RESEARCH PAPER

5-HT₃ receptors promote colonic inflammation via activation of substance P/neurokinin-1 receptors in dextran sulphate sodium-induced murine colitis

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BACKGROUND AND PURPOSE

5-HT (serotonin) regulates various physiological functions, both directly and via enteric neurons. The present study investigated the role of endogenous 5-HT and 5-HT₃ receptors in the pathogenic mechanisms involved in colonic inflammation, especially in relation to substance P (SP) and the neurokinin-1 (NK₁) receptor.

EXPERIMENTAL APPROACH

The effects of 5-HT₃ and NK₁ receptor antagonists were examined in dextran sulphate sodium (DSS)-induced colitis in mice. Inflammatory mediator expression and the distribution of 5-HT₃ and NK₁ receptors were also determined.

KEY RESULTS

Daily administration of ramosetron and ondansetron (5-HT₃ antagonists) dose-dependently attenuated the severity of DSS-induced colitis and up-regulation of inflammatory mediator expression. Immunohistochemical analysis showed 5-HT₃ receptors are mainly expressed in vesicular ACh transporter-positive cholinergic nerve fibres in normal colon. DSS increased the number of colonic nerve fibres that were double positive for 5-HT₃ receptors and SP but not of those that were double positive for 5-HT₃ receptors and vesicular ACh transporter. DSS increased colonic SP levels and SP-positive nerve fibres; these responses were attenuated by ramosetron. DSS-induced colitis and up-regulation of inflammatory mediators were attenuated by aprepitant, an NK₁ antagonist. Immunohistochemical studies further revealed that DSS treatment markedly increased NK₁ receptor expression in CD11b-positive cells.

CONCLUSIONS AND IMPLICATIONS

These findings indicate that the 5-HT/5-HT₃ receptor and SP/NK₁ receptor pathways play pathogenic roles in colonic inflammation. 5-HT acts via 5-HT₃ receptors to up-regulate inflammatory mediators and promote colonic inflammation. These effects may be further mediated by activation of macrophage NK₁ receptors via SP released from 5-HT₃ receptor-positive nerve fibres.

Abbreviations

5-FU, 5-fluorouracil; DAI, disease activity index; DSS, dextran sulphate sodium; EC, enterochromaffin; IBD, inflammatory bowel disease; iNOS, inducible NOS; MPO, myeloperoxidase; NK₁ receptor, neurokinin-1 receptor; SP, substance P; VACHT, vesicular ACh transporter

Tables of Links

TARGETS	
GPCRs^a	Enzymes^d
NK ₁ receptor	iNOS
Ligand-gated ion channels^b	Transporters^e
5-HT ₃ receptor	VACHT
Catalytic receptors^c	
CD11b (integrin, alpha M subunit)	

LIGANDS	
5-fluorouracil	IL-17A
5-HT	Ondansetron
Aprepitant	Ramosetron
IFN- γ	Substance P
IL-10	TNF- α

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (^{a,b,c,d,e}Alexander *et al.*, 2015a,b,c,d,e).

Introduction

5-HT (serotonin), a monoamine neurotransmitter, is synthesized from L-tryptophan by tryptophan hydroxylase in central and peripheral neurones and in enterochromaffin (EC) cells of the gastrointestinal tract (Talley, 2001; Berger *et al.*, 2009). 5-HT has a variety of physiological functions in the gastrointestinal, pulmonary and cardiovascular systems, as well as in the central and peripheral nervous systems (Talley, 2001; Berger *et al.*, 2009). The multiple actions of 5-HT are mediated by specific 5-HT receptors; these are classified into 14 subfamilies, which belong to seven major families, 5-HT₁ to 5-HT₇ (Hannon and Hoyer, 2008).

The 5-HT₃ receptor is a ligand-gated cation channel and is widely distributed in brain and spinal cord neurones, as well as in the gastrointestinal tract (Farber *et al.*, 2004). In the gastrointestinal tract, the 5-HT₃ receptor is involved in secretory, peristaltic, emetic and pain responses (Siriwardena *et al.*, 1993; Jackson and Yakel, 1995; Hansen, 2003). Indeed, 5-HT₃ receptor antagonists have been used clinically for the treatment of chemotherapy-induced nausea/emesis and irritable bowel syndrome with diarrhoea (Minami *et al.*, 1997; Kozłowski *et al.*, 2000; Fukudo *et al.*, 2014). We recently found that 5-HT₃ receptor antagonists such as ramosetron and ondansetron ameliorated intestinal injuries induced by the anticancer agent, 5-fluorouracil (5-FU), and the non-steroidal anti-inflammatory drug, indomethacin (Kato *et al.*, 2012; Yasuda *et al.*, 2013). Several studies further showed that 5-HT₃ receptor antagonists reduced the severity of post-operative ileus and colitis (Mousavizadeh *et al.*, 2009; Motavallian *et al.*, 2013; Maehara *et al.*, 2015). These findings suggest that endogenous 5-HT has pro-inflammatory effects that are mediated via 5-HT₃ receptors in the gastrointestinal tract. However, the underlying mechanisms and localisation of the 5-HT₃ receptor involved in the pathogenesis of gut inflammation have yet to be defined.

Inflammatory bowel disease (IBD), caused by conditions such as ulcerative colitis and Crohn's disease, is a chronic, progressive and recurrent inflammatory condition that affects the lower gastrointestinal tract in particular. The global prevalence of IBD has increased dramatically in recent decades (Molodecky *et al.*, 2012). Although the precise pathogenesis of IBD still remains unclear, an increasing number of studies have shown that dysregulation of mucosal immune

responses, impairment of epithelial barrier functions and several environmental factors (including microbiota) are involved (Kaser *et al.*, 2010). Ghia *et al.* (2009) reported that the severity of experimental colitis was significantly reduced in mice deficient in tryptophan hydroxylase-1 or in those pretreated with a tryptophan hydroxylase inhibitor, suggesting that endogenous 5-HT exacerbates colitis. Thus, it is possible that activation of 5-HT₃ receptors by 5-HT may be important in the pathogenesis of colonic inflammation.

Similar to 5-HT, substance P (SP) plays an important role in the regulation of various gastrointestinal functions such as motility, vascular permeability, epithelial ion transport and immune responses via the neurokinin-1 receptor (NK₁ receptor) (Holzer, 1998; Renzi *et al.*, 2000; O'Connor *et al.*, 2004). Several studies have shown that NK₁ receptor antagonists reduce the severity of experimentally-induced murine colitis, suggesting a role for SP/NK₁ receptors in the pathogenesis of colonic inflammation (Rijnierse *et al.*, 2006; Gad *et al.*, 2009; Engel *et al.*, 2011). Furthermore, the relationship between 5-HT/5-HT₃ receptor and SP/NK₁ receptor signalling regulates gastrointestinal functions such as contractile responses (Saria *et al.*, 1991; Ramirez *et al.*, 1994; Yamano and Miyata, 1996). However, the relationship between these pathways in the pathogenesis of colonic inflammation has not been explored.

The present study aimed to investigate the role of 5-HT/5-HT₃ receptors in the pathogenesis of colonic inflammation and to identify the mechanisms involved, particularly in relation to SP/NK₁ receptors, using a murine model of dextran sulphate sodium (DSS)-induced colitis.

Methods

Animals

Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015). The protocols were approved by the committee on the Ethics of Animal Research of Kyoto Pharmaceutical University (permit number: 14-12-034). Male C57BL/6 mice (9–12 weeks old) weighing 20–24 g were purchased from Japan SLC Inc. (Shizuoka, Japan). All mice were maintained in plastic cages with free access to food and water and housed at 22 ± 1°C with a 12 h light/dark cycle. The experiments were performed using 5–8 un-anaesthetized mice per group.

Induction of colitis

Colitis was induced in mice by p.o. administration of 2.5% DSS (molecular weight: 5000; Wako, Osaka, Japan) to the drinking water for 9 or 10 days. Mice were assigned randomly to six groups for assessment of ramosetron and ondansetron: normal (not treated with DSS), control (DSS treated), ramosetron (0.01, 0.03 and 0.1 mg·kg⁻¹) and ondansetron (5 mg·kg⁻¹); and four groups for assessment of effects of NK₁ receptor antagonist: normal, control and aprepitant (1 and 3 mg·kg⁻¹). Normal mice received drinking water only. Ramosetron and ondansetron were administered p.o. twice daily for 10 days, while aprepitant was administered i.p. once daily for 9 days. The drug volume administered corresponded to 0.1 mL 10 g⁻¹ body weight. Control mice (DSS) received the same volume of drug vehicle (0.5% carboxymethyl cellulose) via the same administration route.

Assessment of disease activity index

The disease activity index (DAI) was expressed as the sum of the scores assigned to reflect the following: body weight loss (0 = <5%, 1 = 5–10%, 2 = 10–15%, 3 = 15–20% and 4 = >20%), stool consistency (0 = normal, 1 = soft but still formed, 2 = very soft, 3 = diarrhoea and 4 = severe diarrhoea) and rectal bleeding (0 = no blood, 1 = occult blood in stool, 2 = traces of blood visible in stool, 3 = grossly bloody stool and 4 = rectal bleeding) (Wirtz *et al.*, 2007; Ghia *et al.*, 2008). Faecal occult blood was detected using the Hemocult II SENSAs test (Beckman Coulter, Fullerton, CA, USA). The minimum DAI score was 0, and maximum score was 12. The DAI scoring was carried out by two investigators who were blinded to the experimental groups.

Macroscopic and histological evaluations

At the end of DSS treatment (day 9 or 10), the entire colon (from the caecum to the anus) was removed, and the colon length was measured. The colonic tissues were then immersed in 10% neutralized formalin overnight, embedded in paraffin, cut into 4 µm sections and stained with haematoxylin and eosin. Histological damage was graded under a light microscope (BX-51; Olympus, Tokyo, Japan) at 200× magnification. Grades of 0 to 4 were assigned, where 0 = normal; 1 = low leukocyte infiltration; 2 = moderate leukocyte infiltration and moderate disruption of epithelium; 3 = high leukocyte infiltration, diffused disruption of epithelium, thickening of the colon wall, moderate goblet cell loss and focal crypt loss; and 4 = transmural infiltrations, massive loss of goblet cells and diffuse loss of crypts (Gonzalez-Rey *et al.*, 2006). All evaluations were carried out by two investigators who were blinded to the experimental groups.

Determination of myeloperoxidase activity

The distal colon was removed from the animals following the indicated treatments. Colonic tissues were rinsed with cold PBS, weighed and homogenized in 50 mM phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (pH 6.0; Wako). Sample protein content was estimated using a spectrophotometric assay kit (Pierce, Rockford, IL, USA). The myeloperoxidase (MPO) activity was determined using *o*-dianisidine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA), as previously described (Yasuda *et al.*, 2011).

Determination of mRNA expression by quantitative real-time RT-PCR

On days 0 (normal; DSS untreated), 3, 7 and 10 following the initiation of DSS treatment, the distal colon was removed, washed with cold PBS and stored in RNeasy lysis buffer (Qiagen, Crawley, UK) at 4°C until use. Total RNA was extracted using RNeasy RNA extraction kit (Qiagen) and reverse transcription was performed using PrimeScript Reverse Transcriptase (Takara, Shiga, Japan). Quantitative PCR was carried out using ABI 7500 (Applied Biosystems, Foster City, CA, USA) with SYBR Premix ExTaq II (Takara). Specific primer sets for β-actin (MA050368), TNF-α (MA097070), inducible NOS (iNOS; MA063888), IFN-γ (MA025911), IL-17A (MA086724) and IL-10 (MA029057) were obtained from the Perfect Real-time Supporting System (Takara). The expression level of each mRNA was calculated using the comparative ΔΔC_T method, where signals were normalized to the mean value on day 0 or to the value observed in the normal group at the same time point.

Immunohistochemical analyses

On days 0 (normal), 3, 7 and 10 following the initiation of DSS treatment, the distal colon was removed, washed with cold PBS and immersed in 4% paraformaldehyde for 2 h at 4°C. The tissues were cryoprotected overnight in 20% sucrose solution prior to embedding in Optimal Cutting Temperature compound (Sakura Finetek, Tokyo, Japan) mounting medium. They were then sectioned on a cryostat (Leica Instruments, Nussloch, Germany) at a thickness of 30 µm and thaw mounted onto Superfrost Plus slides (Matsunami, Osaka, Japan). Immunohistochemical procedures were performed as previously described by Matsumoto *et al.* (2009). In brief, the slide-mounted sections were treated with 10% normal donkey serum for 1 h at room temperature and then incubated with a goat polyclonal anti-5-HT antibody (Immunostar, Hudson, WI, USA), a rabbit polyclonal anti-5-HT₃ receptor antibody (Calbiochem, Darmstadt, Germany), a rat monoclonal anti-CD11b antibody (R&D Systems, Minneapolis, MN, USA), a goat polyclonal anti-vesicular ACh transporter (VACHT) antibody (Phoenix, Burlingame, CA, USA), a guinea pig anti-SP antibody (Abcam, Cambridge, UK) or a rabbit polyclonal anti-NK₁ receptor antibody (Millipore, Billerica, MA, USA) for 40 h at room temperature. To visualize the target protein expression, the sections were incubated with the appropriate secondary antibody (biotin-SP-conjugated donkey anti-rabbit, fluorescein isothiocyanate-labelled donkey anti-rabbit and tetramethyl rhodamine isothiocyanate-labelled donkey anti-guinea pig, anti-goat or anti-rat; Jackson, West Grove, PA, USA) for 4 h. No specific immunostaining was observed in any of the control sections. Immunofluorescence was observed using confocal microscopes (FV-1000; Olympus, and A1R⁺; Nikon, Tokyo, Japan) with an excitation wavelength appropriate for fluorescein isothiocyanate (488 nm) or tetramethyl rhodamine isothiocyanate (543 nm). Images were collected, and 50–60 optical sections were typically taken at intervals of 0.5 µm. Multiple images in Z-stacks were projected onto a single plane and reconstructed using Fluoview version 1.7a software (Olympus). The number of immunopositive cells and nerve fibres in horizontal sections of the mucosa was counted in 100 µm squares at 200× magnification under a confocal microscope.

Figure 1

Effects of Ramosetron (Ramo) and Ondansetron (Onda) on DSS-induced colitis. Animals were exposed to 2.5% DSS for 10 days, while Ramo (0.01–0.1 mg·kg⁻¹) or Onda (5 mg·kg⁻¹) were administered p.o. twice daily. (A) DAI was determined on days 0, 3, 7 and 10. (B) Colon length, (C) histological score and (D) MPO activity were examined on day 10. Data are presented as the mean $\pm$ SEM, eight mice per group. Statistical analyses were performed using one-way ANOVA followed by Holm–Sidak’s multiple comparison test for colon length and MPO activity and using Kruskal–Wallis one-way ANOVA followed by Dunn’s multiple comparison test for DAI and histological scores. * $P < 0.05$ for the comparison with control mice (DSS); # $P < 0.05$ for the comparison with normal mice (DSS untreated). (E) Representative images of haematoxylin and eosin staining in the colon ($\times 100$).

The lengths of immunopositive nerve fibres were measured in horizontal sections using ImageJ 1.48v software in 100 μm squares at 200 $\times$ magnification. All counting was conducted by two investigators who were blinded to the experimental groups.

Determination of SP in the colon

On day 7 of DSS treatment, the distal colon was removed, washed with cold PBS and weighed. Colonic tissues were homogenized in PBS and centrifuged at 10 000 $\times g$ for 10 min at 4°C. The level of SP in the resulting mucosal sac supernatant was determined by enzyme immunoassay (Cayman, Ann Arbor, MI, USA).

Statistical analyses

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). Data are presented as the mean $\pm$ SEM. Statistical analyses were performed by GraphPad Prism 6.0f (GraphPad Software, La Jolla, CA, USA) using one-way ANOVA followed by Holm–Sidak’s multiple

comparison test or Student’s *t*-test for parametric data (colon length, MPO activity, the number of nerve fibres and cells and the length of nerve fibres) while using Kruskal–Wallis one-way ANOVA followed by Dunn’s multiple comparison test for non-parametric data (DAI, histological score and mRNA expression). $P < 0.05$ was regarded as statistically significant.

Drugs

Ramosetron was kindly supplied by Asteralls Pharma Inc. (Tokyo, Japan). Ondansetron and aprepitant (EMEND®) were purchased from LKT Laboratories (St. Paul, MN, USA) and Ono Pharmaceutical Co. Ltd (Osaka, Japan), respectively. Carboxymethyl cellulose was purchased from Nacalai Tesque (Kyoto, Japan). Ramosetron was dissolved, while ondansetron and aprepitant were suspended, in 0.5% carboxymethyl cellulose solution immediately prior to administration, as described. The Ramosetron and Ondansetron doses were selected based on our previous study (Yasuda *et al.*, 2013), while those selected for aprepitant were based on the study reported by Millan *et al.* (2010).

Figure 2

Effect of ramosetron (Ramo) and ondansetron (Onda) on colonic inflammatory mediator mRNA expression. Animals were exposed to 2.5% DSS for 10 days, while Ramo (0.1 mg·kg⁻¹) or Onda (5 mg·kg⁻¹) were administered p.o. twice daily. The expression of inflammatory mediators was determined by real-time RT-PCR. (A) Expression levels of each mRNA were standardized to that of β -actin mRNA and normalized to the mean value for day 0 or normal (DSS untreated) mice at each time point. mRNA expression of (B) iNOS, (C) TNF- α , (D) IFN- γ , (E) IL-17A and (F) IL-10 on day 7. Data are presented as the mean $\pm$ SEM, eight mice per group. Statistical analyses were performed using the Kruskal–Wallis one-way ANOVA followed by Dunn's multiple comparison test. * P < 0.05 for the comparison with control (DSS); # P < 0.05 for the comparison with normal (DSS untreated).

Figure 3

The number of 5-HT-immunoreactive cells in the mouse colonic mucosa. (A) Animals were exposed to 2.5% DSS for 10 days, and the number of 5-HT-immunoreactive cells was determined immunohistochemically using an anti-5-HT antibody on days 0, 3, 7 and 10. Data are presented as the mean $\pm$ SEM, eight mice per group. (B) Representative images showing immunohistochemical detection of 5-HT-immunoreactive EC cells on days 0 and 7. Scale bars: 50 μ m.

Results

Effect of 5-HT₃ receptor antagonists on DSS-induced colitis

To investigate the role of 5-HT₃ receptors in the pathogenesis of colonic inflammation, we examined the effects of ramosetron and ondansetron on DSS-induced colitis. Addition of 2.5% DSS to drinking water produced diarrhoea followed by rectal bleeding and loss of body weight and produced an evident increase in DAI after 7 days (Figure 1A). Twice-daily administration of ramosetron (0.01–0.1 mg·kg⁻¹) attenuated this increase in DAI during DSS exposure in a dose-dependent manner, with a significant effect observed at 0.03 and 0.1 mg·kg⁻¹. Ondansetron (5 mg·kg⁻¹) also significantly attenuated the DSS-induced increase in DAI, producing the same effect as 0.1 mg·kg⁻¹ ramosetron. After 10 days of DSS exposure, colon length was significantly reduced, and a marked increase in colonic MPO activity was observed (Figure 1B and D). It is generally accepted that colon length is inversely correlated with the severity of colitis, while MPO activity provides a marker of leukocyte infiltration into the mucosa. Indeed, histological examination revealed the development of severe colitis, characterized by extensive disruption of the epithelium, massive loss of goblet cells and diffuse loss of crypts; these changes were accompanied by marked leukocyte infiltration into the mucosa and submucosa (Figure 1E), resulting in a marked increase in histological score (Figure 1C). Twice-daily administration of ramosetron (0.01–0.1 mg·kg⁻¹) reduced these DSS-induced effects on colon length and histology in a dose-dependent manner, with significant effects observed at 0.03 and 0.1 mg·kg⁻¹. A similar attenuation of DSS-induced changes was observed in animals receiving twice-daily

Figure 4

Expression of 5-HT₃ receptors (5-HT₃R) in the mouse colonic mucosa. Animals were exposed to 2.5% DSS for 7 days, and the expression of 5-HT₃ receptors was examined immunohistochemically. (A) Expression of 5-HT₃ receptors in the colonic mucosa of normal (DSS untreated) and control (DSS) mice on transverse and horizontal sections. Scale bars: 20 μ m. Arrows indicate 5-HT₃ receptor-positive cells. (B) The numbers and lengths of 5-HT₃ receptor-positive nerve fibres and cells were determined in 100 μ m squares on transverse and horizontal sections respectively. Data are presented as the mean $\pm$ SEM, five mice per group. Statistical analyses were performed using Student's *t*-test. $P < 0.05$ for the comparison of normal and control mice. (C) Double staining of 5-HT₃ receptors (green) and CD11b (red) in a horizontal section of the colonic mucosa in a control mouse. Arrows indicate typical double-positive cells for 5-HT₃ receptors and CD11b.

administration of ondansetron (5 mg·kg⁻¹) (Figure 1A–C). The increase in MPO activity induced by DSS treatment was also significantly reduced by twice-daily administration of Ramosetron (0.1 mg·kg⁻¹) or ondansetron (5 mg·kg⁻¹) (Figure 1D).

Effect of 5-HT₃ receptor antagonists on mRNA expression of inflammatory mediators in DSS-induced colitis

Several previous studies have demonstrated the pathogenic role of various inflammatory mediators in DSS-induced colitis, including iNOS, TNF- α , IFN- γ and IL-17 (Obermeier *et al.*, 1999;

Naito *et al.*, 2003; Takedatsu *et al.*, 2008). In the present study, we also observed a marked up-regulation of TNF- α , IFN- γ , IL-17A and iNOS mRNA levels in the colon of DSS-exposed mice on day 7 (Figure 2A). The up-regulation of iNOS and TNF- α mRNA was further augmented on day 10, while that of IFN- γ and IL-17A was reduced. However, the significant DSS-induced up-regulation of iNOS, TNF- α , IFN- γ and IL-17A on day 7 was not observed in mice treated with Ramosetron (0.1 mg·kg⁻¹) or ondansetron (5 mg·kg⁻¹) (Figure 2B–E). In contrast, the expression of IL-10 mRNA did not change during DSS exposure and was not affected by the administration of these 5-HT₃ receptor antagonists (Figure 2F).

Figure 5

Characterisation of 5-HT₃ receptor (5-HT₃R)-positive nerve fibres in the colonic mucosa. Animals were exposed to 2.5% DSS for 7 days prior to immunohistochemical analysis. Representative images of double staining are shown for (A) 5-HT₃ receptors (green) and VACht (red) and (B) 5-HT₃ receptors (green) and SP (red) in normal (DSS untreated) and control (DSS) mice. Scale bars: 20 μm. Arrows indicate double-positive nerve fibres, while arrow heads indicate nerve fibres that were single positive for 5-HT₃ receptors. (C) The total numbers and lengths of nerve fibres positive for 5-HT₃ receptors and double positive for VACht and 5-HT₃ receptors. (D) The total numbers and lengths of nerve fibres positive for 5-HT₃ receptors and double positive for SP and 5-HT₃ receptors. Immunopositive nerve fibres were determined in 100 μm squares in normal and control mice, and data are presented as the mean ± SEM, five mice per group. The number and length of double-positive nerve fibres were also expressed as a percentage of the total number and length of 5-HT₃ receptor-positive nerve fibres respectively. Statistical analyses were performed using Student's *t*-test. **P* < 0.05 for the comparison between normal and control mice.

Figure 6

Effect of aprepitant (Aprep) on DSS-induced colitis. Animals were exposed to 2.5% DSS for 9 days, while Aprep (1 and 3 mg·kg⁻¹) was administered i.p. once daily. (A) DAI was determined on days 0, 3, 5, 7 and 9. (B) Colon length, (C) histological score and (D) MPO activity were examined after 9 days. Data are presented as the mean ± SEM, six mice per group. Statistical analyses were performed using one-way ANOVA followed by Holm–Sidak’s multiple comparison test for colon length and MPO activity and using the Kruskal–Wallis one-way ANOVA followed by Dunn’s multiple comparison test for DAI and histological scores. **P* < 0.05 for the comparison with control mice (DSS); #*P* < 0.05 for the comparison with normal mice (DSS untreated). (E) Representative images of haematoxylin and eosin staining in the colon (×100) after 9 days of the indicated treatments.

Changes in the number of mucosal 5-HT-immunoreactive cells in DSS-induced colitis

To investigate the involvement of endogenous 5-HT in the pathogenesis of DSS-induced colitis, we examined the number of colonic 5-HT-immunoreactive cells using an anti-5-HT antibody. 5-HT-immunoreactive cells were mostly detected in the colonic epithelium and were spindle shaped, suggesting that most of these were EC cells (Figure 1A) (Oshima *et al.*, 1999). The number of 5-HT-immunoreactive EC cells was clearly increased in animals exposed to DSS, reaching a maximum on day 7 (Figure 3A and B).

Expression of 5-HT₃ receptors in the colonic mucosa in DSS-induced colitis

We examined the expression of 5-HT₃ receptors in the colons of normal (DSS untreated) and control (DSS exposed) animals immunohistochemically using an anti-5-HT₃ receptor antibody. On transverse sections of normal colons, the expression of 5-HT₃ receptors was mostly detected in submucosal nerve fibres and marginally in cell bodies located in the lamina propria (Figure 4A). The number of 5-HT₃ receptor-positive nerve fibres was markedly increased in both the mucosal and submucosal regions of control colons after 7 days of DSS exposure. In horizontal sections of the basal region of

the mucosa, the number of 5-HT₃ receptor-positive nerve fibres was significantly increased in control animals, as compared with normal animals, but the number of 5-HT₃ receptor-positive cells was not altered (Figure 4B). We previously reported that the 5-HT₃ receptor is mostly expressed in macrophages located in the lamina propria of the normal small intestine (Yasuda *et al.*, 2013). The present double immunostaining study also showed that most of the 5-HT₃ receptor-positive cells detected in control colons were also stained by the macrophage marker, CD11b (Figure 4C).

To characterize the 5-HT₃ receptor-positive nerve fibres, we performed double immunostaining for 5-HT₃ receptors and either the cholinergic neuronal marker, VACHT, or the neuropeptide, SP, on horizontal sections of the basal region of the mucosa in normal (DSS untreated) and control (DSS treated) mice. In normal animals, the immunoreactivity of 5-HT₃ receptors was mainly co-localized with that of VACHT (90.3% and 85.9% of the number and length respectively) (Figure 5A and C) and minimally with SP immunoreactivity (19.7% and 19.4% of the number and length respectively) (Figure 5B and D). Although DSS exposure for 7 days increased the total number and length of 5-HT₃ receptor-positive nerve fibres, the number and length of VACHT-positive nerve fibres did not increase, and the percentage of 5-HT₃ receptor-expressing fibres that were VACHT positive decreased to 60.0 and 56.5% respectively (Figure 5C).

In contrast, the total number and length of SP-immunoreactive nerve fibres increased in DSS-exposed (control) colons, and the percentage of 5-HT₃ receptor-expressing fibres that also expressed SP increased to 44.2 and 44.9% of the number and length respectively (Figure 5D).

Effect of aprepitant on DSS-induced colitis

To investigate the involvement of endogenous SP and the NK₁ receptor in the pathogenesis of colonic inflammation, we examined the effects of the NK₁ receptor antagonist, aprepitant, on DSS-induced colitis. In this experiment, severe weight loss, diarrhoea and rectal bleeding were observed earlier than in the aforementioned experiments using 5-HT₃ receptor antagonists. We therefore investigated various parameters on day 9 (instead of day 10) of DSS exposure. The increase in DAI during DSS exposure was dose-dependently attenuated by a single daily administration of aprepitant (1–3 mg·kg⁻¹), with a significant effect observed at 1 mg·kg⁻¹ (Figure 6A). Colon shortening, increased MPO activity and the histological scores on day 9 of DSS treatment were also dose-dependently reduced by a single daily administration of aprepitant, with a significant effect observed at 3 mg·kg⁻¹ (Figure 6B–E). In addition, the significant DSS-induced up-regulation of iNOS, TNF- α and IFN- γ was not observed in mice treated with aprepitant (3 mg·kg⁻¹) (Figure 7A–C). In this experiment, DSS treatment did not significantly increase IL-17A mRNA expression, although it tended towards an increase and this was also prevented by aprepitant (Figure 7D). In contrast, the expression of IL-10 mRNA did not change during DSS treatment and was not affected by the administration of aprepitant (Figure 7E).

Effects of ramosetron and aprepitant on mucosal immunoreactivities of 5-HT and SP in DSS-induced colitis

To confirm the relationship between the 5-HT/5-HT₃ receptor and SP/NK₁ receptor pathways in the pathogenesis of colonic inflammation, we examined the effect of ramosetron and aprepitant on DSS-induced increases in SP-positive nerve fibres and 5-HT-immunoreactive cells respectively. In horizontal sections of the basal region of the mucosa, the number and length of SP-positive nerve fibres apparently increased after 7 days of DSS exposure. These responses were significantly attenuated by twice-daily administration of ramosetron (0.1 mg·kg⁻¹) (Figure 8A and C). Similarly, the level of SP in the colonic tissue, determined by enzyme immunoassay, increased significantly after exposure to DSS for 7 days. This response was significantly reduced to the level observed in normal animals by twice-daily administration of ramosetron (0.1 mg·kg⁻¹) (Figure 8D). In contrast, DSS exposure for 7 days increased the number of 5-HT-positive cells in the mucosa, and this response was not affected by daily administration of aprepitant (3 mg·kg⁻¹) (Figure 8E).

Expression of NK₁ receptors in the colonic mucosa in DSS-induced colitis

Finally, we examined the colonic expression of NK₁ receptors in normal (DSS untreated) and control (DSS exposed) animals immunohistochemically using an anti-NK₁ receptor antibody. In transverse sections of normal mice, NK₁ receptor immunoreactivity was detected mostly in cell bodies located in the lamina propria of the colonic mucosa (Figure 9A). DSS treatment for 7 days (control) dramatically increased

Figure 7

Effect of aprepitant (Aprep) on colonic inflammatory mediator mRNA expression. Animals were exposed to 2.5% DSS for 7 days, and Aprep (3 mg·kg⁻¹) was administered i.p. once daily for 7 days. The mRNA expression of (A) iNOS, (B) TNF- α , (C) IFN- γ , (D) IL-17A and (E) IL-10 was determined by real-time RT-PCR. Expression levels of each mRNA were standardized to that of β -actin mRNA and normalized to the mean value in normal (DSS untreated) mice. Data are presented as the mean $\pm$ SEM, six mice per group. Statistical analyses were performed using the Kruskal–Wallis one-way ANOVA followed by Dunn's multiple comparison test. [#] $P < 0.05$ for the comparison with control (DSS); ^{*} $P < 0.05$ for the comparison with normal (DSS untreated) mice.

Figure 8

Effects of ramosetron (Ramo) and aprepitant (Aprep) on DSS-induced increases in SP-positive nerve fibres and 5-HT-positive cells in the colonic mucosa. Animals were exposed to 2.5% DSS for 7 days prior to immunohistochemical analysis. Ramo ($0.1 \text{ mg}\cdot\text{kg}^{-1}$) was administered p.o. twice daily, while Aprep ($3 \text{ mg}\cdot\text{kg}^{-1}$) was administered i.p. once daily. Representative images showing immunohistochemical detection of (A) SP, scale bars: $20 \mu\text{m}$, and (B) 5-HT, scale bars: $50 \mu\text{m}$. (C) The total numbers and lengths of SP-positive nerve fibres. (D) The SP level in murine colonic tissue. (E) The total number of 5-HT-positive (EC) cells. Nerve fibres and cells for immunopositive for 5-HT₃ receptors were determined in $100 \mu\text{m}$ squares, while cells immunopositive for 5-HT were determined in a 1 mm length. Data are presented as mean $\pm$ SEM values, eight mice per group. The colonic SP level was determined by enzyme immunoassay, and data are presented as mean $\pm$ SEM values in normal ($n = 7$), control (DSS, $n = 8$) and Aprep-treated ($n = 8$) groups. Statistical analyses were performed using one-way ANOVA followed by Holm–Sidak’s multiple comparison test. ^{*} $P < 0.05$ for the comparison with control (DSS); [#] $P < 0.05$ for the comparison with normal (DSS untreated) mice.

Figure 9

Expression of NK₁ receptors (NK1R) in the colonic mucosa. Animals were exposed to 2.5% DSS for 7 days, and the expression of NK₁ receptors was examined immunohistochemically. (A) Expression of NK₁ receptors in the colonic mucosa of normal (DSS untreated) and control (DSS) mice on transverse section. Scale bars: 20 μ m. (B) Double staining of NK₁ receptors (green) and CD11b (red) in the colonic mucosa of normal (DSS untreated) and control (DSS treated) mice on horizontal section. Scale bars: 20 μ m. (C) The number of cells positive for NK₁ receptors and double positive for NK₁ receptors and CD11b were determined in 100 μ m squares in normal and control mice. The number of double-positive cells is also expressed as a percentage of the total number of NK₁ receptor-positive cells. Data are presented as the mean $\pm$ SEM, six mice per group. Statistical analyses were performed using Student's *t*-test. **P* < 0.05 for the comparison between normal and control mice.

the number of NK₁ receptor-positive cells. To characterize these cells, we double immunostained for NK₁ receptors and CD11b. On horizontal sections of normal colons, 70.5% of the NK₁-positive cells were also positive for CD11b (Figure 9B and C). DSS treatment increased the number of both NK₁ receptor- and CD11b-positive cells, and there was no change in the proportion of double-positive macrophages (79.0%).

Discussion and conclusions

There is increasing evidence that endogenous 5-HT modulates inflammatory and immune responses in the gastrointestinal tract (Wang *et al.*, 2007a; Ghia *et al.*, 2009; Li *et al.*, 2011). However, the 5-HT receptor subtypes involved in these responses have not been fully elucidated. In the present study, we found that the 5-HT₃ receptor antagonists, ramosetron and ondansetron,

significantly reduced the severity of DSS-induced macroscopic and histological injuries to the colon, accompanied by an increase in colonic MPO activity. These findings extend previous research by our group and others showing that 5-HT₃ receptor antagonists ameliorated drug-associated and post-operative ileus-associated intestinal and colonic inflammation (Mousavizadeh *et al.*, 2009; Kato *et al.*, 2012; Motavallian *et al.*, 2013; Yasuda *et al.*, 2013; Maehara *et al.*, 2015). These findings strongly suggested that the pro-inflammatory actions of endogenous 5-HT are mediated by activation of 5-HT₃ receptors in both the small intestine and the colon.

Although the pathogenesis of IBD is not fully understood, it is generally accepted that various inflammatory mediators are involved and may coordinate abnormal immune responses to host microbiota antigens in genetically susceptible individuals (Cross and Wilson, 2003; Andoh *et al.*, 2008; Abraham and Medzhitov, 2011). These inflammatory mediators are also involved in the pathogenesis of DSS-induced murine colitis (Obermeier *et al.*, 1999; Naito *et al.*, 2003; Takedatsu *et al.*, 2008). In the present study, we observed that DSS induced a marked up-regulation of TNF- α , IFN- γ , IL-17A and iNOS mRNA in the colon, and this effect peaked on day 7 of DSS exposure. The expression of TNF- α and iNOS mRNA was further augmented on day 10, while that of IFN- γ and IL-17A mRNA was reduced at this time point. A similar time course of cytokine expression was identified by Nishikawa *et al.* (2014) in DSS-induced colitis. IFN- γ and IL-17 may therefore act as regulatory cytokines, inducing production of downstream pro-inflammatory cytokines, chemokines and iNOS-derived NO. Indeed, Iwakura *et al.* (2011) demonstrated that these regulatory cytokines stimulated the production of pro-inflammatory cytokines and chemokines, resulting in leukocyte infiltration into inflammatory sites. The present study showed that the up-regulation of TNF- α , IFN- γ , IL-17A and iNOS mRNA induced by 7-day DSS treatment was significantly attenuated by daily administration of ramosetron and ondansetron. This indicates that in DSS-induced colitis, endogenous 5-HT acts via 5-HT₃ receptors to induce colonic inflammation via up-regulation of these inflammatory mediators.

Margolis *et al.* (2014) recently reported that 5-HT derived from EC cells plays a critical role in intestinal inflammation, rather than in peristaltic or secretory responses. Peripheral tryptophan hydroxylase inhibitors decreased 5-HT levels in the gastrointestinal mucosa and blood but not in the nerve fibres of the myenteric plexus or in the brain. Furthermore, these inhibitors significantly reduced the severity of trinitrobenzene sulfonic acid-induced colitis, without affecting gastrointestinal motor activities. Several studies have demonstrated that the number of EC cells in the colon is increased in patients with IBD (El-Salhy *et al.*, 1997) and in experimental DSS-induced and trinitrobenzene sulfonic acid-induced colonic inflammation (Oshima *et al.*, 1999; Linden *et al.*, 2003; Khan *et al.*, 2006; Bertrand *et al.*, 2010). We also reported that plasma 5-HT levels were significantly increased in a tryptophan hydroxylase-inhibitable manner in the presence of intestinal inflammation induced by indomethacin (Kato *et al.*, 2012). In the present study, we observed that the number of 5-HT-immunoreactive EC cells in the colonic mucosa was significantly increased by DSS treatment; the maximal increase was detected on day 7 of DSS exposure,

and this response was diminished on day 10. This reduction in EC cell number on day 10 may reflect an elimination of EC cells, caused by the severe and extensive colonic injuries induced by DSS. However, the mechanisms underlying the increase in EC cell number and the release of 5-HT associated with intestinal and colonic inflammation remain unclear. Bacteria, their products, and several inflammatory cytokines may be involved in these responses (Khan *et al.*, 2006; Wang *et al.*, 2007b; Kidd *et al.*, 2009).

To investigate the role of the 5-HT/5-HT₃ receptor pathway in these processes further, we examined 5-HT₃ receptor immunohistochemically in colons from normal and DSS-treated mice. In normal animals, the 5-HT₃ receptor was mostly expressed in nerve fibres located in the submucosa and marginally in cell bodies located in the lamina propria. Interestingly, DSS treatment increased the number and length of 5-HT₃ receptor-positive nerve fibres in the mucosa and submucosa but did not increase the number of 5-HT₃ receptor-positive cells in the lamina propria. These results indicate that 5-HT₃ receptors are mostly expressed in nerve fibres in both normal and inflamed colonic mucosa. In contrast, we previously found that 5-HT₃ receptor expression was mostly detected in cell bodies and only weakly in nerve fibres of the small intestine (Yasuda *et al.*, 2013). In addition, the number of both 5-HT₃ receptor-positive cells and nerve fibres was reportedly increased in 5-FU-induced intestinal mucositis (Matsumoto *et al.*, 2013). It is therefore likely that the distribution and expression of 5-HT₃ receptors differ between the small intestine and the colon. Double immunohistochemical analyses further showed that 5-HT₃ receptor-positive nerve fibres were mostly double positive for VAcHt but rarely for SP in normal colons, suggesting that the 5-HT₃ receptor is mainly expressed in cholinergic nerve fibres.

Interestingly, DSS exposure increased the number and length of SP-positive nerve fibres, and these were also double positive for 5-HT₃ receptors. As shown in our previous study, 5-HT₃ receptor-positive nerve fibres in the colonic mucosa were mostly extrinsic spinal and vagal afferents (Matsumoto *et al.*, 2012). These findings strongly suggest that DSS up-regulated the expression of 5-HT₃ receptors in SP-positive non-cholinergic sensory afferent nerve fibres. Therefore, it is possible that 5-HT released from EC cells may act via 5-HT₃ receptors to increase SP release from sensory afferent nerve fibres in DSS-induced colitis. Indeed, Saria *et al.* (1991) showed that 5-HT increased SP release from the rat spinal cord via activation of 5-HT₃ receptors. Furthermore, 5-HT/5-HT₃ receptor-induced contractile responses were prevented by NK₁ antagonists in the guinea-pig ileum and colon. These findings indicated that 5-HT releases SP from nerve fibres in the colon via activation of 5-HT₃ receptors (Ramirez *et al.*, 1994; Yamano and Miyata, 1996). We previously observed that the number of SP-positive nerve fibres was not changed, although the number of SP-positive cells was increased, in DSS-induced colonic inflammation (Matsumoto *et al.*, 2012). This difference between our previous studies and the present findings may be due to a disparity in the severity of colitis. The colitis was much more severe in our previous studies, and it is therefore possible that nerve fibre SP may be depleted under these conditions. Further studies are needed to confirm this hypothesis.

SP may be involved in the pathogenesis of colonic inflammation. Several studies have demonstrated that colonic SP levels are increased in IBD patients and in experimental models of colitis (Mantyh *et al.*, 1995; Watanabe *et al.*, 1998) and NK₁ receptor antagonists reduce the severity of colonic inflammation (Rijnierse *et al.*, 2006; Gad *et al.*, 2009; Engel *et al.*, 2011). In the present study, we also observed that daily administration of the NK₁ receptor antagonist, aprepitant, significantly reduced the severity of DSS-induced colitis and attenuated the up-regulation of inflammatory mediators. These results, taken together with the present immunohistochemical observations, strongly suggest a pathogenic link between 5-HT/5-HT₃ receptor and SP/NK₁ receptor pathways in colonic inflammation. The pro-inflammatory effects mediated by SP/NK₁ receptors may thus lie downstream of 5-HT/5-HT₃ receptors. Indeed, in the present study it was found that DSS-induced increases in SP-positive nerve fibres and SP levels in the colon were significantly diminished by ramosetron treatment. In contrast, the DSS-induced increase in 5-HT-positive cells in the colonic mucosa was not affected by aprepitant treatment. These findings clearly support the proposal that the 5-HT/5-HT₃ receptor interaction promotes colonic inflammation via the SP/NK₁ receptor pathway.

The NK₁ receptor is expressed in enteric neurons, interstitial Cajal cells, the epithelium, vasculature and the immune system (Holzer and Holzer-Petsche, 2001). Matsumoto *et al.* (2013) further showed that the NK₁ receptor was localized in CD11b-positive inflammatory cells (mostly macrophages) in the lamina propria of the small intestine and that the number of NK₁ receptor-positive cells increased during 5-FU-induced inflammation. The present study revealed that DSS treatment markedly increased NK₁ receptor immunoreactivity in CD11b-positive cells of the colonic lamina propria. It is therefore possible that the SP/NK₁ receptor pathway may regulate macrophage immune and inflammatory responses during colonic inflammation. Further studies are required to clarify the mechanism(s) regulating SP/NK₁ receptor responses.

In conclusion, these results demonstrate that the 5-HT/5-HT₃ receptor and SP/NK₁ receptor pathways play a pathogenic role in colonic inflammation. Increased 5-HT levels acted via 5-HT₃ receptors to up-regulate the expression of inflammatory mediators following exposure to DSS. These effects were further mediated by SP, released from sensory afferent nerve fibres and acting via NK₁ receptors. These findings advance our understanding of the influence of 5-HT on the mechanisms underlying colonic inflammation.

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Author contributions

S.K. and D.U. planned and designed the experiments. D.U. and K.M. performed the experiments. S.K., D.U. and K.M. analysed the data. D.U. and S.K. wrote the manuscript. S.K., D.U., K.M., K.A. and S.H. reviewed and discussed the data.

Conflict of interest

The 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 pre-clinical research recommended by funding agencies, publishers and other organizations engaged with supporting research.

References

- Abraham C, Medzhitov R (2011). Interactions between the host innate immune system and microbes in inflammatory bowel disease. *Gastroenterology* 140: 1729–1737.
- Alexander SPH, Davenport AP, Kelly E, Marrion N, Peters JA, Benson HE *et al.* (2015a). The Concise Guide to PHARMACOLOGY 2015/16: G protein-coupled receptors. *Br J Pharmacol* 172: 5744–5869.
- Alexander SPH, Fabbro D, Kelly E, Marrion N, Peters JA, Benson HE *et al.* (2015b). The Concise Guide to PHARMACOLOGY 2015/16: Catalytic receptors. *Br J Pharmacol* 172: 5979–6023.
- Alexander SPH, Fabbro D, Kelly E, Marrion N, Peters JA, Benson HE *et al.* (2015c). The Concise Guide to PHARMACOLOGY 2015/16: Enzymes. *Br J Pharmacol* 172: 6024–6109.
- Alexander SPH, Kelly E, Marrion N, Peters JA, Benson HE, Faccenda E *et al.* (2015d). The Concise Guide to PHARMACOLOGY 2015/16: Transporters. *Br J Pharmacol* 172: 6110–6202.
- Alexander SPH, Peters JA, Kelly E, Marrion N, Benson HE, Faccenda E *et al.* (2015e). The Concise Guide to PHARMACOLOGY 2015/16: Ligand-gated ion channels. *Br J Pharmacol* 172: 5870–5903.
- Andoh A, Yagi Y, Shioya M, Nishida A, Tsujikawa T, Fujiyama Y (2008). Mucosal cytokine network in inflammatory bowel disease. *World J Gastroenterol* 14: 5154–5161.
- Berger M, Gray JA, Roth BL (2009). The expanded biology of serotonin. *Annu Rev Med* 60: 355–366.
- Bertrand PP, Barajas-Espinosa A, Neshat S, Bertrand RL, Lomax AE (2010). Analysis of real-time serotonin (5-HT) availability during experimental colitis in mouse. *Am J Physiol Gastrointest Liver Physiol* 298: G446–G455.
- Cross RK, Wilson KT (2003). Nitric oxide in inflammatory bowel disease. *Inflamm Bowel Dis* 9: 179–189.
- Curtis MJ, Bond RA, Spina D, Ahluwalia A, Alexander SPA, Giembycz MA *et al.* (2015). Experimental design and analysis and their reporting: new guidance for publication in BJP. *Br J Pharmacol* 172: 3461–3471.
- El-Salhy M, Danielsson A, Stenling R, Grimelius L (1997). Colonic endocrine cells in inflammatory bowel disease. *J Intern Med* 242: 413–419.
- Engel MA, Becker C, Reeh PW, Neurath MF (2011). Role of sensory neurons in colitis: increasing evidence for a neuroimmune link in the gut. *Inflamm Bowel Dis* 17: 1030–1033.

- Farber L, Haus U, Spath M, Drechsler S (2004). Physiology and pathophysiology of the 5-HT₃ receptor. *Scand J Rheumatol Suppl* 119: 2–8.
- Fukudo S, Ida M, Akiho H, Nakashima Y, Matsueda K (2014). Effect of ramosetron on stool consistency in male patients with irritable bowel syndrome with diarrhea. *Clin Gastroenterol Hepatol* 12: 953–959. e954
- Gad M, Pedersen AE, Kristensen NN, Fernandez Cde F, Claesson MH (2009). Blockage of the neurokinin 1 receptor and capsaicin-induced ablation of the enteric afferent nerves protect SCID mice against T-cell-induced chronic colitis. *Inflamm Bowel Dis* 15: 1174–1182.
- Ghia JE, Blennerhassett P, Collins SM (2008). Impaired parasympathetic function increases susceptibility to inflammatory bowel disease in a mouse model of depression. *J Clin Invest* 118: 2209–2218.
- Ghia JE, Li N, Wang H, Collins M, Deng Y, El-Sharkawy RT *et al.* (2009). Serotonin has a key role in pathogenesis of experimental colitis. *Gastroenterology* 137: 1649–1660.
- Gonzalez-Rey E, Chorny A, Delgado M (2006). Therapeutic action of ghrelin in a mouse model of colitis. *Gastroenterology* 130: 1707–1720.
- Hannon J, Hoyer D (2008). Molecular biology of 5-HT receptors. *Behav Brain Res* 195: 198–213.
- Hansen MB (2003). The enteric nervous system III: a target for pharmacological treatment. *Pharmacol Toxicol* 93: 1–13.
- Holzer P (1998). Implications of tachykinins and calcitonin gene-related peptide in inflammatory bowel disease. *Digestion* 59: 269–283.
- Holzer P, Holzer-Petsche U (2001). Tachykinin receptors in the gut: physiological and pathological implications. *Curr Opin Pharmacol* 1: 583–590.
- Iwakura Y, Ishigame H, Saijo S, Nakae S (2011). Functional specialization of interleukin-17 family members. *Immunity* 34: 149–162.
- Jackson MB, Yakel JL (1995). The 5-HT₃ receptor channel. *Annu Rev Physiol* 57: 447–468.
- Kaser A, Zeissig S, Blumberg RS (2010). Inflammatory bowel disease. *Annu Rev Immunol* 28: 573–621.
- Kato S, Matsuda N, Matsumoto K, Wada M, Onimaru N, Yasuda M *et al.* (2012). Dual role of serotonin in the pathogenesis of indomethacin-induced small intestinal ulceration: pro-ulcerogenic action via 5-HT₃ receptors and anti-ulcerogenic action via 5-HT₄ receptors. *Pharmacol Res* 66: 226–234.
- Khan WI, Motomura Y, Wang H, El-Sharkawy RT, Verdu EF, Verma-Gandhu M *et al.* (2006). Critical role of MCP-1 in the pathogenesis of experimental colitis in the context of immune and enterochromaffin cells. *Am J Physiol Gastrointest Liver Physiol* 291: G803–G811.
- Kidd M, Gustafsson BI, Drozdov I, Modlin IM (2009). IL1 β - and LPS-induced serotonin secretion is increased in EC cells derived from Crohn's disease. *Neurogastroenterol Motil* 21: 439–450.
- Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). NC3Rs Reporting Guidelines Working Group. *Br J Pharmacol* 160: 1577–1579.
- Kozłowski CM, Green A, Grundy D, Boissonade FM, Bountra C (2000). The 5-HT₃ receptor antagonist alosetron inhibits the colorectal distention induced depressor response and spinal c-fos expression in the anaesthetised rat. *Gut* 46: 474–480.
- Li N, Ghia JE, Wang H, McClemens J, Cote F, Suehiro Y *et al.* (2011). Serotonin activates dendritic cell function in the context of gut inflammation. *Am J Pathol* 178: 662–671.
- Linden DR, Chen JX, Gershon MD, Sharkey KA, Mawe GM (2003). Serotonin availability is increased in mucosa of guinea pigs with TNBS-induced colitis. *Am J Physiol Gastrointest Liver Physiol* 285: G207–G216.
- Maehara T, Matsumoto K, Horiguchi K, Kondo M, Iino S, Horie S *et al.* (2015). Therapeutic action of 5-HT₃ receptor antagonists targeting peritoneal macrophages in post-operative ileus. *Br J Pharmacol* 172: 1136–1147.
- Mantyh CR, Vigna SR, Bollinger RR, Mantyh PW, Maggio JE, Pappas TN (1995). Differential expression of substance P receptors in patients with Crohn's disease and ulcerative colitis. *Gastroenterology* 109: 850–860.
- Margolis KG, Stevanovic K, Li Z, Yang QM, Oravec T, Zambrowicz B *et al.* (2014). Pharmacological reduction of mucosal but not neuronal serotonin opposes inflammation in mouse intestine. *Gut* 63: 928–937.
- Matsumoto K, Lo MW, Hosoya T, Tashima K, Takayama H, Murayama T *et al.* (2012). Experimental colitis alters expression of 5-HT receptors and transient receptor potential vanilloid 1 leading to visceral hypersensitivity in mice. *Lab Invest* 92: 769–782.
- Matsumoto K, Kurosawa E, Terui H, Hosoya T, Tashima K, Murayama T *et al.* (2009). Localization of TRPV1 and contractile effect of capsaicin in mouse large intestine: high abundance and sensitivity in rectum and distal colon. *Am J Physiol Gastrointest Liver Physiol* 297: G348–G360.
- Matsumoto K, Nakajima T, Sakai H, Kato S, Sagara A, Arakawa K *et al.* (2013). Increased expression of 5-HT₃ and NK 1 receptors in 5-fluorouracil-induced mucositis in mouse jejunum. *Dig Dis Sci* 58: 3440–3451.
- McGrath JC, Lilley E (2015). Implementing guidelines on reporting research using animals (ARRIVE etc.): new requirements for publication in BJP. *Br J Pharmacol* 172: 3189–3193.
- Millan MJ, Dekeyne A, Gobert A, Mannoury la Cour C, Brocco M, Rivet JM *et al.* (2010). S41744, a dual neurokinin (NK)₁ receptor antagonist and serotonin (5-HT) reuptake inhibitor with potential antidepressant properties: a comparison to amitriptyline (MK869) and paroxetine. *Eur Neuropsychopharmacol* 20: 599–621.
- Minami M, Nemoto M, Endo T, Hamaue N, Kohno Y (1997). Effects of talipexole on emesis-related changes in abdominal afferent vagal activity and ileal serotonin metabolism in rats. *Res Commun Mol Pathol Pharmacol* 95: 67–82.
- Molodecky NA, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G *et al.* (2012). Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* 142: 46–54 e42; quiz e30.
- Motavallian A, Minaiyan M, Rabbani M, Andalib S, Mahzouni P (2013). Involvement of 5HT₃ receptors in anti-inflammatory effects of tropisetron on experimental TNBS-induced colitis in rat. *Bioimpacts* 3: 169–176.
- Mousavizadeh K, Rahimian R, Fakhfour G, Aslani FS, Ghafourifar P (2009). Anti-inflammatory effects of 5-HT receptor antagonist, tropisetron on experimental colitis in rats. *Eur J Clin Invest* 39: 375–383.
- Naito Y, Takagi T, Handa O, Ishikawa T, Nakagawa S, Yamaguchi T *et al.* (2003). Enhanced intestinal inflammation induced by dextran sulfate sodium in tumor necrosis factor- α deficient mice. *J Gastroenterol Hepatol* 18: 560–569.

- Nishikawa K, Seo N, Torii M, Ma N, Muraoka D, Tawara I *et al.* (2014). Interleukin-17 induces an atypical M2-like macrophage subpopulation that regulates intestinal inflammation. *PLoS One* 9: e108494.
- O'Connor TM, O'Connell J, O'Brien DI, Goode T, Bredin CP, Shanahan F (2004). The role of substance P in inflammatory disease. *J Cell Physiol* 201: 167–180.
- Obermeier F, Kojouharoff G, Hans W, Scholmerich J, Gross V, Falk W (1999). Interferon-gamma (IFN-gamma)- and tumour necrosis factor (TNF)-induced nitric oxide as toxic effector molecule in chronic dextran sulphate sodium (DSS)-induced colitis in mice. *Clin Exp Immunol* 116: 238–245.
- Oshima S, Fujimura M, Fukimiya M (1999). Changes in number of serotonin-containing cells and serotonin levels in the intestinal mucosa of rats with colitis induced by dextran sodium sulfate. *Histochem Cell Biol* 112: 257–263.
- Pawson AJ, Sharman JL, Benson HE, Faccenda E, Alexander SP, Buneman OP *et al.* (2014). The IUPHAR/BPS guide to PHARMACOLOGY: an expert-driven knowledge base of drug targets and their ligands. *Nucleic Acids Res* 42: D1098–D1106.
- Ramirez MJ, Cenarruzabeitia E, Del Rio J, Lasheras B (1994). Involvement of neurokinins in the non-cholinergic response to activation of 5-HT₃ and 5-HT₄ receptors in guinea-pig ileum. *Br J Pharmacol* 111: 419–424.
- Renzi D, Pellegrini B, Tonelli F, Surrenti C, Calabro A (2000). Substance P (neurokinin-1) and neurokinin A (neurokinin-2) receptor gene and protein expression in the healthy and inflamed human intestine. *Am J Pathol* 157: 1511–1522.
- Rijnierse A, van Zijl KM, Koster AS, Nijkamp FP, Kraneveld AD (2006). Beneficial effect of tachykinin NK₁ receptor antagonism in the development of hapten-induced colitis in mice. *Eur J Pharmacol* 548: 150–157.
- Saria A, Javorsky F, Humpel C, Gamse R (1991). Endogenous 5-hydroxytryptamine modulates the release of tachykinins and calcitonin gene-related peptide from the rat spinal cord via 5-HT₃ receptors. *Ann N Y Acad Sci* 632: 464–465.
- Siriwardena AK, Budhoo MR, Smith EP, Kellum JM (1993). A 5-HT₃ receptor agonist induces neurally mediated chloride transport in rat distal colon. *J Surg Res* 55: 55–59.
- Takedatsu H, Michelsen KS, Wei B, Landers CJ, Thomas LS, Dhall D *et al.* (2008). TL1A (TNFSF15) regulates the development of chronic colitis by modulating both T-helper 1 and T-helper 17 activation. *Gastroenterology* 135: 552–567.
- Talley NJ (2001). Serotonergic neuroenteric modulators. *Lancet* 358: 2061–2068.
- Wang H, Steeds J, Motomura Y, Deng Y, Verma-Gandhu M, El-Sharkawy RT *et al.* (2007a). CD4+ T cell-mediated immunological control of enterochromaffin cell hyperplasia and 5-hydroxytryptamine production in enteric infection. *Gut* 56: 949–957.
- Wang J, Kodali S, Lee SH, Galgoci A, Painter R, Dorso K *et al.* (2007b). Discovery of platencin, a dual FabF and FabH inhibitor with in vivo antibiotic properties. *Proc Natl Acad Sci U S A* 104: 7612–7616.
- Watanabe T, Kubota Y, Muto T (1998). Substance P containing nerve fibers in ulcerative colitis. *Int J Colorectal Dis* 13: 61–67.
- Wirtz S, Neufert C, Weigmann B, Neurath MF (2007). Chemically induced mouse models of intestinal inflammation. *Nat Protoc* 2: 541–546.
- Yamano M, Miyata K (1996). Investigation of 5-HT₃ receptor-mediated contraction in guinea-pig distal colon. *Eur J Pharmacol* 317: 353–359.
- Yasuda M, Kawahara R, Hashimura H, Yamanaka N, Iimori M, Amagase K *et al.* (2011). Dopamine D₂-receptor antagonists ameliorate indomethacin-induced small intestinal ulceration in mice by activating α 7 nicotinic acetylcholine receptors. *J Pharmacol Sci* 116: 274–282.
- Yasuda M, Kato S, Yamanaka N, Iimori M, Matsumoto K, Utsumi D *et al.* (2013). 5-HT(3) receptor antagonists ameliorate 5-fluorouracil-induced intestinal mucositis by suppression of apoptosis in murine intestinal crypt cells. *Br J Pharmacol* 168: 1388–1400.