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## **Organoids as an *ex vivo* model for studying the serotonin system in the murine small intestine and colon epithelium**

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## **Abstract**

Intestinal organoids were recently established as an *ex vivo* model of the intestinal epithelium. The present study investigated the serotonin (5-hydroxytryptamine, 5-HT) system using organoids. Organoids from murine small intestinal and colonic crypts were successfully cultured. Reverse transcription–polymerase chain reaction (RT-PCR) analysis showed that small intestinal and colonic organoids express mRNAs encoding tryptophan hydroxylase-1 (TPH1) (the rate-limiting enzyme of 5-HT synthesis), serotonin reuptake transporter (SERT), 5-HT receptor (HTR)2A, HTR2B, and HTR4. SERT mRNA levels were significantly higher in the small intestine than in the colon in both the mucosal tissues and organoids, as estimated by quantitative real-time RT-PCR. Although the 5-HT concentration and levels of chromogranin A (CgA) (an enteroendocrine cell marker), TPH1, and HTR4 mRNAs were significantly higher in the colonic mucosa than the small intestinal mucosa, they were the same in small intestinal and colonic organoids. There were no significant differences in HTR2A and HTR2B mRNA levels between the small intestine and colon in either the mucosal tissues or organoids. Immunofluorescence staining showed that the number of CgA-positive cells in the colonic organoids appeared to increase upon culturing with acetate. Acetate supplementation significantly increased CgA, TPH1, and HTR4 mRNA levels in the colonic organoids. We propose that organoids are useful for investigating the 5-HT system in the intestinal epithelium, even though colonic organoids may require gut microbiota–derived factors such as short-chain fatty acids.

**Keywords:** Intestinal organoid; Enterochromaffin cell; Serotonin; Short-chain fatty acid

## **1. Introduction**

Although the intestinal epithelium has been difficult to model in culture, the establishment of a system for culturing primary stem cell–derived intestinal organoids has overcome this difficulty [1-3]. Intestinal organoids can be produced from isolated intestinal crypts that include stem cells by three-dimensional Matrigel culturing in medium containing a combination of Wnt, R-spondin, and Noggin [1-3]. Small intestinal organoids consist of a polarized epithelium that is patterned into villus-like regions containing differentiated enterocytes, goblet cells, and enteroendocrine (EE) cells and crypt-like proliferative zones containing stem cells, transit-amplifying cells, and Paneth cells [1]. Thus, small intestinal organoids recapitulate critical *in vivo* characteristics, such as the cellular composition and self-renewal kinetics of the small intestine epithelium [1].

Enterochromaffin (EC) cells are a subset of EE cells and are responsible for the synthesis, storage, and release of serotonin (5-hydroxytryptamine, 5-HT), a sensory mediator in the intestine (for reviews, see references [4] and [5]). 5-HT is released from EC cells in response to chemical and mechanical stimuli in the intestinal lumen and mediates many intestinal functions, including peristalsis, secretion, and vasodilation, through activation of a diverse family of 5-HT receptors (HTRs) on intrinsic and extrinsic sensory neurons located in the lamina propria of the intestinal mucosa. The actions of 5-HT are terminated by uptake via the serotonin reuptake transporter (SERT) into intestinal epithelial cells. 5-HT can also act directly on epithelial cells via HTR2 [6] and HTR4 [7, 8]. Thus, intestinal epithelial cells play a central role in the 5-HT system (i.e., in the synthesis, release, reuptake, and recognition of 5-HT). The present study aimed to test whether intestinal organoids are useful for studying the 5-HT system in the intestinal epithelium.

## **2. Materials and methods**

### *2.1. Animal care*

Female C57BL/6N mice (age 5 weeks) were purchased from Japan SLC and housed in

standard plastic cages in a temperature-controlled ( $23 \pm 2^\circ\text{C}$ ) room under a 12-h light/12-h dark cycle and were allowed free access to tap water and standard laboratory rodent feed (Oriental Yeast). All study protocols were approved by the Animal Use Committee of Hokkaido University (approval no. 08-0139). Animals were maintained in accordance with the Hokkaido University guidelines for the care and use of laboratory animals.

## 2.2. In vivo study

Mice were euthanized by cervical dislocation under sevoflurane anesthesia. A laparotomy was made, and the entire length of the small intestine and colon was excised. The luminal contents were thoroughly washed out with ice-cold PBS, the small intestine and colon were opened longitudinally, and the mucosa was scraped off using a glass slide. In addition, a craniotomy was made, and the whole brain was excised. The mucosa and brain were snap-frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  for RNA isolation and 5-HT measurement.

## 2.3. Ex vivo study

Murine small intestinal and colonic crypts were isolated according to the protocol of Sato *et al.* [1, 2]. In brief, mice were euthanized by cervical dislocation under sevoflurane anesthesia. A laparotomy was made, and the entire length of the small intestine and colon was excised. The luminal contents were thoroughly washed out with ice-cold PBS, and the small intestine and colon were opened longitudinally. The tissue was cut into approximately 5-mm pieces and further washed with ice-cold PBS. The small intestinal and colonic tissue fragments were incubated in chelation buffer (2 mM EDTA/PBS for the small intestine and 5 mM EDTA/PBS supplemented with 43.4mM sucrose, 54.9 mM D-sorbitol, and 0.5 mM DL-dithiothreitol for the colon) for 30 min and 2 h, respectively, at  $4^\circ\text{C}$ . After sedimentation, the tissue fragments were resuspended in the respective chelation buffer. After vigorous shaking and sedimentation, the supernatant was passed through a 70- $\mu\text{m}$  cell strainer (BD Biosciences), followed by centrifugation at  $200 \times g$  for 3 min. The resultant precipitate was regarded as the crypt fraction. The crypts were cultured according to the protocol of Miyoshi *et al.* [3] with some

modifications. Crypts were embedded in Matrigel (BD Biosciences) in each well of a 48-well plate (1000 crypts/well) and overlaid with basal culture medium (Advanced DMEM/F12 supplemented with penicillin/streptomycin, 10 mM HEPES, Glutamax [all from Invitrogen], 1  $\mu$ M *N*-acetylcysteine [Sigma], and 20% (v/v) fetal bovine serum [Corning]) supplemented with the following growth factor combinations: 50% (v/v) L-RN cell-conditioned medium (CM) containing R-spondin 3 and Noggin for small intestinal crypts and 50% (v/v) L-WRN cell-CM containing Wnt-3a, R-spondin 3, and Noggin for colonic crypts. L-RN and L-WRN cells were obtained by engineering their parental lines, L cells (ATCC CRL-2648) and L-Wnt3a cells (ATCC CRL-2647), respectively, according to the protocol of Miyoshi *et al.* [3]. Y-27632 (10  $\mu$ M; Sigma) was included in the culture medium for the first 2 days to avoid anoikis. For colonic crypts, the culture medium was exchanged on day 3 with basal medium supplemented with 50% (v/v) L-RN cell-CM to induce differentiation. On day 5, colonic crypts were cultured in medium supplemented with either 0.1, 1, or 10 mM short-chain fatty acids (SCFAs; sodium acetate, sodium propionate, and sodium butyrate) or 1, 10, or 100 ng/mL lipopolysaccharide (LPS, from *Salmonella enterica* serotype abortus equi; Sigma) for 24 h. In a separate experiment, colonic crypts were cultured in medium supplemented with 0.1 or 1 mM SCFAs throughout the culture period. On day 6, the organoids were subjected to RNA isolation, 5-HT measurement, and immunofluorescence staining, as described below.

#### 2.4. Isolation and analysis of RNA

Total RNA was isolated from small intestinal and colonic mucosa samples and organoids using an RNeasy Mini Kit (Qiagen), and first-strand cDNA was synthesized using ReverTra Ace qPCR RT Master Mix (Toyobo) according to the manufacturer's instructions.

Conventional polymerase chain reaction (PCR) was performed in a 25- $\mu$ L reaction solution containing 2.5  $\mu$ L of 10 $\times$  universal buffer, 160  $\mu$ M dNTPs, 100 nM gene-specific primers (**Table 1**), 0.25  $\mu$ L of Gene *Taq* (Nippon Gene), and 1  $\mu$ L of first-strand cDNA sample. The PCR conditions were as follows: 95°C for 2 min, followed by 40 cycles at 95°C for 30 s, 60°C for 60 s, and 68°C for 60 s, with a final extension at 68°C for 5 min. The PCR products

were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide. Quantitative real-time PCR (qRT-PCR) was performed using a Thermal Cycler Dice Real-Time System (Takara). The qRT-PCR reaction was performed in a 25- $\mu$ L reaction solution containing 12.5  $\mu$ L of SYBR Premix Ex *Taq* (Takara), 200 nM gene-specific primers (**Table 1**), and 2  $\mu$ L of first-strand cDNA sample. The qRT-PCR conditions were as follows: 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, 60°C to 65°C for 40 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. The fluorescent products were detected at the last step of each cycle. The relative mRNA expression levels were normalized to that of glyceraldehyde-3-phosphate dehydrogenase.

### *2.5. Determination of 5-HT*

Small intestinal and colonic mucosa samples and organoids were homogenized in buffer composed of 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.1% (w/v) SDS, 1% (w/v) Triton X-100, and 1% (w/v) sodium deoxycholate. After centrifugation, the concentration of 5-HT was measured in the supernatant using a serotonin ELISA kit (Abnova) according to the manufacturer's instructions. The protein concentration in the samples was also measured using a microplate BCA protein assay kit (Pierce) according to the manufacturer's instructions.

### *2.6. Immunofluorescence staining*

Small intestinal and colonic organoids were removed from the Matrigel on day 6 of culture using Cell Recovery Solutions (BD Biosciences) according to the manufacturer's instructions. After pelleting by centrifugation at  $200 \times g$  for 3 min, the organoids were attached to the glass slide using a gelatinizing agent (Smear Gell, Geno Staff) according to the manufacturer's instructions. The samples were fixed in 4% paraformaldehyde/PBS for 30 min at room temperature, followed by incubation in 0.5% Triton X-100/PBS for 15 min at room temperature. After blocking with 1% bovine serum albumin/PBS for 1 h, the samples were incubated with anti-chromogranin A (CgA) antibody (ab45179, Abcam) at a 1:200 dilution

overnight at 4°C. After washing with PBS, the samples were incubated with anti-rabbit IgG conjugated with Alexa Fluor 568 (Life Technologies) at a 1:1,000 dilution for 2 h at room temperature while protected from light. After washing with PBS, the slides were immediately coverslipped with ProLong Gold antifade reagent with DAPI (Life Technologies).

Fluorescence microscopy was performed using a laser confocal microscope (TCS SP5, Leica).

### *2.7. Statistical analyses*

Results are presented as means and SEM. An unpaired *t*-test was used to compare means between two groups. Tukey-Kramer's test or Dunnett's test following one-way or two-way analysis of variance was used for multiple comparisons. Data were analyzed using GraphPad Prism for Macintosh (version 6, GraphPad Software). *P* values <0.05 were considered to indicate statistical significance.

## **3. Results**

### *3.1. Small intestinal and colonic organoids express 5-HT-related genes*

We successfully cultured murine small intestinal and colonic organoids (**Fig. 1A**). On day 5 of culture, we observed the typical structure of mature intestinal organoids, consisting of a central cyst structure and surrounding crypt-like budding structures in both the small intestine and colon. Organoids were used for the experiments on day 6 of culture. Ethidium bromide staining of PCR products separated by electrophoresis on a 2% agarose gel showed that both the small intestinal and colonic mucosa and organoids expressed TPH1, SERT, HTR2A, HTR2B, and HTR4 mRNAs (**Fig. 1B**). In contrast, HTR2C, HTR3A, and HTR3B mRNAs were expressed only in the small intestinal mucosa. HTR3B mRNA was not detected in the brain.

### *3.2. Organoids do not reflect the expression profiles of 5-HT-related genes and 5-HT levels in the small intestine and colon*

CgA and TPH1 mRNA levels estimated by qRT-PCR were significantly higher in the colonic mucosa than the small intestinal mucosa (**Fig. 2A** and **2B**, respectively). In the organoids, however, there were no differences in levels of these mRNAs between the small intestine and colon, and the levels were similar to those in the small intestinal mucosa. SERT mRNA levels were significantly lower in the colon than in the small intestine in both the mucosa and organoids (**Fig. 2C**). HTR2A mRNA levels tended to be lower in the organoids than in the mucosa in both the small intestine and colon (ANOVA *P* value, 0.0923), and the levels were the same in the small intestine and colon in both the mucosa and organoids (**Fig. 2D**). There were no differences in HTR2B mRNA levels in the small intestinal and colonic mucosa and organoids (**Fig. 2E**). HTR4 mRNA levels were significantly higher in the colonic mucosa than the small intestinal mucosa, and the inverse trend was observed in the organoids (**Fig. 2F**). 5-HT concentrations were significantly higher in the colonic mucosa than in the small intestinal mucosa (**Fig. 2G**) and were the same in the small intestinal and colonic organoids (**Fig. 2H**).

### *3.3. SCFA but not LPS promotes 5-HT production and expression of the CgA and TPH1 genes in the colonic organoids*

On day 5, colonic organoids were cultured in medium supplemented with different concentrations of SCFAs or LPS for 24 h. After culture, 5-HT levels were significantly higher in organoids cultured with 0.1 mM sodium acetate and sodium propionate as compared with those cultured without SCFA supplementation (**Fig. 3A**). Sodium butyrate and higher concentrations (1 and 10 mM) of sodium acetate and sodium propionate had no effect on the 5-HT level. qRT-PCR revealed significantly higher levels of CgA mRNA in organoids cultured with 10 mM sodium butyrate compared with organoids cultured without SCFA supplementation, whereas sodium acetate and propionate had no effect (**Fig. 3B**). TPH1 mRNA levels were significantly higher in organoids cultured with 10 mM sodium acetate, propionate, or butyrate compared with those cultured without SCFA supplementation (**Fig. 3C**). LPS supplementation had no significant effect on 5-HT level or CgA and TPH1 mRNA

levels in the colonic organoids (**Fig. 3D-3F**).

### *3.4. Acetate increases the number of EE cells in the colonic organoids*

Culturing colonic organoids with 0.1 or 1 mM sodium acetate for 6 days significantly increased CgA and TPH1 mRNA levels (**Fig. 4A** and **4B**). Sodium acetate (0.1 mM) also increased HTR4 mRNA levels (**Fig. 4C**). Data for organoids cultured with 1 mM sodium butyrate were not included due to reduced organoid viability (data not shown).

Immunofluorescence staining revealed CgA-positive EE cells in the colonic organoids (**Fig. 4D**). As compared with organoids cultured without SCFA, the number of CgA-positive cells tended to be higher in organoids cultured with 0.1 mM SCFAs for 6 days. In particular, the number of CgA-positive cells was clearly higher in organoids cultured with sodium acetate.

## **4. Discussion**

The present study examined the expression of mRNAs encoding 5-HT-related proteins in intestinal mucosal tissues and organoids. We found that TPH1 mRNA is expressed in both the small intestinal and colonic organoids as well as the mucosal tissues, suggesting that epithelial cells express TPH1 mRNA. This result is reasonable, because EC cells that express the TPH1 mRNA are a subset of EE cells located in the intestinal epithelium. In the same manner, our data suggest that SERT mRNA is expressed in the epithelial cells, which is consistent with previous studies in which SERT protein was detected in rat small intestinal epithelial cells by immunohistochemical examination [9]. Therefore, intestinal organoids could be considered a useful *ex vivo* model for investigating the synthesis, secretion, and reuptake of 5-HT in intestinal epithelial cells. Indeed, we found that intestinal organoids contain 5-HT.

We also found that mRNAs encoding HTR2A, HTR2B, and HTR4 are expressed in both small intestinal and colonic organoids, suggesting that epithelial cells express these 5-HT receptors. Because HTR2C, HTR3A, and HTR3B mRNAs were detected in the mucosal tissues but not organoids, it is reasonable that these receptors are expressed in non-epithelial

cells such as sensory neurons and immune cells located in the lamina propria of the intestinal mucosa. Previous studies involving pharmacological experiments using receptor agonists and antagonists have suggested that intestinal epithelial cells express some 5-HT receptors. For instance, in one study, 5-HT induced active electrogenic ion transport in intestinal mucosal preparations; this transport was preserved following neural conduction blockade by tetrodotoxin and inhibited by HTR2 antagonists, suggesting the presence of HTR2-like receptors in the intestinal epithelial cells [6]. In contrast, two studies directly demonstrated the expression of HTR4 in intestinal epithelial cells. Using immunohistochemistry analysis, Hoffman *et al.* showed that HTR4 is expressed in murine small intestinal and colonic epithelial cells, including EC and goblet cells [7]. Using RT-PCR analysis, Tuo *et al.* demonstrated that HTR4 mRNA is expressed in epithelial cells isolated from murine duodenum [8]. Therefore, it is conceivable that 5-HT released from EC cells acts on intestinal epithelial cells in both an autocrine and paracrine fashion. In fact, HTR4 activation reportedly elicits 5-HT release and mucus secretion in isolated distal colon of mice and guinea pigs, suggesting autocrine regulation of EC cell function and paracrine regulation of goblet cell function by 5-HT [7].

In the present study, 5-HT levels were higher in the colon than in the small intestine of mice, consistent with the results of a previous study [10]. We also found higher mRNA levels for CgA and TPH1 (markers for EE and EC cells, respectively) in the colon than the small intestine. Although it is possible that 5-HT synthesis in individual EC cells is higher in the colon than the small intestine, these findings suggest that EC cells are more abundant in the colon than the small intestine. In fact, previous immunohistochemical examinations showed that the density of 5-HT-containing EC cells is higher in the colonic mucosa than the small intestine [11]. In addition, higher levels of HTR4 mRNA in the colon suggest that the colon contains a greater number of EC cells, because HTR4 is reportedly expressed by EC cells [7]. However, we found no differences in 5-HT levels and CgA, TPH1, and HTR4 mRNA levels between small intestinal organoids and colonic organoids. Thus, it seems that the colonic

organoids do not reflect the synthesis of 5-HT and/or differentiation and maturation of EC cells *in vivo*. This may be due to the absence of gut microbiota in the culture of organoids. Previous studies demonstrated that colonic 5-HT levels and TPH1 mRNA levels are lower in germ-free (GF) mice than conventionally raised (CR) mice [10, 12, 13]. In the small intestine, however, there is no difference in 5-HT level between GF and CR mice [10]. Therefore, organoids derived from the colon, but not those derived from the small intestine, may require gut microbiota-derived factors for the synthesis of 5-HT and/or the differentiation and maturation of EC cells.

SCFAs are the principal products of microbial fermentation of dietary saccharides in the colon. Previous studies demonstrated that SCFAs stimulate 5-HT synthesis and release in EC cells. Fukumoto *et al.* [14] reported that intraluminal perfusion of a mixture of acetate, propionate, and butyrate increases the luminal release of 5-HT in isolated rat colon. Yano *et al.* [10] showed that supplementation with butyrate and propionate for 1 h promotes 5-HT release and TPH1 mRNA expression in RIN14B cells, an EC cell model derived from rat pancreatic islets [15]. Reigstad *et al.* [12] showed that supplementation with acetate and butyrate, but not LPS, for 24 h increases TPH1 mRNA levels in BON cells, another EC cell model derived from human pancreatic carcinoids [16]. In line with these reports, we found that incubation with 0.1 mM sodium acetate or propionate, but not butyrate, for 24 h increased the 5-HT level in colonic organoids. We also observed an increase in TPH1 mRNA levels by culturing with 10 mM sodium acetate, propionate, or butyrate. LPS, another microbiota-derived substance, had no effect on levels of 5-HT or TPH1 and CgA mRNAs. Our results suggest that colonic organoids respond to acetate and propionate with increased 5-HT synthesis, which may not be mediated by pretranslational upregulation of TPH1. In another set of experiments, we found that supplementation with acetate during the development of colonic organoids from isolated crypts increased CgA, TPH1, and HTR4 mRNA levels. In addition, immunohistochemical examination showed that the number of CgA-positive cells increased in colonic organoids following acetate supplementation. These findings suggest that acetate promotes the

differentiation and maturation of EC cells in colonic organoids. SCFAs may also be required for developing colonic organoids suitable as an *ex vivo* model for investigating the 5-HT system in the colonic epithelium. However, other luminal substances, such as  $\alpha$ -tocopherol, bile acids, *p*-aminobenzoate, and tyramine, reportedly promote 5-HT release and TPH1 mRNA expression in RIN14B cells [10]. We need to test whether these substances affect the 5-HT system in colonic organoids.

Currently, BON cells and RIN14B cells are widely used as models of EC cells. However, these cell lines were originally derived from the pancreas rather than the intestinal epithelium. The present study demonstrated that cultured organoids derived from murine small intestine and colon contain 5-HT-producing EC cells and express SERT and 5-HT receptors. We therefore propose that organoids are useful for investigating the 5-HT system in the intestinal epithelium, even though further studies are needed to identify the required factors in order to more accurately reflect the *in vivo* 5-HT system.

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## Figure legends

**Figure 1** *A*, Light-microscopic visualization of murine small intestinal and colonic organoids. Mature organoids were obtained after incubating small intestinal and colonic crypts for 6 days. Scale bars indicate 100  $\mu\text{m}$ . *B*, Expression of mRNAs encoding tryptophan-hydroxylase 1 (TPH1), serotonin reuptake transporter (SERT), and serotonin receptors 2A, 2B, 2C, 3A, 3B, and 4 (HTR2A, HTR2B, HTR2C, HTR3A, HTR3B, and HTR4, respectively) in murine small intestinal and colonic mucosa and cultured small intestinal and colonic organoids. PCR products were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide. *Lane 1*, brain; *lane 2*, small intestinal mucosa; *lane 3*, colonic mucosa; *lane 4*, small intestinal organoids; *lane 5*, colonic organoids; *lane 6*, negative control (water). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as a positive internal control.

**Figure 2** *A-F*, Comparison of mRNA levels in murine small intestinal and colonic mucosa and cultured small intestinal and colonic organoids. mRNA levels were estimated by quantitative real-time PCR and were normalized to that of glyceraldehyde-3-phosphate dehydrogenase. Data are shown relative to the levels in the small intestinal mucosa, which were set to 1. *G* and *H*, Serotonin (5-HT) levels in the intestinal mucosa and organoids, respectively. White and black bars represent the small intestine and colon, respectively. Data are expressed as means and SEM ( $n = 6$ ). In charts *A-F*, values not sharing the same letters are significantly different ( $P < 0.05$ ). In charts *G* and *H*, values with an asterisk are significantly different vs. the small intestine ( $P < 0.05$ ). CgA, chromogranin A. Other abbreviations are the same as those used in **Fig. 1**.

**Figure 3** *A* and *D*, Serotonin (5-HT) levels in murine colonic organoids. *B*, *C*, *E*, and *F*, comparison of mRNA levels in murine colonic organoids. Organoids were cultured with different concentrations (0.1, 1, or 10 mM) of sodium acetate (Ace), propionate (Pro), or butyrate (But) or different concentrations (1, 10, or 100 ng/mL) of lipopolysaccharide (LPS)

for 24 h. mRNA levels were estimated by quantitative real-time PCR and were normalized to that of glyceraldehyde-3-phosphate dehydrogenase. Data are shown relative to the levels in the small intestinal mucosa, which were set to 1. Data are expressed as means and SEM (n = 6). Values with an asterisk are significantly different vs. without supplementation (No add) ( $P < 0.05$ ). Abbreviations are the same as those used in **Fig. 1**.

**Figure 4 A-C**, comparison of mRNA levels in murine colonic organoids. Organoids were cultured with different concentrations (0.1 or 1 mM) of sodium acetate (Ace), propionate (Pro), or butyrate (But) throughout development (6 days). mRNA levels were estimated by quantitative real-time PCR and were normalized to that of glyceraldehyde-3-phosphate dehydrogenase. Data are shown relative to the levels in the small intestinal mucosa, which were set to 1. Data are expressed as means and SEM (n = 6). Values with an asterisk are significantly different vs. without supplementation (No add) ( $P < 0.05$ ). Abbreviations are the same as those used in **Fig. 1**. **D**, immunofluorescent staining of chromogranin A (CgA) in colonic organoids. Organoids were cultured with 0.1 mM sodium acetate (Ace), propionate (Pro), or butyrate (But) throughout development (6 days). Scale bars indicate 50  $\mu\text{m}$ . Blue, DAPI; magenta, CgA.

**Table 1.** Primer sequences used for PCR analyses.

Target	Forward primer	Reverse primer
CgA	cacagcagctttgaggatga	atgggggactcttggttagg
TPH1	aacaaagaccattcctccgaaag	tgtaacaggctcacatgattctc
SERT	tgggcgctctactacctcat	atgtgtcctgggcgaagta
HTR2A	cgaagcctcgaactggacaat	ccgcaatggtgagaataatcacg
HTR2B	acctgatcctgactaaccgtt	tgggtattatcaccgcgagtat
HTR2C	agccattgctgatatgctggtg	tgcatgatggacgcagttga
HTR3A	ctgtggcgatcaccggaag	ggctgactgcgtagaataaagg
HTR3B	atgattcttctgtggtcctgcctc	gtggtggcctcagcccagttgt
HTR4	aattcagccacaactctaactc	ccagcaccatgaggagaa
GAPDH	tcaccacatggagaaggc	gctaagcagttggtggtgca

CgA, chromogranin A; TPH1, tryptophan-hydroxylase 1; SERT, serotonin reuptake transporter; HTR2A, serotonin receptor 2A; HTR2B, serotonin receptor 2B; HTR2C, serotonin receptor 2C; HTR3A, serotonin receptor 3A; HTR3B, serotonin receptor 3B; HTR4, serotonin receptor 4; GAPDH, glyceraldehyde 3-phosphate hydroxylase

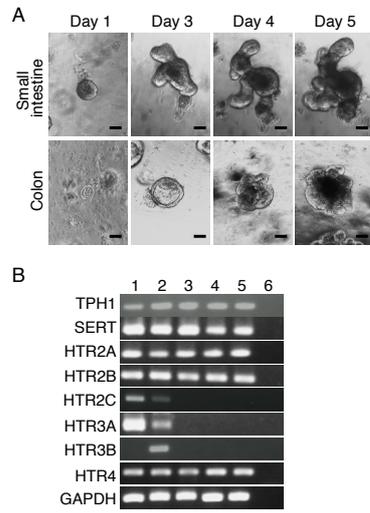


Fig. 1 Tsuruta *et al.*  
(single column)

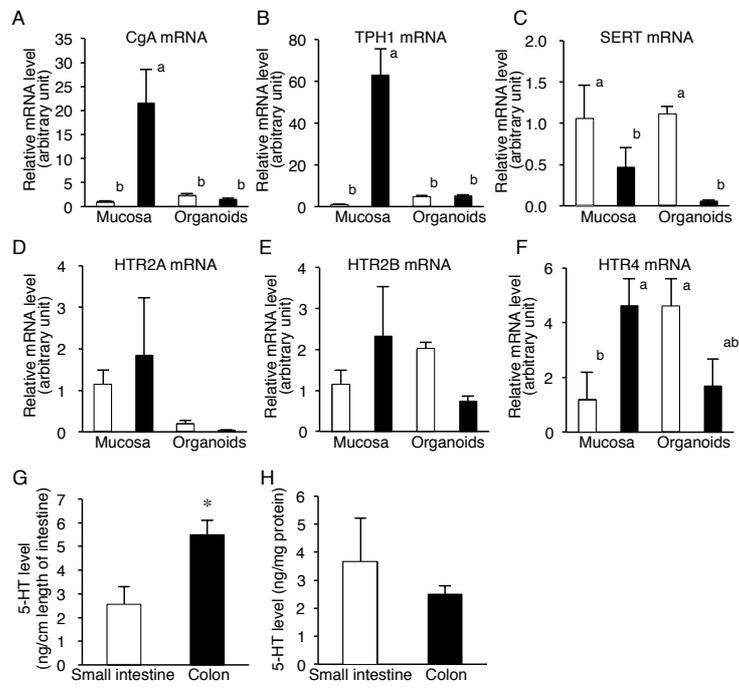


Fig. 2 Tsuruta *et al.*  
(2 column)

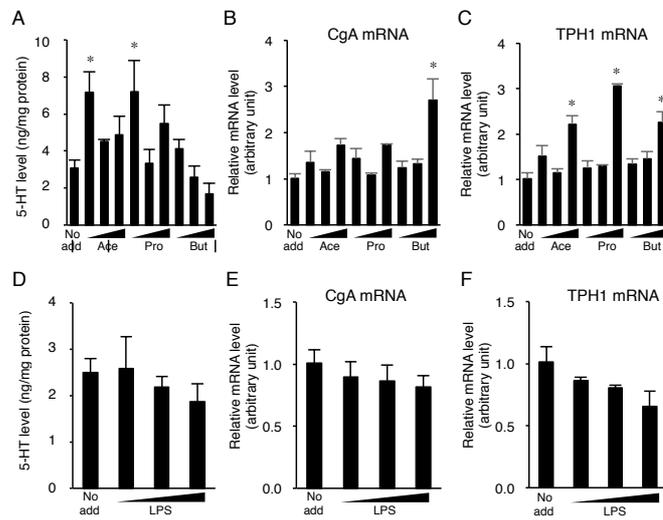


Fig. 3 Tsuruta *et al.*  
(2 column)

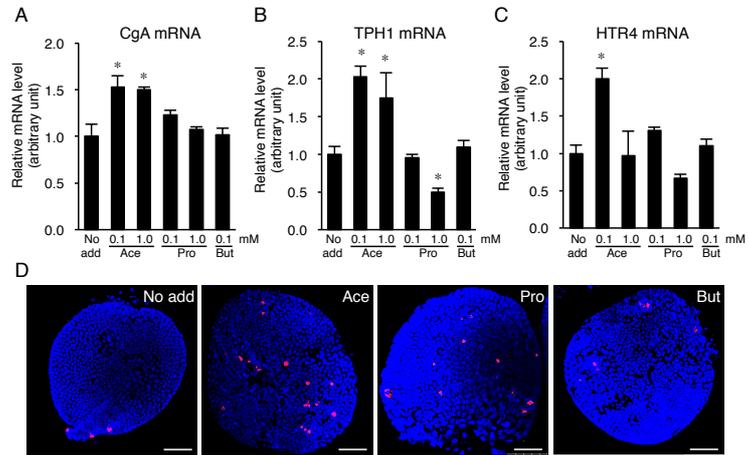


Fig. 4 Tsuruta *et al.*  
(2 column)