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Loperamide Inhibits Tachykinin NK_3-Receptor-Triggered Serotonin Release Without Affecting NK_2-Receptor-Triggered Serotonin Release From Guinea Pig Colonic Mucosa

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Abstract. The effect of loperamide on tachykinin NK_2- and NK_3-receptor-mediated 5-HT outflow from guinea pig colonic mucosa was investigated in vitro. The selective tachykinin NK_2-receptor agonist [β-Ala^8]-neurokinin A_{4-10} (βAla-NKA) or the selective NK_3-receptor agonist senktide elicited an increase in 5-HT outflow from whole colonic strips, but not from mucosa-free muscle layer preparations. The enhancing effect of βAla-NKA and senktide was prevented by the selective NK_2-receptor antagonist GR94800 or the selective NK_3-receptor antagonist SB222200. Loperamide concentration-dependently suppressed the senktide-evoked 5-HT outflow, but failed to affect the βAla-NKA-evoked 5-HT outflow. The κ-opioid receptor antagonist nor-binaltorphimine or the δ-opioid receptor antagonist naltrindole displaced the concentration-response curve for the suppressant action of loperamide to the right without significant depression of the maximum. However, the μ-opioid receptor antagonist CTOP did not affect the suppressant effect of loperamide. We concluded that the NK_3 receptor-triggered 5-HT release from colonic mucosa is suppressed by loperamide-sensitive mechanisms, whereas the NK_2-receptor-triggered 5-HT release is loperamide-insensitive. Our data also suggest that the suppressant effect of loperamide is probably mediated by the activation of κ- and δ-opioid receptors located on intrinsic neurons.

Keywords: colon, serotonin, loperamide, tachykinin, opioid receptor

Introduction

5-Hydroxytryptamine (serotonin, 5-HT) has long been recognized as an important messenger substance, which regulates colonic motility or secretion by acting via multiple receptor subtypes (1 – 4). In the colon, 5-HT is found predominantly in the enterochromaffin (EC) cells of the mucosa (5), but the precise mechanism controlling the release of 5-HT from the colonic EC cells remains poorly understood. As an enhanced release of 5-HT from EC cells has been linked to diarrhea or accelerated colonic transit in patients with carcinoid tumors (6), a comprehensive understanding of the regulatory mechanism(s) of 5-HT release may lead to new ways to control defecation or diarrhea in bowel disorders.

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Loperamide is a widely used antidiarrhoeal that primarily acts at nanomolar concentrations through activation of opioid receptors in the intestinal tract (7). At somewhat higher concentrations, loperamide blocks calmodulin activity and calcium channels (8). The gastroenterologic use of loperamide has recently been extended to the alleviation of diarrhea in patients with irritable bowel syndrome (9). However, whether loperamide affects the release of 5-HT from the EC cells is still largely unclear.

We have recently shown that isolated guinea pig proximal colon is a useful preparation for studying regulatory mechanisms of 5-HT release from the colonic mucosa (10 – 12). In addition, we reported that using the isolated guinea pig proximal colon, the selective NK_2-receptor agonist [βAla^8]-neurokinin A_{4-10} (βAla-NKA) is capable of inducing tetrodotoxin-resistant 5-HT release from the colonic mucosa, whereas the selective

NK_3-receptor agonist senktide is capable of inducing 5-HT release from the colonic mucosa through an action on myenteric neurons (13). We were thus interested in investigating whether loperamide affects the tachykinin NK_2/NK_3-receptors-mediated 5-HT release from guinea pig colonic mucosa.

To accomplish this goal, we tested whether loperamide affects the release of 5-HT from guinea pig colonic mucosa, evoked by the selective NK_2-receptor agonist βAla-NKA and the selective NK_3-receptor agonist senktide.

Materials and Methods

Tissue preparation

All procedures were performed in accordance with the Dokkyo University School of Medicine animal care guidelines, which confirm to the Guide for the Care and Use of Laboratory animals (NIH publication No. 85-23, revised 1985). Male Dunkin-Hartley guinea pigs (250 – 500 g body weight) were purchased from Shizuoka Laboratory Animal Center, Inc. (Shizuoka). Guinea pigs were anesthetized with enflurane and bled via the femoral artery. A segment of the proximal colon, 3 – 6-cm distal from the caecum was removed, and the luminal contents were washed out with a modified Tyrode's solution (136.8 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.05 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, 5.56 mM glucose, and 0.06 mM $EDTANa_2$). Two preparations were used in this study. The first preparation was the whole intact colon (1.0 cm in length), which contained all layers of the intestinal wall. The second preparation consisted of a mucosa-free longitudinal/circular muscle with an intact adherent myenteric plexus, which was obtained by removal of the underlying mucosa, as described in a previous study (1). These two distinct isolated preparations were suspended in a longitudinal direction under a 4.9-mN load in 2-ml tissue baths filled with modified Tyrode's solution at 37°C and were aerated with 95% O_2 / 5% CO_2. To minimize endogenous monoamine oxidase A activity, Tyrode's solution contained 1 μM clorgyline. The tissue preparations were allowed to equilibrate for 60 min with fresh replacement of the bathing medium every 5 min. Following the equilibration period, the experiments were conducted by collecting the bathing medium every 10 min. The medium obtained during the first 60 – 70 min was discarded. βAla-NKA or senktide was added to the incubation medium from 90 to 110 min. Antagonists were added to the incubation medium 30 min before the start of the collection period. In some experiments, the inhibitory effect of loperamide on senktide-evoked 5-HT outflow was expressed as the % change from the control response. At the end of the collection period, the tissue preparations were blotted and weighed.

Measurement of 5-HT and 5-hydroxyindoleacetic acid

The collected medium was lyophilized, dissolved in 0.4 M perchloric acid (200 μl), and passed through a 0.45-μm filter (Dismic-13CP; Advantec, Tokyo). 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) levels in the filtrate were measured by high-performance liquid chromatography (HPLC) with electrochemical detection (ECD-300; Eicom, Kyoto) as described previously (10). Known concentrations of 5-HT and 5-HIAA (Sigma, St Louis, MO, USA) were used as standards. The limit of detection was between 50 fmol and 100 fmol for 5-HT and between 25 fmol and 50 fmol for 5-HIAA per injection. The separation of 5-HT and 5-HIAA was achieved by a reverse-phase column (length: 110 mm, inner diameter: 4 mm, C-18: 3 μm; BAS), using a mobile phase consisting of 0.1 M monochloroacetic acid, 1 mM EDTA, 60 $mg \cdot l^{-1}$ sodium octylsulfate, and 8% acetonitrile (pH 3.2) at a flow rate of 0.5 $ml \cdot min^{-1}$. Aliquots (20 μl) of the filtrate were injected directly into the HPLC column. The levels of 5-HT and 5-HIAA in the incubation medium are expressed in units of $pmol \cdot g^{-1} \cdot 10\ min^{-1}$. The results are expressed as a percentage of the mean outflow observed during the first two collection samples (70 – 90 min of incubation) of the individual experiments.

Drugs

βAla-NKA, CTOP, GR94800, loperamide hydrochloride, naltrindole hydrochloride, nor-binaltorphimine dihydrochloride, and SB222200, senktide were purchased from Sigma Chemicals. Tetrodotoxin was purchased from Wako (Osaka). All drugs were dissolved in distilled water with the following exceptions: GR94800 (100 μM) and SB222200 (100 μM) were dissolved in 100% dimethylsulphoxide. All subsequent dilutions of the drugs were made with distilled water. The vehicles had no effects on agonists-evoked 5-HT outflow.

Analyses of data

Data are expressed as means ± S.E.M. from n experiments. The significance of the differences between two mean values was assessed using Student's *t*-test. For the comparison of one control with several experimental groups, the significance of differences was evaluated by one-way ANOVA, followed by the Newman-Keuls post hoc test. A value of $P<0.05$ was considered statistically significant. The pEC_{50} was the negative logarithm of the molar concentration of agonist causing 50% of the maximal effect. The pEC_{50} values were calculated according to the method of Van Rossum (14).

Results

General

The mean spontaneous outflow of 5-HT and 5-HIAA from the whole colonic strips incubated in modified Tyrode's solution (contained 1 μM clorgyline, a monoamine oxidase A inhibitor) in the absence of test compounds (determined between 70 and 90 min of incubation) amounted to 135.9 ± 15.8 and 38.5 ± 4.9 pmol $\cdot$ g^{-1} $\cdot$ 10 min^{-1}, respectively (n = 15). Similar to previous observations (13), the spontaneous outflow of 5-HT from the whole colonic strips did not change significantly during the observation period in control experiments (Fig. 1). Addition of the selective NK_3-receptor agonist senktide to the incubation medium (100 nM, the maximally effective concentration, from 90 to 110 min) caused a transient increase in the outflow of 5-HT; 5-HT outflow was enhanced to 307.7 ± 47.8 pmol $\cdot$ g^{-1} $\cdot$ 10 min^{-1} (n = 8, 240.6 ± 24.4% compared to the initial outflow) (Fig. 1). The senktide-evoked 5-HT outflow was significantly reduced to 158.9 ± 13.6 pmol $\cdot$ g^{-1} $\cdot$ 10 min^{-1} (n = 4, $P<0.01$, from 90 to 100 min), when the selective NK_3-receptor antagonist SB222200 (300 nM) (15) was present from the start of incubation. The senktide-evoked 5-HT outflow and basal 5-HT outflow were not detectable after removal of the underlying mucosa (n = 4) (Fig. 1).

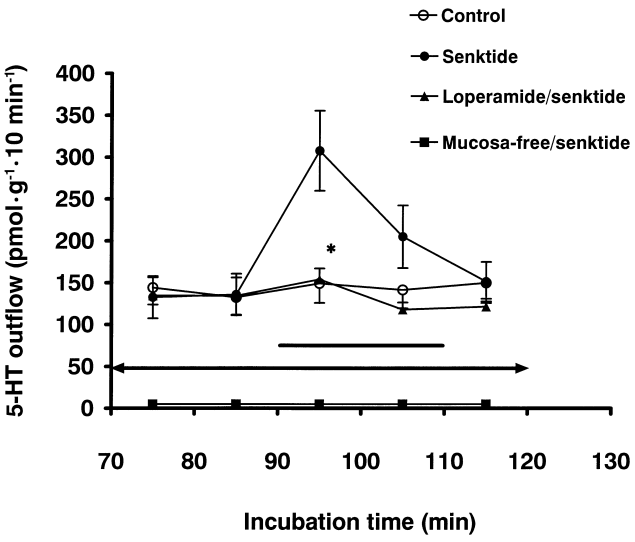

Fig. 1. Effects of senktide (100 nM) in the absence or presence of loperamide (10 nM) on the outflow of 5-HT from the guinea pig isolated whole colonic strips and mucosa-free muscle layer preparations. Senktide was present from 90 to 110 min of incubation, as indicated by the horizontal bar. Loperamide was present from 70 to 120 min of incubation, as indicated by the arrowhead bar. Ordinate scale: outflow of 5-HT, expressed as pmol $\cdot$ g tissue^{-1} $\cdot$ 10 min^{-1}. Each point represents the mean $\pm$ S.E.M. (vertical bars) from four to eight experiments. *$P<0.01$: senktide alone *vs* senktide/loperamide.

Addition of the selective NK_2-receptor agonist βAla-NKA to the incubation medium (1 μM, the maximally effective concentration, from 90 to 110 min) caused a sustained increase in the outflow of 5-HT; 5-HT outflow was enhanced to 220.3 ± 26 pmol $\cdot$ g^{-1} $\cdot$ 10 min^{-1} (n = 9) (Fig. 3). The βAla-NKA-evoked 5-HT outflow was significantly reduced to 153 ± 7.7 pmol $\cdot$ g^{-1} $\cdot$ 10 min^{-1} (n = 4, $P<0.05$, from 100 to 110 min), when the selective NK_2-receptor antagonist GR94800 (30 nM) (16) was present from the start of incubation. The βAla-NKA-evoked 5-HT outflow and basal 5-HT outflow were not detectable after removal of the underlying mucosa (n = 4) (Fig. 3).

Effects of loperamide

When added 20 min before the senktide-stimulus, loperamide (0.01 – 100 nM) elicited a concentration-dependent and significant decrease in the senktide (100 nM)-evoked maximal 5-HT outflow from the whole colonic strips ($pEC_{50} = 9.89 \pm 0.2$, n = 6) (Figs. 1 and 2). Loperamide, applied at the concentrations of 10 and 100 nM, suppressed the senktide-evoked maximal 5-HT outflow to 123.3 ± 16.2% and 123.1 ± 12.6%, respectively. In contrast, loperamide (100 nM) failed to affect the βAla-NKA (1 μM)-evoked maximal 5-HT outflow (Fig. 3).

Several antagonists with some degree of selectivity for the different opioid receptor subtypes were tested against the suppressant effect of loperamide. None of the antagonists investigated had a significant influence on basal 5-HT outflow. As shown in Fig. 4, the selective

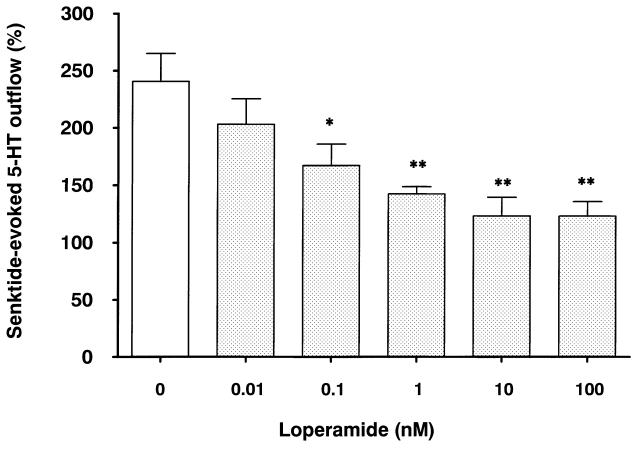

Fig. 2. Effects of increasing concentration of loperamide on the maximal outflow of 5-HT from the whole colonic strips evoked by senktide (100 nM). Height of columns: senktide-evoked maximal 5-HT outflow, expressed as % of the mean outflow of first two collections (70 – 90 min). Mean values $\pm$ S.E.M. (vertical bars) from six to seven experiments are shown. Significance of differences from the control: *$P<0.05$, **$P<0.01$.

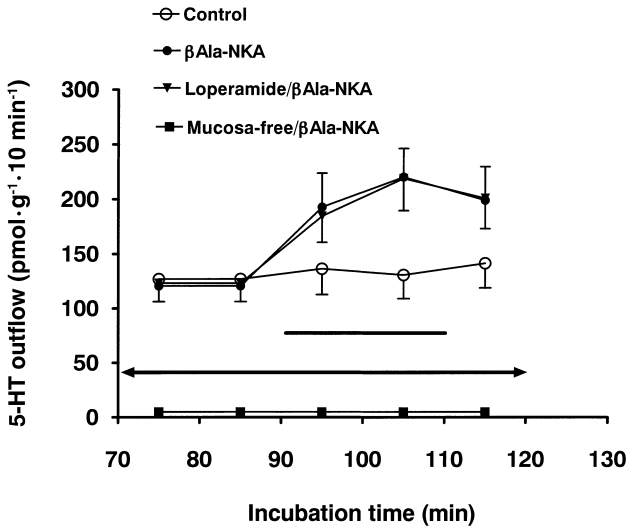

Fig. 3. Effects of [βAla^8]-neurokinin A_{4-10} (βAla-NKA, 1 μM) in the absence or presence of loperamide (100 nM) on the outflow of 5-HT from the whole colonic strips and mucosa-free muscle layer preparations. βAla-NKA was present from 90 to 110 min of incubation, as indicated by the horizontal bar. Loperamide was present from 70 to 120 min of incubation, as indicated by the arrowhead bar. Ordinate scale: outflow of 5-HT, expressed as pmol · g tissue^{-1} · 10 min^{-1}. Each point represents the mean ± S.E.M. (vertical bars) from six to nine experiments are shown.

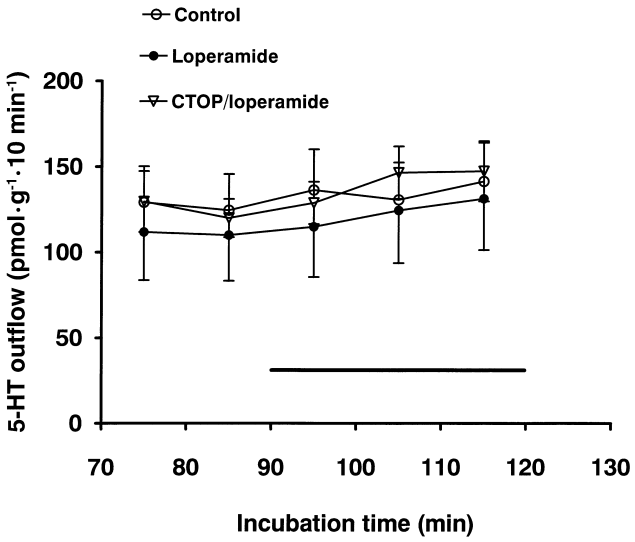

Fig. 5. Effects of loperamide (100 nM) on the spontaneous outflow of 5-HT from the whole colonic strips in the absence or presence of CTOP (100 nM). Loperamide was present from 90 to 120 min of incubation, as indicated by the horizontal bar. Ordinate scale: outflow of 5-HT, expressed as pmol · g tissue^{-1} · 10 min^{-1}. Each point represents the mean ± S.E.M. (vertical bars) of six experiments. Loperamide did not significantly affect the spontaneous 5-HT outflow from the colonic strips.

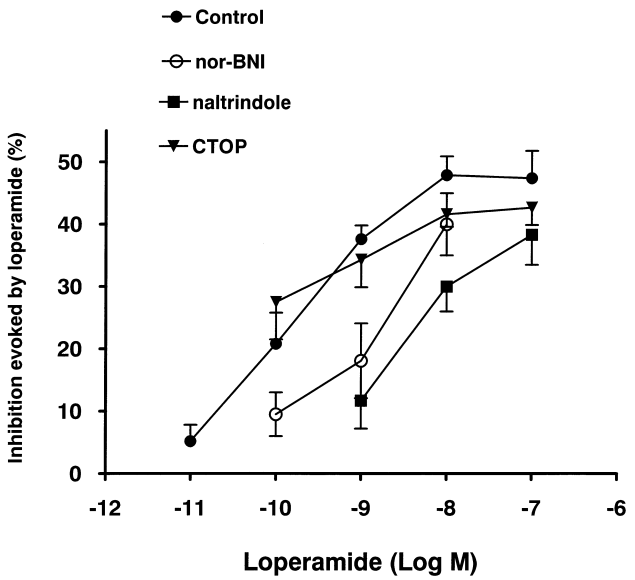

Fig. 4. Effects of increasing concentrations of loperamide in the absence or presence of nor-binaltorphimine (norBNI, 1 nM), naltrindole (10 nM) or CTOP (100 nM) on the senktide-evoked maximal outflow of 5-HT from the whole colonic strips. Each point represents the mean ± S.E.M. (vertical bars) of six experiments.

κ-opioid receptor antagonist nor-binaltorphimine (1 nM) displaced the concentration-response curve to loperamide to the right without significant depression of the maximum. The pEC_{50} value for loperamide was significantly reduced from 9.89 ± 0.2 in the absence of nor-binaltorphimine to 8.86 ± 0.3 (n = 6, P<0.05) in the presence of nor-binaltorphimine. Likewise, the selective δ-opioid receptor antagonist naltrindole (10 nM) also displaced the concentration-response curve to loperamide to the right without significant depression of the maximum (Fig. 4). The pEC_{50} value for loperamide was significantly reduced from 9.89 ± 0.2 in the absence of naltrindole to 8.18 ± 0.4 (n = 6, P<0.01) in the presence of naltrindole. The selective μ-opioid receptor antagonist CTOP (100 nM) did not affect the concentration-response curve to loperamide (Fig. 4).

Loperamide (100 nM) did not affect basal 5-HT outflow from the whole colonic strips in the absence or presence of the selective μ-opioid receptor antagonist CTOP (100 nM) (Fig. 5).

Discussion

The results of the present study confirm our recent observations: the activation of tachykinin NK_2/NK_3 receptors induces an increase in 5-HT release from guinea pig colonic mucosa (13). In contrast to our results, Ginap and Kilbinger (17) have shown that 5-HT release from the vascularly perfused small intestine of the guinea pig was indirectly inhibited by neuronal NK_3

receptors whose stimulation leads to the release of endogenous tachykinins. Although the cause of this discrepancy is not clear, the different role of endogenous tachykinins or enterochromaffin cells in different regions of the intestine may be involved.

The present study deals with the question of whether the antidiarrhoeal agent loperamide affects the tachykinin NK_2- and NK_3-receptor-mediated 5-HT outflow from guinea pig colonic mucosa. As the first main finding of the present study, loperamide suppressed the 5-HT outflow evoked by the selective NK_3-receptor agonist senktide, whereas loperamide failed to affect the 5-HT outflow evoked by the selective NK_2-receptor agonist βAla-NKA, thus indicating that loperamide suppresses the NK_3-receptor-triggered 5-HT release without affecting the NK_2-receptor-triggered 5-HT release. These observations imply that the suppressant effect of loperamide on the NK_3-receptor-triggered 5-HT release is not due to a direct action on colonic enterocytes because tetrodotoxin prevented the NK_3-receptor-triggered 5-HT release without affecting the NK_2-receptor-triggered 5-HT release (13). 5-HT is an important mediator of intestinal water and electrolyte secretion (2, 18), and the activation of tachykinin NK_3 receptors induces a neurally mediated (involving 5-HT release) Cl^- secretion by guinea pig colonic mucosa (19). Therefore, the suppressant effect of loperamide on the NK_3-receptor-triggered 5-HT release contributes to the antisecretory effect of loperamide.

We have also examined the effects of κ- and μ-opioid receptor antagonists in order to elucidate the role of these receptors in the suppressant effect of loperamide. As the second main result in the present study, the selective κ-opioid receptor antagonist nor-binaltorphimine ($pA_2 = 9.83$ in guinea pig colon, 20) displaced the concentration-response curve to loperamide to the right without depression of the maximum effect, suggesting an involvement of the κ-opioid receptors in the suppressant effect of loperamide. These observations are consistent with the observations made in isolated guinea pig colonic mucosa wherein the antisecretory effect of loperamide was prevented by blockade of κ-opioid receptors (21). However, the role of μ-opioid receptors in the suppressant effect of loperamide is questionable because the suppressant effect of loperamide was not affected by the selective μ-opioid receptor antagonist CTOP ($pA_2 = 7.85$ *vs* endomorphin-2, 22). It has also been demonstrated that μ-opioid receptors have a pro-secretory action on the guinea pig colonic mucosa (21). However, the possibility that loperamide may facilitate 5-HT release from the colonic mucosa through the activation μ-opioid receptors is unlikely because loperamide failed to affect basal 5-HT outflow in the absence or presence of CTOP.

We have also made use of the selective δ-opioid receptor antagonist naltrindole to define a role for δ-opioid receptors since the coexpression of δ- and κ-opioid receptors has been documented in the myenteric neurons of porcine small intestine (23). As the third important finding, δ-opioid receptors also played a role in the suppressant effect of loperamide since naltrindole (K_D value = 0.12 nM in porcine proximal colon, 24) displaced the concentration-response curve to loperamide to the right without significant depression of the maximum effect. There have been a number of studies demonstrating that opioid receptor activation leads to presynaptic inhibition of transmitter release in the enteric nervous system. Therefore, these findings lead us to speculate that the activation of κ- and δ-opioid receptors by loperamide can modulate the release of acetylcholine or tachykinins from enteric neurons which are in NK_3-receptor-activated pathways that enhance 5-HT release from colonic mucosa. In contrast, it is unlikely that the inhibitory κ- and δ-opioid receptors are expressed in NK_2-receptor-activated non-neuronal pathways. Interestingly, tachykinins appear to be the major agent involved in castor oil diarrhea, and loperamide prevented the diarrhea induced by castor oil (25).

In conclusion, our findings show that the NK_3-receptor-triggered 5-HT release from colonic mucosa is suppressed by loperamide-sensitive mechanisms, whereas the NK_2-receptor-triggered 5-HT release is loperamide-insensitive. Moreover, our data suggest that the suppressant effect of loperamide is probably mediated by the activation of κ- and δ-opioid receptors located on intrinsic neural plexuses and that these receptors might participate in neuroregulation of 5-HT release from colonic enterochromaffin cells.

Acknowledgment

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