British Journal of Pharmacology

British Journal of Pharmacology (2017) **174** 3370–3381 3370

RESEARCH PAPER

Ameliorative effect of chlorpromazine hydrochloride on visceral hypersensitivity in rats: possible involvement of 5-HT_{2A} receptor

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Received 7 March 2017; **Revised** 13 July 2017; **Accepted** 17 July 2017

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BACKGROUND AND PURPOSE

Visceral hypersensitivity is responsible for pathogenesis of irritable bowel syndrome (IBS). Therefore, its prevention can help avoid abdominal pain and discomfort in IBS. To find candidate drugs for visceral hypersensitivity, we screened existing medicines for their ability to prevent visceral sensitivity induced by colorectal distension (CRD) in rats and identified chlorpromazine, a typical antipsychotic drug, as a candidate compound. In this study, we investigated the effect of chlorpromazine on visceral hypersensitivity.

EXPERIMENTAL APPROACH

Visceral sensitivity (visceromotor response) was monitored by measuring the electrical activity of the abdominal external oblique muscle contraction in response to CRD using a barostat apparatus. Visceral hypersensitivity was induced by a colonic instillation of sodium butyrate or acetic acid in neonates.

KEY RESULTS

Oral administration of chlorpromazine suppressed butyrate-induced visceral hypersensitivity to CRD. Interestingly, atypical antipsychotic drugs, quetiapine and risperidone, ameliorated butyrate-induced visceral hypersensitivity, whereas the typical antipsychotic drugs, haloperidol and sulpiride, did not. Pharmacological analysis using specific inhibitors showed that a selective 5-HT_{2A} receptor antagonist, ketanserin, suppressed butyrate-induced visceral hypersensitivity, whereas a selective dopamine D_2 receptor antagonist, L-741626, did not. Furthermore, the 5-HT_{2A} receptor agonist AL-34662 stimulated visceral sensitivity to CRD in healthy control rats but not in butyrate-treated rats. These findings suggest that increased 5-HT levels in the colon contribute to the induction of visceral hypersensitivity.

CONCLUSIONS AND IMPLICATIONS

Our results indicate that chlorpromazine ameliorates visceral hypersensitivity and that the 5-HT_{2A} receptor is a potential therapeutic target for treating abdominal pain and discomfort in IBS.

Abbreviations

7OH-Cpz, 7-hydroxy chlorpromazine; ASIC, acid-sensing ion channel; CRD, colorectal distension; DRG, dorsal root ganglion; IBS, irritable bowel syndrome; *p*-CPA, *p*-chlorphenylalanine; TRPV1, transient receptor potential vanilloid 1; VMR, visceromotor response

DOI:10.1111/bph.13960

Introduction

Irritable bowel syndrome (IBS) is a complex functional gastrointestinal disorder characterized by chronic, recurrent abdominal pain and altered bowel habits (diarrhoea, constipation or alternating diarrhoea/constipation). Its prevalence in the general population is remarkably high (~11% of the world's population) (Chang *et al.,* 2014). Although not life-threatening, it imposes a large burden on global healthcare and considerably reduces patient quality of life (Canavan *et al.,* 2014). However, no satisfactory pharmacological therapy has been identified for IBS.

Psychiatric comorbidities are highly common in IBS patients (Ladabaum *et al.,* 2012). Centrally acting agents including antidepressants, anxiolytics, antipsychotics and sedatives are two to four times more likely to be prescribed to presumed IBS patients compared to other patients (Grover and Drossman, 2011). Atypical antipsychotic drugs have been effective in patients with severe IBS and abdominal pain (Grover *et al.,* 2009; Martin-Blanco *et al.,* 2010; Pae *et al.,* 2013). However, the mechanisms underlying the efficacy of these drugs in ameliorating pain are unclear.

Visceral hypersensitivity plays an important role in IBS pathogenesis, and IBS patients have been shown to present visceral hypersensitivity in response to colorectal distension (CRD) (Azpiroz *et al.,* 2007). Therefore, suppression of this pathology could be an effective treatment option for IBS. The mechanisms responsible for visceral hypersensitivity are not completely understood. However, several contributing factors have been suggested, including enhanced visceral perception, altered intestinal microbiota, post-inflammatory changes in gastrointestinal function and enhanced immunological reactivity (Keszthelyi *et al.,* 2012; Matricon *et al.,* 2012; Simren *et al.,* 2013; Brierley and Linden, 2014). In particular, butyrate, a short-chain fatty acid produced by bacterial fermentation in the colon, appears to be important in the development of visceral hypersensitivity. Increased faecal butyrate levels have been observed in IBS patients with diarrhoea, and rectal instillation of butyrate increases visceral sensitivity to colonic distension in rats (Treem *et al.,* 1996; Bourdu *et al.,* 2005). **5-HT** (serotonin) is another key molecule regulating visceral perception (Cremon *et al.,* 2011). In fact, a 5-HT_3 receptor antagonist (alosetron) and 5-HT_4 receptor agonist (tegaserod) have been approved for the clinical treatment of diarrhoea- and constipation-predominant IBS respectively. However, adverse effects, such as ischaemic colitis and cardiac toxicity, limit the use of these drugs (Pasricha, 2007). The role of other 5-HT receptor subtypes, including **5-HT_{2A} receptor**, in visceral sensitivity, has not been fully investigated.

The 5-HT_{2A} receptor is mainly located in the brain, spinal cord, sensory neurons and intestines. Recently, the 5-HT_{2A} receptor antagonist cyproheptadine has been found effective against abdominal pain in children with IBS (Madani *et al.,* 2016). Moreover, the 5-HT_{2A} receptor antagonist **ketanserin** showed an antinociceptive effect on abdominal pain in the acetic acid-induced writhing test (Alhaider, 1991). These findings indicate that the 5-HT_{2A} receptor is involved in visceral pain sensitivity. In addition to visceral pain, the 5-HT_{2A} receptor is associated with the potentiation of pain sensation. Further, activation of the 5-HT_{2A} receptor enhances transient receptor potential vanilloid 1 (TRPV1) function in colon sensory neurons and acid-sensing ion channels (ASICs) in dorsal root ganglion (DRG) neurons *in vitro* (Sugiuar *et al.,* 2004; Qiu *et al.,* 2012). These results suggest that the 5-HT_{2A} receptor participates in peripheral sensitization.

We previously screened a compound library of clinically available drugs for their ability to prevent the visceral pain response to noxious CRD (Asano *et al.,* 2017) and identified aminophylline, a bronchodilator, as a potential candidate. As **chlorpromazine** strongly suppressed visceral sensitivity to noxious CRD in the same phenotypic screen, it was also considered a candidate drug for abdominal pain. Chlorpromazine is traditionally used for treating schizophrenia, nausea and vomiting and severe hiccups (Adams *et al.,* 2005) and has antagonistic activity at various receptors, including the dopamine **D_2 receptor** and 5-HT_{2A} receptors (Horacek *et al.,* 2006). Chlorpromazine's antagonistic activity against the dopamine D_2 and 5-HT_{2A} receptors is involved in its efficacy in regulating positive or negative symptoms and cognitive symptoms in schizophrenia respectively (Horacek *et al.,* 2006).

In this study, we investigated the effect of chlorpromazine on visceral hypersensitivity to CRD. Our results suggest that 5-HT_{2A} receptor activation and increased 5-HT levels in the colon are involved in visceral hypersensitivity.

Methods

Animals

Male Wistar rats (4 to 5 weeks old, 150–200 g) or primiparaous late pregnant Wistar female rats were obtained from Charles River Laboratories Japan (Yokohama, Japan). Animals were housed under conditions of controlled temperature (22–24°C) and light (12 h light: 12 h dark cycle) for 1–2 weeks before experiments. The standard pellet diets (Rodent Diet CE-2, CLEA Japan, Inc.) were fed to the experimental animals ad libitum. The experiments and procedures described here were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health (NIH, USA) and were approved by the Animal Care Committees of Keio University and St. Marianna University. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.,* 2010; McGrath and Lilley, 2015).

Assessment of visceromotor response (VMR) to CRD

VMR to CRD was monitored as described previously (Asano *et al.,* 2017). Briefly, rats were deeply anaesthetized with an intraperitoneal injection of mixture of medetomidine chloride (0.5 $mg \cdot kg^{-1}$), midazolam (2.5 $mg \cdot kg^{-1}$) and butorphanol tartrate (2.5 $mg \cdot kg^{-1}$). We ensured adequate depth of anesthesia by testing pedal withdrawal and palpebral reflexes. The skin was incised (1 cm-long incision), and the external oblique muscle of the abdomen was exposed. Electromyography electrodes (Starmedical, Tokyo, Japan) were sutured into the external oblique muscle to record the EMG. Electrode leads were tunnelled s.c. and exteriorized at the nape of the neck for future access. Incised skin was closed with nylon sutures, and the rats were given butorphanol tartrate

(2.5 mg·kg^{-1}) and viccillin (30 mg·kg^{-1}) s.c. After surgery, rats were housed individually and allowed to recuperate for 5 to 7-days before being used in the CRD study. During the recovery period, several rats broke the EMG electrodes and were excluded from VMR assessment. On the day of the CRD test, rats were restrained in a plastic conical tube cage (diameter, 6 cm; height, 15 cm), 15 min before electromyography. Rats were not habituated to the tube prior to experiments. A polyethylene barostat balloon (length, 2 cm; maximum diameter, 1.5 cm; volume, 2–3 mL) was inserted in the colon, positioned 1 cm proximal to the rectum and connected to a balloon catheter, which was anchored with tape to the base of the tail. Pressure and volume of the balloon were controlled and monitored by a barostat device (Distender Series II; G & J Electronics, Toronto, Canada) connected to it. Conscious rats were subjected to graded phasic CRD (10, 20, 40, 60 and 80 mmHg; each distension in triplicate; duration, 20 s; interstimulus interval, 150 s) to estimate drug activity.

EMG data were collected and analysed using the 8 STAR software package (Star Medical, Tokyo, Japan). EMG amplitude (mV·s) resulting from contraction of the external oblique muscle was quantified by calculating the AUC of the voltage alteration graph during CRD. EMG amplitude data were corrected by subtracting the baseline EMG data, which were collected 20 s before each CRD. The AUC (mmHg·mV·s) of EMG amplitude (mV·s) – balloon pressure (mmHg) curve – was also determined.

Colonic compliance (pressure–volume curves) was monitored during phasic CRD in control rats and presented as change in cylinder volume and balloon pressure.

Chlorpromazine, **sulpiride**, **haloperidol**, **risperidone** or **quetiapine** were administered p.o. 2 h before the CRD test. Chlorpromazine was dissolved in saline, whereas other drugs were suspended in 1% methylcellulose. Ketanserin, **L-741,626**, AL-34662 and 7OH-Cpz were also suspended in 1% methylcellulose but were administered i.p. 15 min before the CRD study. In the 5-HT depletion experiment, rats were administered **p-chlorphenylalanine** (*p*-CPA; 300 mg·kg^{-1}, p.o.) with 0.1% Tween-80 or vehicle 30 min before butyrate application, once daily for 3 days.

Sodium butyrate- or acetic acid-induced visceral hypersensitivity to CRD

A butyrate enema was performed using a previously described method with some modifications (Asano *et al.*, 2012). Briefly, rats were instilled with 1 mL sodium butyrate solution (110 mg·mL^{-1}, pH 6.9) or saline into the colon twice daily for 3 days (day 1 to 3). On day 4, rats were subjected to CRD experiments.

Acetic acid-induced colonic hypersensitivity was induced as described previously (Winston *et al.*, 2007). Primiparous late pregnant Wistar female rats were individually housed for about a week prior to giving birth (10–15 pups per rat). The 10-day-old rat pups were subjected to intracolonic injection of 0.2 mL acetic acid (0.5% in saline) 2 cm from the anus; control rats received an equal volume of saline. At 5–6 weeks of age, VMR to CRD was measured in both groups of male rats.

Western blot analysis

Rats were killed by decapitation, and tissue samples from the sigmoid colon and L5-S1 dorsal root ganglia were collected, frozen with liquid nitrogen and stored at −80°C until Western blot analysis. Tissue samples were homogenized in RIPA buffer [50 mM Tris–HCl (pH 7.2), 150 mM NaCl, 1% sodium deoxycholate, 1% SDS, 1% NP-40] containing protease inhibitor cocktail (Sigma, St. Louis, MO, USA) and phosphatase inhibitor cocktail (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Samples were centrifuged at 15 000 × *g* at 4°C, and the supernatants were collected. Total protein concentration of the sample was determined using the Bradford method. Samples were subjected to electrophoresis on 10% polyacrylamide SDS gel (Thermo Fisher Scientific, Rockford, IL, USA), after which the separated proteins were transferred to PVDF membranes. Membranes were blocked for 30 min with Tris phosphate buffer containing 0.1% Tween-80 (T-TBS) and 5% skimmed milk and incubated overnight at 4°C with T-TBS containing the appropriate primary antibodies. Antibodies, including mouse monoclonal anti-5-HT_{2A} receptor (5-HT_{2A} R) antibody (MABN1595, Merck Millipore, Darmstadt, Germany), rabbit polyclonal anti-phosphorylated **ERK1/2** antibody (#9101, Cell Signalling Technology), rabbit polyclonal anti-ERK1/2 antibody (#9102, Cell Signalling Technology) and mouse monoclonal anti-β-actin antibody (Sigma, St. Louis, MO, USA) were used at 1:2000, 1:2000, 1:2000 and 1:1000 dilutions respectively. The membranes were washed three times with T-TBS, incubated for 60 min at room temperature with horseradish peroxidase-conjugated secondary antibodies in T-TBS containing 2% skimmed milk and washed three times with T-TBS. Signal was developed in ECL™ Prime solution (GE Healthcare) and imaged using ImageQuant™ LAS4000 image analyser (GE Healthcare). Intensity of immunoreactive bands was quantified using Image J software (NIH) and normalized to β-actin levels. Data are expressed as the fold change over saline groups.

Measurement of 5-HT levels in colonic tissues

After the 3 day butyrate treatment, the rats were killed and colon tissues were collected to determine tissue 5-HT levels using an enzyme immunoassay kit (Beckman Coulter, Inc., Fullerton, CA, USA), according to the manufacturer's instructions.

Statistical analyses

Data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). In all animal studies, a co-worker blinded to the experimental protocol randomized animals into groups. The values presented were derived from at least two independent experiments. All data are expressed as mean ± SEM values. Normalization was performed for the quantitative analysis of Western blots. Statistical analyses were performed using SPSS 24.0 software (IBM, Armonk, NY, USA). One or two-way ANOVA followed by Dunnett's test or Student's *t*-test for unpaired results was used to evaluate differences between more than two groups or between two groups respectively. Two-way ANOVA with repeated measures followed by Bonferroni's method was used for the phasic CRD test. Differences were considered significant at $P < 0.05$.

Chemicals

7-Hydroxy chlorpromazine (7OH-Cpz) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Methylcellulose, sodium butyrate, haloperidol and quetiapine were obtained from Wako Pure Chemical Industries (Osaka, Japan). Medetomidine chloride (Domitor®) and butorphanol tartrate (Vetorphale®) were purchased from Meiji Seika Pharma Co., Ltd. (Tokyo, Japan). Midazolam was purchased from SANDOZ (Tokyo, Japan). 5-HT, *p*-chlorphenylalanine, sulpiride and chlorpromazine hydrochloride (chlorpromazine) were purchased from Sigma (St. Louis, MO, USA). Risperidone (Ris) was obtained from LKT laboratories, Inc. (St Paul, MN, USA). AL-34662 was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). L-741,626 and ketanserin tartrate (ketanserin) were obtained from Abcam (Cambridge, UK).

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org/, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.,* 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.,* 2015a,b).

Results

Effect of chlorpromazine on butyrate-induced visceral hypersensitivity in rats

Effect of oral administration of chlorpromazine on butyrate-induced visceral hypersensitivity, which is a model of IBS, was investigated in rats. Rats subjected to butyrate treatment showed increase in VMR to CRD compared with that in saline-treated control rats (Figure 1A, B). Oral pre-administration of chlorpromazine significantly decreased this response in a dose–response manner (Figure 1A, B). Interestingly, chlorpromazine did not affect visceral sensitivity in healthy control rats except at a high dose of 40 $mg \cdot kg^{-1}$ (Figure 1C) nor was colonic compliance affected (Figure 1D). In a preliminary experiment, we observed that 12 $mg \cdot kg^{-1}$ chlorpromazine significantly induced a cataleptic response in healthy rats (an adverse effect). However, there was no significant difference between inhibitory actions of 4 and 12 $mg \cdot kg^{-1}$ chlorpromazine in Figure 1A, B. Thus, we decided to use 4 $mg \cdot kg^{-1}$ chlorpromazine for subsequent experiments. We also assessed the effect of chlorpromazine on visceral hypersensitivity induced by colonic instillation of dilute acetic acid to neonatal rats, another model of IBS. As shown in Figure 1E, chlorpromazine restored visceral sensitivity stimulated by acetic acid to the levels observed in control rats.

The effect of antipsychotics other than chlorpromazine on visceral hypersensitivity to CRD was also evaluated. Typical antipsychotic drugs sulpiride and haloperidol and atypical antipsychotics quetiapine and risperidone were analysed. In addition to inhibiting the dopamine D_2 receptor, quetiapine and risperidone are 5-HT_{2A} receptor antagonists. According to a previous report, sulpiride, haloperidol, quetiapine and risperidone show <500, 20, 0.13 and 0.33 5-HT_{2A}/D_2 K_i ratios respectively (Horacek *et al.,* 2006). We examined whether the inhibition of 5-HT_{2A} receptor by antipsychotic drugs is associated with the suppression of visceral hypersensitivity. As shown in Figure 2A, B, quetiapine and risperidone suppressed butyrate-induced visceral hypersensitivity to CRD, whereas sulpiride and haloperidol did not.

Role of 5-HT_{2A} receptor in visceral hypersensitivity

Next, it was investigated whether 5-HT_{2A} or dopamine D_2 receptor antagonists could inhibit sodium butyrate-induced increase in visceral sensitivity. Results showed that a selective 5-HT_{2A} receptor antagonist, ketanserin, ameliorated butyrate-induced visceral hypersensitivity to CRD, whereas a selective dopamine D_2 receptor antagonist, L-741,626, did not. To confirm these findings, the effect of 7OH-Cpz, which does not bind to 5-HT_{2A} receptors, was also assessed. 7OH-Cpz (4.2 $mg \cdot kg^{-1}$) was administered p.o. to rats at a dose equivalent to that of chlorpromazine. 7OH-Cpz did not suppress butyrate-induced visceral hypersensitivity to CRD (Figure 3A, B).

Moreover, we investigated whether a 5-HT_{2A} receptor agonist stimulated visceral sensitivity to CRD in healthy rats to study the involvement of 5-HT_{2A} receptor activation in the stimulation of visceral sensitivity. AL-34662, a selective 5-HT_{2A} receptor agonist, stimulated visceral sensitivity to CRD in control rats (Figure 3C). Additionally, we investigated the effect of AL-34662 on visceral sensitivity in rats treated with butyrate to determine the sensitivity of 5-HT_{2A} receptor in butyrate-treated rats. AL-34662 did not affect the VMR to CRD in butyrate-treated rats (Figure 3D) and significantly restored butyrate-induced visceral hypersensitivity to CRD in the presence of chlorpromazine (Figure 3E).

Mechanisms underlying the inhibitory effect of chlorpromazine on visceral hypersensitivity

Activation of ERK1/2 in DRG neurons plays an important role in butyrate-induced colonic hypersensitivity to CRD (Xu *et al.,* 2013). We examined the effect of chlorpromazine on the expression of phospho-ERK1/2 in DRG neurons after butyrate treatment. As shown in Figure 4A, B, administration of sodium butyrate increased the expression of phospho-ERK1/2, whereas oral pre-administration of chlorpromazine inhibited this effect. To determine whether 5-HT_{2A} receptor antagonism is involved in the attenuation of ERK1/2 activation, we examined the effects of ketanserin and L-741626 on the expression of phospho-ERK1/2 in butyrate-treated rats. Application of ketanserin but not L-741626 to butyrate-treated rats decreased phospho-ERK1/2 expression in DRG neurons (Figure 4C, D).

Moreover, expression of 5-HT_{2A} receptors in DRG neurons and colon was evaluated and shown not to change after butyrate treatment (Figure 5A–C). Next, we determined colonic 5-HT levels. Butyrate treatment significantly increased 5-HT levels in colonic tissues (Figure 5D). Subsequently, we investigated the effect of a 5-HT synthesis inhibitor *p*-CPA on butyrate-induced hypersensitivity (Qin *et al.,* 2010). After oral administration of *p*-CPA for 3 days, colonic 5-HT levels significantly decreased in butyrate-treated rats (Figure 6A), whereas stimulated visceral sensitivity to CRD improved (Figure 6B).

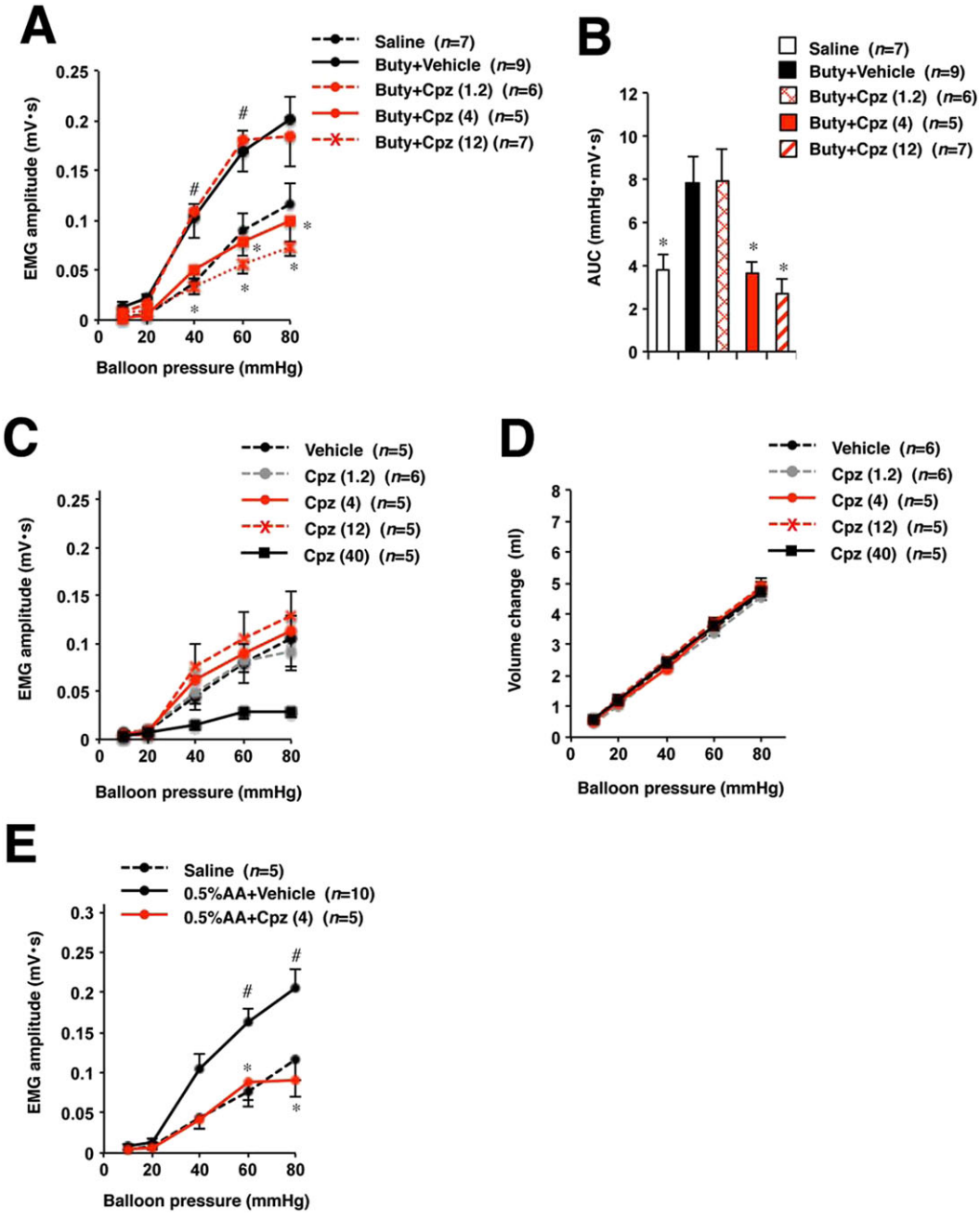

Figure 1

Effects of chlorpromazine on visceral sensitivity to CRD in rats without or with butyrate or acetic acid treatment. Rats were administered saline, sodium butyrate (Buty) or 0.5% acetic acid (0.5% AA) in neonates (A, B, E). Rats were orally administered the indicated doses of chlorpromazine (Cpz) (mg·kg^{-1}) or vehicle (saline), then two hours later, the CRD study was performed (A–E). (A) Visceral sensitivity to CRD (EMG amplitude) was determined. (B) Collective AUC data from (A). (C, D) Visceral sensitivity to CRD and colonic compliance in healthy control rats were examined. Values are mean ± SEM; * or $^{\#}P < 0.05$ (*, vs. vehicle; $^{\#}$, vs. saline).

Discussion

Various types of drugs including psychoactive agents are prescribed to IBS patients, and many target molecules for IBS drugs have been proposed. However, an appropriate pharmacological therapy has not yet been established. Furthermore, although some antipsychotic drugs are prescribed to treat pain symptoms in IBS, their pain amelioration mechanisms are unclear. Previously, in a phenotypic screening, we identified aminophylline as a drug that suppressed visceral sensitivity response to repeated CRD. Aminophylline suppressed stress-induced visceral hypersensitivity by inhibiting adenosine A_2 receptors (Asano *et al.*, 2017). In the same screen, chlorpromazine showed similar effects. Chlorpromazine has been used clinically as an antipsychotic drug for a long time. However, the effects of chlorpromazine on IBS have not been

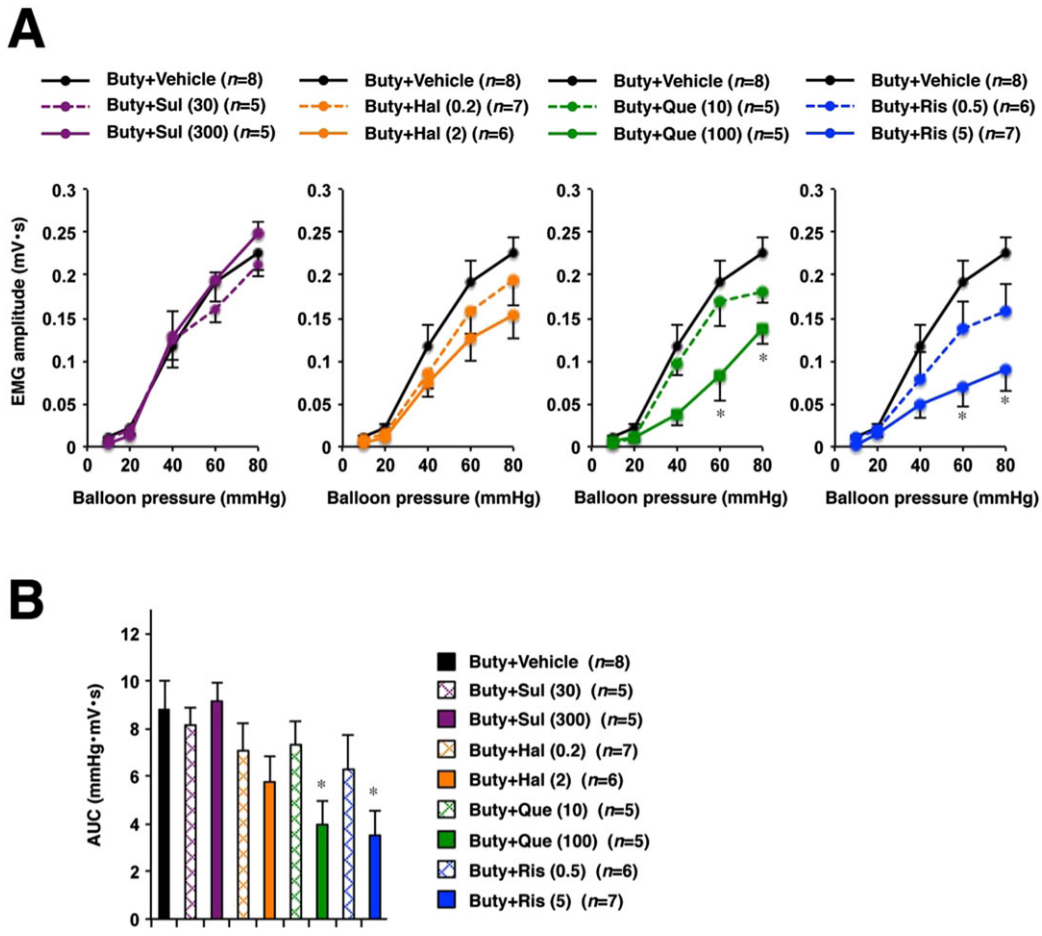

Figure 2

Effects of antipsychotic drugs on butyrate-induced visceral hypersensitivity to CRD in rats. The indicated doses (mg·kg^{-1}) of sulpiride (Sul), haloperidol (Hal), quetiapine (Que), risperidone (Ris) or vehicle (1% methylcellulose) were administered p.o. to butyrate (Buty)-treated rats. Two hours later, the CRD study was performed (A, B). (B) shows the collective AUC data from (A). Values are mean ± SEM; $^{*}P < 0.05$ vs. vehicle. Each graph of Buty + Vehicle group in four panels represents the same data as in (A).

investigated in any non-clinical or clinical study. Herein, we demonstrated that chlorpromazine suppressed visceral hypersensitivity in an IBS animal model, by inhibiting 5-HT$_{2A}$ receptor signalling.

Oral administration of chlorpromazine prevented butyrate- or acetic acid-induced visceral hypersensitivity to CRD without affecting visceral sensitivity under normal conditions or affecting colonic compliance. This activity profile suggests that chlorpromazine could be beneficial for IBS patients. Furthermore, atypical antipsychotic drugs risperidone and quetiapine improved colonic hypersensitivity in rats. Whereas sulpiride and haloperidol, which did not show this effect in our analysis, are preferential dopamine D$_2$ receptor antagonists, quetiapine and risperidone inhibit both 5-HT$_{2A}$ receptor and dopamine D$_2$ receptor. Therefore, 5-HT$_{2A}$ receptor inhibition may be involved in the suppression of visceral hypersensitivity. Although previous studies have suggested the possibility of atypical antipsychotics as a treatment option for IBS (Pae *et al.*, 2013), this is the first report in an animal model.

Pre-administration of a subtype-specific 5-HT$_{2A}$ receptor antagonist significantly suppressed visceral sensitivity to CRD in rats subjected to butyrate treatment. In contrast, preferential dopamine D$_2$ receptor inhibitor did not affect visceral hypersensitivity. This result does not conflict with previous reports suggesting that central activation of dopamine D$_2$ receptor produces antinociceptive action against CRD (Nozu *et al.*, 2016; Okumura *et al.*, 2016). Our results indicate that chlorpromazine-induced amelioration of visceral hypersensitivity could have been mediated by 5-HT$_{2A}$ receptor antagonism. To confirm this, we examined 7OH-Cpz, which does not bind to 5-HT$_{2A}$ receptor (Suzuki *et al.*, 2013), and AL-34662, a subtype-specific 5-HT$_{2A}$ receptor agonist, in the presence of chlorpromazine. As 7OH-Cpz does not inhibit 5-HT$_{2A}$ receptor but does bind to D$_2$ receptor (Suzuki *et al.*, 2013), we anticipated that 7OH-Cpz treatment would have no inhibitory effect on visceral hypersensitivity. As expected, 7OH-Cpz did not suppress butyrate-induced visceral hypersensitivity to CRD, indicating that 5-HT$_{2A}$ receptor antagonism was involved in chlorpromazine-induced inhibition of visceral hypersensitivity. Moreover, AL-34662 induced visceral hypersensitivity in response to CRD in normal rats, but not in butyrate-treated rats, suggesting that the activation of 5-HT$_{2A}$ receptor might be the mechanism

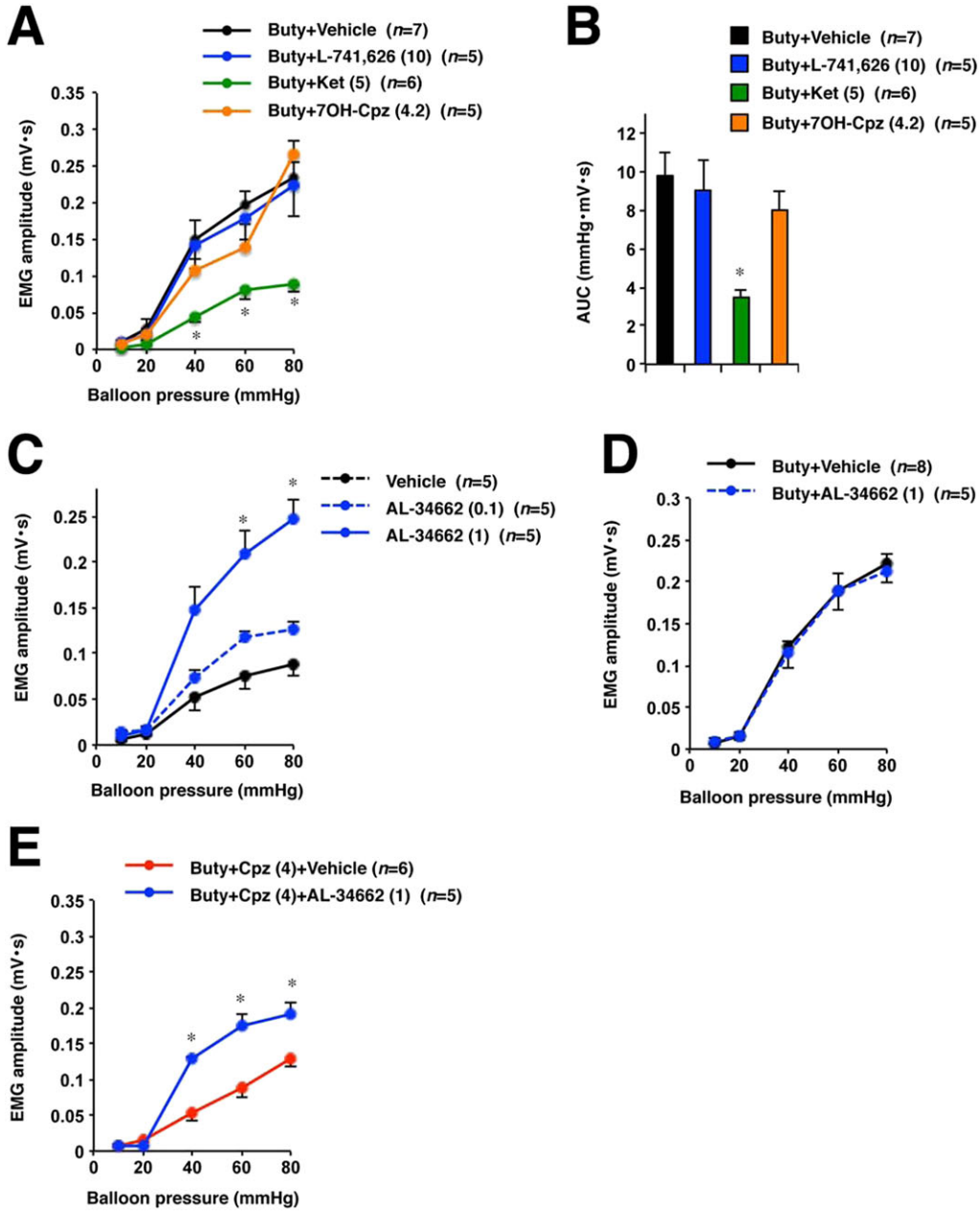

Figure 3

Pharmacological study of role of the 5-HT_{2A} receptor in visceral sensitivity to CRD. Rats treated with sodium butyrate (Buty) for 3 days were administered, i.p., the indicated doses (mg·kg^{-1}) of L-741626 (a selective dopamine D_2 receptor antagonist) (A, B), ketanserin (Ket) (a selective 5-HT_{2A} receptor antagonist) (A, B), AL-34662 (a selective 5-HT_{2A} receptor agonist) (D, E) or vehicle (1% methylcellulose), 15 min before the CRD study. The indicated doses (mg·kg^{-1}) of chlorpromazine (Cpz) (E) or 7OH-Cpz (A, B) were administered, p.o., to butyrate-treated rats, 2 h before the CRD test. (C) The indicated doses of AL-34662 (mg·kg^{-1}) or vehicle (1% methylcellulose) were administered, i.p., to healthy control rats. Fifteen minutes later, the CRD study was performed. (B) Collective AUC data from (A). Values are mean ± SEM; $^{*}P < 0.05$, vs. vehicle.

responsible for sodium butyrate-induced visceral hypersensitivity to CRD. Lack of visceral sensitivity to CRD stimulation by AL-34662 in butyrate-treated rats may imply that the 5-HT_{2A} receptor was already fully occupied by 5-HT produced due to butyrate treatment. Additionally, as AL-34662 is unable to cross the blood–brain barrier (Sharif *et al.*, 2007), 5-HT_{2A} receptors expressed in peripheral tissues (such as the colon) rather than those in the central nervous system appear to be involved in the inhibitory effects of chlorpromazine. However, our data do not exclude the possibility of a central mechanism of chlorpromazine, as 5-HT_{2A} receptors are expressed in the brain and spinal cord and chlorpromazine is a centrally acting agent. Furthermore, inhibition of spinal 5-HT_{2A} receptors was reported to decrease nociceptive responses to noxious stimuli (Rahman *et al.*, 2011), and chlorpromazine was shown to elicit a spinal blockade of

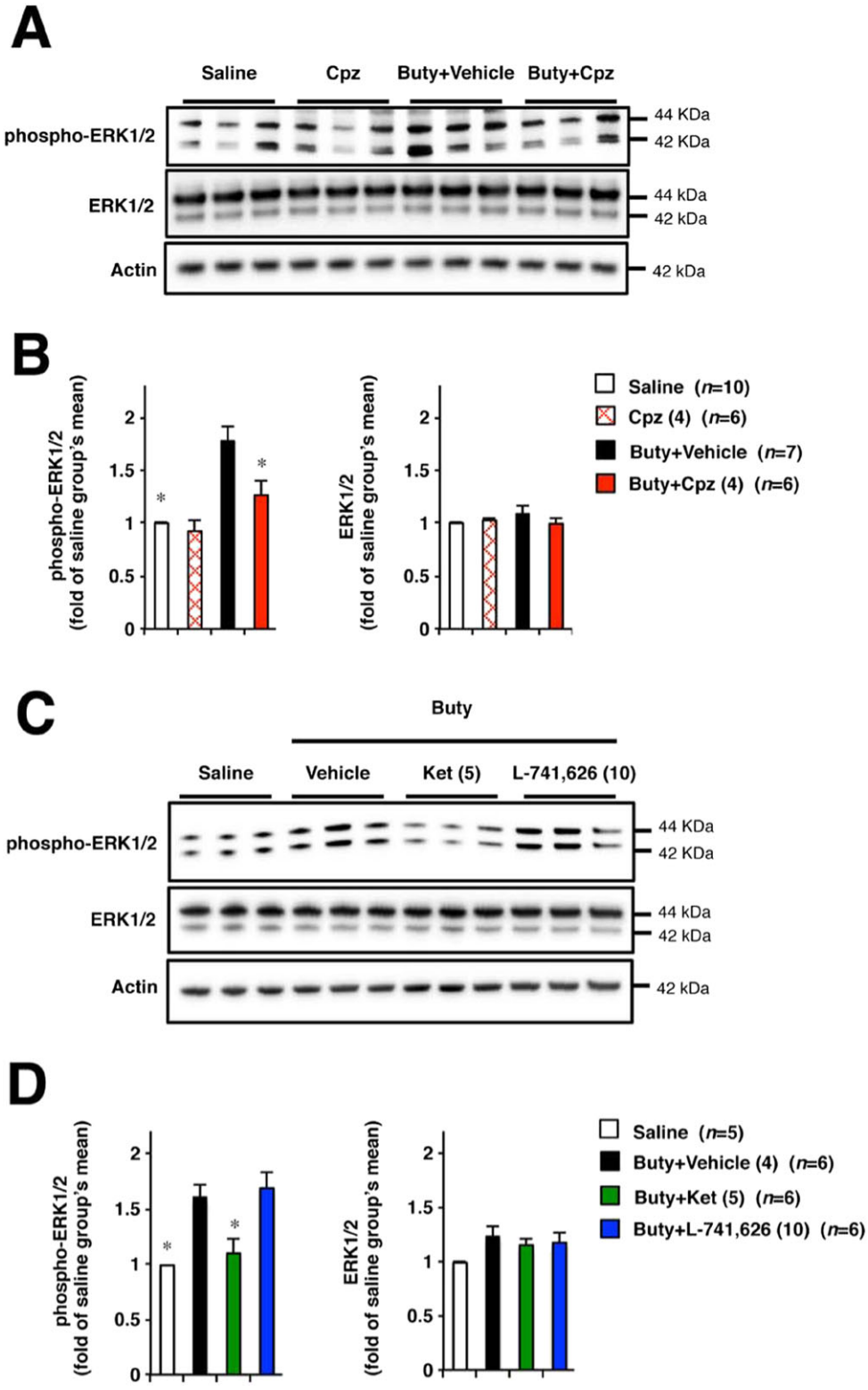

Figure 4

Effect of chlorpromazine, ketanserin or L-741626 on the butyrate-induced activation of ERK1/2 in DRG neurons. Rats were intracolonically administered sodium butyrate or saline twice daily for 3 days (A–D). On the next day after the last administration, chlorpromazine (4 mg·kg^{-1}) or vehicle (saline) was administered p.o. to these rats (A, B). Rats treated with butyrate were given ketanserin (5 mg·kg^{-1}), L-741626 (10 mg·kg^{-1}) or vehicle (1% methylcellulose) (C, D), i.p. Two hours (A, B) or 15 min (C, D) later, the L5-S1 DRG were removed from rats and tissues were subjected to Western blot analysis. (A) and (C) show representative data of immunoblotting. (B) and (D) show the relative data of band intensity at (A) and (C) respectively. Values are mean ± SEM; $^{*}P < 0.05$, vs. vehicle.

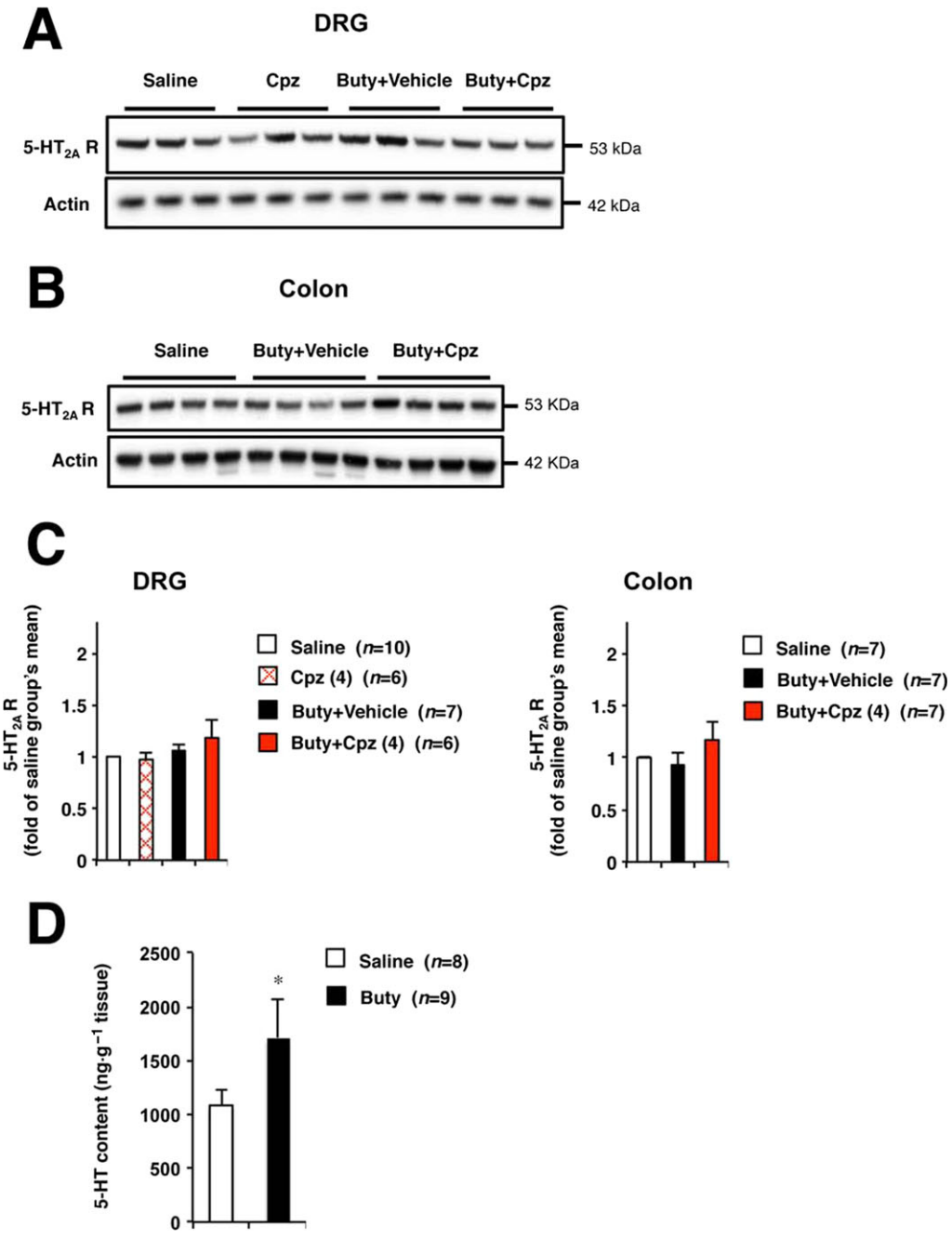

Figure 5

The expression of 5-HT_{2A} receptors in DRG neurons and colon, and colonic 5-HT contents. Rats were administered sodium butyrate or saline *via* colonic instillation twice daily for 3 days (A–D). After the last administration, chlorpromazine (4 mg·kg^{-1}) or vehicle (saline) was administered p.o. Two hours later, the L5-S1 DRG or colon was removed from the rats and tissues were subjected to Western blot analysis (A–C) or 5-HT ELISA assay (D). (A) and (B) show representative data of immunoblotting. (C) The relative data of band intensity at (A) and (B). Values are mean ± SEM; $^{*}P < 0.05$ vs. saline.

nociception in rats (Chen *et al.*, 2012). Thus, further studies are required to determine whether the amelioration of colonic hypersensitivity by chlorpromazine originates from peripheral or central actions of this drug.

As demonstrated earlier, 5-HT_{2A} receptor antagonists have an analgesic effect on inflammatory pain (Nitanda *et al.*, 2005; Cervantes-Duran *et al.*, 2016). In the present study, we demonstrated the inhibitory effect of a 5-HT_{2A} antagonist on the visceral pain response in non-inflammatory models (Bourdu *et al.*, 2005). Inflammatory pain models, such as neuropathic pain and formalin stimulation, were shown to increase the expression of 5-HT_{2A} receptors in DRG neurons (Okamoto *et al.*, 2002; Cervantes-Duran *et al.*, 2016); however, colonic butyrate application did not alter the expression

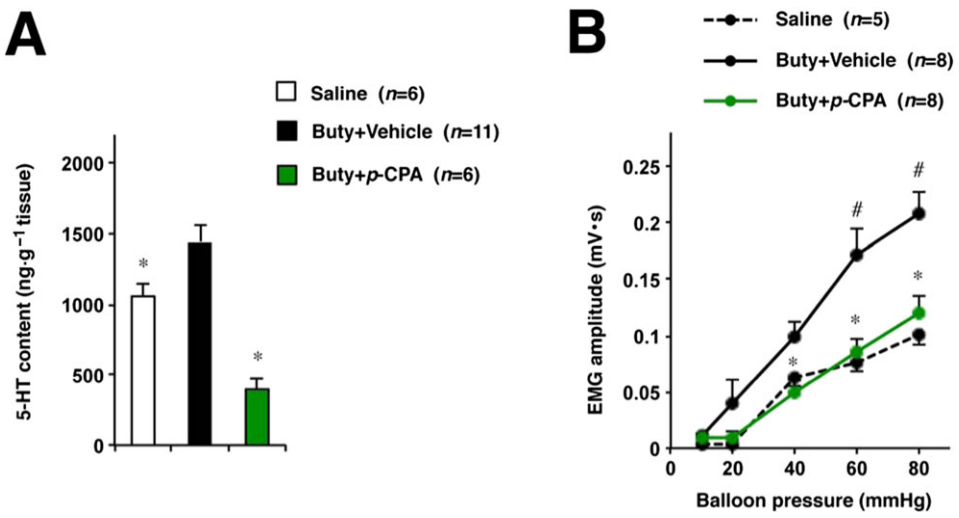

Figure 6

Effects of *p*-CPA on butyrate-induced visceral hypersensitivity to CRD in rats. Rats were administered 300 mg·kg^{-1} *p*-CPA (a 5-HT synthesis inhibitor) or vehicle (0.1% Tween-80), p.o., once daily for 3 days. Thirty minutes after each treatment, sodium butyrate or saline was administered *via* colonic administration. The next day, after the last administration of butyrate, the CRD test was performed (A). Colonic tissues removed from rats were subjected to 5-HT ELISA assay (B). Values are mean ± SEM; * or $^{\#}P < 0.05$ (*, vs. vehicle; $^{\#}$, vs. saline).

of 5-HT$_{2A}$ receptors in this study. This may be because the pathophysiology of pain sensation differs between our models and previous inflammatory pain models.

In rat DRG neurons, butyrate-induced colonic hypersensitivity is mediated by ERK1/2 activation (induction of ERK1/2 phosphorylation) (Xu *et al.,* 2013). Prevention of ERK1/2 phosphorylation in DRG neurons by an MEK inhibitor attenuated the butyrate-induced enhancement of DRG neuronal excitability and colonic hypersensitivity (Xu *et al.,* 2013). However, mechanisms based on an overexpression of acid-sensing ion channels (ASICs), the T-type calcium channel ($Ca_v3.2$) or phosphorylated voltage-gated potassium channel subunit 4.2 ($pK_v4.2$) in colonic sensory neurons have also been proposed (Marger *et al.,* 2011; Xu *et al.,* 2013). These changes would result in the stimulation of neuronal firing, leading to colonic hypersensitivity. In this study, butyrate treatment increased the phosphorylation of ERK1/2, whereas chlorpromazine prevented this activation without altering 5-HT$_{2A}$ receptor expression in DRG neurons. Moreover, ketanserin but not L-741626 suppressed butyrate-induced ERK1/2 activation, suggesting that 5-HT$_{2A}$ receptors may be involved in ERK1/2 activation in DRG neurons. Additionally, chlorpromazine improved visceral hypersensitivity induced by intracolonic injection of dilute acetic acid in neonatal rats. Recent study using the acetic acid model has shown that increased expression of 5-HT and 5-HT$_{2A}$ receptors in colon and DRG neurons is involved in the induction of visceral hypersensitivity (Chen *et al.,* 2016). Present study focused on the role of 5-HT$_{2A}$ receptor and 5-HT in butyrate-induced visceral hypersensitivity, as these mechanisms have not been investigated previously in this model.

Expression of 5-HT$_{2A}$ receptors in DRG neurons and colon did not change after butyrate instillation, in agreement with AL-34662 not stimulating visceral pain response in rats treated with butyrate. Therefore, we focused on colonic 5-HT levels. Butyrate treatment increased 5-HT levels in the colon, in agreement with the findings of a previous study (Grider and Piland, 2007), which showed that butyrate causes 5-HT release in the rat colon. These findings indicate that the change in 5-HT$_{2A}$ receptor expression does not contribute to the inhibitory effect of chlorpromazine on visceral hypersensitivity, whereas increased levels of 5-HT may be responsible for inducing visceral hypersensitivity. To confirm this hypothesis, we examined the effect of *p*-CPA, an inhibitor of 5-HT synthesis, on visceral hypersensitivity. 5-HT depletion *via p*-CPA treatment attenuated butyrate-induced colonic hypersensitivity. As elevated 5-HT levels or 5-HT$_{2A}$ receptor expression in the colon and DRG neurons are associated with acetic acid-induced visceral hypersensitivity (Chen *et al.,* 2016), increased colon 5-HT levels may be a visceral hypersensitivity mechanism shared by butyrate and acetic acid models. Moreover, inhibition of 5-HT$_{2A}$ receptor signalling by chlorpromazine may account for antinociceptive effects in these models. As ASIC3 and TRPV1 are required for visceral sensitivity to CRD, and 5-HT$_{2A}$ receptor signalling enhances TRPV1 and ASICs activity in sensory neurons (Sugiuar *et al.,* 2004; Jones *et al.,* 2005; Qiu *et al.,* 2012), activation of 5-HT$_{2A}$ receptors in our study may also be associated with modulation of these channels. However, we could not exclude the possibility that other effects of chlorpromazine, including histamine H_1 receptor antagonism and anti-calmodulin activity, may also be involved in the inhibitory effect observed in this study (Horacek *et al.,* 2006; Olah *et al.,* 2007). Taken together, our results indicate that butyrate-induced increase in colonic 5-HT levels potentiates visceral sensitivity *via* the 5-HT$_{2A}$ receptor, with chlorpromazine potentially preventing the binding of released 5-HT to 5-HT$_{2A}$ receptors.

In conclusion, we propose that the ameliorating effects of chlorpromazine on visceral hypersensitivity may be beneficial for IBS patients with abdominal pain and discomfort. The inhibitory effect of chlorpromazine on visceral hypersensitivity is likely to be mediated by inhibition of 5-HT$_{2A}$

receptor signalling, and we suggest the 5-HT_{2A} receptor as a potential therapeutic target protein for abdominal pain and discomfort in IBS.

Acknowledgements

We gratefully acknowledge Ayumi Kanada for technical assistance with animal experiments. We would like to thank Editage for English language editing. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Health, Labour, and Welfare of Japan, as well as the Center of Innovation Program from Japan Science and Technology Agency, Scientific Technique Research Promotion Program for Agriculture, Forestry and Food Industry, and Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Author contributions

T.A., K.T., M.T. and T.M. conceived and designed the experiments; T.A., A.T., K.T., R.T., H.S. and H.M. carried out the analysis and interpretation; and T.A performed the drafting and critical revising of the manuscript.

Conflict of interest

T.M. is the chairman and director of LTT Bio-Pharma Co., Ltd. T.A. and M.T. belong to an endowed research division of LTT Bio-Pharma Co., Ltd. The other authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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