Neurochemical features of endomorphin-2-containing neurons in the submucosal plexus of the rat colon

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Abstract

AIM: To investigate the distribution and neurochemical phenotype of endomorphin-2 (EM-2)-containing neurons in the submucosal plexus of the rat colon.

METHODS: The mid-colons between the right and left flexures were removed from rats, and transferred into Kreb's solution. For whole-mount preparations, the mucosal, outer longitudinal muscle and inner circular...
In the submucosal plexus of the mid-colon, many EM-2-immunoreactive (IR) and NSE-IR neuronal cell bodies were found in the submucosal plexus of the rat mid-colon. Approximately 6 ± 4.2 EM-2-IR neurons aggregated within each ganglion and a few EM-2-IR neurons were also found outside the ganglia. The EM-2-IR neurons were also immunopositive for ChAT, SP, VIP or NOS. EM-2-IR nerve fibers coursed near ChAT-IR neurons, and some of these fibers were even distributed around ChAT-IR neuronal cell bodies. Some EM-2-IR neuronal cell bodies were surrounded by SP-IR nerve fibers, but many long processes connecting adjacent ganglia were negative for EM-2 immunostaining. Long VIP-IR processes with many branches coursed through the ganglia and surrounded the EM-2-IR neurons. The percentages of the EM-2-IR neurons that were also positive for ChAT, SP, VIP or NOS were approximately 91% ± 2.6%, 36% ± 2.4%, 44% ± 2.5% and 44% ± 4.7%, respectively, but EM-2 did not co-localize with CGRP.

CONCLUSION: EM-2-IR neurons are present in the submucosal plexus of the rat colon and express distinct neurochemical markers.

Key words: Endomorphin-2; Submucosal plexus; Enteric neuron; Colon; μ-opioid receptor

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INTRODUCTION

It is well known that morphine is a selective exogenous agonist of the μ-opioid receptor (MOR) and is a widely used analgesic drug in the clinic. Unfortunately, morphine can cause gastrointestinal disorders, such as constipation, nausea and emesis, when used for a long period of time[1-4]. To explore the mechanism of the gastrointestinal disorders caused by morphine and endogenous opioid peptides, pharmacological and morphological studies have been performed in the gastrointestinal tract; however, the regulatory mechanisms of morphine and endogenous opioid peptides relating to gastrointestinal activity remain unclear.

The results of many previous studies have shown that there are at least three types of opioid receptors, i.e., MORs, δ-opioid receptors (DORs) and κ-opioid receptors (KORs), and the endogenous ligands of these receptors, including endomorphin (EM), enkephalin and dynorphin, are widely distributed in the gastrointestinal tract[5-8]. In the gastrointestinal tract, MOR immunoreactivity is the most abundant compared to other opioid receptors, particularly in the colon[1,9,10]. After the MOR was knocked out in the mouse, the effects of both MOR agonists and DOR or KOR agonists on gastrointestinal disorders nearly disappeared[11]. Endomorphin-1 (EM-1) and endomorphin-2 (EM-2), two endogenous ligands for the MOR, selectively bind to the MOR with high affinity[12]. The distribution of EM-immunoreactive (IR) structures is generally similar to that of the MOR in the nervous system, including the myenteric plexus[13,14], but not the submucosal plexus[14,15] of the enteric nervous system (ENS). Exploring the distribution and chemical nature of the EM-IR structures in the submucosal plexus is fundamental to clarifying the regulatory mechanisms of morphine and opioid peptides. In our previous study, we reported that only EM-2-containing neurons, but not EM-1-IR neurons, were present in the submucosal plexus of the rat colon. Therefore, we examined the distribution patterns and neurochemical phenotypes of submucosal EM-2-containing neurons in the rat colon with immunofluorescence histochemical staining of whole-mount preparations of the rat mid-colon.

MATERIALS AND METHODS

Experimental procedures
Male Sprague-Dawley rats, weighing 220-250 g, were
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provided by the Experimental Animal Center of the Fourth Military Medical University (Xi’an, China). All the protocols described below were approved by the Committee of Animal Use for Research and Education of the Fourth Military Medical University. In accordance with the ethical guidelines for animal research, all efforts were made to minimize the number of animals used and the suffering of animals[16].

**Tissue collection**

After the rats (n = 7) were anesthetized with ether, their abdominal cavities were opened, and the mid-colons between the right and left flexures were removed and stored in iced Kreb’s solution containing the following (in mmol/L): 115.5 NaCl, 4.16 KCl, 1.16 NaH2PO4, 21.9 NaHCO3, 11.1 glucose, 1.16 MgSO4 and 2.5 CaCl2. Thereafter, colonic segments were immersed in Kreb’s solution at room temperature after rinsing. Finally, the mesentery was trimmed.

**Organotypic culture**

The dissected mid-colons were transferred into Kreb’s solution containing 50 mg/mL gentamicin, 2.5 mg/mL amphotericin B, 10% fetal bovine serum, and 4 mmol/L nicardipine and bubbled with 95% O2 and 5% CO2 at 37°C for 2 h, followed by incubation for 5 to 6 h in the same medium with 1% colchicine added to the mixed medium to enhance EM-2 immunoreactivities in the neuronal somata. Thereafter, the intestinal lumen was distended with a fixative solution containing 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). The sections were immersed into the same fixative for 6 to 8 h at 4°C and were then opened longitudinally.

**Tissue preparation**

For whole-mount preparations, the mucosal, outer longitudinal muscle and inner circular muscle layers of the tissues were separated from the submucosal layer attached to the submucosal plexus. The whole-mount preparations from each rat mid-colon were mounted onto seven gelatin-coated glass slides.

**Immunofluorescence histochemical double-staining**

The whole-mount sections (n = 7) obtained from 7 male rats were processed for immunofluorescence histochemical double-staining of EM-2 with calcitonin gene-related peptide (CGRP), choline acetyltransferase (ChAT), nitric oxide synthetase (NOS), neuron-specific enolase (NSE), substance P (SP) and vasoactive intestinal peptide (VIP). The immunofluorescence histochemical double-staining was performed by incubating the slides with the following solutions: (1) The respective mixture of rabbit antiserum against EM-2 (AB5104, 1:200; Chemicon, Billerica, MA, United States) with mouse antiserum against NSE (MAB314, 1:200; Chemicon), goat antiserum against ChAT (IHCR1008-6, 1:500; Chemicon), rat antiserum against SP (MAB356, 1:500; Chemicon), mouse antiserum against VIP (SC-25347, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, United States), mouse antiserum against NOS (N2280, 1:200; Sigma, Saint Louis, MO, United States) or goat antiserum against CGRP (ab36001, 1:200; Abcam, Cambridge, United Kingdom) in 0.01 M phosphate-buffered saline (PBS, pH 7.4) containing 5% (v/v) normal goat serum (NGS), 0.3% (v/v) Triton X-100, 0.05% (w/v) NaN3 and 0.25% (w/v) carrageenan (PBS-NGS, pH 7.4) for 72 hr at 4°C; (2) The respective mixture of biotinylated donkey anti-rabbit IgG (BA-1000, 1:200; Vector, Burlingame, CA, United States) to stain EM-2 and Alexa 594-labeled donkey anti-mouse IgG (A21203, 1:500; Invitrogen, Eugene, OR, United States) for NSE, VIP and NOS staining, Alexa 594-labeled donkey anti-goat IgG (A-11058, 1:500; Invitrogen) for ChAT and CGRP staining, or Cy3-labeled donkey anti-rat IgG (AP189C, 1:200; Chemicon) for SP staining in PBS-NGS for 4 hr at 4°C; and (3) Fluorescein isothiocyanate (FITC)-labeled avidin D (A-2001, 1:1000; Vector) in PBS containing 0.3% Triton X-100 (PBS-X, pH 7.4) for 4 h at room temperature.

The sections were cover-slipped with 0.01 M PBS containing 50% (v/v) glycerin and 2.5% (w/v) triethylene diamine (an anti-fading reagent).

**Control test**

The rabbit antiserum against EM-2 was prepared against a full-length synthetic peptide (Tyr-Pro-Phe-Phe-NH2) from EM-2 conjugated to bovine serum albumin (BSA). We tested the specificity of the antibody by incubating it with either 2 μmol/L EM-2 or 2 μmol/L EM-1 peptide, as previously described[17,18]. Normal mouse serum was used to confirm the specificities of the mouse antibodies against NSE, VIP and NOS. Using replacement tests, normal goat serum was used to confirm the specificities of the goat antibodies against ChAT and CGRP, and normal rat serum were used to confirm the specificity of the rat antibody against SP. In the present study, when the anti-EM-2 antibody was pre-absorbed with either synthetic homologous or heterologous peptides and normal mouse, goat and rat sera were used to replace the mouse, goat and rat antibodies against NSE, VIP and NOS, ChAT and CGRP, and SP, respectively, there was no immunopositive staining observed in the sample preparations. The antibodies were thus considered to be specific and reliable (data not shown).

**Cell quantification**

After staining, all the fluorescence-labeled sections were observed with a confocal laser scanning microscope (Olympus FV1000; Tokyo, Japan) equipped with the appropriate filters for FITC (excitation 490 nm; emission 520 nm), Alexa 594 (excitation 590 nm; emission 617 nm) and Cy5 (excitation 650 nm; emission 667 nm). To estimate the extent of the co-
neuronal cell bodies that expressed both markers.

**Statistical analysis**
The results are expressed as the mean ± SD.

**RESULTS**

**Distribution of EM-2-IR neurons in the submucosal plexus of the rat colon**

In the present study, many EM-2-IR and NSE-IR neuronal cell bodies were found in the submucosal plexus of the rat mid-colon (Figure 1). The EM-2-IR proteins were localized predominantly in the cytoplasm of the neuronal cell bodies. The EM-2-IR neuronal cell bodies commonly had an oval-shaped cell body with several thick processes protruding from the soma and a long process, which could extend up to 95 μm or considerably longer (Figure 1A). The average length and width of the EM-2-IR neuronal cell bodies were 34.6 ± 9.8 μm and 16.5 ± 5.2 μm, respectively. The antiserum against NSE labeled all the neuronal components, including neuronal cell bodies and varicose and non-varicose nerve fibers (Figure 1B). In the submucosal layer, the ganglia were quite dispersed. The relative quantification of the number of perikarya expressing EM-2 was estimated by counting the number of positive cells per ganglion in the colonic submucosal layer. Approximately 6 ± 4.2 EM-2-IR neurons aggregated within each ganglion, and a few EM-2-IR neurons were also found outside the ganglia (Figure 1C).

The results of the immunofluorescence histochemical double-staining revealed that nearly all of the EM-2-IR neurons also contained NSE-immunopositive products in their neuronal cell bodies (Figure 1C). However, only approximately 68% ± 1.5% of the NSE-IR neuronal cell bodies had EM-2-immunopositive staining in the submucosal ganglia (Table 1). Some of the long EM-2-IR processes with or without varicosities connecting adjacent ganglia formed a nerve fiber network between each ganglion. These results indicated that all the EM-2-IR cells were neurons and that approximately 2/3 of localization of EM-2 with CGRP, ChAT, NOS, NSE, SP, and VIP, 6 sets of representative whole-mount sections from the mid-colons were selected (n = 6). Ganglia, which have a clear boundary and neuronal cell outline, were randomly selected from each specimen for this analysis. In each specimen, approximately 6-7 ganglia were selected to quantify the neuronal cells, and the total number of ganglia was approximately 35 in each set. Within each ganglion in the submucosal plexus, we counted the number of EM-2-IR neuronal cell bodies, the number of neuronal cell bodies labeled with the other markers, and the number and percentage of neuronal cell bodies that expressed both markers.

### Table 1  Percentages of submucosal neurons with the co-localization of endomorphin-2 and various markers

| Marker pair     | Submucosal ganglion neurons (n1, n2, mean ± SD) | Double-labeled neurons (n1, mean ± SD) | Percentage (%) (mean ± SE) |
|-----------------|-----------------------------------------------|---------------------------------------|---------------------------|
| CGRP/EM-2       | 1.58 ± 1.4 and 5.0 ± 1.1                       | 0                                     | CGRP of EM-2 (n1/n2)      |
| ChAT/EM-2       | 6.0 ± 2.4 and 3.5 ± 1.6                        | 3.2 ± 1.2                             | ChAT of EM-2 (n1/n2)      |
| NOS/EM-2        | 2.3 ± 1.1 and 5.2 ± 1.4                        | 2.3 ± 1.1                             | NOS of EM-2 (n1/n2)       |
| NSE/EM-2        | 6.2 ± 3.7 and 4.2 ± 2.9                        | 4.2 ± 2.9                             | NSE of EM-2 (n1/n2)       |
| SP/EM-2         | 2.4 ± 1.0 and 5.0 ± 1.2                        | 1.8 ± 0.6                             | SP of EM-2 (n1/n2)        |
| VIP/EM-2        | 1.5 ± 0.5 and 5.0 ± 1.3                        | 1.5 ± 0.5                             | VIP of EM-2 (n1/n2)       |

VIP: Vasoactive intestinal peptide; EM-2: Endomorphin-2; SP: Substance P; NSE: Neuron-specific enolase; NOS: Nitric oxide synthetase; CGRP: Calcitonin gene-related peptide; ChAT: Choline acetyltransferase.

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the total number of neuronal cell bodies in the ganglia contained EM-2.

Co-localization of EM-2 with cytochemical markers within submucosal neurons
The co-localization of EM-2 with ChAT, SP, VIP, NOS and CGRP in submucosal neurons was investigated. ChAT immunoreactivities were localized predominantly in the neuronal soma. EM-2-IR nerve fibers coursed near ChAT-IR neurons, and some of these fibers were even distributed around ChAT-IR neuronal cell bodies (Figure 2B and E). A few EM-2-IR neuronal cell bodies were surrounded by ChAT-IR nerve fibers and terminals. SP-IR neurons exhibited a slightly round or oval-shaped neuronal cell body with one long process and several short and lamellar processes around the somata. Some EM-2-IR neuronal cell bodies were surrounded by SP-IR nerve fibers, but many long processes connecting adjacent ganglia were negative for EM-2 immunostaining (Figure 2A and D). In the submucosal layer, a few VIP-IR neuronal cell bodies were labeled. VIP-containing neurons had a slightly oval-shaped neuronal cell body with several short and lamellar processes as well as one long process around their somata. Long VIP-IR processes with many branches coursed through the ganglia and surrounded the EM-2-IR neurons (Figure 3A and D). The anti-NOS antibody staining revealed homogeneous immunoreactivity of the neuronal cell bodies without nuclear labeling. The NOS-IR neurons were peripherally located in the submucosal ganglia. NOS-IR processes were only labeled at their origin and could not be followed over a long distance (Figure 3B and E). In the submucosal plexus, the percentages of the EM-2-IR neurons that were also positive for ChAT, SP, VIP or NOS were approximately 91% ± 2.6%, 36% ± 2.4%, 44% ± 2.5% and 44% ± 4.7%, respectively (Table 1). In contrast, EM-2 was detected in ChAT-, SP- and VIP-IR neurons in 53% ± 2.8%, 75% ± 3.4% and 73.3% ± 2.6% of the cells, respectively, and all the NOS-IR neurons co-localized with EM-2-immunoreactivities (Table 1).

The co-localization of EM-2 and CGRP was not found in submucosal neurons, but CGRP-IR nerve fibers were observed to make close contacts with the EM-2-IR neuronal cell bodies and the large processes of these cells (Figure 4).

After the antiserum against EM-2 was pre-absorbed with synthetic homologous or heterologous peptides, there was no positive EM-2-IR immunostaining observed in the submucosal layer (Figure 5B).

DISCUSSION
In the gastrointestinal tract, submucosal neurons principally control the mucosal absorption and secretion of water and electrolytes[19-21]. To meet the needs of digestion, submucosal neurons provide a
precise and complex regulation of epithelial function and blood flow, such that the absorption and secretion of water and electrolytes maintain a balance.\(^{[22]}\)

Previously, the general explanation for the constipating effect of opioids was that opioids suppress propulsive motility and gastrointestinal transit, thereby extending the time of water and electrolyte absorption. However, this explanation is currently not fully accepted because opioids can modulate both gastrointestinal motility and mucosal secretion as well as water/electrolyte metabolism.\(^{[8,23-25]}\)

In the colon, MOR-IR products are found in neurochemically distinct classes of enteric neurons, and the proportion of MOR-labeled perikarya in the submucosal plexus is more abundant than that in the myenteric plexus.\(^{[15,26,27]}\) MOR agonists can inhibit colon water and electrolyte secretion, which can contribute to opioid-induced constipation. Submucosal secretomotor neurons release acetylcholine and VIP, which can activate epithelial cell chloride channels via muscarinic and VIP receptors on the basolateral surface of enterocytes. Chloride moves from the enterocyte cytoplasm into the gut lumen, and $H_2O$ follows the chloride via an osmotic mechanism. The MOR on secretomotor neurons is activated by opioid drugs and suppresses acetylcholine and VIP release.

Figure 3 Immunofluorescence histochemical double-staining of the whole-mount sections of the rat mid-colon. A and D: The endomorphin-2 (EM-2)-immunoreactive (IR) neurons; B and E: Vasoactive intestinal peptide (VIP)-IR neurons (B) and nitric oxide synthetase (NOS)-IR neurons (E) in the submucosal layer; C and F: Merged images of (A, B) and (D, E). Arrows point to neurons containing both EM-2 and VIP or NOS. Scale bar = 45 μm.

Figure 4 Immunofluorescence histochemical double-staining of the whole-mount sections of the rat mid-colon. A and B: Distributions of endomorphin-2-immunoreactive (IR) neurons (A) and calcitonin gene-related peptide-IR neurons (B) in the submucosal layer; C: Merged images of (A) and (B). Scale bar = 45 μm.
resulting in a decrease in chloride secretion and osmotic water movement\(^\text{[29,30]}\). However, to date, the source of the endogenous ligand of the MOR in the gastrointestinal tract has remained unclear.

In the present study, EM-2-IR cells were found in the submucosal layer of the rat mid-colon, and all the EM-2-IR cells co-localized with the general neuronal marker NSE, which labeled all the neuronal components\(^\text{[35]}\). This result confirmed that the EM-2-IR cells were enteric neurons, which account for a large proportion of submucosal neurons. EM-2-IR neurons, similar to cells found in the myenteric plexus of the colon, usually exhibit a round or oval cell body with short and thick dendrites protruding from the stomata and a long axonal process\(^\text{[14]}\).

The present double-staining results indicated that EM-2 co-localized with ChAT, SP or VIP in the perikarya of submucosal neurons. The EM-2-IR nerve fibers coursed near ChAT-IR neurons, and some of these fibers even surrounded ChAT-IR neuronal cell bodies. In the submucosal plexus, approximately half of the neurons expressing ChAT are secretomotor neurons to epithelial cells\(^\text{[30-35]}\). Similarly, VIP and SP are also predominantly located in secretomotor neurons\(^\text{[20,28,35-38]}\). Acetylcholine and VIP are released by secretomotor neurons at neuroepithelial junctions\(^\text{[25]}\). An autoradiographic localization of the MOR binding sites has revealed that the MOR is also expressed by nerve terminals in the mucosal layers, as well as in the submucosal plexus, of the gastrointestinal tract\(^\text{[10,26]}\); and MOR activation inhibits acetylcholine and VIP release in submucosal neurons of the rat colon and cat intestine\(^\text{[32,36,39]}\). Based on the results of these studies, we speculated that the EM-2 released by submucosal neurons may modulate the mucosal absorption and secretion of water and electrolytes via secretomotor neurons\(^\text{[29,30]}\). Functional and electrophysiological studies provide evidence to support our hypothesis. Functional data obtained from rat intestines suggest that the MOR participates in the regulation of fluid and electrolyte transport\(^\text{[40]}\). In addition, ChAT and SP also transmit sensory information of the intestinal lumen\(^\text{[20,38,41]}\). The present results have demonstrated that some EM-2-IR neurons are surrounded by SP- and ChAT-IR nerve fibers. These nerve fibers may be primary afferent fibers originating from neurons in the submucosal ganglia.

EM-2 is also co-localized with NO in the perikarya of submucosal neurons. NO has been found in interneurons or inhibitory neurons in the submucosal plexus, and has been shown to participate in modulating the circular muscle\(^\text{[38]}\). The MOR is expressed by enteric neurons, but not on smooth muscle membranes of the rat intestine\(^\text{[42,43]}\). EM-2-IR neurons may also modulate the contraction of circular muscle similar to interneurons. However, the EM-2-IR neurons did not co-localize with CGRP, a marker for primary afferent neurons in the rat colon\(^\text{[44,45]}\), but the CGRP-IR nerve fibers surrounded these EM-2-IR neurons, indicating that the EM-2-IR neurons may be regulated by CGRP. In addition, MOR proteins have also been observed on nerve fibers surrounding blood vessels\(^\text{[10,26]}\). This result leads to the hypothesis that EM-2 is involved in the regulation of blood flow via nerve ending mechanisms, particularly in the submucosal and mucosal layers.

In conclusion, EM-2-IR neurons are present in the submucosal plexus of the rat colon, and EM-2 co-localized with several neurochemical makers in distinct populations of submucosal neurons. Therefore, EM-2 may mediate the secretory response of the rat colon via secretomotor neurons, which endogenously express the specific opioid receptor, MOR.

**COMMENTS**

**Background**

Morphine is a selective exogenous agonist of the \(\mu\)-opioid receptor (MOR) and is a widely used analgesic drug in the clinic. Unfortunately, morphine can cause gastrointestinal disorders when used for a long period of time. However, the regulatory mechanisms of morphine and MOR relating to gastrointestinal activity remain unclear. Previous studies have showed that MORs are widely distributed in the gastrointestinal tract, particularly in the colon, and modulate the gastrointestinal activity, but the neural pathway is unclear. Endomorphins (EMs) are the endogenous ligands for the MOR. The distribution of EM-immunoreactive (IR) structures is generally similar to that of the MOR in the nervous system, but not the submucosal plexus of the enteric nervous system.
Research frontiers
The authors aimed to explore the distribution and chemical nature of the EM-IR structures in the submucosal plexus to clarify the regulatory mechanisms of MOR.

Innovations and breakthroughs
This study demonstrates for the first time that EM-2-IR neurons are present in the submucosal plexus of the rat colon, and EM-2 co-localized with several neurochemical markers in distinct populations of submucosal neurons.

Applications
The authors hypothesize that EM-2 may mediate the secretory response of the rat colon via secretomotor neurons, which endogenously express the specific opioid receptor, MOR.

Terminology
The ENS is an intrinsic neuronal network within the gut wall, extending over the entire length of the gastrointestinal tract. The ENS is responsible for the intrinsic control and coordination of motility, blood flow and secretion to support normal digestion. It consists of two ganglionated plexuses: the submucosal plexus is located near the luminal side between the mucosa and circular muscle layer, and the myenteric plexus is embedded between the outer longitudinal and inner circular muscle layer of the intestine.

Peer-review
This is a good descriptive study in which the authors explored the distribution and chemical nature of the EM-IR structures in the submucosal plexus. To clarify the regulatory mechanisms of MOR, it provides the morphological foundation.

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