The P2Y₁ Receptor in the Colonic Submucosa of Rats and Its Correlation with Opioid-Induced Constipation

Yuqiong Zhao
Ningxia Medical University

Xiaojie Ren
Ningxia Medical University

Fan Li
xiantao first people's hospital affiliated to yangze university

Binghan Jia
Ningxia Medical University

Dengke Wang
Ningxia Medical University

Xuwen Jiao
Ningxia Medical University

Lixin Wang
Ningxia Medical University

junping li (✉ lijp7221@163.com)
Ningxia Medical University  https://orcid.org/0000-0002-8840-7811

Research Article

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Abstract

Aims: To explore the expression changes of P2Y$_1$ in the distal colonic submucosa of opioid induced constipation (OIC) rats and its correlation with the occurrence of OIC.

Methods: OIC model was generated by intraperitoneal injection of loperamide hydrochloride, a selective agonist of the $\mu$-opioid receptor (MOR). Seven days later, the model was assessing by detecting the fecal traits and calculating the fecal water content. The distribution of MOR-containing neurons and P2Y$_1$-containing neurons in colonic submucosal plexus of rat were demonstrated by immunofluorescence histochemistry. Western Blot was used to evaluate the expression changes of MOR, P2Y$_1$ and ATP synthase subunit beta (ATPB) in colonic submucosa, while the RT-PCR analysis was performed to determine the relative mRNA expression of MOR, P2Y$_1$ and ATPB.

Results: After seven days, the feces of OIC rats had an appearance of like sausage-shaped pieces, and the fecal water content, stool weight of OIC rats were decreased. Immunofluorescence histochemistry showed the co-expression of MOR and ATPB, P2Y$_1$ and calbindin (CB) in the nerve cells of distal colonic submucosal plexus. RT-PCR showed that MOR mRNA levels were significantly increased in the distal colonic submucosa of OIC rats, while the mRNA levels of P2Y$_1$ were decreased. Western blot results showed that MOR protein expression was increased, and the P2Y$_1$ protein expression was significantly decreased in the distal colonic submucosa of OIC rats.

Conclusion: P2Y$_1$ is associated with the occurrence of OIC in rats, and the expression of MOR and P2Y$_1$ and OIC are correlated with each other.

1 Introduction

Opioid preparations such as morphine are clinically effective in relieving acute, chronic and intractable pain etc; however, long-term use of opioids can induce adverse effects such as respiratory depression, constipation and so on[1–5]. Among them, constipation, also known as opioid-induced constipation (OIC), is characterized by decreased frequency of bowel movements, changes in stool characteristics and incomplete excretion. OIC, can seriously impact the patient's quality of life, resulting in discontinuation of medication by patients, which affects the desired analgesic effect[6–8]. Statistically, about 90% of patients experience gastrointestinal dysfunction such as constipation after taking opioid analgesics[9–12].

The effect of opioid preparations such as morphine on the body include the central nervous system and the peripheral nervous system; the $\mu$-opioid receptor (MOR) is the main action site of opioid preparations in the periphery[13–16]. It has been reported that OIC is mainly due to the activation of the MOR in the gastrointestinal tract, thereby reducing the sensitivity of sensory information transmission in the colon defecation reflex pathway, and inhibiting the release of inhibitory neurotransmitters in colonic nerve cells. This in turn inhibits long-distance transport of the colon, causing intestinal contents to stay in the
intestinal cavity of the colon for too long, resulting in excessive absorption of water and electrolytes[7, 8, 13, 17, 18].

The biological actions of purine signaling have been recognized since 1929. The purine receptors include subsets of P1 and P2. P2 receptors can be further sub-classified into two major families: P2X and P2Y[4, 19]. The P2Y receptor family is subdivided into the P2Y₁-like receptor subtypes (P2Y₁, P2Y₂, P2Y₄, P2Y₆ and P2Y₁₁) and the P2Y₁₂-like receptor subtypes (P2Y₁₂₋₁₄)[19, 20]. Among them, P2Y₁ is widely expressed in the enteric nervous system of the gastrointestinal tract, and is closely related to the diastolic function of gastrointestinal smooth muscle induced by non-cholinergic and non-adrenergic neurotransmitters[1, 10, 11, 21]. It has been reported that the inhibitory neurotransmitters involved in the regulation of gastrointestinal motility are mainly non-cholinergic and non-adrenergic inhibitory neurotransmitters, such as adenosine triphosphate (ATP), nitric oxide (NO) etc, which can stimulate the hyperpolarization of smooth muscle cells, leading to relaxation of smooth muscles[13, 22].

In vitro experiments have shown that electrical stimulation of colonic circular muscle cells can record two different functional potentials: excitatory neuromuscular junction potential (EJP) and inhibitory neuromuscular junction potential (IJP). EJP can cause colonic smooth muscle contraction, while IJP is mainly induced by inhibitory neurotransmitters such as ATP, NO etc. Further, IJP comprises fast inhibitory junction potential (fIJP) and the following slow-acting neuromuscular junction potential (sIJP)[11, 22, 23]. Experimental studies have shown that purine-dependent fIJP induces transient phase relaxation of gastrointestinal smooth muscle, while NO-dependent sIJP induces sustained relaxation of gastrointestinal smooth muscle[9, 22]. The literature reports that fIJP is highly sensitive to MRS2500, which is a selective antagonist for the P2Y₁ receptor, while sIJP is more sensitive to the NO synthase inhibitor NG-nitro-L-arginine methyl ester (L-NAME)[10, 22].

Endomorphin-2 (Tyr-Pro-Phe-Phe-Nh2, EM2), the endogenous ligands of MOR, which is selectively bind to MOR at a high affinity and involved in the regulation of visceral information transmission[23]. The previous experimental study of our group found that the endogenous receptor agonist endorphine-2 (EM2) of MOR has no obvious effect on the sIJP of colonic circular muscle cells, but it can completely block fIJP, which is similar to the effect of the P2Y₁ receptor selective receptor antagonist MRS2500 on the colon fragment.

Therefore, this study was designed to determine the correlation of P2Y₁ and OIC by observing the distribution characteristics of P2Y₁ in the distal colonic submucosal plexus and its expression changes in the distal colonic submucosa of OIC rats.

### 2 Materials And Methods

#### 2.1 Animals
Sprague-Dawley male rats weighing 180-200g were selected from the Experimental Animal Center of Ning Xia Medical University (Yin Chuan, China). All protocols described below were approved by the Committee of Animal Use for Research and Education of the Ning Xia Medical University. We let all the animals adapt to the experimental environment 2–3 days before experiment. In accordance with the ethical guidelines for animal research, all efforts were made to minimize the number of animals used, and the suffering of the animals.

Rats were randomly divided into opioid induced constipation group (OIC; \(n = 20\)), normal saline group (NSG; \(n = 20\)) and normal control group (NCG; \(n = 20\)). For the OIC, rats were given an intraperitoneal injection of loperamide hydrochloride (4mg/kg, 1ml/100g)\([24]\) twice a day for 7 days, loperamide hydrochloride dissolved in 0.9% normal saline; in the NSG group, the rats were injected with 0.9% normal saline(1ml/100g) twice a day for 7 days, while the rats in NCG group did not receive any treatment.

### 2.2 Model Evaluation

(1) On the first day of modeling, the traits of rat feces were observed and recorded daily. According to the Rome II classification criteria, the rat feces were scored: dispersed hard block = 1; small sausage-like pieces = 2; presence of cracks on the sausage-like surface = 3; the sausage-like surface was smooth and soft = 4; soft lumps but clearly defined = 5; pasty but unclear = 6; watery feces sample = 7\([25]\). 1 and 2 are considered constipation; 3 and 4 are considered normal stool; 5–7 are considered diarrhea. (2) Fecal fecal content: After modeling, collect the fresh feces of each group of rats. The electronic balance weighs the quality of each group of fresh feces as wet weight. After baking in a microwave oven for 5 minutes, the dry weight is weighed to calculate the fecal moisture content. Fecal moisture content = (wet weight-dry weight) / wet weight × 100%.

### 2.3 Immunofluorescent Staining

4 untreated male rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (3ml/kg), and an incision was made along the midline of the abdomen to expose the colon. The left colic flexure was used for the sector to retain the distal parts. The section was washed with 0.01mol/L PBS and fixed with paraformaldehyde. Both ends were ligated and stored at 4°C for 8 hours. After 24 hours in a 30% sucrose solution, the preparations were cut along the mesentery margin and stored in a 30% sucrose solution. The mucosal and muscular layers were then removed under a dissecting microscope, while the submucosa that was left behind was collected in the 0.01mol/L PBS.

Divide the prepared submucosal tissue specimens into 10 groups and detect the co-existence of MOR, P2Y\(_1\), ATPB, EM2 and NeuN; ATPB and MOR, EM2; MOR and CB, CGRP; P2Y\(_1\) and CB, CGRP in the distal colonic submucosal of rats. After rinsing the tissue samples with 0.01 mol/L PBS for 3 times, 1 mol/L hydrochloric acid was added to repair at room temperature, and then rinsed with 0.01 mol/L PBS for 3 times, newborn calf serum was added to block for 1 h at room temperature. The corresponding primary antibodies were added to the 10 groups of tissue samples: rabbit anti-MOR polyclonal antibody (1:300, abcam, ab10275) and mouse anti-NeuN polyclonal antibody (1:200, abcam, ab104224), rabbit anti-P2Y\(_1\)
polyclonal antibody (1:200, NOVUS, NBP1-30741) and small Mouse anti-NeuN polyclonal antibody (1:200, abcam, ab104224), mouse anti-ATPB monoclonal antibody (1:200, ab14730, abcam) and rabbit anti-NeuN polyclonal antibody (1:200, abcam, ab104225), rabbit anti-EM2 polyclonal antibody (1:200, abcam, ab10289) and small Mouse anti-NeuN polyclonal antibody (1:200); mouse anti-ATPB monoclonal antibody (1:200) and rabbit anti-MOR polyclonal antibody (1:300); mouse anti-ATPB monoclonal antibody (1:200) and Rabbit anti-EM2 polyclonal antibody (1:200); rabbit anti-MOR polyclonal antibody (1:300) and mouse anti-CB monoclonal antibody (1:300, abcam, ab11426), rabbit anti-MOR polyclonal antibody (1:300) and small Mouse anti-CGRP monoclonal antibody (1:50, abcam, ab81887); rabbit anti-P2Y1 polyclonal antibody (1:200) and mouse anti-CB monoclonal antibody (1:300); rabbit anti-P2Y1 polyclonal antibody (1:200) and small Mouse anti-CGRP monoclonal antibody (1:50) was incubated at room temperature for 1 hour and then placed at 4°C for 48 hours, rinsed with 0.01 mol/L PBS 3 times, and then the corresponding fluorescein-labeled secondary antibodies were added to the 10 groups of tissue samples: Alex488 labeled donkey anti-rabbit serum (1:500, abcam, ab6978) and Alex594 labeled goat anti-mouse serum (1:500, ab150116), Alex488 labeled donkey anti-rabbit serum (1:500) and Alex594 labeled goat anti-mouse serum (1:500), Alex488 labeled donkey anti-mouse serum (1:500, abcam, ab6816) and Alex594 labeled goat anti-rabbit serum (1:500, abcam, ab150080), Alex488 labeled donkey anti-rabbit serum (1:500) and Alex594 labeled goat anti-mouse serum (1:500); Alex488 labeled donkey anti-mouse serum (1:500) and Alex594 labeled goat anti-rabbit serum (1:500); Alex488 labeled donkey anti-mouse serum (1:500) and Alex594 labeled goat anti-mouse serum (1:500) at room temperature. After incubating for 2 hours, rinse again with 0.01 mol/L PBS for 3 times. Finally, the slices were placed on the glass slides and covered by coverslips, with a fluorescent encapsulating agent used as a mounting medium. The specimens were imaged by a fluorescent microscope (OLYMPUS-BX51).

2.4 Real Time-PCR(RT-PCR)

Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (3 ml/kg), and the colon was exposed along the midline of the abdomen. The distal parts (left colic flexure is used for the sector) were used for this experiment. The mucosal layer was retained and the muscle layer was removed. RNA was extracted using an RNA extraction kit, and SYBR Green Bestar™ qPCR MasterMix (Bioscience) was used for the RT-PCR reaction. Primer sequences for the specific genes are as follows (Forward-Reverse): MOR (5'-CATGGCCCTTCGGAACCATC-3'/5'-TGGCAGACAGCAATGTAGCG); P2Y1 (5'-TTATGTGCAAGCTGCAGAGG-3'/5'-CTGCCCAGAGACTTGAGAGG-3'); ATPB (5'-TTGGCAGATGAATGAACCGC-3'/3'-GCAGGACATCTTGGCCTTCC-5'); CB (5'-CGACGCTGATGGAAGTGGTTACC-3'/5'-GGTGATAGCTCCAATCCAGCCTTC-3'); CGRP (5'-GTGAAGAAGCTCGCTACTGG-3'/5'-CTTCAGCCCCCTGTTCCTCCTC-3'); β-actin (5'-AGCAATGTAGCC); P2Y1 (5'-TTATGTGCAAGCTGCAGAGG-3'/5'-CTGCCCAGAGACAGAC-5')
CAGGAGGCATTGCTGATGAT-3' / 5' GAAGGCTGGGGCTCATTT-3'). The primer sequences are all from Sangon Biotech.

### 2.5 Western Blot

Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (3 ml/kg), and the colon was exposed along the midline of the abdomen. The distal parts (left colic flexure is used for the sector) were used for this experiment. 10 µl of each group tissue sample was pipetted into a well of a 10% polyacrylamide gel and gel electrophoresis. The proteins were transferred to a PVDF membrane at 200 mA constant flow. For detection of MOR protein, P2Y₁ protein, ATPB protein, CB protein and β-actin, the PVDF membrane was incubated with the corresponding rabbit anti-MOR polyclonal antibody (1:1000, abcam, ab10275), rabbit anti-P2Y₁ polyclonal antibody (1:1000, abcam, ab85896), mouse anti-ATPB monoclonal antibody (1:1000, abcam, ab14730), mouse anti-CB monoclonal antibody (1:1000, abcam, ab11426), mouse anti-β-actin monoclonal antibody (1:1000, TA09, ZSGB-BIO), respectively, for 1 hour at room temperature, and allowed to stand at 4°C for 12 hours overnight. Then, the membrane was incubated with the secondary antibody—either HRP-goat anti-mouse IgG (1:3000, ZB-2305, ZBGB-BIO) or HRP-goat anti-rabbit IgG (1:3000, ZB-2301, ZBGB-BIO)—for 1 h at room temperature, and washed with TBST 3 times for 10 minutes each time. Ultrasensitive chemiluminescent reagents were used to visualize protein bands, the images were captured by Amersham lamger 600 chemical image system, and the gray value of each strip was measured. The ratio of the gray value of the target protein band to the internal reference protein band was used to determine the relative expression of the target protein.

### 3 Data Analysis

The results of the fecal water content, stool weight changes, RT-PCR and Western Blot were analyzed by one-way ANOVA and using the SPSS 17.0 statistical software. P < 0.05 was considered to be statistically significant.

### 4 Results

#### 4.1 Establishment of an OIC model

| Group     | OIC          | NSG          | NCG          |
|-----------|--------------|--------------|--------------|
| Stool weight(g) | 1.5 ± 0.13<sup>ab</sup> | 3.1 ± 0.09   | 3.0 ± 0.17   |
| Fecal water content(%) | 49.2 ± 1.6<sup>ab</sup>  | 55.6 ± 4.9   | 58.3 ± 3.1   |

Table 1 Compared with NSG and NCG, the gastrointestinal transit ratio, stool water content and stool weight of OIC rats was significantly decreased. <sup>a</sup>P < 0.05 vs NSG; <sup>b</sup>P < 0.05 vs NCG.
With the prolonged modeling time, the stool score of OIC rats gradually decreased. The stool of OIC rats became smaller and harder, which was close to the small sausage-like pieces with a score of 2; the stool of NCG and NSG rats were similar, with a sausage-like appearance but a smooth and soft surface with a score of 4 (Fig. 1). Statistical analysis showed that compared with NCG and NSG that on the 7th day of modeling, the fecal water content and the stool weight of the OIC rats were significantly lower than the NCG and NSG rats in the same time period, and the difference was statistically significant ($P<0.05$, Table 1).

### 4.2 Immunofluorescent staining

Immunofluorescence histochemistry results showed that a large number of MOR, P2Y₁, ATPB and EM2-positive nerve cells aggregated in the colonic submucosal plexus of rats to form ganglia (Fig. 2A, B, C, D). In the ganglion, MOR, P2Y₁, ATPB and CB were observed to co-localize with NeuN positive markers in intestinal cells respectively, suggesting that all these positive cells are neurons (Fig. 2A2, B2, C2, D2). Most of the MOR-positive cells were oval, and their positive markers were mainly located in the cell bodies and process. A large number of MOR-positive fibers were found to travel through the ganglia (Fig. 2A). The P2Y₁-positive nerve cell body was round or elliptical, and the positive marker was mainly located in the cell bodies and process, which emits a long protrusion from the cell body (Fig. 2B). The ATPB-staining markers were mainly located on the cell membrane (Fig. 2C). In the ganglion, it also could be seen that MOR and ATPB positive markers are co-expressed in intestinal nerve cells, and a large number of MOR-positive nerve fibers surrounded the cell bodies of ATPB-positive cells (Fig. 3A, A1, A2). The CB-positive nerve cell body was round or elliptical, and the positive marker was mainly located in the cytoplasm, and several protrusions were emitted from the cell body (Fig. 4A1; Fig. 4C1). In the ganglion, CGRP protuberances were observed in the submucosal plexus (Fig. 4B1; Fig. 4D1). In addition, CB and CGRP could be found in the colonic submucosal ganglia and co-localized with MOR and P2Y₁ positive markers respectively (Fig. 4).

### 4.3 RT-PCR

RT-PCR results showed that compared with the expression levels in NCG and NSG rats, the mRNA expression levels of MOR and CGRP in the submucosal layer of OIC rats were increased, while the ATPB, P2Y₁ and CB were decreased, with a statistically significant difference ($P<0.05$, Fig. 5).

### 4.4 Western Blot

Western blot results showed the expression levels of MOR protein in the submucosal layer of OIC rats was increased, while the expression levels of ATPB, P2Y₁ and CB proteins were significantly decreased compared with NCG and NSG rats with statistically significant differences ($P<0.05$, Fig. 6).

### 5 Discussion

#### 5.1 Evaluation of OIC rat model
Studies have shown that effective analgesic sites for opioids such as morphine are in the central nervous system, and the main cause of constipation is the activation of the peripheral MOR, leading to gastrointestinal dysfunction[14, 15, 26–30]. Loperamide hydrochloride is an opioid receptor agonist whose main mechanism is to reduce water and electrolyte secretion by binding to the MOR in the gastrointestinal tract, inhibiting long-distance transport of the gastrointestinal tract, and causing intestinal contents to remain in the intestinal lumen[31]. In this experiment, an OIC rat model was generated by intraperitoneal injection of loperamide hydrochloride solution into rats. The OIC rat model was evaluated according to the Rome II stool typing criteria[18, 25, 32–34]. After 7 days of modeling, the stool of OIC rats was close to a small sausage-like piece with a score of 2, the fecal water content and stool weight of OIC rats were significantly lower than the NCG and NSG rats; this was consistent with the characteristics of OIC stool, indicating that the OIC rat model was successfully established in this study.

5.2 Distribution of MOR and P2Y\textsubscript{1} receptor in rat colonic submucosal plexus

Gastrointestinal functions, such as smooth muscle contraction and electrolyte secretion, are controlled by the intrinsic enteric neurons and extrinsic innervation. Intrinsic innervation of the gut is provided by the enteric nervous system, an independent neural network system in the gastrointestinal tract, which mainly includes the submucosal plexus between the mucosa and the submucosa and the myenteric plexus located between the circular muscle and the longitudinal muscle[13, 18, 35–37]. According to morphological and electrophysiological characteristics, enteric nerve cells can be divided into intrinsic primary afferent or sensory neuron, intermediate nerve cells, and motor nerve cells[13]. The intrinsic primary afferent nerve cells are the first neurons in reflex pathways activated by mucosal stimulation. These neurons have cell bodies in the myenteric and submucosal ganglia, and one of the process projecting into the mucosal villi and a second process synapsing with intermediate nerve cells and motor nerve cells in submucosal or myenteric ganglia, and transmits various mechanical and chemical stimuli information to the middle and motor nerve cell body in the intestinal plexus, thereby regulating the gastrointestinal tract smooth muscle movement[13, 35, 38, 39]. MOR is a kind of opioid receptor, which belongs to the G-protein-coupled-receptor family and is widely distributed in enteric nerve cells in the gastrointestinal tract. It is the main site action of morphine and other opioid preparations in the periphery[13]. Compared with other opioid receptors, MOR is not only widely distributed in the gastrointestinal tract, but also has obvious regional differences in expression. Specifically, it is more densely distributed in the colon than in the stomach and small intestine[13, 16]. EM2, an endogenous ligand with high affinity for MOR, is similarly distributed in the gastrointestinal tract, especially in the colon[16, 23, 40]. In our study, the immunofluorescence histochemistry showed that there were MOR and EM2 double-positive nerve cells in the distal submucosal plexus of rats, and MOR was co-expressed with CB and CGRP in intestinal nerve cells. According to reports in the literature, CB belongs to the family of calcium-binding proteins. Studies have shown that CB is mainly expressed in primary sensory afferent neurons and participates in sensory information transmission in the gastrointestinal tract[36, 39, 41, 42]. CGRP, an important neurotransmitter, is mainly released by submucosal plexus cells that transmit various
mechanical and chemical irritative sensory information in the gastrointestinal tract[39, 43, 44]. Therefore, it is suggested that MOR may be closely related to the transmission of sensory information in rat colon.

The movement of gastrointestinal smooth muscle is mainly dominated by motor nerve cells, including excitatory and inhibitory nerve cells, in which inhibitory motor neurons are mainly non-cholinergic and non-adrenergic inhibitory nerve cells[45]. ATP, an important non-cholinergic non-adrenergic inhibitory neurotransmitter, belongs to the class of purine neurotransmitters that transmits inhibitory neurological information in the gastrointestinal tract, which is widely distributed in colonic nerve cells[44, 46]. In addition, it is a high-affinity ligand for the P2Y₁ receptor[22, 44]. The P2Y₁ receptor belongs to the purine receptor family and also is a member of the G protein conjugate receptor family. It is abundantly expressed in gastrointestinal smooth muscle cells and is involved in regulating the transmission of neural information. [46–49]. Immunofluorescence histochemistry showed that the P2Y₁ receptor and ATPB were abundantly expressed in the submucosal plexus of the distal colon of rats, and the P2Y₁ receptor is also co-expressed with CB and CGRP in the nerve cells. As reported in the literature, the P2Y₁ receptor is highly sensitive to stimulatory sensory signals such as mechanical stimuli in gastrointestinal dysfunction disorders[48]. It is therefore suggested that the P2Y₁ receptor is distributed in the distal colonic submucosal plexus cells, and the P2Y₁ receptor may be involved in the transmission of neural information in the colon of rats.

5.3 Correlation between MOR and P2Y₁ receptor in the development of OIC in rats

The results of RT-PCR and western blot showed that the mRNA and protein expression levels of MOR increased in the distal submucosa of OIC rats, while the mRNA expression levels of P2Y₁ receptor, ATPB, CB and CGRP significantly decreased. It is also shown that the protein expression levels of P2Y₁ receptor, ATPB and CB is consistent with the mRNA expression levels. Studies have shown that long-term use of opioid preparations such as morphine may induce disorders of MOR function in the colorectal region, leading to constipation[13, 14, 26]. Constipation induced by gastrointestinal dysfunction is closely related to abnormal colorectal nerve signaling[50]. In the colon, MOR is distributed on the surface of nerve cells in the submucosal plexus. By regulating the release of intestinal neurotransmitters, MOR further regulates the movement of the colonic smooth muscle[13]. Immunofluorescence histochemistry showed that MOR and ATPB were co-expressed in the distal colonic submucosal plexus of rats. ATP is an important extracellular messenger in the colon, mainly released by non-cholinergic non-adrenergic neurons in the enteric nervous system, and regulates the functional activities of the colon by activating receptors such as P2Y₁ in the colon[45, 48]. In our study, we detected that P2Y₁ is distributed in the nerve cells of the colonic submucosal plexus. In the colon, P2Y₁ is the dominant purine receptor and closely related to the transmission of sensory information in the colonic submucosa[26, 48]. This indicates that MOR, ATP and P2Y₁ receptors have morphological basis for the formation of intact neuromodulation pathways in the rat colonic nervous system. It is also suggested that the P2Y₁ receptor expression is associated with the
occurrence of OIC in rats, and that the expression of MOR and P2Y₁ receptors, and the occurrence of OIC, are related to each other.

6 Conclusion

P2Y₁ is distributed in rat colonic submucosal plexus cells and associated with the occurrence of OIC.

Declarations

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Data availability statement

The data that support the fundings of this study are available from the corresponding author upon reasonable request.

Author Contribution

The corresponding author is responsible for ensuring that the descriptions are accurate and agreed by all authors.

Junping Li: conceptualization; Xuwen Jiao and Dengke Wang: methodology; Binghan Jia: resources; Yuqiong Zhao; Fan Li and Xiaojie Ren: investigation; Yuqiong Zhao: writing-original draft; Lixing Wang: writing-review & editing.

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Figures
The graphs show rat fecal consistence.
The immunofluorescence histochemistry indicated the coexistence of various factors in submuscoaal plexus of normal rat colon. A: shows the MOR staining; A1: shows the NeuN immunoreactivity; A2: shows the co-localization of MOR with NeuN. B: shows the P2Y1 staining; B1: shows the NeuN staining; B2: shows the co-localization of P2Y1 with NeuN. C: shows the ATPB staining; C1: shows the NeuN immunoreactivity; C2: shows the co-localization of ATPB with NeuN. D: shows the EM2 staining; D1: shows the NeuN immunoreactivity; D2: shows the co-localization of EM2 with NeuN. The arrowheads
indicate double-labeled neurons of MOR with NeuN (A2), P2Y1 with NeuN (B2), ATPB with NeuN (C2), and EM2 with NeuN (D2). Bar=20μm

Figure 3

The immunofluorescence histochemistry indicated the coexistence of various factors in the submuscoal plexus of normal rat colon. A: shows the ATPB immunoreactivity; A1: shows the MOR staining; A2: shows the co-localization of MOR with ATPB. B: shows the ATPB staining; B1: shows the EM2 immunoreactivity; B2: shows the co-localization of EM2 with ATPB. The arrowheads indicate double-labeled neurons of MOR with ATPB (A2) and EM2 with ATPB (B2). Bar=20μm
Figure 4

The immunofluorescence histochemistry indicated the co-expression of various factors in submucosal plexus of normal rat colon. A: showing the MOR immunoreactivity; A1: shows the CB staining; A2: shows the co-localization of MOR with CB. B: shows the MOR staining; B1: shows the CGRP immunoreactivity; B2: shows the co-localization of MOR with CGRP. C: shows the P2Y1 staining; C1: shows the CB immunoreactivity; C2: shows the co-localization of P2Y1 with CB. D: shows the P2Y1 immunoreactivity; D1: shows the CGRP staining; D2: shows the co-localization of P2Y1 with CGRP. The arrowheads indicate
double-labeled neurons of MOR with CB (A2), MOR with CGRP (B2); P2Y1 with CB (C2) and P2Y1 with CGRP (D2). Bar=20μm

Figure 5

-PCR showed the relative mRNA expression of MOR, ATPB, P2Y1, CB and CGRP in rat colonic submucosa among the three groups. aP < 0.05 vs NSG, bP < 0.05 vs NCG.
Figure 6

Western blot showed the relative protein expression levels of MOR in rat colonic submucosa among the three groups. aP < 0.05 vs NSG, bP < 0.05 vs NCG.