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An Enhanced cAMP Pathway Is Responsible for the Colonic Hyper-secretory Response to 5-HT in Acute Stress Rats

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Short title： 5-HT-evoked rat colonic ΔI_{SC} in stress condition

Summary

5-Hydroxytryptamine (5-HT) is involved in the stress-induced alteration of colonic functions, specifically motility and secretion, but its precise mechanisms of regulation remain unclear. In the present study, we have investigated the effects of 5-HT on rat colonic mucosal secretion after acute water immersion restraint stress, as well as the underlying mechanism of this phenomenon, using short circuit current recording (I_{SC}), real-time polymerase chain reaction, Western blot analysis, and enzyme-linked immunosorbance assays. After 2h of water immersion restraint stress, the baseline I_{SC} and 5-HT-induced I_{SC} responses of the colonic mucosa were significantly increased. Pretreatment with selective 5-HT_4 receptor antagonist, SB204070, inhibited the 5-HT-induced colonic I_{SC} response by 96% in normal rats and 91.2% in acute-stress rats. However, pretreatment with the selective antagonist of 5-HT_3 receptor, MDL72222 or Y-25130, had no obvious effect on 5-HT-induced I_{SC} responses under either set of conditions. Total protein expression of both the mucosal 5-HT_3 receptors and the 5-HT_4 receptors underwent no significant changes following acute stress. Both colonic basal cAMP levels and foskolin-induced I_{SC} responses were significantly enhanced in acute stress rats. 5-HT significantly enhanced the intracellular cAMP level via 5-HT_4 receptors in the colonic mucosa from both control and

stressed animals, and 5-HT-induced cAMP increase in stressed rats was not more than that in control rats. Taken together, the present results indicate that acute water immersion restraint stress enhances colonic secretory responses to 5-HT in rats, a process in which increased cellular cAMP accumulation is involved.

Key words

5-Hydroxytryptamine • Colon• Ion transport • cAMP • Stress

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Introduction

5-hydroxytryptamine (5-HT, serotonin) is a well-known neurotransmitter in central nervous system, but most of 5-HT is from intestinal enterochromaffin (EC) cells and very small part of 5-HT is from enteric nervous system. Enteric 5-HT plays an important role in the gastrointestinal tract as a bioactive mediator or signal molecular by neurotransmitter and paracrine fashions. 5-HT exerts significant effects on regulating sensory, motor and secretory functions of the digestive system through interacting with the different receptor subtypes (5-HT_1-5-HT_7) (Gershon and Tack 2007). It is a potent paracrine secretagogue acting on the intestines of all

studied species, including humans (Hansen and Witte 2008). Studies show that 5-HT stimulates intestinal secretion primarily through the 5-HT_3 receptor and 5-HT_4 receptor (Nagakura *et al.* 1997, Ning *et al.* 2004, Yang *et al.* 2010). 5-HT_3 receptors belong to the ion-channel-linked receptor superfamily, whereas 5-HT_4 receptors belong to G protein-coupled receptor superfamily that stimulate cAMP production in response to 5-HT (Gerald *et al*. 1995).

Various types of psychological and physical stressors have a significant impact on several components of intestinal mucosal function, which is thought to contribute to symptoms in chronic inflammatory diseases and functional disorders of the gastrointestinal tract (Kim *et al*. 2010, Konturek *et al*. 2011). *In vivo* and *in vitro* studies suggest that 5-HT is related to stress-induced alterations of colonic secretion, and peripheral 5-HT_3 and 5-HT_4 receptors are involved in this process (Goldhill *et al*. 1998, Hirata *et al*. 2008, Kiso *et al*. 1997, Li *et al*. 2011). Furthermore, in the clinical practice, both 5-HT_3 receptor (Fujita *et al*. 2005) and 5-HT_4 receptor agonists (Camilleri *et al*. 2008) have proved effective in treating constipation. However, the precise mechanisms by which 5-HT regulates colonic mucosal secretion during stress remain unclear. The aim of the present study is to investigate the alterations of 5-HT-induced rat colonic ion transport following acute stress, and to determine the underlying mechanisms of this phenomenon.

Animals and Methods

Animals and water immersion restraint stress models

The animal use protocol, which is based on NIH guidelines, was approved by the

Animal Care and Use Committee of Capital Medical University. Adult male Sprague-Dawley rats (Laboratory Animal Services Center, Capital Medical University) ranging 200-300g had free access to standard rodent laboratory food and water until the day of the experiments. The rats were randomly divided into the following two groups: control and water-immersion restraint stress (WIRS), and kept for at least 7d before starting experiments. The WIRS group rats, were tied to a wooden board, and their entire bodies, except for their heads, were immersed vertically to the level of the xiphoid process in a water bath maintained at 19±1°C for 2h as previously described (Li *et al*. 2006, Li *et al*. 2011). Animals were conscious during the stress procedure. At the end of the experimental period, the animals were euthanized by cervical dislocation.

Colonic mucosa preparation

The distal colon was removed and defined as an approximately 7 cm-long segments proximal to the lymph node of anus. As previous studies (Yang *et al*. 2006) demonstrated that different segments of the distal colon have diverse physiological properties, the distal colon was divided into four segments, which were termed DC1 (adjacent to the lymph node), DC2, DC3 and DC4. DC3 and DC4 were chosen for the study, due to their consistent and similar responses to 5-HT. Each tissue was cut along the mesenteric border into a flat sheet and flushed with ice-cold oxygenated Krebs-Henseleit solution (K-HS) containing the following ingredients: 117mM NaCl, 4.7mM KCl, 1.2mM $MgCl_2 \cdot 6H_2O$, 1.2mM NaH_2PO_4, 25mM $NaHCO_3$, and 2.5mM $CaCl_2 \cdot 2H_2O$. Additionally, as described in our previous study (Li *et al*. 2011), endogenous prostaglandins are released during tissue preparation, and the cyclooxygenase (COX) pathway plays a major role in the mediation of the secretory response to exogenous 5-HT *in vitro*, indomethacin (10 μM), a

COX inhibitor, was routinely added to the K-HS. The tissue was pinned flat with the mucosal side facing up in a petri-dish containing ice-cold oxygenated K-HS. The colonic mucosa-only preparations were obtained using dissecting forceps under a dissecting microscope.

Short circuit current measurement

Short circuit current (I_{SC}) was measured *in vitro* in Ussing chambers. A flat sheet of the colonic mucosa-only preparation was mounted between two halves of a modified Ussing chamber, in which the total cross-sectional area was 0.5 cm^2. The basal and apical surfaces of the tissue were bathed in 5 ml of K-HS maintained at 37℃ via by recirculation from a reservoir during the experiments. The K-HS was bubbled with 95% O_2 and 5% CO_2 to maintain the pH of the solution at 7.4. The trans-epithelial potential difference of each preparation of colonic mucosa was measured with an Ag/AgCl reference electrodes (Physiologic Instruments, San Diego, CA, USA; P2020S) connected to a preamplifier that was, in turn, connected to a voltage-clamp amplifier (Physiologic Instruments, San Diego, CA, USA; VCC MC6). The tissue was continuously voltage-clamped to a zero potential difference by the application of external current, with compensation for fluid resistance. The tissues were allowed to rest for approximately 30 min to stabilize. Drugs were added directly to either the basolateral side of the epithelial sheets. Responses were continuously recorded by a computer. The transepithelial resistance (Ω cm^2) was measured by altering the membrane potential in a stepwise fashion (-0.1 mV) and applying Ohm's law. The baseline value of the electrical parameters was determined as the mean over the 3 min immediately prior to drug administration. The change in I_{SC} response (ΔI_{SC}) was calculated on the basis of the values before and after stimulation and was normalized as current per unit area of

epithelium ($\mu A \cdot cm^{-2}$), which allowed the area under the curve for 15 min to be calculated ($\mu A \cdot min$). A positive I_{SC} corresponded to the net eletrogenic anion secretion (such as Cl^-) or cation absorption (such as Na^+).

Real time RT-PCR

Total RNA from the colonic mucosa preparations was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the protocol of the manufacturer. The first strand of cDNA was synthesized following the protocol of the superscript first-strand synthesis system for RT-PCR (Invitrogen). Transcripts encoding 5-HT_3 receptors, 5-HT_4 receptors and β-actin in samples of WIRS and control rat colonic mucosa were comparatively quantified by real time PCR with the Brilliant SYBR Green QPCR Master Mix kit (Stratagene, La Jolla, CA,USA), using a Light Cycler instrument (Stratagene). Expression of β-actin was used as an internal control for normalization. Amplifications were performed in a final volume of 20 μl of reaction mixture according to the manufacturer's instructions. The primers were used at a final concentration of 0.2 μM, and 0.3 μl of cDNA prepared from tissue were added to the mixture. Data were analyzed with MxPro QPCR software (version 3.0, Mx3000P system, Stratagene). Primer sequences used for amplifications were as follows: β-actin, forward: 5′-TTC AAC ACC CCA GCC ATG T-3′, reverse: 5′-GTG GTA CGA CCA GAG GCA TAC A-3′; 5-HT_3 receptor, forward: 5′-TGC ATA CCA TCC AGG ACA TCA-3′, reverse: 5′-CTC TTG TCC GAC CTC ACT TCT TC-3′; 5-HT_4 receptor, forward: 5′-GCT GGG TCA TTC CCA TGT TT -3′, reverse: 5′-CAA CTA TGC CGA TGT TGT TCC A-3′.

Western blotting

Parts of the colonic mucosa (0.1~0.2g) were harvested from control and WIRS rats prior to short circuit current experiments. All samples were washed with cold phosphate-buffered saline (PBS) and homogenized in 300 μl of cold lysis buffer, pH 7.5, containing Nonidet P-40 (1%), Tris-HCl (10 mM, pH8.0), NaCl(150 mM), SDS(0.1%), EDTA(1 mM), leupeptin (5 μg/ml), aprotinin (5 μg/ml), PMSF(1 mM), deoxycholic acid (0.5%) and sodium orthovanadate (1mM), all of which were purchased from the Sigma Company. Total tissue homogenates were sonicated to dissolve them completely and then centrifuged at 12,000 rpm for 30 min at 4°C to separate the membrane-containing fraction (pellet) from the cytosol. The supernatant was collected, and its protein level was quantified by the BCA assay according to the manufacturer's protocol (Thermo, Rockford, IL, USA). Proteins (100 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis. Following electrophoresis and the transfer of proteins onto the nitrocellulose membrane (Millipore, Billerica, MA, USA), the membrane was blocked in TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% v/v Tween-20) containing 10% nonfat milk for 1h at room temperature. The membrane was then incubated with primary polyclonal antibodies to the 5-HT_3 receptor (Santa Cruz/sc-28958, diluted 1:400), 5-HT_4 receptor (Novus/NBP1-19627, diluted 1:600), GAPDH (Sigma/G9545, diluted 1:10000) or Actin (Sigma/A 5060, diluted 1:10000) overnight at 4°C. After being washed 3 times in TBST, for 10 min each wash, the membrane was incubated with the appropriate secondary antibodies (goat anti-rabbit IgG, Rockland/16747, diluted 1:10000) for 1 h at room temperature, followed by washing as performed previously. The protein bands were visualized via scanning by Odyssey Infrared Imager (LI-COR, NE, USA), and analyzed by Odyssey software (version 1.2).

cAMP measurement

Rat colonic mucosa samples (about 150 mg) were incubated in K-HS solution for 30 min for equilibration, and all samples were pretreated with indomethacin (10 μM), TTX(1 μM). The samples were pretreated with vehicle (saline), 5-HT_4 receptor antagonists (SB204070, 1 μM) for 5 min before adding 5-HT (10 μM, 20 min). All colonic mucosa-only samples from the WIRS and control rats were frozen in liquid nitrogen immediately and ground into a fine powder under liquid nitrogen by a stainless steel mortar. After the liquid nitrogen evaporated, the frozen tissues were weighed and homogenized in 10 volumes of 0.1 M HCl before being centrifuged at 600 × g at room temperature for 5 min. The supernatant was subsequently collected for cAMP measurement. Intracellular levels of cAMP were determined using the Enzyme Immunoassay direct cyclic AMP kit according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, Mo.). The optical density (OD), which was inversely proportional to the concentration of cAMP in both the standards and the samples, was read at 405 nm on a microplate reader (Bio-Rad).

Drugs

5-hydroxytryptamine (5-HT), 3-tropanyl-3, 5-dichlorobenzoate (MDL72222), (1-Butyl-4-piperidinyl) methyl-8-amino-7-chloro-1, 4-benzodioxane-5-carboxylate hydrochloride (SB204070), GR113808, indomethacin, and tetrodotoxin (TTX) were obtained from Sigma Chemical Company (St. Louis, MO, USA). Y-25130 hydrochloride (Y-25130) were obtained from Tocris bioscience (Tocris Cookson Ltd, UK). Drug concentrations were chosen based on previous studies. Stock solutions of indomethacin and MDL72222 were dissolved in dimethyl sulphoxide

(DMSO), and the final DMSO concentrations never exceeded 0.1% (v/v). The other drugs were dissolved in aqueous stock solution.

Statistical analysis

The values are presented as means ± S.E.M. (standard error of mean): "*n*" refers to the number of rats or the number of pairs used. The statistics and graphs were generated using GraphPad Prism, version 5.0 (GraphPad Software Inc., San Diego, CA, USA). Statistical analysis was conducted using Student's paired or unpaired t-test. "*P*" values less than 0.05 denoted a statistically significant difference.

Results

Alterations of basic characteristics of electrophysiology and 5-HT-induced ion transport in the rat distal colonic mucosa after water immersion restraint stress

After an equilibration time of about 30 min, the mean basal I_{SC} and the transepithelial resistance (*Rte*) of the colonic epithelium were 42.2±4.0 $\mu A \cdot cm^{-2}$ and 72.9±3.9 $\Omega \cdot cm^2$ in control rats, and 60.2±4.2 $\mu A \cdot cm^{-2}$ and 68.8±3.7 $\Omega \cdot cm^2$ in rats exposed to 2 h of WIRS, respectively. The baseline I_{SC} in WIRS rats was significantly higher than that of the control rats, as it was 42.2±4.0 $\mu A \cdot cm^{-2}$ for the control rats and 60.2±4.2 $\mu A \cdot cm^{-2}$ for the WIRS rats (n=26, P<0.01, Fig. 1A1), but no significant difference in *Rte* was observed between the control and the WIRS rats (n=26, P>0.05, Fig. 1A2).

Based on our previous studies (Ning *et al*. 2004, Li *et al*. 2011), indomethacin (10 μM) was routinely added to subsequent experiments to abolish the effects of endogenous prostaglandin，and the nerve conduction blocker, tetrodotoxin (TTX) (1 μM), was later added to block any effects of residual nerve activity. Then, 10 μM of 5-HT was added to the basolateral side of the tissue. The area under the 15 min period of I_{SC} recording was calculated to represent the total charge transfer per unit area for the given period.

The 5-HT-induced ΔI_{SC} in colonic mucosa in WIRS rats (1651.0±195.5μA·min) was significantly higher than in control rats (999.0±115.1μA·min) (*n*=8, *P*<0.01, Fig. 1 B1, B2).

Role of 5-HT$_3$ receptors in the enhanced 5-HT-induced ion transport in rat colonic mucosa under water immersion restraint stress condition

As shown in a previous study, 5-HT induces colonic ion transport by acting on 5-HT$_3$ and 5-HT$_4$ receptors, which are located on colonic epithelial cells (Budhoo and Kellum 1994, Glatzle *et al*. 2002, Hirata e*t al*. 2008). We first tested the role of 5-HT$_3$ receptors in the setting of enhanced 5-HT-induced ion transport in WIRS rats. Highly seceletive 5-HT$_3$ receptor antagonists, MDL72222 (10 μM) and Y-25130 (5 μM), were chosen (Yang *et al.* 2010, Lee *et al*. 2005). Short circuit current results showed that pretreatment with MDL72222 (10 μM) or Y-25130 (5 μM) did not affect 5-HT-induced ion transport under either normal or WIRS conditions (*n*=5, Fig. 2 A1, A2, A3, A4). Real time RT-PCR results showed that the mRNA expression of 5-HT$_3$ receptors in WIRS rats, was reduced by 43% compared with normal rats (100%) (*n*=4, *P*<0.05, Fig. 2B1).Western blot results showed no obvious alterations in the protein expression of 5-HT$_3$ receptors in WIRS rats compared with control rats (*n*=6, *P* >0.05, Fig. 2B2).

Role of 5-HT$_4$ receptors in the enhanced 5-HT-induced ion transport in rat colonic mucosa under water immersion restraint stress condition

To determine the role of 5-HT$_4$ receptors in 5-HT-induced colonic ion transport in WIRS rats, the 5-HT$_4$ receptor specific antagonist, 1 μM of SB204070 and 1 μM of GR113808, was administered (Yuan, *et al*. 2011, Ning, *et al*. 2004). Short circuit current results indicated that pretreatment with SB204070 (1μM) inhibited 5-HT (10μM) -induced ΔI_{SC} by 96% in control rats (n=5, P<0.01, Fig. 3 A1 A3), and by 91.2% in WIRS rats (n=5, P<0.01, Fig. 3 A2 A3). Pretreatment with another 5-HT$_4$ receptor specific antagonist, GR113808 (1 μM), also almost totally inhibited 5-HT (10 μM)-induced ΔI_{SC} in both group rats (n=4, Fig. 3 A4 A5). Real time RT-PCR results showed a 45% reduction in mRNA expression of 5-HT$_4$ receptors in WIRS rats compared with control rats (n=6, P<0.001, Fig.3 B1), but Western blot analysis revealed no significant alterations in protein expression of 5-HT$_4$ receptors in the colonic mucosa of WIRS rats (n=6, P>0.05, Fig. 3 B2).

Role of the cAMP pathway in enhanced 5-HT-induced ion transport in rat colonic mucosa under water immersion restraint stress condition

To measure the change in cellular cAMP levels, an ELISA was used. The results showed that the basal cAMP level in colonic epithelial cells of WIRS rats was 11.5±0.15 pmol/mg, which was significantly higher than that of control rats (8.31±0.43 pmol/mg) (n=5, P<0.001, Fig. 4A). 5-HT increased intracellular cAMP level of colonic mucosa in control rats by 38.5% (n=5, P<0.05, Fig. 4A) and in WIRS rats by 19.1% (n=5, P<0.01, Fig. 4A). Pretreatment with 5-HT$_4$ receptor

antagonist SB204070 (1 μM) almost totally block 5-HT-induced increase in colonic intracellular cAMP of both control and WIRS rats (*n*=5, Fig.4A). Forskolin, an adenylate cyclase activator that does not require receptor mediation, was chosen to compare the effect of direct stimulation on colonic ion transport in both the WIRS and control groups, which imitate the effect of 5-HT. Basal addition of forskolin (1 μM) stimulated an I_{SC} increase, 197.5±8.476 μA·min in WIRS rats, which was much higher than that the 149.5±17.02 μA·min increase observed in control rats (*n*=6, *P*<0.05, Fig. 4 B1 B2).

Discussion

Increasing evidence supports a prominent role of stress in the pathophysiology and clinical presentation of irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) (Reber 2012, Chang 2011). 5-HT acts as a mediator of stress-induced colonic dysfunction, the symptoms of which include an increase in stool excretion, diarrhea, and abdominal pain (Sanger *et al*. 2000, Julio-Pieper *et al*. 2012). Additionally, 5-HT has also been implicated in many gastrointestinal disorders, such as IBS and IBD (Manocha and Khan 2012). A combined blockade of 5-HT_3 and 5-HT_4 receptors can completely inhibits 5-HT-induced diarrhea in mice and rats (Nagakura *et al*. 1997). However, the mechanism of stress-induced alterations of 5-HT regulatory pathways in colonic ion transport is not clear. This study has investigated the mechanism underlying 5-HT-enhanced colonic ion transport under stressful conditions.

Stress induces hyper-secretion in the human intestine (Barclay and Turnberg 1987). Goldhill *et al*. (1998) have also reported that the *in vitro* response of the colonic epithelium to

5-HT is altered by prior whole-body stress, and have detected a bimodal effect. Mucosal tissue from approximately 50% of studied animals displayed a reduced responsiveness to 5-HT, which may be indicative of a reduction in receptor number, desensitization of receptors or induction of inhibitory pathways. In our previous studies, we have observed that activation of submucosal neural 5-HT_3 receptors elicited a somatostatin-dependent inhibition of ion secretion in rat colon (Yang *et al*. 2010). This neural 5-HT_3 receptor-mediated somatostatin-dependent secretoinhibitory pathway is suppressed in the acute water-immersion restraint stressed rats (Li *et al*, 2011), which may contribute to the acute stress-induced increase in colonic secretion. However, we do not know whether non-neural 5-HT_3 or 5-HT_4 receptors are involved in the colonic hyper-secretion in acute stress. Our results showed that the baseline I_{SC} of colonic mucosa tissues was significantly elevated in WIRS rats. And almost all colonic mucosa tissues from WIRS rats were hyper-responsive to 5-HT, which can be almost completely blocked by pretreatment with a 5-HT_4 receptor antagonist (SB204070 or GR113808). However, the protein level of 5-HT_4 receptors in stressed distal colonic mucosa of rats was not significant affected. This finding suggests that the increased ion transport induced by 5-HT is predominantly mediated by 5-HT_4 receptors in WIRS rats, but acute stress may have little impact on the number of 5-HT_4 receptors in colonic mucosa. Our results also showed that the mRNA level of both 5-HT_3 and 5-HT_4 receptors were significantly decreased in WIRS rats. This inconsistency between mRNA and protein might be the consequence of negative feedback regulation in rat colonic mucosa against the hyper-responsiveness to 5-HT. It has been also reported that the absence of mRNA-protein correlation for the investigated genes suggests that the relation between mRNA and protein is not strictly linear (de SOUSA *et al*. 2009), and different regulation mechanisms (such as synthesis and

degradation rates) acting on both the synthesized mRNA and protein affect the amount of the two molecules differentially.

We suppose that the increased 5-HT-induced I_{SC} response may be due to an upregulated second messenger system (cAMP) in stressed colonic epithelia, a finding supported by several pieces of evidence. First, previous investigations in our lab showed that 5-HT can elicits Cl^- and HCO_3^- anion secretion and Na^+ absorption by acting directly on colonic epithelial cells via 5-HT_4 receptors, a process mediated by the cystic fibrosis transmembrane conductance regulator (CFTR) (Ning *et al*. 2004). Second, the 5-HT_4 receptor is a G protein-coupled receptor that stimulates cAMP production in response to serotonin, and CFTR functions as a cyclic adenosine 3',5'-monophosphate (cAMP)-regulated chloride channel, which is expressed at high levels in the crypts of adult colon (Crawford *et al*. 1991). Third, stress-related mediators (corticotropin releasing hormone, 5-HT, acetylcholine) (Larauche *et al*. 2009, Stengel and Tache 2009, Karantanos *et al*. 2010) directly act on their respective receptors during stress, and elevate the level of intracellular second messenger, such as Ca^{2+} and cAMP, which may induce more CFTR chloride channel activity (Prince *et al.* 1994) and result in a basal hyper-secretion condition within the stressed intestine.

To verify this hypothesis, cellular cAMP level was measured. The results showed that WIRS colonic mucosa had higher basal level of cAMP than the mucosa of control rats, which was consistent with our expectation. Since studies (Soderholm and Perdue 2001) have reported that stress could enhance baseline Cl^- secretion of distal colon resulted in higher level of colonic baseline $I_{SC,}$ while Cl^- secretion is mainly mediated by the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-dependent Cl^- channel. So, the higher level of cAMP

might activate more CFTR and contribute to the elevated baseline I_{SC} in WIRS rats. 5-HT significantly increased the intracellular cAMP level via 5-HT_4 receptor in the colonic mucosa from both control and WIRS rats, but 5-HT-induced cAMP increase in WIRS rats was not more than that in control rats. Forskolin increased intracellular levels of cAMP by activating the enzyme adenylyl cyclase, which mimicked the effects of 5-HT on colonic ion transport by participating in the post-5-HT_4 receptor signaling pathway. Short circuit current results also showed that the forskolin-induced I_{SC} response was consistent with the effects of 5-HT in stressed rats.

In conclusion, we have demonstrated that the ion transport of colonic mucosa induced by 5-HT is increased in an animal model of acute stress via increases in intracellular cAMP, which may contribute to stress-related hyper-secretion in humans. Whether the secretory responsiveness of the epithelium under acute stress condition undergoes a general change or is merely a specific phenomenon must be studied further by analyzing other secretagogues. This study may help us better understand the mechanisms underlying the gastrointestinal symptoms and discomfort associated with stress.

Conflict of Interest

There is no conflict of interest.

Acknowledgments

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Figures and Figure Legends

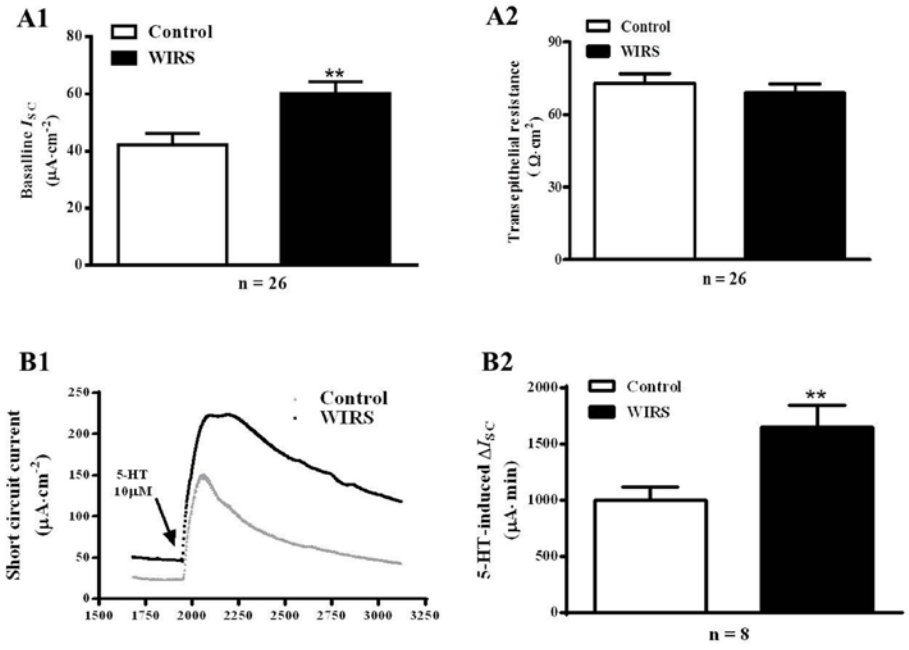

Fig.1. Basic characteristics of electrophysiology and 5-HT-induced ΔI_{SC} in the distal colonic mucosa of control and WIRS rats. (A1) Baseline I_{SC}. (A2) Transepithelial resistance. (B1) Representative I_{SC} recording with arrow indicates the application of 5-HT (10μM, basolateral side) to the colonic mucosa. (B2) Summary of 5-HT-induced ΔI_{SC} in colonic mucosa. Columns show the mean ± S.E.M., ** P<0.01. WIRS: water immersion restraint stress; I_{SC}: short circuit current.

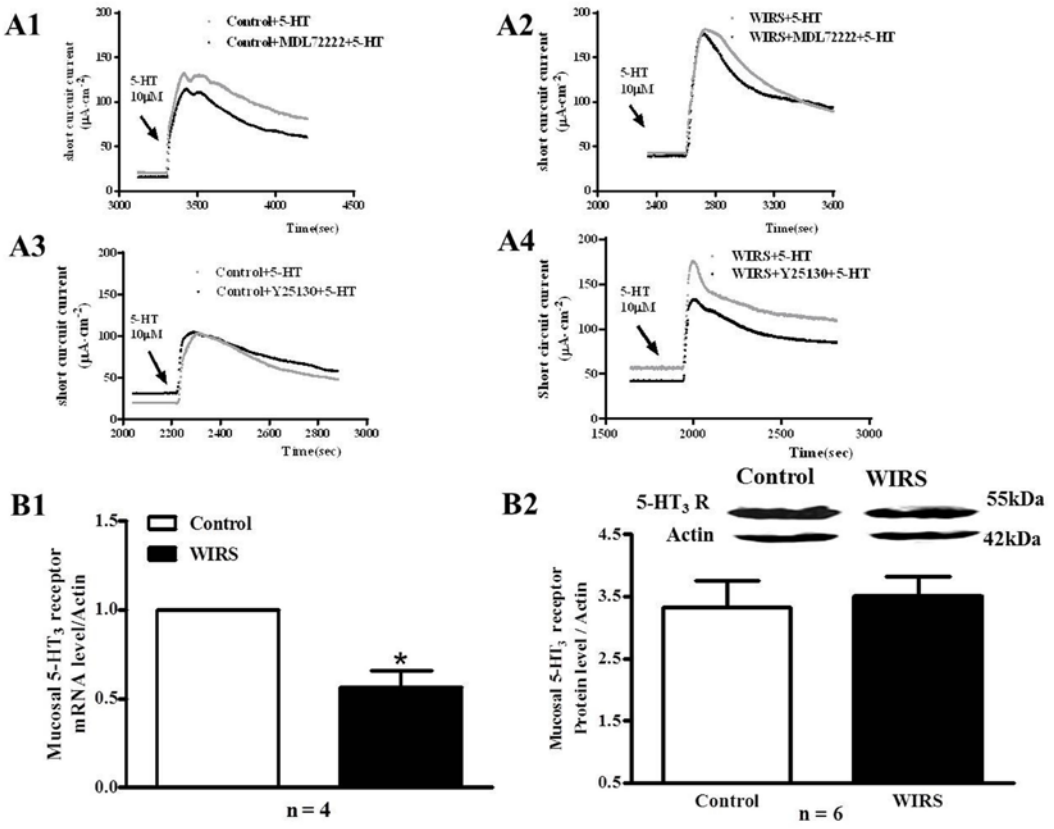

Fig.2. Role of 5-HT_3 receptors in the enhanced 5-HT-induced ΔI_{SC} in distal colonic mucosa of WIRS rats. Representative I_{SC} recording with arrows indicating the time for the basolateral application of 5-HT (10μM) to control (A1, A3) or WIRS (A2, A4) rats mucosa basolateral pretreated with MDL72222 (10μM) (A1, A2) or Y-25130 (5μM) (A3, A4), respectively. The mRNA (B1) and protein (B2) levels of mucosal 5-HT_3 receptors. The difference in expression levels is reflected by variations in the Y-axes. Columns show the mean ± S.E.M., * P<0.05. WIRS: water immersion restraint stress; I_{SC}: short circuit current.

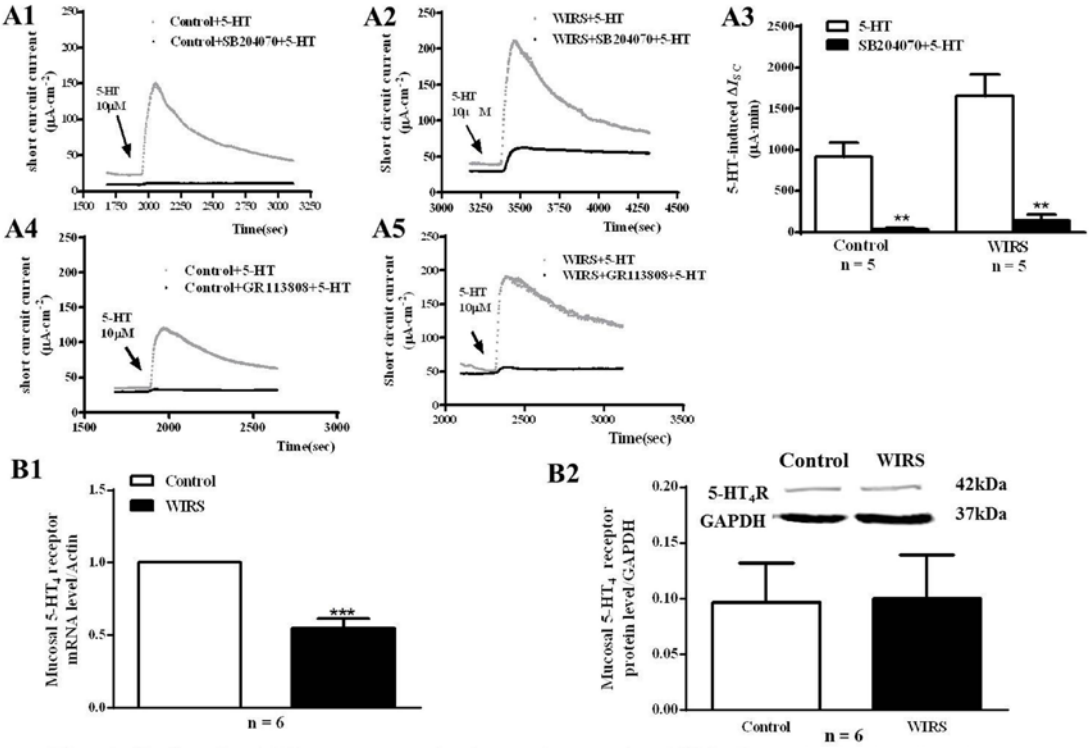

Fig.3. Role of 5-HT_4 receptors in the enhanced 5-HT-induced ΔI_{SC} in distal colonic mucosa of WIRS rats. Representative I_{SC} recording with arrows indicating the time for the basolateral application of 5-HT (10μM) to control (A1, A4) or WIRS (A2, A5) rats mucosa basolateral pretreated with SB204070 (1μM) (A1, A2) or GR113808 (1μM) (A4, A5), respectively. Summary of the effects of SB204070 on 5-HT-induced ΔI_{SC} (A3). The mRNA (B1) and protein (B2) levels of mucosal 5-HT_4 receptors. The difference in expression levels is reflected by variations in the Y-axes. Columns show the mean ± S.E.M., $^{**}P < 0.01$, $^{***}P < 0.001$. WIRS: water immersion restraint stress; I_{SC}: short circuit current.

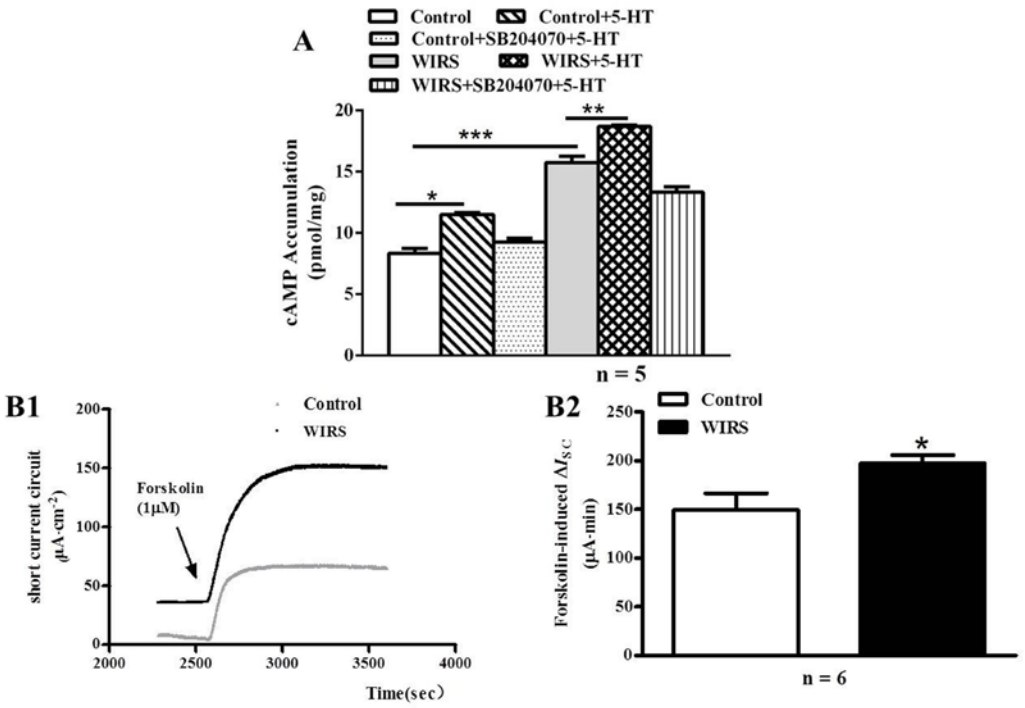

Fig.4. Role of the cAMP pathway in the enhanced 5-HT-induced ΔI_{SC} in distal colonic mucosa of WIRS rats. (A) Summary of the basal intracellular cAMP level and effects of 5-HT_4 receptor antagonists, SB204070 (1μM) on 5-HT (10μM)-stimulated intracellular cAMP production in the colonic mucosa preparations of control and WIRS rats. (B1) Representative I_{SC} recording with arrow indicates the application of forskolin (1μM, basolateral side) to the mucosa. (B2) Summary of forskolin-induced ΔI_{SC} in the colonic mucosa. Columns show the mean ± S.E.M., *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. WIRS: water immersion restraint stress; I_{SC}: short circuit current.