalin inhibits release of mediators of ascending contraction (acetylcholine)[32] and descending relaxation (vasoactive intestinal polypeptide)[6] from isolated guinea pig ileum and rat colon, respectively. By releasing excitatory and inhibitory motoneurons from the inhibitory influence of endogenous opioids, DOR antagonists augment ascending contraction and descending inhibition of mouse colon.[5] The effects of enkephalins on inhibitory junction potentials in the dog duodenum are neurogenic and DOR-dependent,[9] suggesting that opioids activate DOR on inhibitory motoneurons that control neuromuscular transmission to circular muscle. The effects of opioids on inhibitory junction potentials are retained in preparations devoid of the myenteric plexus, supporting an action at inhibitory motor nerves within the circular muscle or deep muscular plexus.[9,33]

Co-expression of Opioid Receptors by Enteric Neurons

Most enteric neurons that express DOR also express MOR, detected immunochemically and by Cy3-dermorphin uptake. DOR and MOR also are co-expressed in myenteric neurons of rat ileum,[34] and DOR colocalizes with KOR in myenteric neurons of the pig ileum.[24] Electrophysiological[35] and pharmacologic[36] studies support DOR/MOR co-expression by enteric neurons. In contrast to extensive DOR/MOR co-expression in enteric neurons, DOR and MOR are expressed by distinct neuronal populations of dorsal root ganglia neurons.[16] Although the functional relevance of DOR/MOR co-expression in enteric neurons remains to be determined, enkephalins may co-activate these receptors, which could amplify their inhibitory effects on motoneurons.[9] DOR/MOR heterodimerization also affects opioid affinity and G-protein signaling.[37] Whether these receptors dimerize in enteric neurons, and the functional relevance of their differential subcellular location (DOR mostly at the plasma membrane and MOR intracellularly or at the plasma membrane), remain to be determined.

Regulation of DOR in the Enteric Nervous System

Neuronal responsiveness to extracellular agonists requires receptor localization at the plasma membrane. Endocytosis attenuates G-protein–mediated signaling by depleting surface receptors, but some receptors interact in endosomes with β-arrestins, which transmit distinct signals.[10] DOR internalizes in the soma and axons of enteric neurons by clathrin/dynamin-mediated mechanisms, and traffics via early endosomes to lysosomes in the soma but to unidentified vesicles in the neurites. Although DOR will be degraded in lysosomes in the soma, the fate of the receptor in axons is unknown. Activated DOR is depleted from the cell surface for prolonged periods and replenishment of DOR at the surface of enteric neurons requires synthesis of new receptors. These observations are consistent with ubiquitination-mediated trafficking of DOR to lysosomes in cell lines.[11] Agonists that induce DOR endocytosis and down-regulation (SNC80) result in sustained tolerance to DOR-mediated analgesia, locomotor activation, and anxiolysis, whereas noninternalizing agonists (ARM390) induce tolerance only to DOR-mediated analgesia.[15] Thus, once activated by agonists that induce DOR endocytosis, enteric neurons would remain unresponsive to further challenges with DOR agonists for prolonged periods. DOR endocytosis and degradation could mediate tolerance to the antisecretory effects of enkephalins in the ileum after prolonged DOR activation.[38] However, chronic morphine induces tolerance to the contractile effects of opioids in the ileum but not the colon.[39] Further studies are required to define the mechanisms that regulate opioid-receptor subtypes in enteric neurons of different intestinal regions after acute and chronic activation.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2011.05.042.

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Daniel Poole and Juan–Carlos Pelayo contributed equally to this study.

Conflicts of interest

The authors disclose no conflicts.

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Supplementary Materials and Methods

Materials

Cycloheximide, Dynasore, SNC80, naloxone hydrochloride, and naltrindole hydrochloride were from Tocris Biologicals (Ellisville, MO); Met-enkephalin was from Bachem (Torrance, CA); LysoTracker DND99 was from Invitrogen (Carlsbad, CA); peptidase and protease inhibitor cocktails were from Calbiochem/EMD Chemicals (Gibbstown, NJ); and other reagents were from Sigma-Aldrich (St. Louis, MO). An analogue of MOR-selective agonist dermorphin[1] was synthesized with Lys and Cys at positions 7 and 8, respectively (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Lys-Cys-NH_2; [Lys,[7] Cys[8]]dermorphin; CPC Scientific, San Jose, CA). Peptide was labeled with Cy3 maleimide (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions. Cy3-dermorphin was purified by reverse-phase high-performance liquid chromatography on a C18 column, and labeled peptide was quantified using unlabeled [Lys,[7] Cys[8] dermorphin standards.[2]

Western Blotting

Tissues were snap-frozen and sonicated in RIPA buffer containing protease inhibitors. Proteins (60 μg) were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (12% gel) and transferred to polyvinylidene difluoride membranes. Membranes were incubated with GFP antibody, washed, and incubated with AlexaFluor680-conjugated secondary antibody (Invitrogen, 1:10,000, 1 h, room temperature). Proteins were detected using an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

Tissue Collection and Immunofluorescence

Tissues were placed in phosphate-buffered saline (PBS) (0.1 mol/L, pH 7.4) containing nicardipine (1 μmol/L), opened, and then fixed in paraformaldehyde (4%, PBS, overnight). Tissue for sectioning was cryoprotected in 30% sucrose (PBS, 0.1% sodium azide, overnight), embedded in optimal cutting temperature compound, and frozen sections were prepared (12 μm). Whole-mounts of myenteric and submucosal plexuses were prepared as described.[3] Sections and whole-mounts were incubated in blocking buffer (10% normal horse serum, 0.1% Triton X-100 in PBS), and then incubated with primary antibodies (Supplementary Table 1). Preparations stained for neurofilament M were preincubated in 0.3% Triton X-100 in PBS for 3 days before antibody application.[4] Tissues were washed, incubated with secondary antibodies coupled to fluorescein isothiocyanate, Rhodamine Red X, or Cy5 (donkey IgG, 1:200, 1 h, room temperature; Jackson Immunoresearch, West Grove, PA), and mounted. LysoTracker was used according to the manufacturer's directions.

Primary Cultures of Myenteric Neurons

Segments of ileum or distal colon were placed in ice-cold Hanks balanced salt solution containing nicardipine (1 μmol/L) and antibiotic/antimycotic solution (1:100 dilution; 25 μg/mL amphotericin B; 10,000 U/mL penicillin, and 10 mg/mL streptomycin). The muscularis externa and myenteric plexus were removed by sharp dissection, and digested with collagenase type IA (1 mg/mL) and DNase IV (0.1 mg/mL, 150 kU) in Dulbecco's modified Eagle medium (1 h, 37°C, constant agitation).[5] Tissue was digested with trypsin (0.25% solution with ethylenediaminetetraacetic acid, 5–10 min, 37°C). Digestion was terminated by adding 20% fetal bovine serum and soybean trypsin inhibitor (10 mg/mL) followed by centrifugation. A single-cell suspension was achieved by trituration. Neurons were plated onto poly-L-lysine/laminin–coated coverslips, and were cultured in Dulbecco's modified Eagle medium supplemented with normal horse serum (5%), fetal bovine serum (5%), L-glutamine (2 mmol/L), antibiotic/antimycotic, and N1 neuronal supplement (1:100). Cytosine arabinoside (1 μmol/L) was included in the culture medium from day 2 onward to inhibit proliferation of non-neuronal cells.

Imaging

Specimens were examined using a Zeiss Axiovert 200M microscope and Zeiss LSM510 META confocal system. Images were captured with 40× (Plan-Neofluar/1.3), 63×, or 100× (Plan-Apochromat/1.4) objectives, with 1024 × 1024 pixel resolution. To directly observe DOReGFP in cultured neurons, coverslips were mounted in a temperature-controlled open chamber containing Hank's balanced salt solution with 0.1% bovine serum albumin and 20 mmol/L HEPES (pH 7.4, 37°C). Expression of GFP relative to the total neuronal population and functional neuronal subtypes was determined by co-labeling with neurochemical markers.[6,7] Counts were made from captured images (≥20 neurons analyzed per preparation, n ≥ 3 mice per marker and region). Neuron diameter was measured along the longest axis using Zeiss LSM Image. The subcellular distribution of DOReGFP was analyzed using ImageJ (National Institutes of Health, Bethesda, MD).[3]

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Supplementary Table 1. Sources and Dilutions of Primary and Secondary Antibodies

Antigen	Species	Conditions	Source
GFP	Chicken	IF, 1:500	Abcam, Inc, Cambridge, MA
GFP	Rabbit	IF, 1:500; WB, 1:1000	Invitrogen, Eugene, OR
ChAT	Goat	IF, 1:200	Millipore, Temecula, CA
CGRP	Goat	IF, 1:200	Abcam, Inc, Cambridge, MA
EEA1	Rabbit	IF, 1:200	Abcam, Inc, Cambridge, MA
Leu-Enkephalin	Rabbit	IF, 1:200	Abcam, Inc, Cambridge, MA
Met-Enkephalin	Rabbit	IF, 1:200	Abcam, Inc, Cambridge, MA
GFAP	Goat	IF, 1:400	NOVUS Biologicals, Littleton, CO
LAMP-1	Rat	IF, 1:400	Developmental Studies Hybridoma Bank, clone 1D4B
MOR	Rabbit	IF, 1:250	C. J. Evans, UCLA
NFM	Chicken	IF, 1:500	Gene Tex, Inc, Irvine, CA
NK_1R	Rabbit	IF, 1:500	CURE, UCLA, #94168
NOS	Goat	IF, 1:500	NOVUS Biologicals, Littleton, CO
NPY	Rabbit	IF, 1:400	CURE, UCLA #8711
PGP9.5	Chicken	IF, 1:500	Abcam, Inc, Cambridge, MA
Somatostatin	Sheep	IF, 1:500	Gift from G. W. Aponte, UC Berkeley, #8903
Somatostatin	Rabbit	IF, 1:500	CURE, UCLA, #8401
SSTR2A	Rabbit	IF, 1:2000	CURE, UCLA, #9431
SP	Guinea pig	IF, 1:200	Neuromics, Edina, MN
VIP	Sheep	IF, 1:200	Abcam, Inc, Cambridge, MA
Chicken, rabbit, goat, sheep, rat IgG conjugated to fluorescein isothiocyanate, rhodamine red X, Cy5	Donkey	IF, 1:200	Jackson Immunoresearch, West Grove, PA

CGRP, calcitonin gene-related peptide; EEA1, early endosomal antigen 1; GFAP, glial fibrillary acidic protein; IF, immunofluorescence; NK_1R, neurokinin 1 receptor; NFM, neurofilament M; NPY, neuropeptide Y; VIP, vasoactive intestinal polypeptide; WB, Western blot.

Supplementary Table 2. Quantification of Neurons in Submucosal Plexus

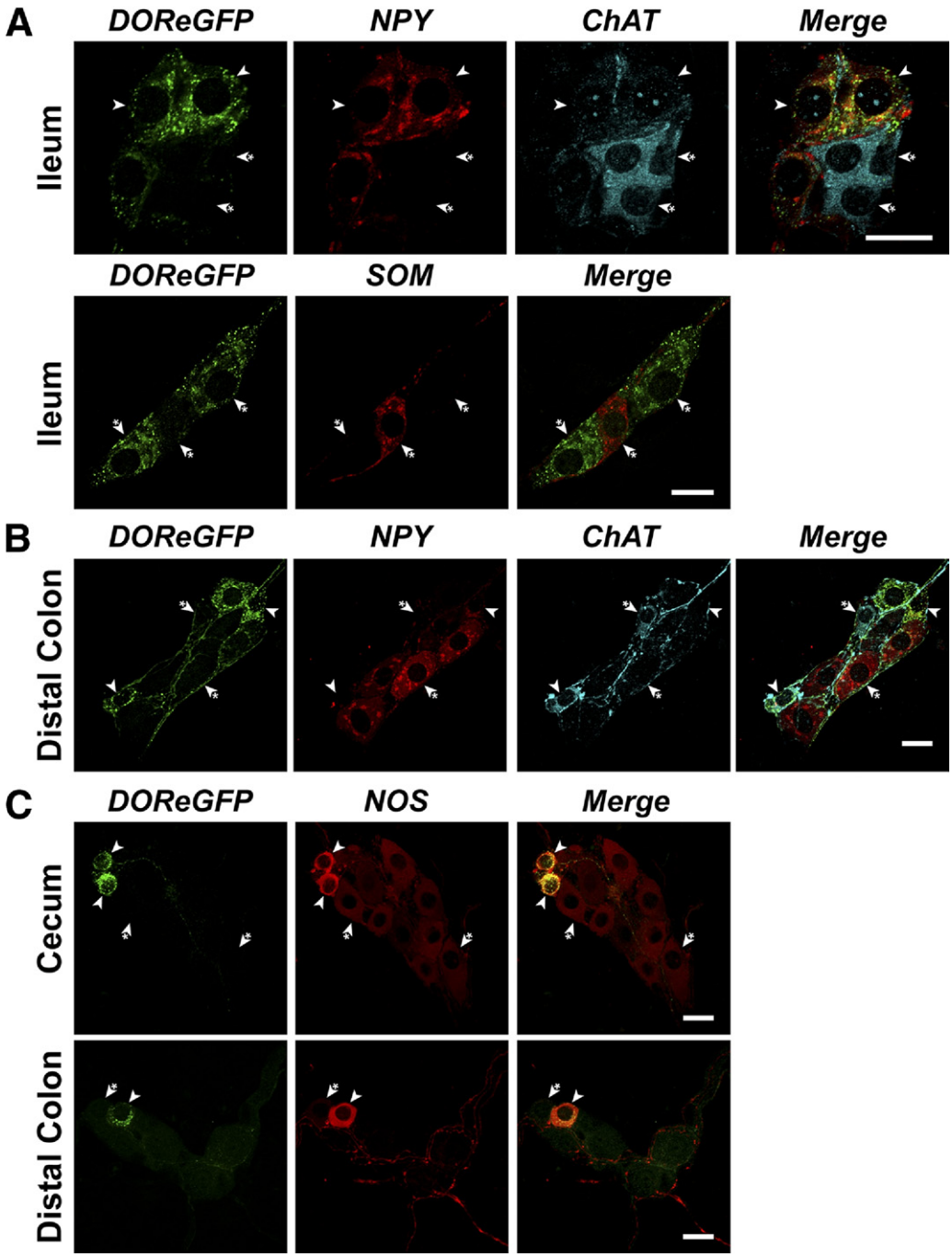
Region	NPY	ChAT	SOM	NOS
Ileum	62/69[a] (90%)	13/171 (8%)	10/92[a] (11%)	NE
	62/62[b] (100%)	13/129 (10%)	9/66[b] (14%)	
Cecum	NE	NE	NE	57/59[a] (96%)
				55/250[b] (22%)
Proximal colon	NE	NE	NE	9/27[a] (33%)
				2/11[b] (18%)
Distal colon	17/44[a] (39%)	6/47[a] (13%)	NE	11/14[a] (79%)
	17/120[b] (12%)	6/40[b] (15%)		11/17[b] (65%)

NOTE. For all regions, n = 3–5 mice. Note that the small numbers of neurons studied in the large intestine reflects the infrequent expression of DOR in submucosal neurons in this tissue.

NE, not examined; NPY, neuropeptide Y.

[a]Number of marker-positive neurons in the DOReGFP-positive population (ie, in ileum, of 69 cells expressing DOReGFP, 62 expressed neuropeptide Y).

[b]Number of DOReGFP-positive cells in the marker-positive population (ie, in ileum, of 62 cells expressing neuropeptide Y, 62 also expressed DOReGFP).



Supplementary Figure 1. Localization of DOR to distinct classes in submucosal neurons of the small and large intestine. (*A*) DOReGFP-IR was detected in neuropeptide Y (NPY)-IR (*arrowheads*) but not ChAT-IR (*arrowheads with asterisk*) neurons in ileum (*top panels*). There was no colocalization of DOReGFP-IR and SOM-IR in ileum (*bottom panels*). (*B* and *C*) DOReGFP-IR rarely was detected in submucosal neurons in the large intestine. In the large bowel DOReGFP-IR was not observed in NPY-IR or ChAT-IR neurons (B), but was co-expressed with NOS-IR in an uncharacterized subclass of submucosal neurons. *Scale bar*, 20 μm.

Supplementary Table 3. Quantification of Neurons in Myenteric Plexus

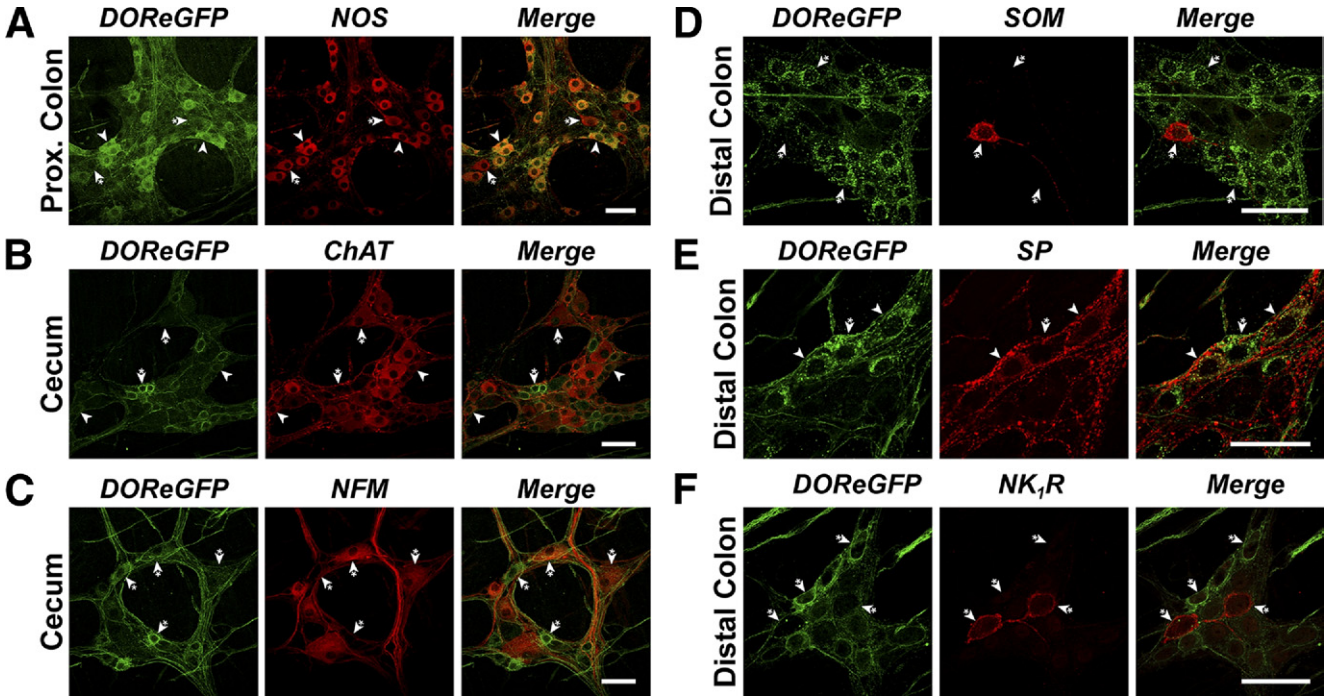
Region	PGP9.5	NOS	NFM	ChAT	SST-R2A	SOM	SP	NK_1R	MOR	Enkephalin
Antrum	130/130[a] (100%)	252/376[a] (67%)	NE	69/180[a] (38%)	NE	NE	NE	NE	NE	NE
	130/208[b] (62.5%)	124/376[b] (33%)	NE	111/180[b] (62%)	NE	NE	NE	NE	NE	NE
Duodenum	173/173[a] (100%)	110/274[a] (40%)	3/86[a] (3%)	53/130[a] (41%)	NE	NE	NE	NE	NE	NE
	173/332[b] (52%)	105/164[b] (64%)	3/30[b] (10%)	52/164[b] (32%)	NE	NE	NE	NE	NE	NE
Ileum	182/182[a] (100%)	165/357[a] (46%)	0/332[a] (0%)	95/213[a] (45%)	NE	12/388[a] (4%)	87/263 (33%)	NE	98/115[a] (85%)	1/137[a] (1%)
	182/390[b] (47%)	172/217[b] (79%)	0/102[b] (0%)	95/308[b] (31%)	NE	12/50[b] (24%)	88/111 (88%)	NE	100/176[b] (57%)	1/35[b] (3%)
Cecum	97/97[a] (100%)	130/202[a] (64%)	3/139[a] (2%)	19/64[a] (30%)	0/100[a] (0%)	NE	NE	NE	NE	NE
	97/186[b] (52%)	130/226[b] (58%)	3/79[b] (4%)	19/97[b] (20%)	0/40[b] (0%)	NE	NE	NE	NE	NE
Proximal colon	186/186[a] (100%)	465/507[a] (92%)	0/300[a] (0%)	20/231[a] (9%)	NE	NE	NE	NE	NE	NE
	186/481[b] (39%)	466/590[b] (79%)	0/115[b] (0%)	20/363[b] (6%)	NE	NE	NE	NE	NE	NE
Distal colon	270/270[a] (100%)	484/738[a] (66%)	2/304[a] (1%)	58/292[a] (20%)	0/150[a] (0%)	11/297[a] (4%)	45/181[a] (25%)	2/94[a] (2%)	119/154[a] (77%)	NE
	270/554[b] (49%)	489/668[b] (73%)	2/113[b] (2%)	58/421[b] (14%)	0/30[b] (0%)	11/62[b] (18%)	45/77[b] (58%)	[B]1/92 (1%)	119/196[b] (61%)	NE

NOTE. For all regions, n = 3–8 mice.

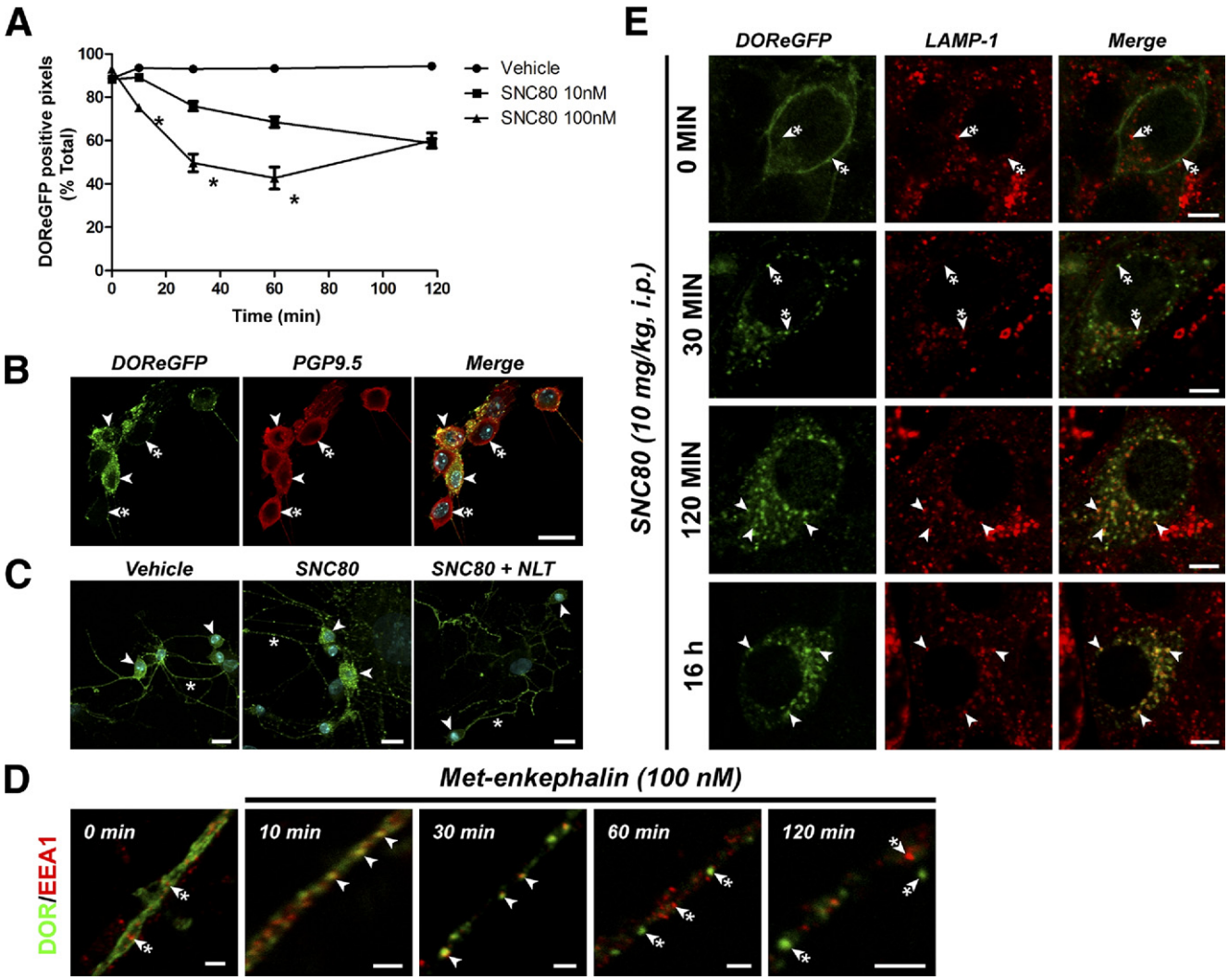
NE, not examined; NFM, neurofilament M; NK_1, neurokinin 1.

[a]Number of marker-positive neurons in the DOReGFP-positive population (ie, in antrum, of 130 cells expressing DOReGFP, 130 expressed PGP9.5).

[b]Number of DOReGFP-positive cells in the marker-positive population (ie, in antrum, of 208 cells expressing PGP9.5, 130 also expressed DOReGFP).



Supplementary Figure 2. Localization of DOReGFP with neurochemical markers, neuropeptides, and their receptors in the myenteric plexus of the large intestine. (*A*) DOReGFP-IR was colocalized extensively with NOS-IR in proximal colon (*arrowheads*). (*B*) DOReGFP-IR colocalized with ChAT-IR in small neurons of the cecum (*arrowheads*). DOReGFP-IR was absent from large ChAT-IR neurons (*arrowheads with asterisks*). (*C*) DOReGFP-IR was not colocalized in cecum with neurofilament M (NFM)-IR. (*D*) DOReGFP-IR and SOM-IR did not colocalize in distal colon. (*E*) DOReGFP-IR and SP-IR frequently were co-expressed in myenteric neurons of the distal colon (*arrowheads*). (*F*) DOReGFP-IR and neurokinin 1 receptor (NK_1R)-IR did not colocalize in the distal colon. *Scale bars*, 50 μm.



Supplementary Figure 3. Agonist-induced trafficking of DOR in myenteric neurons. (*A*) Quantitative analysis of DOReGFP-IR at the plasma membrane of myenteric neurons in organotypic preparations treated with vehicle or SNC80 (10 and 100 nmol/L) for 0–120 minutes. $^*P < .05$ compared to vehicle. n = 18–40 neurons from 3 mice per data point. (*B*) DOReGFP-IR was detected in approximately 50% of PGP9.5-IR myenteric neurons in culture. Neurons positive for both DOReGFP-IR and PGP9.5-IR are indicated by an *arrowhead* and neurons positive for PGP9.5-IR only are indicated by an *arrowhead with asterisk*. *Scale bars*, 20 μm. (*C*) In vehicle-treated cultured neurons, DOReGFP-IR was detected at the plasma membrane (*arrowheads*, *left panel*). SNC80 (100 nmol/L, 30 min, *middle panel*) induced DOReGFP-IR trafficking to endosomes in the soma and neurites (*asterisks*). Naltrindole (NLT, *right panel*) abolished DOReGFP-IR endocytosis. *Scale bars*, 20 μm. (*D*) In the neurites of cultured neurons, Met-enkephalin stimulated trafficking of DOReGFP-IR (*green*) to early endosomal antigen 1 (EEA1)-positive endosomes (*red*) after 10 and 30 minutes (*arrowheads*), but after 60 and 120 minutes DOReGFP-IR was in distinct vesicles (*arrowhead with asterisk*). *Scale bar*, 2 μm. (*E*) SNC80-induced trafficking of DOReGFP-IR to lysosomes of myenteric neurons in the intact animal. In unstimulated neurons, DOReGFP-IR was at the plasma membrane. At 30 minutes after IP injection of SNC80 (10 mg/kg), DOReGFP was in endosomes that did not colocalize with LAMP1 (*arrowhead with asterisk*). At 120 minutes and 16 hours after SNC80, DOReGFP-IR was detected in LAMP1-positive lysosomes. *Scale bars*, 5 μm.