

N-Methyl-D-Aspartate Receptors Mediate Endogenous Opioid Release in Enteric Neurons After Abdominal Surgery

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Background & Aims: We tested the hypothesis that N-methyl-D-aspartate (NMDA) receptors mediate surgery-induced opioid release in enteric neurons. **Methods:** We used μ opioid receptor (μ OR) internalization as a measure of opioid release with immunohistochemistry and confocal microscopy. μ OR internalization was quantified in enteric neurons from nondenervated and denervated ileal segments of guinea pig after abdominal laparotomy with and without pretreatment with NMDA-receptor antagonists acting at different recognition sites (+)-5-methyl-10, 11-dihydro-5H-dibenzo [a, b] cyclohepten-5, 10-imine (MK-801) or (D) 2-amino-5-phosphopenoic acid (AP-5) at .5, 1 mg/kg; 8-chloro-4-hydroxy-1-oxo-1, 2-dihydropyridazinol [4,5]-quinoline-5-oxide choline (MRZ 2/576) or 8-chloro-1, 4-dioxo-1,2,3,4-tetrahydropyridazinol [4,5]-quinoline choline salt (MRZ 2/596) at .3, 1 mg/kg, or with an antagonist for the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, 6-cyano-7-nitroquinoxaline-2, 3-dione (1, 3 mg/kg). To determine whether NMDA stimulation induces opioid release, (1) ilea were exposed to NMDA (100 μ mol/L) and D-serine (10 μ mol/L) with or without the antagonist MK-801 or AP-5 (50 μ mol/L); and (2) neuromuscular preparations of the ileum were stimulated electrically (20 Hz, 20 min) with or without MK-801 or AP-5 (50 μ mol/L). **Results:** μ OR endocytosis induced by abdominal laparotomy was inhibited significantly by NMDA-receptor antagonists in nondenervated and denervated ileal segments, but not by the AMPA-receptor antagonist. μ OR endocytosis in neurons exposed to NMDA or electrical stimulation was prevented by NMDA-R antagonists. **Conclusions:** Abdominal laparotomy evokes local release of glutamate that results in endogenous opioid release through the activation of peripheral NMDA receptors. This suggests an interaction between the glutamatergic and opioid systems in response to the noxious and perhaps mechanosensory stimulation of surgery.

μ opioid receptors (μ ORs) are G protein-coupled receptors that mediate the effects of endogenous opioid peptides and of structurally distinct opiates. These are powerful analgesic drugs with many undesirable side effects including tolerance, respiratory depression, and pronounced delay of gastrointestinal transit, which can result in severe constipation and abdominal discomfort.^{1–6} In the gastrointestinal tract, μ ORs are expressed predominantly by enteric neurons,^{7–10} and they have been implicated in the regulation of secretion and motility.^{11,12} In the guinea pig, μ OR immunoreactivity is localized to functionally distinct populations of Dogiel type I myenteric neurons, which comprise ascending and descending motor neurons that control smooth muscle activity, and interneurons that transmit information to other enteric neurons, but not to intrinsic primary afferent neurons.¹⁰ In the guinea pig enteric nervous system, the distribution of μ OR immunoreactivity closely matches that of the opioid peptide, enkephalin.^{10,13} In addition, μ OR and enkephalin immunoreactivities colocalize in some myenteric neurons and fibers distributed to the circular muscle and the deep muscular plexus.¹⁰ Activation of μ OR resulting in coupling with its effector systems attenuates neuronal activity by inhibiting neurotransmitter release and changing neuronal excitability by pre- and postsynaptic mechanisms.^{14–16}

Abbreviations used in this paper: AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AP-5, (D) 2-amino-5-phosphopenoic acid; CNQX, 6-cyano-7-nitroquinoxaline-2, 3-dione; IPAN, intrinsic primary afferent neurons; LMMP, longitudinal muscle-myenteric plexus; MK-801, (+)-5-methyl-10, 11-dihydro-5H-dibenzo [a, b] cyclohepten-5, 10-imine; μ OR, μ opioid receptor; MRZ 2/576, 8-chloro-4-hydroxy-1-oxo-1, 2-dihydropyridazinol [4,5]-quinoline-5-oxide choline; MRZ 2/596, 8-chloro-1, 4-dioxo-1,2,3,4-tetrahydropyridazinol [4,5]-quinoline choline salt; NMDA, N-methyl-D-aspartate; NOS, nitric oxide synthase; TH, tyrosine hydroxylase.

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μ OR immunoreactivity is localized predominantly at the cell surface in unstimulated neurons and cell lines, but it translocates to endosomes after acute activation with selective μ OR ligands.^{9,17–21} Ligand-induced endocytosis of μ OR occurs in myenteric neurons of guinea pig ileum in vivo and in vitro in response to exogenously administered selective μ OR agonists, and to endogenously released opioids.^{9,20–24} μ OR internalization is concentration dependent, thus it can serve as a measure of opioid release in response to stimulation and it is a reliable indication of opioid-induced activity.^{20,21} We previously have shown that μ OR endocytosis occurs in functionally distinct myenteric neurons of the guinea pig ileum in response to abdominal surgery and that the stimulus responsible for the endogenous opioid release resulting in μ OR internalization is likely to be the noxious stimulation induced by the surgery.²⁴ Indeed, endogenous opioid release has been reported in response to the stress of surgery,^{25–28} and noxious stimulation has been shown to evoke local release of endogenous opioids from the nervous system.^{29,30}

There is evidence that *N*-methyl-D-aspartate (NMDA)-type glutamate receptors mediate noxious transmission and regulate peptide release from neurons.^{31–35} NMDA receptors are ligand-gated, ion-channel, glutamate receptors that are distributed abundantly in the nervous system, including primary afferent neurons innervating the viscera and the enteric nervous system.^{35–38} These observations formed the basis of our hypothesis that NMDA receptors mediate opioid release and μ OR endocytosis in enteric neurons induced by the noxious stimulation of abdominal surgery.

The aims of the present study were to determine whether (1) μ OR endocytosis in myenteric neurons induced by abdominal surgery was prevented by NMDA receptor blockade in vivo, (2) extrinsic denervation of ileal segments affected laparotomy-induced μ OR endocytosis and NMDA-receptor blockade of μ OR endocytosis in myenteric neurons induced by abdominal surgery, (3) NMDA-receptor activation induced opioid release in enteric neurons in vitro, and (4) μ OR endocytosis induced by opioid release in response to electrical stimulation of longitudinal muscle–myenteric plexus (LMMP) preparations of the guinea pig ileum was prevented by NMDA-receptor antagonists. Because functional inhibition of NMDA receptors might be achieved by acting at different recognition sites, we used different NMDA-receptor antagonists, including compounds acting at the glutamate or glycine site or blocking the ion channel.^{39–41}

Materials and Methods

Experimental Animals

Animal care and procedures were in accordance with the National Institutes of Health recommendations for the humane use of animals. All experimental procedures were reviewed and approved by the appropriate Animal Use Committee of the University of California Los Angeles and Departments of Veterans Affairs Greater Los Angeles Healthcare. The number of animals used was kept to the minimum necessary for meaningful interpretation of the data. Male albino Porcellus guinea pigs weighing 200–300 g (Simonsen Labs, San Diego, CA) were used.

Guinea pigs were kept in a 12-hour light-dark cycle (lights on at 6:00 AM). Anesthesia was induced with .5%–3% isoflurane. After aseptic preparation of the abdomen, laparotomy incision in the abdominal midline skin, muscle layers, and peritoneum was performed followed by exteriorization of the intestine. The viscera gently were returned and retained within the abdominal cavity. Animals were allowed to recover for 30 minutes and then were euthanized with an overdose of sodium pentobarbital (100 mg/kg, intraperitoneally). Functional inhibition of NMDA receptors was achieved by using compounds that act at different recognition sites, including the primary transmitter (glutamate) site or strychnine-insensitive glycine site (glycine B), or by blocking the associated ion channel.^{39–41} Experimental groups included (1) animals subjected to abdominal surgery alone; (2) animals that received an intraperitoneal injection of the noncompetitive ion-channel blocker, NMDA-receptor antagonist, (+)-5-methyl-10, 11-dihydro-5H-dibenzo [*a*, *b*] cyclohepten-5, 10-imine (MK-801, .5 or 1 mg/kg; Sigma, St. Louis, MO); (3) animals that received an intraperitoneal injection of the competitive NMDA receptor, (D) 2-amino-5-phosphopenoic acid (AP-5, .5 or 1 mg/kg; Tocris, Ellisville, MO) acting at the glutamate site; (4) animals that received an intraperitoneal injection of the noncompetitive NMDA-receptor antagonist acting at glycine B site, 8-chloro-4-hydroxy-1-oxo-1, 2-dihydropyridazinol [4,5-] quinoline-5-oxide choline (MRZ 2/576) (.3 or 1 mg/kg); (5) animals that received an intraperitoneal injection of the noncompetitive NMDA-receptor antagonist acting at glycine B site, 8-chloro-1, 4-dioxo-1,2,3,4-tetrahydropyridazinol [4,5-] quinoline choline salt (MRZ 2/596) (1 mg/kg); or (6) animals that received an intraperitoneal injection of the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor antagonist, 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX, 1 or 3 mg/kg; Tocris). MRZ 2/576 and MRZ 2/596 were provided by Merz & Co (GmbH, Frankfurt, Germany).⁴² Because the NMDA-receptor antagonists MRZ 2/576 and MRZ 2/596 acting at the glycine B site have a half-life of about 20 minutes, and the surgical procedure lasts 10–15 minutes and the animals are euthanized 30 minutes after surgery, these compounds were injected 2 minutes before as well as 5–10 minutes after abdominal laparotomy. The other NMDA-receptor antagonists were injected 3 minutes before laparotomy. Drugs were dissolved in .9% sterile sodium chlo-

ride or double-distilled water. Each experimental group comprised 3–10 animals.

Denervation Procedures

To determine whether opioid release and μ OR endocytosis in enteric neurons induced by abdominal surgery and NMDA-receptor blockade involved central or peripheral neural pathways (or both), extrinsic denervation of ileal segments was performed before laparotomy with and without NMDA-receptor blockade. The denervation surgeries, which were a combination of extrinsic denervation and myotomy,⁴³ were performed on 8 male guinea pigs (weight range, 250–350 g) anesthetized with .5%–3% isoflurane. A loop of intestine, 10–15 cm in length, supplied by a single mesenteric artery and its branches, was selected. All nerve fiber bundles that could be detected under a dissecting microscope and after swabbing with 80% alcohol in distilled water, which makes the nerves appear white, were dissected away from the artery and its accompanying vein. On each side adjacent to the denervation, a circumferential myotomy surgery was performed consisting of cuts through the external muscle to the depth of the submucosa, around the full circumference of the intestine. The denervated region was marked by a loose ligature around its vascular supply and was returned to the abdominal cavity. The abdomen was closed by sutures and the animals were allowed to recover from anesthetic and then supplied with food and water ad libitum. The guinea pigs underwent abdominal laparotomy 10 days later, with or without pretreatment with the NMDA-receptor antagonist acting at glycine B site (MRZ 2/596, 1 mg/kg), and were euthanized 30 minutes later as described earlier. This NMDA-receptor antagonist was chosen for these experiments because it has been shown to act predominantly peripherally at the dose and route of administration used in this study.^{41,44,45} Segments of the denervated and nondenervated intestine were collected and processed for immunohistochemistry.

Immunohistochemistry

The distal ileum was dissected, washed with ice-cold saline, opened along the longitudinal axis, pinned flat, and fixed in 4% paraformaldehyde in .1 mol/L phosphate buffer, pH 7.4 for 2 hours at room temperature and stored in .1 mol/L phosphate buffer with .1% sodium azide. Whole mounts of the longitudinal muscle with the myenteric plexus attached were prepared and processed for immunohistochemistry as described.²⁴ Briefly, tissues were incubated in phosphate buffer containing .5% Triton X-100 (3 periods of 30 min), followed by 5% normal goat serum in .5% Triton X-100/phosphate buffer for 60 minutes, and then rabbit polyclonal affinity-purified antibody directed to the C-terminus region of rat μ OR (384–398) (ImmunoStar Inc., Hudson, WI) (1:3000) for 48 hours at 4°C, washed and incubated with affinity-purified donkey anti-rabbit immunoglobulin G conjugated to ALEXA 488 (1:1000; Molecular Probes, Eugene, OR) for 2 hours at room temperature. The μ OR antibody has been characterized previously.²⁰ To test the effectiveness of extrinsic

denervation, we used the immunohistochemical localization of tyrosine hydroxylase (TH), a marker of extrinsic noradrenergic axons with a mouse anti-TH antibody (1:250, Chemicon International, Temecula, CA). Only ileal denervated segments whose TH staining was abolished were considered for μ OR immunostaining. To test whether myotomy lesions completely interrupted the myenteric nerve pathways, immunohistochemistry for nitric oxide synthase (NOS), the enzyme that produces nitric oxide, was used with mouse anti-NOS (1:50, Transduction Laboratories, Lexington, KY), because NOS-immunoreactive neurons are abundant in the myenteric plexus. Whole-mount preparations were used as described earlier under immunohistochemistry.

In Vitro Experiments

Segments of the distal ileum, pinned flat as described earlier, were placed in sterile Krebs' solution (5.9 mmol/L KCl, 118 mmol/L NaCl, 2.5 mmol/L CaCl₂, 1.2 mmol/L MgSO₄, 1.4 mmol/L NaH₂PO₄, 5 mmol/L glucose, 2.5 μ g/mL fungizone, 100 IU/mL penicillin, and 100 μ g/mL streptomycin), bubbled with 95% O₂:5% CO₂, pH 7.4, washed, and then incubated for 30 minutes at 37°C in Dulbecco's modified Eagle medium containing 10% vol/vol fetal bovine serum, penicillin, streptomycin, and fungizone with 95% O₂:5% CO₂, pH 7.4. The ileum was incubated for 1 minute at 37°C in Dulbecco's modified Eagle medium containing NMDA (100 μ mol/L) and D-serine (10 μ mol/L) in the presence or absence of the noncompetitive NMDA-receptor antagonist, MK-801 (50 μ mol/L), or the competitive NMDA-receptor antagonist, AP-5 (50 μ mol/L), then moved to drug-free medium and incubated for 30 minutes at 37°C to allow internalization. These experiments were performed in duplicate.

LMMP Preparations

Segments of the distal ileum were dissected from guinea pigs killed by CO₂ inhalation and then immersed in standard Tyrode solution composed of (in mmol/L): 136.9 NaCl, 2.7 KCl, 1.8 CaCl₂, 1.04 MgCl₂, .4 NaH₂PO₄, 11.9 NaHCO₃, 5.5 glucose. A total of 4 animals were used for these studies. LMMP preparations were obtained by teasing the longitudinal muscle with the intact myenteric plexus from the underlying circular muscle.²⁰ For endogenous release of opioids, LMMP preparations were stimulated electrically at a frequency of 20 Hz (a frequency that has been shown to induce release of enkephalins⁴⁶ and μ OR internalization²⁰) for 20 minutes. The protease inhibitors, phosphoramidone (1 μ mol/L), captopril (10 μ mol/L), and amastatin (1 μ mol/L) (Sigma) were added to the bathing solution for 20 minutes before stimulation to prevent peptide degradation. Some tissues were incubated with the NMDA-receptor antagonists, MK-801 or AP-5, at 50 μ mol/L for 5 minutes before and after stimulation. Tissues were fixed in 4% paraformaldehyde in phosphate buffer for 2 hours at room temperature and were processed for immunohistochemistry to determine the subcellular distribution of μ OR immunoreactivity.

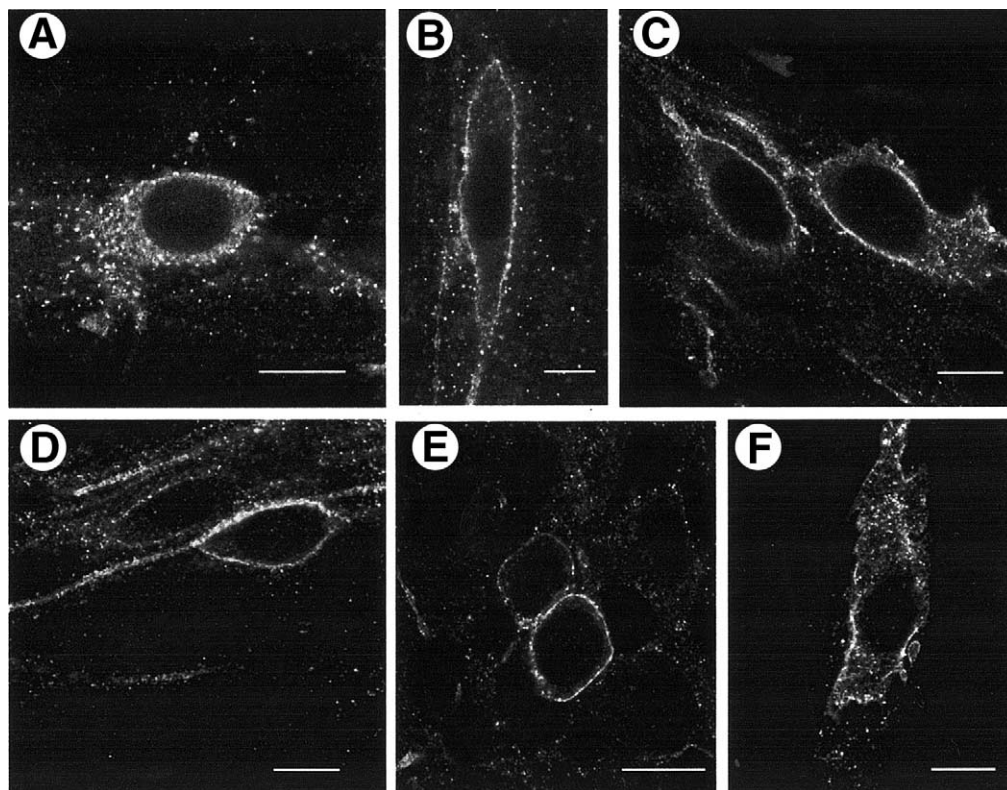


Figure 1. Single confocal images. μ OR immunoreactivity in myenteric neurons from guinea pigs that underwent laparotomy with or without pretreatment with NMDA- or AMPA-receptor antagonists. μ OR immunoreactivity is mostly in the cytoplasm in neurons from (A) a guinea pig that underwent laparotomy and from (F) a guinea pig pretreated with intraperitoneal injection of the AMPA-receptor antagonist CNQX (3 mg/kg) before laparotomy. μ OR immunoreactivity is mostly at the cell surface in neurons from guinea pigs that received intraperitoneal injection of the NMDA-receptor antagonists (B) MK-801, (C) AP-5, (D) MRZ 2/576, or (E) MRZ 2/596 at 1 mg/kg. Calibration bars: (A, E) 7 μ m, (B) 4 μ m, and (C, D, F) 5 μ m.

Microscopy and Quantification

TH and NOS distribution was examined with a Zeiss Axioplan 2 research microscope for fluorescence with axiocam color digital camera fluorescence microscope (Carl Zeiss Inc., Thornwood, NY), equipped with a fluorescein isothiocyanate cube. μ OR immunoreactivity distribution was analyzed with either a Zeiss 410 laser scanning confocal microscope equipped with a krypton/argon laser and attached to a Zeiss Axiovert 100 microscope or a Zeiss 510 Meta laser scanning confocal microscope equipped with both HeCd and argon lasers and attached to a Zeiss Axioplan 2 microscope with a 100 \times Plan Apo 1.4 numerical aperture objective (Carl Zeiss Inc). Images of 512 \times 512 pixels were collected at a magnification zoom of 1.5 \times . Typically, 10–20 optical sections were taken at the z-axis at .5- to .75- μ m intervals through the cells. Images were processed and labeled using Adobe Photoshop 6.0 (Adobe Systems, Mountain View, CA), as described.^{20,24}

The levels of μ OR internalization were quantified by National Institutes of Health Image software using single confocal images that included the nucleus and a large area of cytoplasm. Fluorescence density, contrast, brightness magnification, and zoom were kept constant throughout the analysis. A line was drawn around the outside of the neuron and the total neuronal fluorescence (surface plus cytoplasm) was measured as the number of pixels with intensity of fixed fluorescence density. To measure the surface and cytoplasmic receptor separately, a second line was drawn inside the cell membrane .5 μ m from the first line and the number of

pixels at the same fluorescence density parameter was measured. The percentage of fluorescence present in the cytoplasm then was calculated from the second measure (immunoreactivity in the cytoplasm only) divided by the first measure (total immunoreactivity).^{21,24} For each data point, internalization was quantified for 30–100 neurons (ie, 10 neurons from each of 3–10 animals). For this analysis, the neurons were chosen randomly and all the images were collected using an identical setting for laser illumination, magnification, and zoom of the sample.

Statistical Analysis

Values are shown as mean \pm SEM. We compared means using 1-way analysis of variance and the corresponding post hoc *t* tests. Statistical significance was assessed using the Tukey–Fisher least-significant difference criterion. Significance was attained with the nominal α value of .05.

Results

μ OR Internalization Induced by Abdominal Surgery In Vivo

As we reported previously,²⁴ there was a pronounced μ OR internalization in myenteric neurons from animals that underwent laparotomy (Figure 1A). By contrast, μ OR was located predominantly at the cell surface in animals that received injection of the noncompetitive NMDA receptor, MK-801 (Figure 1B), the com-

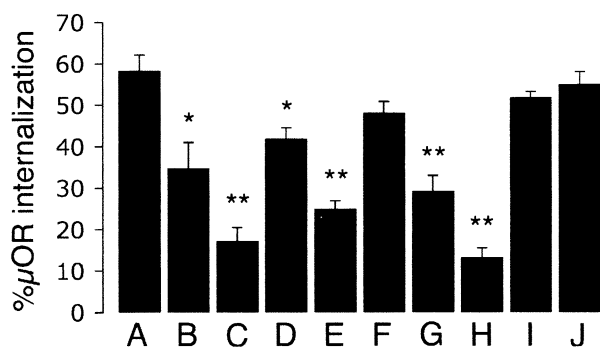


Figure 2. Quantification of μ OR immunofluorescence in neurons from guinea pigs that underwent laparotomy with or without pretreatment with the NMDA- or AMPA-receptor antagonists. Levels of μ OR internalization in neurons from guinea pigs that (A) underwent laparotomy or laparotomy preceded by intraperitoneal injection of the NMDA receptor antagonists (B, C) MK-801 (.5 or 1 mg/kg), (D, E) AP-5 (.5 or 1 mg/kg), (F, G) MRZ 2/576 (.3 or 1 mg/kg), or (H) MRZ 2/596 (1 mg/kg), or by (I, J) intraperitoneal injection of the AMPA-receptor antagonist CNQX (1 or 3 mg/kg). μ OR internalization is expressed as the percent of immunoreactivity translocated into the cytoplasm. * $P < .001$ and ** $P < .05$ compared with controls.

petitive NMDA-receptor antagonist, AP-5 (Figure 1C), or the glycine B site NMDA-receptor antagonists, MRZ 2/576 (Figure 1D), and MRZ 2/596 (Figure 1E). By contrast, μ OR immunoreactivity was predominantly in the cytoplasm in neurons from animals that received injection of the AMPA/kainate-receptor antagonist CNQX before laparotomy (Figure 1F).

Quantification of μ OR immunoreactivity in enteric neurons from the different groups of animals showed that μ OR endocytosis was $58.2\% \pm 3.9\%$ (Figure 2, column A) 30 minutes after laparotomy, and that the levels of μ OR endocytosis were decreased significantly in neurons from animals that received the noncompetitive NMDA-receptor antagonist, MK-801 (.5 or 1 mg/kg) ($34.7\% \pm 6.4\%$ and $17.1\% \pm 3.4\%$, Figure 2, columns B and C, $P < .05$ and $P < .001$ vs laparotomy alone, respectively)

or the competitive NMDA-receptor antagonist acting at the glutamate site, AP-5 (.5 or 1 mg/kg) ($41.8\% \pm 2.7\%$ and $24.8\% \pm 2.2\%$, Figure 2, columns D and E, $P < .05$ and $P < .001$, respectively) before laparotomy. Similarly, μ OR internalization induced by laparotomy was prevented by pretreatment with the NMDA-receptor antagonists acting at the glycine B site, MRZ 2/576 (1 mg/kg) ($29.1\% \pm 2.5\%$, Figure 2, column G, $P < .001$ vs laparotomy alone) or MRZ 2/596 (1 mg/kg) ($13.2\% \pm 1.6\%$, Figure 2, column H, $P < .001$). MRZ 2/576 at .3 mg/kg only slightly reduced the levels of μ OR internalization ($48.0\% \pm 2.9\%$, Figure 2, column F, $P =$ not significant). The levels of μ OR internalization in animals that were pretreated with the AMPA/kainate receptor antagonist CNQX before laparotomy (1 and 3 mg/kg) ($51.7\% \pm 3.2\%$ and $54.8\% \pm 6.0\%$, Figure 2, columns I and J, respectively) were not significantly different from those observed in neurons from animals that underwent laparotomy without any pretreatment.

μ OR Internalization Induced by Abdominal Surgery In Vivo After Extrinsic Denervation

There was a pronounced μ OR internalization in myenteric neurons from animals that underwent laparotomy 10 days after extrinsic denervation of an ileal loop (Figure 3A) that was comparable with the internalization observed in nondenervated animals. μ OR was located predominantly at the cell surface in extrinsically denervated animals that received injection of MRZ 2/596 before laparotomy (Figure 3B), similarly to what was observed with this treatment in nondenervated animals. Quantification of μ OR immunoreactivity in enteric neurons from denervated animals showed that μ OR endocytosis was $78.3\% \pm 1.4\%$ 30 minutes after laparotomy and that the levels of μ OR endocytosis were decreased

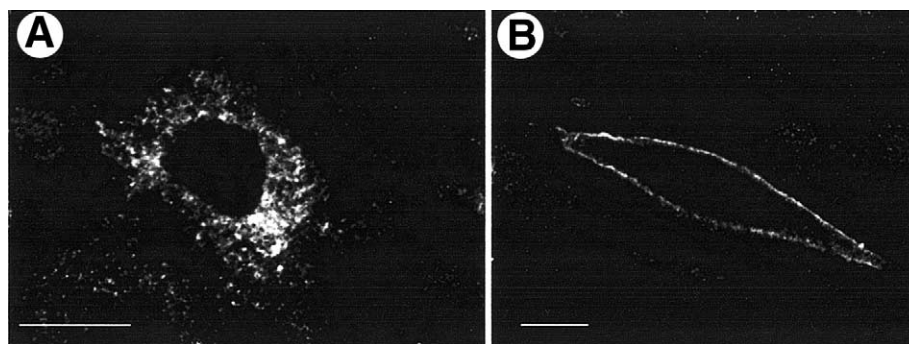


Figure 3. Single confocal images. μ OR immunoreactivity in myenteric neurons from extrinsically denervated ileal segments of guinea pigs that underwent laparotomy with or without pretreatment with NMDA-receptor antagonist. (A) μ OR immunoreactivity is in the cytoplasm in neurons from denervated guinea pig that underwent laparotomy. (B) μ OR immunoreactivity is at the cell surface in neurons from guinea pigs that received intraperitoneal injection of the NMDA-receptor antagonist MRZ 2/596 at 1 mg/kg before and after abdominal laparotomy. Calibration bars: (A) 9 μ m and (B) 4 μ m.

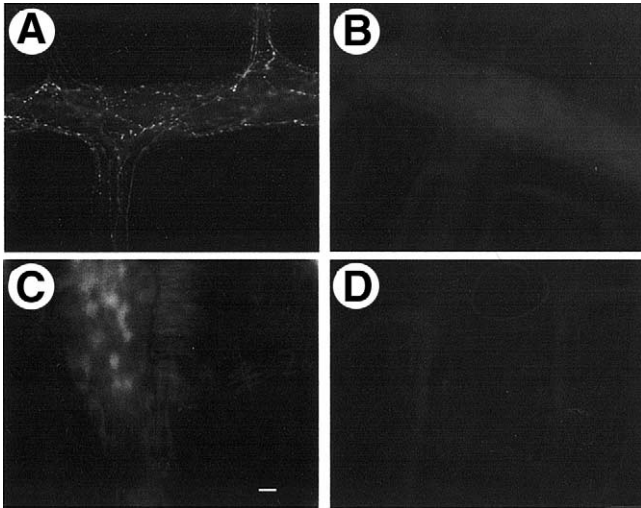


Figure 4. Whole-mount preparations showing dense networks of TH immunoreactive fibers in the myenteric plexus of (A) nondenervated ileal segment and (B) the disappearance of TH immunoreactive fibers in a myenteric plexus of an extrinsically denervated ileal segment. Whole-mount preparations of the myenteric plexus showing numerous NOS immunoreactive neurons in (C) a denervated but not myotomized segment of the ileum and (D) the disappearance of NOS staining at the site of myotomy proving that myotomy was successful in ablating the myenteric plexus, thus severing nerves entering the denervating region from above or below the extrinsic denervation site. Calibration bars: 50 μm .

significantly in neurons from denervated animals that received MRZ 2/596 (1 mg/kg) $19.7\% \pm 2.3\%$, ($P < .001$).

The effectiveness of extrinsic denervation was verified by the localization of TH immunoreactivity that served as a marker for the visualization of extrinsic, sympathetic postganglionic (noradrenergic) axons. Although networks of TH immunoreactive fibers were located in the myenteric ganglia in control but not denervated loops of ileum (Figure 4A), there was a virtual disappearance of TH immunoreactivity in all denervated regions (Figure 4B), and only occasional fibers could be visualized in some cases. Furthermore, the lack of NOS immunoreactive cells at sites of myotomy compared with the dense NOS-containing neurons in denervated but not myotomized ileum, confirmed that the myenteric plexus was severed in each site of the denervation, thus indicating that fibers previously entering the denervated loop between the myotomies had been interrupted (Figure 4C, D).

μOR Internalization in Enteric Neurons of the Ileum In Vitro

μOR internalization was observed in neurons from guinea pig ileum exposed to NMDA (100 $\mu\text{mol/L}$) and D-serine (10 $\mu\text{mol/L}$), 2 co-agonists that activate NMDA receptors (Figure 5A). The exposure of organo-

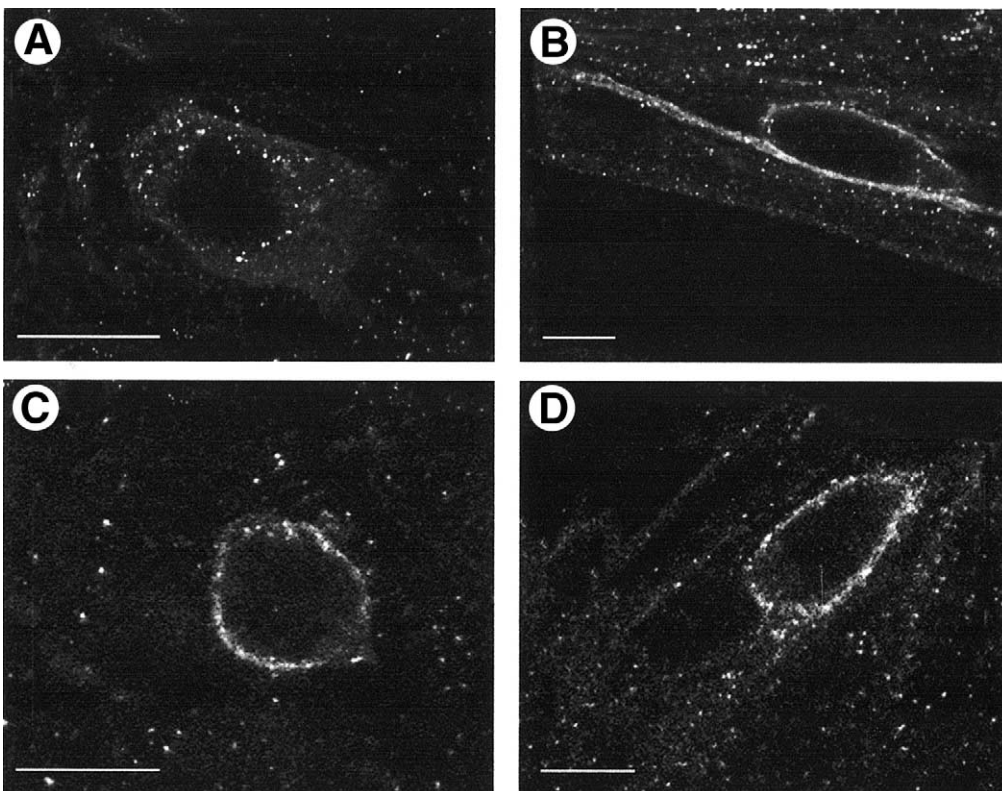


Figure 5. Single confocal images showing μOR immunoreactivity in neurons from guinea pig ileum exposed to (A) NMDA (100 $\mu\text{mol/L}$) plus D-serine (10 $\mu\text{mol/L}$), (B) medium only, (C) NMDA and D-serine plus MK-801 (50 $\mu\text{mol/L}$), or (D) NMDA and D-serine plus AP-5 (50 $\mu\text{mol/L}$). μOR immunoreactivity is predominantly at the cell surface of enteric neurons from preparations not incubated with NMDA plus D-serine or treated with the NMDA-receptor antagonists MK-801 or AP-5 before NMDA stimulation. Calibration bars: (A, C) 10 μm , (B) 5 μm , and (D) 7 μm .

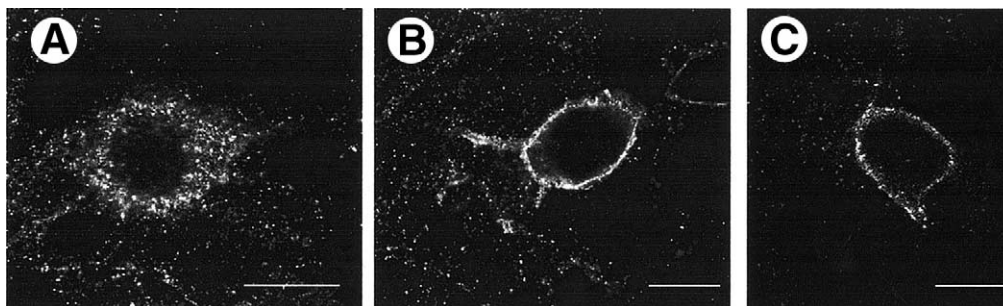


Figure 6. Single confocal images showing μ OR immunoreactivity in neurons from electrically stimulated LMMP preparations. μ OR immunoreactivity is predominantly in (A) the cytoplasm in a neuron stimulated at 20 Hz, whereas it is mostly at the cell surface in neurons from preparations pretreated with the NMDA-receptor antagonist (B) AP-5 or (C) MK-801 at 50 μ mol/L. Calibration bars: (A) 7 μ m and (B, C) 5 μ m.

typic cultures to NMDA and D-serine was kept short (1 min) to minimize possible cell damage caused by neurotoxicity and was followed by 30 minutes of incubation in drug-free medium to allow internalization to occur. No significant or detectable internalization was observed in neurons that either were not exposed to NMDA stimulation (Figure 5B) or were pretreated with the NMDA-receptor antagonist MK-801 (Figure 5C) or AP-5 (Figure 5D) before NMDA stimulation.

Electrically Induced μ OR Endocytosis in LMMP Preparations of the Guinea Pig Ileum

In enteric neurons from LMMP preparations exposed to electrical stimulation (20 Hz), μ OR immunoreactivity was detected predominantly in the cytoplasm (Figure 6A). Pretreatment of LMMP preparations for 15 minutes with 50 μ mol/L of either AP-5 or MK-801 prevented the electrically induced μ OR internalization in myenteric neurons (Figure 6B, C). This indicates that NMDA-receptor antagonists block the release of endogenous opioid induced by electrical stimulation at 20 Hz.

Discussion

The present study shows that (1) μ OR internalization in enteric neurons of the guinea pig induced by abdominal laparotomy occurred in both denervated and nondenervated ileal segments; (2) blockade of NMDA receptors, but not of AMPA/kainate receptors, prevented μ OR internalization in both nondenervated and denervated ileal segments; (3) NMDA stimulation triggered μ OR internalization in enteric neurons from organotypic cultures of the guinea pig ileum, indicating opioid release, which was prevented by NMDA-receptor antagonists; and (4) NMDA-receptor antagonists prevented μ OR internalization in enteric neurons induced by electrical stimulation, indicating inhibition of opioid release. These findings are consistent with our hypothesis that

peripheral NMDA receptors mediate local release of endogenous opioids induced by abdominal surgery, resulting in μ OR internalization in enteric neurons.

The occurrence of translocation of a receptor from the cell surface to the cytoplasm requires agonist-receptor interaction, thus the internalization of μ OR observed in myenteric neurons *in vivo* after surgery is a clear indication of endogenous opioid release.²⁴ The levels of receptor internalization reflect the amount of opioids available for binding to the receptor because μ OR endocytosis is concentration dependent, thus μ OR internalization can serve as a measure of endogenous opioid release induced by μ OR agonists that are capable of triggering receptor endocytosis.²¹ Because the levels of μ OR internalization in denervated and nondenervated ileal segments were comparable, we can conclude that abdominal laparotomy induces local release of opioids through a peripheral neural pathway. The endogenous opioids responsible for μ OR endocytosis in enteric neurons are likely to be mainly enkephalins, which are expressed by myenteric neurons with a distribution similar to the μ OR immunoreactivity.^{10,13} Enkephalins, which have high affinity for the μ OR,⁴⁶ are capable of triggering μ OR endocytosis in enteric neurons *in vitro*.²¹ Furthermore, enkephalins are the opioids almost exclusively released from enteric neurons when electrically stimulated.^{46–48}

Indeed, we showed that electrical stimulation of LMMP preparations at the frequencies used to induce enkephalin release results in massive μ OR internalization in enteric neurons that is blocked by μ OR antagonists.²⁰ μ ORs can be activated by other endogenous opioids including dynorphins, endorphins, and the recently discovered endomorphins 1 and 2.^{49,50} Dynorphins, similar to enkephalins, are localized to enteric neurons⁵¹ but display higher selectivity for k opioid receptors, even though it can bind μ OR with lower affinity compared with enkephalins.^{47,49} By contrast, en-

dorphins, which bind to μ OR and Δ opioid receptors with similar affinity,^{47,49} appear to be confined to endocrine cells of the gut,^{52,53} so they are not likely to be involved in the μ OR internalization in enteric neurons. Finally, endomorphins 1 and 2, which have the highest selectivity and affinity for the μ OR compared with the other opioid receptors and have been proposed as the endogenous μ OR ligands,⁵⁰ have not been reported in the gastrointestinal tract to date.

Functional inhibition of the glutamate NMDA receptors can be obtained through actions at different recognition sites including the primary transmitter (glutamate) site, the ion-channel site, the strychnine-insensitive glycine B site, and the polyamine site (selective for a subtype of NMDA receptors).^{39,54} The prevention of surgery-induced endogenous opioid release by the blockade of NMDA receptors achieved by antagonists acting at different sites (glutamate, glycine B, and ion channel) provides strong evidence that opioid release from enteric neurons is mediated by these receptors. These receptors are likely to be located peripherally as indicated by several lines of evidence. Indeed, even though 2 of the compounds used to block NMDA receptors (ie, the channel-blocking antagonist MK-801 and the glycine B antagonist MRZ 2/576) cross the blood-brain barrier with very good and moderate permeation coefficients, respectively, the other antagonists do not. AP-5 crosses the blood-brain barrier poorly and the glycine B antagonist MRZ 2/596 practically is impermeable at the doses and means of administration used in the present study, strongly suggesting a peripheral effect.^{40,41,44,45,55} It is worthy to point out that the latter antagonist, MRZ 2/596, was the most effective compound in blocking the surgery-induced opioid release as indicated by the marked reduction of the levels of μ OR internalization measured in these animals, which was within the range of μ OR endocytosis observed in normal unstimulated neurons.^{21,24} This is consistent with an involvement of peripheral NMDA receptors in the prevention of opioid release induced by surgery. Furthermore, the participation of a central neural pathway is not likely based on the denervation experiments showing that NMDA-receptor blockade obtained with the peripherally acting MRZ 2/596 occurs in both denervated and nondenervated ileal segments. The lack of effect of CNQX, a selective antagonist for the AMPA/kainate receptors on μ OR internalization induced by abdominal surgery, further supports the concept that the glutamate receptors involved in regulation of local opioid release are NMDA receptors. This is in agreement with previous results showing that NMDA and not AMPA receptors are involved in peptide release in the central nervous system^{32,33,56} Two additional lines of evidence support the hypothesis that NMDA receptors mediate endogenous opioid release in the enteric

nervous system: first, the occurrence of μ OR internalization in myenteric neurons from NMDA stimulated organotypic cultures, which is antagonized by NMDA-receptor antagonists; second, endogenous opioid release due to electrical stimulation is abolished by NMDA-receptor blockade. The observation that NMDA stimulation induces μ OR internalization in enteric neurons caused by local endogenous opioid release confirms that NMDA receptors in the enteric nervous system are functional. These functional data provide indirect evidence for the localization of NMDA receptors on enteric neurons containing and releasing endogenous opioids. Assembly of functional NMDA receptors requires the expression of the NR1 subunit with 1 of 4 NR2 subunits.⁵⁷ The NMDA-receptor subunits NR1 and NR2A/B have been reported to be localized to almost all enteric neurons in the guinea pig,³⁸ thus it is reasonable to assume that opioid-containing enteric neurons also express NMDA receptors.

Glutamate, the major excitatory neurotransmitter in the central nervous system,⁵⁸ is localized to enteric neurons, as well as the vesicular glutamate transporter and the ionotropic glutamate receptors, NMDA, AMPA, and kainate receptors.^{38,59} NMDA receptors mediate cholinergic neurogenic muscle contractions and acetylcholine release in the guinea pig ileum and colon myenteric plexus.^{60–64} Furthermore, both NMDA and AMPA receptors appear to affect peristalsis, with the NMDA receptors mediating inhibition of peristalsis and the AMPA receptors enhancing peristalsis efficiency.^{64,65} NMDA receptors have been implicated in the transmission of noxious stimuli and regulation of neuropeptide release in the central and peripheral nervous system.^{31,33–35,37,66} In our experimental model, the noxious challenge of the surgical procedure and perhaps the mechanosensory stimulation caused by the touching of the eviscerated intestine to return it into the abdominal cavity are likely to be the main stimuli responsible for opioid release resulting in μ OR internalization²⁴ because the tissue was harvested at 30 minutes after surgery, whereas inflammatory response appears to be much more pronounced at later times.⁶⁷ This supports a role of NMDA receptors in the processing of peripheral noxious and perhaps mechanosensory stimuli and it is in agreement with previous reports of endogenous opioid release in other regions of the nervous system in response to the stress of surgery and to noxious stimulation.^{25,26,29,30,68} As stated earlier, these NMDA receptors are likely to be peripheral because μ OR internalization, and thus endogenous opioid release induced by abdominal surgery, is prevented by NMDA-receptor blockade obtained with a peripherally acting NMDA receptor antagonist in extrinsically denervated animals.

In summary, these studies identify an interaction between the peripheral glutamate NMDA receptors and

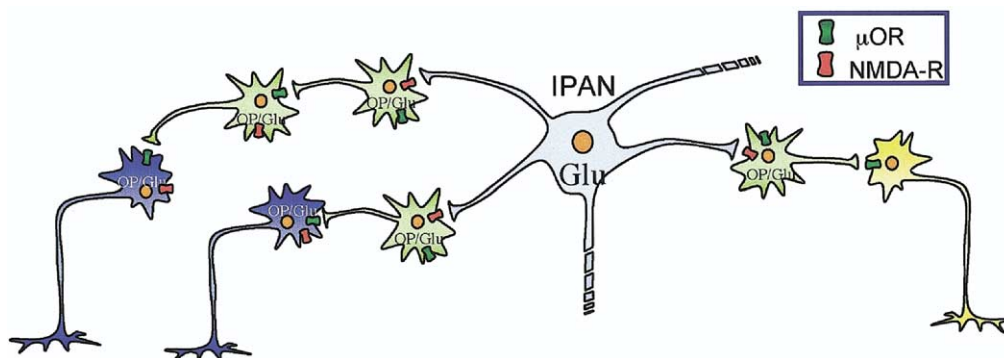


Figure 7. Proposed model of NMDA-induced opioid release. We hypothesize that abdominal surgery with evisceration and manipulation to return the viscera into the abdominal cavity induce distortion of the IPAN processes, thus resulting in depolarization and action potential initiation leading to glutamate (Glu) release from IPANs. This in turn will activate ascending and descending interneurons (shown in *green*) and motor neurons (the ascending shown in *dark blue*, and the descending shown in *yellow*) (either directly or indirectly through interneurons) expressing opioids (OP) and NMDA receptors, thus inducing opioid release, which then will result in μ OR internalization.

μ ORs after the noxious and mechanosensory stimulation of abdominal surgery. Figure 7 shows a model of NMDA-induced opioid release after abdominal surgery. Glutamate is expressed in all cholinergic neurons, which include ascending motor neurons and interneurons, intrinsic primary afferent neurons (IPAN), and descending interneurons.³⁸ Endogenous opioids also are found in ascending and descending motor neurons and interneurons.⁶⁹ μ OR is expressed by about 50% of neurons expressing enkephalin, the main endogenous opioid that activates μ OR in the gut, and we have morphologic evidence that it is expressed by both ascending and descending neurons.¹⁰ Finally, our functional data shown here indicate that NMDA receptors are expressed by opioid neurons. Indeed, NMDA stimulation of organotypic cultures of the ileum induces μ OR internalization, a phenomenon that requires the presence of μ OR ligand, thus indicating the occurrence of endogenous opioid release. Furthermore, NMDA-receptor blockade prevents μ OR endocytosis induced by electrical stimulation of neuromuscular preparations of the ileum, which is caused by endogenous opioid (likely enkephalin) release (see Figures 5 and 6). We hypothesize that abdominal surgery with evisceration and gentle touching of the intestine, which is required to return the viscera into the abdominal cavity, induce distortion of the IPAN processes, thus resulting in depolarization and action potential initiation leading to glutamate release.⁷⁰ Indeed, serosal as well as mucosal distortion have been shown to elicit reflexes resulting in peristaltic movements of the small intestine.⁷¹ IPAN activation in turn will activate ascending and descending interneurons and motor neurons (either directly or indirectly through interneurons) expressing opioids and NMDA receptors, thus inducing endogenous opioid release, which then will result in

μ OR internalization. Blockade of NMDA receptors will inhibit opioid release, thus preventing μ OR internalization. Interactions between NMDA receptors and the opioid system have been reported previously in the nervous system as exemplified by the ability of antagonists acting at NMDA receptors to prevent tolerance to the opioid antinociceptive effect.^{72,73} A combination of peripherally acting NMDA receptors and μ OR antagonists might provide a useful therapeutic approach for preventing the many side effects of abdominal surgery that range from alterations of motility with abdominal pain and discomfort to inflammation and more serious complications such as the paralytic ileus as well as visceral pain with or without inflammation.

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