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The opioid system in the gastrointestinal tract

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Abstract *μ-, δ- and κ-opioid receptors (ORs) mediate the effects of endogenous opioids and opiate drugs. Here we report (1) the distribution of μOR in the guinea-pig and human gastrointestinal tract in relation to endogenous ligands, to functionally distinct structures in the gut and to δOR and κOR; and (2) the ligand-induced μOR endocytosis in enteric neurones using* in vitro *and* in vivo *models. In the guinea pig, μOR immunoreactivity is confined mainly to the myenteric plexus. μOR myenteric neurones are most numerous in the small intestine, followed by the stomach and the proximal colon. μOR immunoreactive fibres are dense in the muscle layer and the deep muscular plexus, where they are in close association with interstitial cells of Cajal. This distribution closely matches the pattern of enkephalin. μOR enteric neurones comprise functionally distinct populations of neurones of the ascending and descending pathways of the peristaltic reflex. In human gut, μOR immunoreactivity is localized to myenteric and submucosal neurones and to immune cells of the lamina propria. δOR immunoreactivity is located in both plexuses where it is predominantly in varicose fibres in the plexuses, muscle and mucosa, whereas κOR immunoreactivity appears to be confined to the myenteric plexus and to bundles of fibres in the muscle. μOR undergoes endocytosis in a concentration-dependent manner,* in vitro *and* in vivo. *Pronounced μOR endocytosis is observed in neurones from animals that underwent abdominal surgery that has been shown to induce delay in gastrointestinal transit. We can conclude that all three ORs are localized to the enteric nervous system with differences among species, and that μOR endocytosis can be utilized as a means to visualize enteric neurones activated by opioids and sites of opioid release.*

Keywords *ascending excitatory neurones, descending inhibitory neurones, G-protein-coupled receptors, interneurones, receptor endocytosis.*

OVERVIEW OF OPIOID RECEPTOR LOCALIZATION AND FUNCTION IN THE GUT

Endogenous opioid peptides and opiate drugs modulate a variety of biological processes, including stress response, immunity, analgesia, motor activity and autonomic functions.[1–3] Endogenous opioids are generated from three different genes, the pro-opiomelanocortin, proenkephalin and prodynorphin genes, which give rise to several biologically active peptides, including *β*-endorphin, enkephalin and dynorphin.[4] These genes and their products have a broad distribution in the central nervous system and periphery, including the gastrointestinal tract.[1,4–7] In recent years, two new opioid tetrapeptides, named endomorphin 1 and endomorphin 2, have been isolated from the brain.[8] Endomorphins have potent biological effects that mimic those of other opioids, including analgesia and inhibition of electrically induced contraction of the guinea-pig ileum[8–10] and have been localized in the central nervous system and sensory ganglia.[11,12] However, the genes producing these peptides have not been identified. Opiate drugs or alkaloids, including morphine and fentanyl – the most potent analgesics used widely in humans for pain control[7,13] – are structurally distinct, nonpeptide ligands that activate the same receptors as native opioids. They have profound side-effects, including respiratory depression, inhibition of gastrointestinal transit and secretion, tolerance and addiction that severely hamper their clinical application.[14,15] It

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should be noted that a molecule corresponding to the opiate morphine has also been localized in the brain and periphery of animals.[16] This endogenous morphine might be released following surgical procedures.[17,18]

Opioid receptors

The diverse effects of opioids and opiates are mediated by the activation of multiple membrane receptors, the opioid receptors (ORs), which belong to the superfamily of seven transmembrane G-protein-coupled receptors.[3,15,19] These include three major classes of receptors, δOR, κOR and μOR, which partly overlap in their distribution and function, but maintain a certain degree of selectivity for the three families of endogenous opioids and differ in their pharmacological profiles.[7,20] For instance, enkephalins are the preferred ligands for δOR, but they also have remarkable affinity for μOR. Dynorphins display some selectivity for κOR, whereas endorphins bind to μOR and δOR with similar affinity, having only low affinity for κOR sites.[21,22] However, endogenous opioids bind all OR subtypes with moderate selectivity. By contrast, endomorphin 1 and 2 have the highest affinity and selectivity for μOR than any other opioid peptides described to date. Endomorphins have a preference for μOR that is several thousand-fold that of δOR and κOR.[8] Similarly, opiates used clinically preferentially activate the μOR[15,20] even though their ligand selectivity profiles are impaired when they accumulate at high concentrations, in which conditions they can interact with other ORs.[22]

Localization of endogenous opioids

In the gastrointestinal tract, endogenous opioid peptides are localized to the neural networks and to endocrine cells.[1,7,23–25] Specifically, in the enteric nervous system, the derivatives of proenkephalins are confined mainly to myenteric neurones projecting to the circular muscle and submucosal plexus,[26–28] whereas prodynorphin-derived peptides are localized to submucosal and myenteric neurones and to fibres originating from the celiac ganglion.[29,30] In the enteric nervous system, enkephalins and dynorphins colocalize with either classical transmitters such as acetylcholine or peptides like substance P, vasoactive intestinal polypeptide, gastrin-releasing peptides, galanin and nitric oxide (NO) – the major transmitter for descending neuronal pathways.[27,30–32] By contrast, endorphin peptides appear to be confined to endocrine cells, including enterochromaffin cells of the intestine, gastrin cells of the antrum and endocrine pancreatic cells;[23,33–35] however, clear evidence for their localization to the neural networks of the gastrointestinal tract is lacking. Furthermore, there is direct and indirect evidence for opioid release from gut tissue *in vitro* and in intact animals, supporting a physiological and pathophysiological role of opioids in regulating gut functions.[36–39] Finally, opioid release appears to occur in response to the stress of surgery, and the opioid system has been implicated in postoperative ileus and opioid bowel dysfunction, which develop in patients who undergo surgery and in those receiving long-term opiate treatment for chronic pain, respectively.[40,41] To date, endomorphins have not been localized in the gastrointestinal tract.

Gastrointestinal functions of opioids

Opioids and opiates affect a variety of gastrointestinal functions, including motility, secretion and transport of electrolytes and fluids by the activation of the three major classes of ORs, δOR, κOR and μOR.[2,3,15,19,25,42] Opioid effects are complex because they are mediated by different classes of cell-surface receptors and they depend upon different sites of action, including the brain, spinal cord, peripheral nervous system and the gastrointestinal region, and also species.[25,43] In the gastrointestinal tract, OR binding sites corresponding to δOR, κOR and μOR have been associated with different structures.[2,43–45] Furthermore, morphological studies using specific antibodies for the cloned OR have provided direct evidence for their presence in the gastrointestinal tract, where they appear to be predominantly localized to neuronal structures.[46–51] μOR and κOR immunoreactivities have been described in enteric neurones of rat, guinea-pig and porcine gastrointestinal tract, whereas δOR immunoreactivity has been reported in the porcine ileum. In the rat, specifically, both μOR and κOR are localized to myenteric and submucosal neurones (which are quite abundant in the stomach and proximal colon), to fibres distributed in the muscle layer, mucosa, blood vessels and lymphatic nodes, and to putative interstitial cells of Cajal (ICC) in the myenteric plexus and deep muscular plexus.[48] By contrast, in the guinea-pig distal ileum, μOR immunoreactivity is localized predominantly to myenteric neurones and dense neuropil in the muscle layer and deep muscular plexus.[46,47,51] In porcine ileum, δORs are localized to neurones of both the submucous and myenteric plexus and to fibres in the muscle and mucosa, whereas κORs are found only in the myenteric plexus.[50] ORs are functionally coupled to different

effector pathways, including those responsible for inhibition of cyclic AMP formation, decreased conductance of voltage-gated Ca^{2+} channels, increases in K^+ current and activation of mitogen-activated protein.[52,53] Activation of these effector systems results in inhibition of neuronal activity and decrease in neurotransmitter release.

Cellular expression of opiate receptors

The elucidation of the exact cellular sites of expression of ORs in relation to their ligands and the functionally distinct types of enteric neurones, as well as the trafficking of the receptors in response to stimulation, are important steps in the process of providing a better understanding of how the opioid system influences gastrointestinal functions in normal conditions and, perhaps, in disease states. μORs are particularly important from a clinical point of view, given their predominant role in the development of tolerance and addiction, and their implication in postoperative ileus and opioid bowel dysfunction – conditions that are characterized by pronounced delay in gastrointestinal transit often resulting in severe constipation and abdominal discomfort.[40,41]

The opioid system in relationship to enteric neural pathways

In the present study, we investigated (1) the regional distribution of μOR immunoreactivity in the guinea-pig gastrointestinal tract and in specimens of the human bowel, (2) the relationship of μOR immunoreactive neurones and fibres with endogenous opioids, and (3) μOR internalization in response to opioids. We also examined the localization of δOR and κOR in the guinea-pig ileum.

MATERIALS AND METHODS

Specimens from the gastrointestinal tract of guinea pigs (stomach, duodenum, ileum and proximal colon) and humans (jejunum, and proximal and distal colon) were used in this study. Care and handling of the animals were in accordance with the National Institutes of Health recommendations for the humane use of animals and were approved by the appropriate Animal Research Committee of the University of California (UCLA) and Veterans Administration Greater Los Angeles Healthcare System where the experiments were performed. Appropriate ethical procedures were followed for the collection of human specimens.

Guinea-pig tissue

Male albino, Porcellus guinea pigs (250–350 g; Simonsen Laboratories, San Diego, CA, USA) were euthanized with an overdose of sodium pentobarbital (100 mg kg^{-1} intraperitoneally). Segments of the stomach (corpus and antrum), proximal duodenum, distal ileum and proximal colon were collected and either prepared for whole mounts or cryostat sections.[51,54] For whole mount preparations, specimens of the gastrointestinal tract were removed, washed thoroughly in ice-cold saline and opened along the longitudinal axis. They were then pinned flat on wax, immersed in 4% paraformaldehyde in 0.1 mol L^{-1} phosphate buffer (PB) pH 7.4 for 2 h, and stored in 0.1 mol L^{-1} PB with 0.1% sodium azide. For cryostat sections, tissues were either collected fresh or following perfusion with 50 mL PB, followed by 500 mL of 4% paraformaldehyde in PB; perfused tissue was then postfixed for 1 h at room temperature, then transferred to 25% sucrose in saline-PB (phosphate buffered saline: PBS) at 4°C. Tissue, that was collected fresh, was pinned flat as for whole-mount preparation, fixed for 2 h in 4% paraformaldehyde in PB at room temperature, then transferred to 25% sucrose/PB. Tissue sections were cut to 12 μm thickness using a cryostat perpendicularly to the lumen and collected onto gelatin-coated slides.

A commercially available rabbit polyclonal antiserum (Incstar Science, Technology and Research, Stillwater, MN, USA), directed to the C-terminal fragment of rat μOR (384–398) was used in this study. This antiserum has been characterized previously with transfected cells and guinea-pig tissue.[10] Immunohistochemistry using the immunofluorescence method was performed as described previously.[51,55] Briefly, tissues were incubated in PB containing 0.5% Triton X-100 (three 30-min periods), followed by 5% normal donkey serum in 0.5% Triton X-100/PB for 60 min, and then μOR antibody (1: 3000) for 48 h at 4°C, washed and incubated with affinity-purified donkey antirabbit immunoglobulin G (IgG) conjugated to fluorescein isothiocyanate (FITC; Jackson Immunoresearch Laboratories, West Grove, PA, USA; 1: 100) or Alexa-FITC (Jackson Immunoresearch Laboratories, USA; 1: 1000) for 2 h at room temperature. δOR and κOR immunoreactivities were detected with a rabbit polyclonal antibody raised to the amino terminus sequence of mouse δOR (Neuromics, Northfield, MN, USA; 1: 500) and a goat polyclonal antiserum raised against the amino terminus region of the human κOR, κOR-1, N-19 (sc-7494, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; 1: 50–100). Tissues were mounted on gelatin-coated slides with glycerol–phosphate buffer containing 2% potassium iodide to retard fading of the immunoreactivity.

Immunoblocking with an excess of the peptide to which each antiserum was raised was performed for δOR and κOR immunostaining specificity.

For double-labelling immunofluorescence, the rabbit μOR antibody was used with mouse monoclonal antibody to enkephalin (MASO83; Harlan Sera Laboratory, Indianapolis, IN, USA).[51] Incubation with the primary antibodies was followed by secondary antibodies with distinct fluorophores. Specifically, donkey antirabbit IgG-FITC or Alexa-FITC was used in combination with donkey antimouse IgG conjugated with rhodamine Red X (Red X; Jackson Immunoresearch Laboratories; 1:300). For enkephalin localization, we used colchicine *in vitro* with organotypic cultures of the ileum to increase the levels of opioid peptides in cell bodies. For this procedure, the distal ileum was removed, opened along the longitudinal axis and washed three times (10-min periods) in Krebs solution (in mM: 5.9 KCl, 118 NaCl, 2.5 $CaCl_2.2H_2O$, 1.2 $MgSO_4.7H_2O$, 1.4 NaH_2PO_4, 22.7 $NaHCO_3$, 1 g L^{-1} D-glucose, pH 7.4) containing 100 μg mL^{-1} streptomycin, 100 IU mL^{-1} penicillin, and 2.5 μg mL^{-1} fungizone.[55] Following removal of the mucosa, tissue was incubated in Dulbecco's modified medium nutrient mixture F-12 HAM containing 10% fetal bovine serum (FBS), 100 μg mL^{-1} streptomycin, 100 IU mL^{-1} penicillin, and 2.5 μg mL^{-1} fungizone, bubbled with 95% O_2 and 5% CO_2 for 30 min at 37°C. The ileum was pinned flat in Krebs' solution containing 100 μM nicardipine to relax the muscle. Ileum specimens were incubated in Dulbecco's modified medium nutrient mixture F-12 HAM containing 10% FBS, 100 μM colchicine for 4–12 h. Organotypic cultures were fixed in 4% paraformaldehyde for 2 h and stored in PB containing 0.1% sodium azide until processing for immunohistochemistry.[55]

Tissue was analysed with a Zeiss Axioplan 2 research microscope for fluorescence with an axiocam colour digital camera system and with either a Zeiss 410 or 510 laser scanning confocal microscope equipped with a krypton/argon laser and attached to a Zeiss Axiovert 100 microscope with an 100× Plan Apo 1.4 na objective (Carl Zeiss Inc., Thornwood, NY, USA). Images of 512 × 512 pixels were collected at a magnification zoom of 1.5×. Typically, 10–20 optical sections were taken at *z*-axis 0.5–0.75 μm intervals through the cells. Images were processed and labelled using Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA, USA), as described.[51,56]

Human tissues

Paraffin-embedded, formalin-fixed tissue blocks of human jejunum ($n = 3$) and colon ($n = 3$) of proximal and distal resection margins (normal bowel from patients undergoing surgery for a variety of conditions) were obtained from Peterborough Hospital Human Research Tissue Bank following appropriate ethical procedures. The mean age of the subjects was 50 years (age range 29–61 years). The tissue was sectioned (3 μm) onto microscope slides (VWR, Lutterworth, Leicestershire, UK). Following de-waxing, heat induced epitope retrieval in citric acid buffer (HD Supplies, Aylesbury, Bucks, UK) was carried out. Endogenous peroxidase activity was inactivated by incubating the slides for 20 min in 3% (v/v) H_2O_2 in distilled water, followed by washes in 0.1 mol L^{-1} PBS. Immunohistochemistry was performed using the same antibody used for guinea-pig tissue (Incstar, USA) in 0.1 mol L^{-1} PBS/0.3% (v/v) Triton X-100 for a minimum of 48 h at a dilution of 1: 1600. Sites of antibody attachment were detected using the LSAB2 kit (Dako Cytomation, Ely, Cambridgeshire, UK) following the manufacturer's instructions. Colour development was carried out using a liquid DAB detection system (Dako Cytomation, UK), followed by counter-staining in Mayer's haematoxylin (VWR, UK). The slides were dehydrated, cleared and mounted following standard protocols. Controls in which the primary antibodies were omitted were included in each study.

Stained sections were photographed and analysed using AnalySIS software connected to an Olympus BX41 microscope with an Olympus U-TV1X digital camera.

Ligand-induced μ-opioid receptor endocytosis

We have shown that μOR undergoes ligand-selective and rapid endocytosis in enteric neurones in response to exogenously administered opioids and opiates such as etorphine and fentanyl, but not morphine *in vivo* and *in vitro* as well as to endogenously released opioids *in vitro*.[47,55,56] For the exogenous application of opioids, we have used organotypic cultures of the distal ileum of the guinea pig.[55,56] The distal ileum was dissected and placed in sterile Krebs' solution containing 2.5 μg mL^{-1} fungizone, 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin), bubbled with 95% O_2, 5% CO_2, pH 7.4, at 37°C, washed and then incubated in culture medium, Dulbecco's Modified Eagle's Medium (DMEM), containing 10% v/v FBS, penicillin, streptomycin and fungizone at 37°C with 95% O_2, 5% CO_2 for 30 min, as described. The ileum was incubated in oxygenated DMEM with FBS, penicillin/streptomycin and fungizone at 37 °C, exposed to the opioids Met-enkephalin or [D-Ala^2, N-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO), or the opiates, etorphine, fentanyl or

morphine (1 nmol L^{-1}–10 μmol L^{-1}) in the absence or presence of opioid antagonists, naloxone or CTOP (1 μmol L^{-1}) for 1 h at 4°C for equilibrium binding of the agonist to the receptor, washed and then incubated in agonist-free medium at 37°C for 0–30 min for internalization.

To investigate μOR endocytosis in response to endogenously released opioids *in vivo*, we used a model of abdominal surgery that has been shown to delay gastrointestinal transit. The occurrence of opioid release in response to the stress of surgery and the finding that selective inhibition of gastrointestinal ORs ameliorates postoperative bowel dysfunction[40,41,57] led us to hypothesize that abdominal surgery resulting in impairment of gastrointestinal transit might induce μOR internalization in enteric neurones.[58] Laparotomy (a midline incision of the abdominal skin, muscle layers and peritoneum, which resulted in partial eventration of the small intestine), followed by intestinal evisceration and manipulation consisting of gentle touching of the caecum or the small intestine for 2–5 min, or simply a midline incision of abdominal skin were performed under isofluorane anaesthesia. Animals were allowed to recover for 30 min and then euthanized with an overdose of sodium pentobarbital (100 mg kg^{-1} intraperitoneally). Animals that received anaesthesia alone or animals that received an intraperitoneal injection of OR antagonists, naloxone (1 mg kg^{-1}; UCLA Pharmacy, Los Angeles, CA, USA) or the selective μOR antagonist, CTOP (1 mg kg^{-1}, Bachem, Torrance, CA, USA) 3 min prior to laparotomy served as control.

μOR internalization was quantified by determining the proportion of μOR fluorescence in the cytoplasm in immunoreactive neurones chosen at random. For this analysis, we used single confocal images that included the nucleus and a large area of cytoplasm, which were analysed using NIH 'image' software.[56,58]

RESULTS

Distribution of μ-opioid receptor immunoreactivity in guinea-pig and human gut

In the guinea pig, μOR immunoreactivity was localized primarily to enteric neurones of the myenteric plexus of the stomach, duodenum, distal ileum and proximal colon, with different densities. μOR immunoreactive neurones were most abundant in the ileum followed by the duodenum and stomach, with no detectable differences between the gastric corpus and the antrum; they were the least abundant in the proximal colon (Fig. 1). In each region examined, μOR immunoreactive neurones had Dogiel type I morphology (Fig. 2), with an ovoid cell body, several thick dendrites protruding from the cell body and a long axonal process. μOR immunoreactivity was predominantly localized at the cell-surface membrane (Fig. 2). μOR immunoreactive fibres were abundant within the myenteric plexus (Fig. 1), in interconnecting strands and in the external muscle layer of each region examined, even though they were more abundant in the small intestine (Fig. 3) than in the stomach and proximal colon (not shown). μOR immunoreactivity was rarely detected in the submucosal plexus of the small intestine and the staining was typically so weak as to make it extremely difficult, if not impossible, to determine with certainty whether it was in cell bodies or fibres, the latter possibility appearing more likely (Fig. 3). μOR immunoreactivity was not detected in the mucosa. Immunostaining was observed in mononuclear cells in the lamina propria of the intestine. However, this staining was also detected in sections when the primary antibody was omitted, indicating non-specificity (not shown). In the small intestine, there was a particularly dense area of μOR immunoreactive staining in the deep muscular plexus, which appeared to be associated with presumed ICCs (Fig. 3). Confocal microscope analysis and double labelling with c-Kit, a marker for ICCs, showed a dense neuropil of μOR immunoreactive fibres wrapped around ICCs (not shown),[51] but μOR immunoreactivity was not localized to the ICCs themselves.

Enkephalin fibres were often in close proximity with μOR neurones and fibres and sometimes they appeared to wrap around μOR enteric neurones (Fig. 4). In addition, a discrete proportion of μOR immunoreactive neurones (~30%) were immunoreactive for the endogenous opioid enkephalin, which has high affinity for, and activates, μOR.[51] We have shown previously that μOR immunoreactivity is localized to a large proportion of cholinergic myenteric neurones (~50%), as well as to numerous neurones immunoreactive for nitric oxide synthase or vasoactive intestinal polypeptide (not shown), markers for descending neurones.[51]

In human gut, μOR immunoreactivity was present in specific regions of the small and large intestine in a consistent and discrete distribution. μOR immunoreactivity was not detected when the primary antibody was omitted. The distribution and density of μOR immunoreactivity were comparable in the human jejunum and colon, as well as between the specimens from different subjects. μOR immunoreactivity was localized to neuronal cell bodies in both submucosal and myenteric ganglia (Fig. 5) and to nerve fibres in the myenteric plexus. μOR immunoreactive fibres were not detected in the mucosa. μOR immunoreactivity was observed in

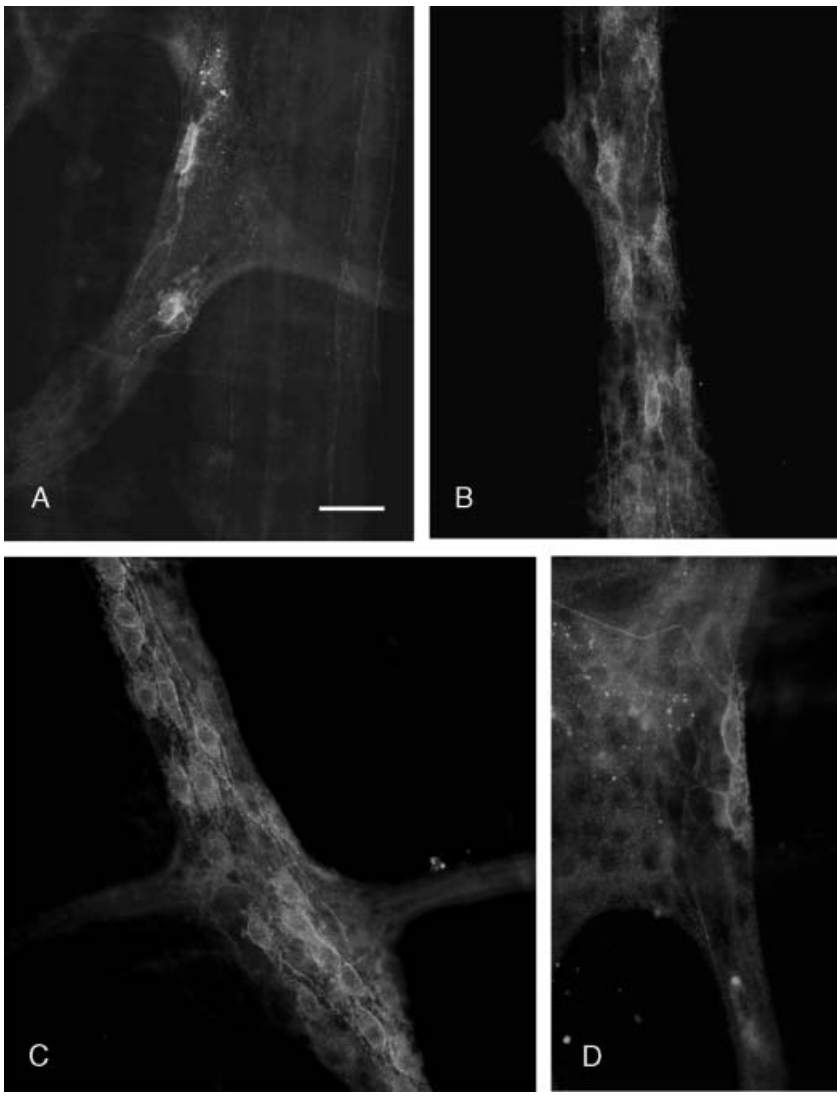

Figure 1 (A) μOR immunoreactivity in myenteric neurones of the guinea-pig stomach, (B) duodenum, (C) distal ileum and (D) proximal colon. Note the abundance of μOR immunoreactive neurones in the ileum. Light microscope image. Calibration bar: 100 μm.

some mononuclear cells in the lamina propria and in neutrophils in the microcirculation (Fig. 5).

Ligand-induced μ-opioid receptor endocytosis

In enteric neurones exposed to enkephalin, the μOR-selective enkephalin analogue, DAMGO or the opiates fentanyl or etorphine, μOR immunoreactivity was concentrated in endosomes, whereas μOR immunoreactivity was confined at the cell surface in unstimulated neurones (Fig. 6), as well as in neurones exposed to morphine (not shown).[56]

μOR internalization was pronounced in most neurones from animals that underwent either laparotomy or laparotomy with intestinal manipulation at 30 min, whereas it was confined mainly at the cell surface of neurones from control animals that received anaesthesia alone (Fig. 7).[58] μOR endocytosis was prevented by treatment with an opioid antagonist prior to abdominal surgery (Fig. 7), indicating specificity of ligand-induced μOR internalization. By contrast, a simple incision of the abdominal skin did not result in significant levels of receptor internalization (not shown).[58]

δ-opioid receptor and κ-opioid receptor immunoreactivities in the distal ileum of the guinea pig

Both δOR and κOR immunoreactivities were also detected in the guinea-pig ileum, where they were localized to enteric neurones and fibres. δOR immunoreactivity was localized to neurones of the myenteric and submucosal plexuses, but was particularly prominent in varicose fibres that closely surrounded un-

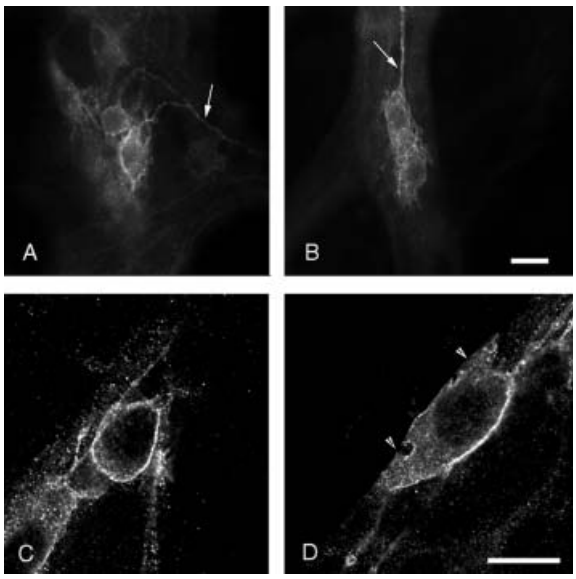

Figure 2 (A, B) The Dogiel type I morphology of μOR immunoreactive neurones in the guinea-pig: (A) duodenum, (B) ileum. (C, D) The predominant localization of μOR immunoreactivity at the cell surface. Arrows point to the axonal process and arrowheads to dendrites. (A) and (B) light microscope images; (C) and (D) confocal images. Calibration bars: 25 μm.

stained cell bodies in both plexuses (Fig. 8). δOR immunoreactive beaded fibres were also observed in the mucosa surrounding the intestinal glands. δOR immunostaining was abolished by preadsorption with the peptide used to raise the antibody (Fig. 9). On the other hand, κOR immunoreactivity appeared to be confined to the myenteric plexus, where it was localized to neurones, and to fibres distributed to the muscle layer including the deep muscular plexus (Fig. 10). κOR immunostaining was also abolished by preadsorption of tissue sections with the respective antigen.

DISCUSSION

μOR immunoreactivity is localized predominantly to enteric neurones in the guinea-pig and human gut with regional differences in density and distribution. In the guinea pig, μOR is confined to Dogiel type I myenteric neurones that are more abundant in the small intestine, particularly in the distal ileum; they are less numerous in the stomach and the least abundant in the colon. μOR immunoreactivity is also prominent in fibres distributed throughout the muscle layer, with a particularly high density in the deep muscular plexus, where these fibres form a dense neuropil closely surrounding ICCs. By contrast, in human small and large intestine μOR immunoreactivity is localized to both myenteric and submucosal neurones as well, as to mononuclear cells in the lamina propria and neutrophils. The distribution of μOR immunoreactivity in the gut of the guinea pig and humans also differs in some aspects from the distribution in the rat, where μOR was localized to both myenteric and submucosal neurones, but was more prominent in the submucosal plexus, and to ICCs, with the highest density in the stomach and colon. Furthermore, there is no evidence for the presence of μOR immunoreactivity in the porcine ileum.[50] Together, these findings indicate the existence of species differences in the expression of μOR in the mammalian gut, even though the possibility that differences in the methodology used and the antibodies, which could detect different forms of μOR, cannot be ruled out. Indeed, two forms of μOR – μOR-1 and μOR-2 – and several isoforms of μOR-1 have been reported, and can be distinguished on the basis of their pharmacological properties and anatomical distribution.[13,59]

Differences also appear to exist in the distribution of κOR immunoreactivity, even though much less

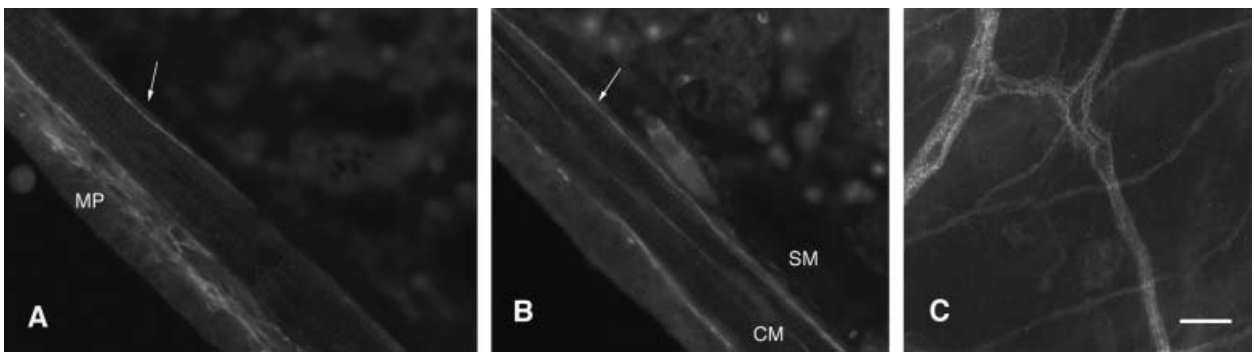

Figure 3 (A, B) μOR immunoreactivity in cryostat sections, and (C) a whole mount preparation of the guinea-pig small intestine showing (C) μOR immunoreactivity in bundles of fibres in the circular muscle and deep muscular plexus and in interconnecting strands (A) and in myenteric neurones. Note the weak μOR immunoreactivity in the submucosal plexus (B). CM, circular muscle; M, mucosa; MP, myenteric plexus; SM, submucosa. Calibration bar: 50 μm.

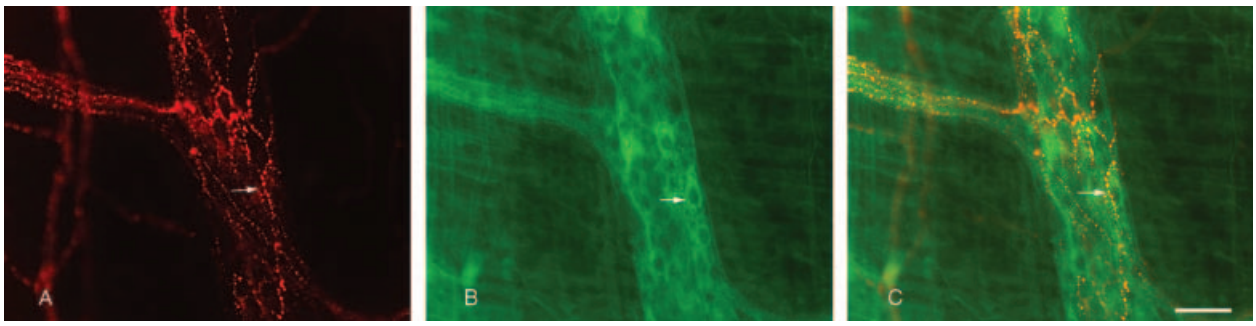

Figure 4 (A–C) The close relationship of enkephalin (red fluorescence) immunoreactive fibres to μOR neurones (green fluorescence). Arrows point to a fibre immunoreactive for enkephalin in close vicinity to a μOR myenteric neurone. (C) An overlay of enkephalin and μOR immunoreactivity. Calibration bar: 50 μm.

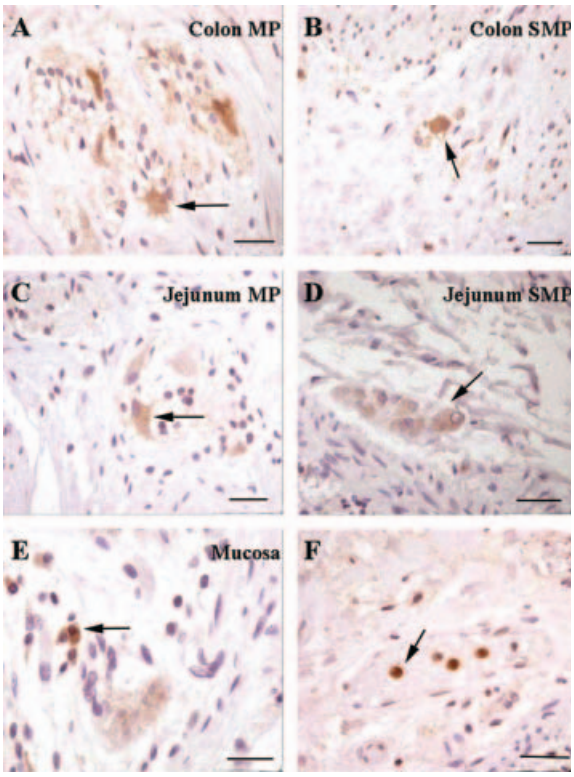

Figure 5 μOR distribution in human specimens. μOR immunoreactivity is found in (A and B) myenteric neurones, (C and D) submucosal neurones, and (E and F) immune cells. Arrows point to μOR in neurones of the myenteric plexus of (A) the colon and (C) the jejunum, of the submucosal plexus of (B) the colon and (D) the jejunum, and in (E and F) immune cells. MP, myenteric plexus; SMP, submucosal plexus. Calibration bars: 50 μm.

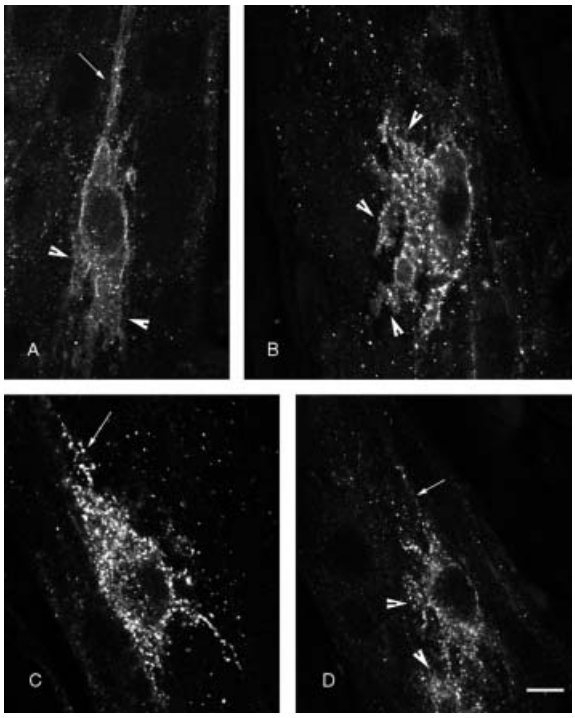

Figure 6 Single confocal images showing μOR immunoreactivity at the cell surface of (A) unstimulated neurones, in endosomes following stimulation with (B) enkephalin (C) DAMGO and (D) fentanyl. Note that internalization occurs in the soma, dendrites (arrowheads) and axonal processes (arrows). Calibration bar: 10 μm.

information is available for this OR. In the rat small intestine and colon, κOR is found prominently in submucosal neurones, even though it is also localized to myenteric neurones and ICCs,[48] whereas in the porcine[50] and guinea-pig ileum (demonstrated in this paper), κOR is confined to the myenteric plexus. By contrast, the distribution of δOR immunoreactivity in the guinea-pig and porcine ileum appear similar, as in both species δOR immunoreactivity was localized to both the myenteric and submucosal plexuses and fibres to muscle and mucosa.[50] Overall, despite some differences noted above, the main feature that is common to all species is that all three ORs are expressed predominantly by neuronal structures.

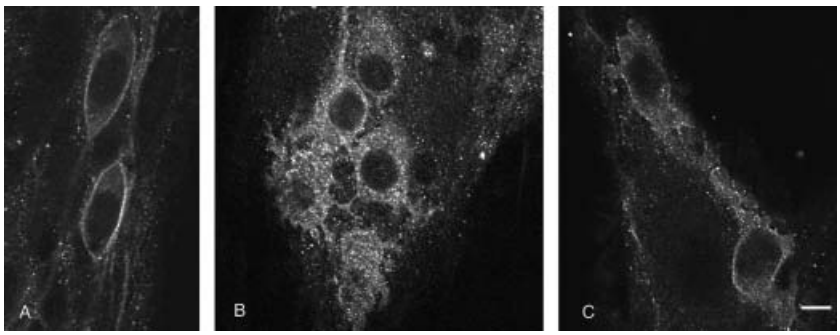

Figure 7 Single confocal microscopic images showing μOR immunoreactivity (A) at the cell surface in neurones from a control animal (anaesthesia alone) or (C) from an animal that was injected with naloxone intraperitoneally prior to laparotomy. (B) μOR immunoreactivity is predominantly in endosomes in neurones from an animal that underwent laparotomy plus manipulation of the intestine. Calibration bar: 5 μm.

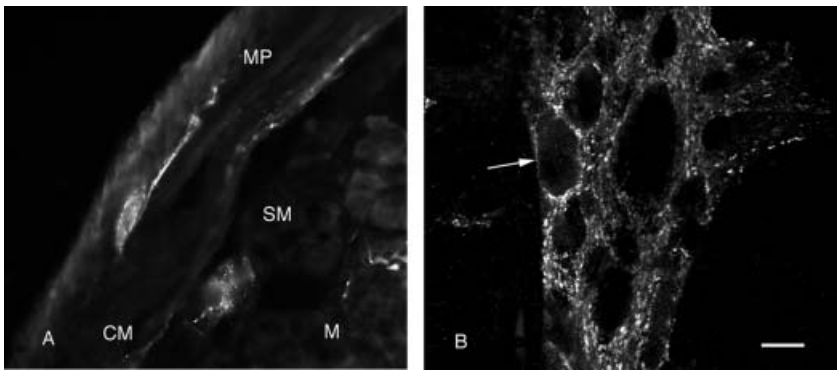

Figure 8 Localization of δOR immunoreactivity in the guinea-pig ileum: (A) cryostat section and (B) whole mount; confocal image. δOR immunoreactivity is localized to the myenteric and submucosal plexus, where it is found in neurones and varicose fibres. Note varicose fibres also in the mucosa (A). Arrows point to a cell body immunoreactive for δOR. CM, circular muscle; M, mucosa; MP, myenteric plexus; SMP, submucosal plexus. Calibration bar: 5 μm.

Interestingly, despite the observation of binding sites for all three ORs in the gastrointestinal smooth muscle and the findings that all three types of receptors produce contractions of smooth muscle cells of the stomach and intestine, immunoreactivity for any of these ORs has not been reported in smooth muscle cells in any of the species studied. Indeed, the neural effects of opioids in intact tissues appear to be more important than direct smooth-muscle effects and the relevance of smooth-muscle ORs to *in vivo* biological actions of opioids is not well understood.[25] Functional and pharmacological evidence indicates that the effects of opioids on intestinal motility induced by the activation of ORs on neuronal structures are mediated predominantly by μOR and κOR.[24,60] Furthermore, the neural circuits controlling peristalsis in the guinea-pig small intestine have been reported to be inhibited by endogenous and exogenous opioids acting via μOR and κOR, but not δOR.[61] In a similar way to μOR, κOR effects are likely to be mediated by inhibition of cholinergic and non-cholinergic neurotransmission.[62] δOR appear to be involved predominantly with neurogenic secretion and perhaps inflammatory processes,[63] even though an involvement of δOR in modulating neurogenic contractions has been reported,[64] and it is likely that all three ORs play a role in gastrointestinal motility.

The μOR immunoreactivity distribution closely matches the distribution of the opioid peptide, enkephalin.[28] μOR and enkephalin immunoreactivities also colocalize in some myenteric neurones,[51] suggesting an autocrine mechanism for the regulation of endogenous opioids. Enkephalins might have a negative feedback on the neuronal activity by activating μOR. In addition, the close vicinity of some enkephalin immunoreactive fibres and myenteric neurones bearing μOR indicates possible sites of ligand release. Enkephalins are likely to be the predominant endogenous opioids that activate neuronal μOR in the gut. Enkephalins are capable of activating μOR[65] and of triggering μOR endocytosis in enteric neurones *in vitro*.[56] In addition, electrical stimulation of longitudinal muscle–myenteric plexus preparations of the guinea-pig ileum, at frequencies that have been shown to release enkephalin almost exclusively, induced massive μOR endocytosis,[55] indicating binding between enkephalin and μORs. Myenteric neurones of the guinea pig also express dynorphin, which binds to μOR with much lower affinity compared with enkephalins and preferentially binds to the κOR, whereas there is no evidence for the localization of β-endorphin or endomorphins in the enteric nervous system.

μOR myenteric neurones comprise functionally distinct types of neurones, including cholinergic

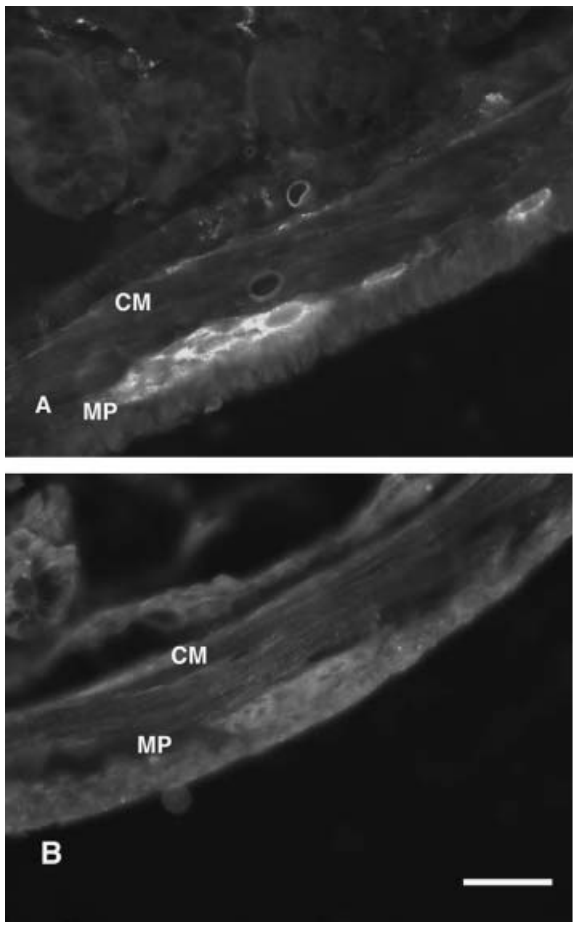

Figure 9 (A) δOR immunoreactivity in the myenteric plexus and circular muscle layer in a cryostat section of the ileum. (B) δOR immunostaining is abolished by preadsorption with an excess of peptide. MP, myenteric plexus; CM, circular muscle layer. Calibration bar: 100 μm.

ascending, excitatory motor neurones to the muscle, descending inhibitory motor neurones and descending interneurones.[51] The presence of μOR on cholinergic ascending excitatory neurones that innervate the muscle is consistent with the functional evidence that opioids and opiates inhibit the electrically evoked release of acetylcholine by acting on enteric neurones primarily via the μOR. This in turn results in inhibition of muscle contraction, which is responsible for the delayed gastrointestinal transit and severe constipation induced by opiates.[2] On the other hand, the presence of μOR on a substantial population of descending neurones is in agreement with a modulatory effect of opioids on the release of vasoactive intestinal polypeptide and the production of NO. Reduced release of inhibitory transmitters would account for the reported excitatory effect of opioids on smooth muscle.[66] This is also in agreement with the opioid inhibitory effect on compliance of the intestinal wall during the preparatory phase of peristalsis in the intact segment of the guinea-pig ileum.[67] Inhibition of vasoactive intestinal polypeptide release and NO production could also suppress descending relaxation and consequently interfere with intestinal propulsion.[68,69]

The involvement of the opioid system in the control of gastrointestinal motility is supported by a large body of anatomical and functional evidence.[2,68,69] Opioids impair gastrointestinal transit by inhibiting neurotransmitter release (including acetylcholine), by changing neuronal excitability[52] and by exerting a modulatory influence on motor neurones that function as a restraining mechanism in conditions of fatigue (i.e. when the intestine empties against high resistance).[67,68] The localization of μOR to neurones in both the ascending and descending pathways[51] is consistent with the hypothesis that the final effect of μOR activation depends on the balance between the inhibition of transmitter release from different neuronal pathways. The close localization of a dense network of μOR immunoreactive fibres to ICCs suggests a new pathway through which opioids might affect gastrointestinal motility, which would involve inhibition of ICC activity via a reduction of transmitter release by excitatory and inhibitory nerves that innervate these cell types.[70] The presence of both δOR and κOR in

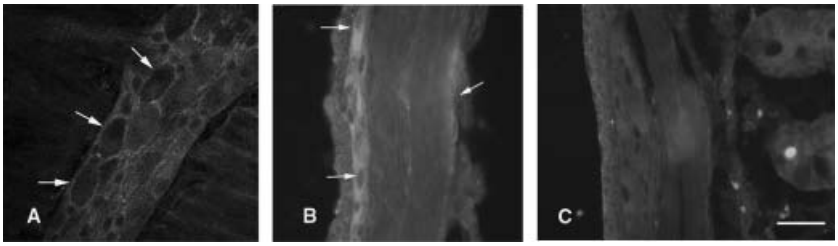

Figure 10 (A) Whole mount preparation showing κOR immunoreactivity in neurones of the myenteric plexus of the guinea-pig ileum; confocal image. In (B) arrows point to κOR immunoreactivity in myenteric neurones and in the deep muscular plexus in a cryostat section. (C) κOR immunostaining is abolished by preadsorption with an excess of peptide. Calibration bar: 50 μm.

myenteric neurones also supports their involvement in the regulation of gastrointestinal motility

In addition to the effects on motility, opioids modulate electrolyte and fluid transport by stimulating absorption of water and electrolytes and modulate gastric acid secretion and bicarbonate secretion.[2] These effects are consistent with the presence of ORs in the submucosal plexus of both human and rat. Furthermore, the presence of μOR in neutrophils and mononuclear cells in humans (demonstrated in this paper) and the close vicinity of μOR fibres to lymphatic nodes in the rat intestine[48] are supportive of an involvement of μOR in neuroimmune interactions. Finally, the predominant localization of δORs in the submucosal plexus and the presence of δOR immunoreactive fibres in the mucosa are consistent with a major role of this receptor in modulating the neural pathway controlling secretomotor functions, even though a role in gastrointestinal motility is also supported by the presence of this receptor in myenteric neurones and processes to the muscle. The main effect of opioids and opiates in the gastrointestinal tract is inhibition of neuronal excitability and transmitter release[52,71] by inhibiting K^+ conductance and Ca^{2+} channels.

In enteric neurones, μOR immunoreactivity is localized predominantly at the cell surface. μOR undergoes specific and ligand-selective endocytosis in a concentration-dependent manner in the soma and neurites of enteric neurones.[56] We have shown that this μOR endocytosis occurs primarily via a clathrin-mediated pathway, and μOR recycles to the cell surface within 6 h of receptor internalization following endosomal acidification. Furthermore, μOR can cycle repetitively between the cell surface and the cytoplasm of enteric neurones, and previous internalization of μOR does not diminish the level of ligand-induced receptor translocation from the plasma membrane to the cytoplasm. Morphine, the prototype of opiate drugs, has high affinity for μOR and uses the same signalling pathway as other opiates, so its failure to induce μOR internalization is intriguing. This functional dissociation between trafficking and signalling and the ligand selectivity of μOR endocytosis support the hypothesis that individual agonists might have differential abilities to regulate biological effects mediated by μOR. Receptor endocytosis and recycling are regulatory processes that are associated with functional diminution of receptor-mediated signalling and with resensitization,[72] but might also influence biological and adaptive responses mediated by activated receptors.[73] Receptor trafficking might modulate opioid- and opiate-mediated neurotransmission and play an important role in the pharmacological effects of opiates, potent analgesics whose clinical use is limited by the development of many side-effects, including tolerance, respiratory depression and profound impairment of gastrointestinal transit.

The occurrence of translocation of a receptor from the cell surface to the cytoplasm requires agonist–receptor interaction, thus the internalization of μOR observed in myenteric neurones *in vitro* following electrical stimulation and *in vivo* following surgery is an indication of endogenous opioid release. The levels of receptor internalization reflect the amount of opioids available for binding to the receptor, because studies with enteric neurones in organotypic culture showed that μOR internalization is concentration-dependent.[56] Laparotomy with or without intestinal manipulation is a potent stimulus for evoking endogenous opioid release, resulting in prominent μOR internalization in enteric neurones. The stimulus responsible for opioid release is likely to be the noxious challenge of surgery and not the mechanical stimulation of the intestine, as the levels of receptor internalization induced by laparotomy with and without intestinal manipulation were comparable.[58]

Impairment of intestinal transit, often resulting in postoperative ileus, is a common complication of abdominal surgery, trauma and peritoneal irritation leading to significant morbidity.[57,74] Different mechanisms appear to cause postoperative ileus[57,75] including the activation of inhibitory neuronal reflex pathways[57,61,76] and the local release of peptides and inflammatory mediators.[77–79] Activation of ORs by endogenous opioids released in response to the stress of surgery and by opiates used as analgesics for surgical pain has been regarded as one of the factors contributing to postsurgical constipation.[57] Release of opioids has been reported in response to stress and surgery,[17,80] even though their contribution to ileus is still unknown. Naloxone does not appear to improve the stress-induced depression of gastrointestinal transit following abdominal surgery in rats or to affect normal intestinal motility.[81–83] By contrast, selective inhibition of gastrointestinal μORs by peripherally acting antagonists with limited oral absorption and poor ability to cross the blood–brain barrier has been shown to reduce the severity and duration of postoperative ileus as well as improve gastrointestinal transit in patients with opioid bowel dysfunction.[40,41,84–86] Opioid bowel dysfunction, which is characterized by pronounced impairment of gastrointestinal transit with severe constipation as observed postsurgically, is a major side-effect of chronic opioid treatments due predominantly to the activation of peripheral ORs.[7,87–89] Taken together, these findings suggest an involvement of the opioid system in these

conditions, which could also include ORs other than the μOR, such as the κOR or δOR.

CONCLUSIONS

The opioid system is complex; opioids and ORs are widespread throughout the body and affect a variety of functions. It is unique in that every one of the endogenous ligands binds each of the receptor subtypes (with different affinities). These receptors are activated by powerful analgesic drugs that have many undesirable side-effects, including addiction, tolerance and pronounced delay of gastrointestinal transit that can result in severe constipation and abdominal discomfort. Knowledge of the distribution and interaction of opioids and ORs and of receptor-mediated processes provides the basis for the development of novel therapies targeted to the specific processes that affect neuronal responsiveness. For instance, μOR endocytosis might play a more complex role than presently thought. Apart from its involvement in the regulation of signal transduction and the maintenance of cellular desensitization through control of cell-surface-receptor expression, it might also regulate physiological responses and biological actions of agonists. This is particularly important for its clinical implications, suggesting that μOR endocytosis might influence the therapeutic action of potent analgesics like the opiate alkaloids.

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