Protective Actions of Epithelial 5-Hydroxytryptamine 4 Receptors in Normal and Inflamed Colon

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Short Title: 5-HT4R stimulation is protective in colitis

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Abbreviations Arbitrary fluorescence units (AFUs), dextran sodium sulfate (DSS), dimethyl sulfoxide (DMSO), disease activity index (DAI), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), fluorescein isothiocyanate (FITC), gastrointestinal (GI), histological damage score (HDS), inflammatory bowel disease (IBD), inhibitory junction potential (IJP), intraperitoneal (IP), phosphate buffered saline (PBS), reverse transcriptase polymerase chain reaction (rtPCR), serotonin (5-HT), sulforhodamine B (SRB), trinitrobenzene sulfonic acid (TNBS)

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ABSTRACT

Background & Aims: The 5-hydroxytryptamine receptor 4 (5-HT4R or HTR4) is expressed in the colonic epithelium but little is known about its functions there. We examined whether activation of colonic epithelial 5-HT4R protects colons of mice from inflammation.

Methods: The 5-HT4R agonist tegaserod (1 mg/kg), the 5-HT4R antagonist GR113808 (1
mg/kg), or vehicle (control) were delivered by enema to wild-type or 5-HT4R knockout mice at the onset of, or during, active colitis, induced by administration of dextran sodium sulfate or trinitrobenzene sulfonic acid. Inflammation was measured using the colitis disease activity index and by histologic analysis of intestinal tissues. Epithelial proliferation, wound healing, and resistance to oxidative stress-induced apoptosis were assessed, as was colonic motility.

Results: Rectal administration of tegaserod reduced the severity of colitis, compared to mice given vehicle, and accelerated recovery from active colitis. Rectal tegaserod did not improve colitis in 5-HT4R knockout mice, and intraperitoneally administered tegaserod did not protect wild-type mice from colitis. Tegaserod increased proliferation of crypt epithelial cells. Stimulation of 5-HT4R increased Caco-2 cell migration and reduced oxidative stress-induced apoptosis; these actions were blocked by co-administration of the 5-HT4R antagonist GR113808. In non-inflamed colons of wild-type mice not receiving tegaserod, inhibition of 5-HT4Rs resulted in signs of colitis within 3 days. In these mice, epithelial proliferation decreased and bacterial translocation to the liver and spleen was detected. Daily administration of tegaserod increased motility in inflamed colons of guinea pigs and mice, whereas administration of GR113808 disrupted motility in animals without colitis.

Conclusions: 5-HT4R activation maintains motility in healthy colons of mice and guinea pigs reduces inflammation in colons of mice with colitis. Agonists might be developed as treatments for patients with inflammatory bowel diseases.

KEY WORDS: IBD; colonic motility; wound healing; mucosal drug action

INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) acts as a neurotransmitter in both the central and peripheral nervous systems, and as a paracrine signaling molecule in the periphery.[1] In the gastrointestinal (GI) tract 5-HT contributes to motility, secretion, vasodilation, and sensation,[2] and it also has both neuro-protective[3] and pro-inflammatory actions in the gut.[4 5] Because of the importance of 5-HT in gut functions and sensation, drugs targeting 5-HT receptors have been developed for the treatment of functional GI disorders and pain.[2]

Several 5-HT receptor subtypes are located in the wall of the gut.[2] The 5-HT_4 receptor, is located on enteric nerve terminals where it mediates presynaptic facilitation of neurotransmitter release when activated.[6 7] 5-HT_4 receptors are also highly expressed in the colonic epithelium, where they appear to be expressed by all epithelial cells.[8] Epithelial 5-HT_4 receptors mediate a variety of responses, including 5-HT release, chloride secretion and goblet cell degranulation, as well as enhanced propulsive motility and reduced visceral hypersensitivity.[8]

5-HT_4 receptor agonists have prokinetic and anti-nociceptive actions and have been used to treat constipation-predominant irritable bowel syndrome,[2] but the functions of these receptors are still being elucidated, and they likely include protective actions. For example, 5-HT_4 receptor agonists can reduce the extent of NSAID-induced inflammation.[9] Furthermore, 5-HT_4 agonists stimulate enteric neurogenesis and promote axon growth *in vitro*,[3] and the restoration of the recto-anal reflex in re-anastomosed preparations *in vivo*.[10] 5-HT_4 receptors also appear to play a role in the development and

survival of enteric neurons, since neuronal density is reduced in the intestines of 5-HT_4 knockout mice.[3]

We tested the hypothesis that mucosal 5-HT_4 receptors exert protective effects in normal and inflamed colon. Using animal models of colitis, we examined whether mucosal 5-HT_4 receptor stimulation affects the extent of colitis as it develops and whether it can improve recovery from colitis once it is established. We examined potential mechanisms of epithelial healing and resistance to oxidative stress. Furthermore, we tested whether the mucosal 5-HT_4 receptor influences the integrity of the epithelial layer and colonic motor function under basal, healthy conditions. Our findings indicate that stimulation of mucosal 5-HT_4 receptors exerts protective and restorative effects in models of colitis, and that these receptors contribute physiologically to mucosal integrity.

METHODS

Animal Preparations

All experimental protocols were approved by the Institutional Animal Care and Use Committees of the University of Vermont and the University of Calgary. Animals were euthanized by isoflurane overdose and exsanguination or cervical dislocation. The following animals were used for these studies: 7-8 week Male CD-1 IGS mice from Charles River, Canada; 7-8 week male and female 5-HT_4R knockout mice and their litter mates on an SV129 background from Dr. Valérie Compan, Université Montpellier, via Dr. David Linden, Mayo Clinic; 250-300 g male Hartley guinea pigs, Charles River, Canada.

Induction of Colitis

Dextran sodium sulfate (DSS) colitis was induced in mice by administering DSS (w/v in water; 3% for SV129 mice and 4% for CD-1, MW: 36,000-50,000, MP Biomedicals, Solon, OH) for 5 days followed by a return to tap water for 2-10 days. 2,4,6-trinitrobenzene sulfonic acid (TNBS; Sigma-Aldrich, St. Louis, MO) colitis was induced by a single colonic enema (mice: 7.5 mg/mL in 50% ethanol, 100 μL; guinea pigs: 25 mg/mL in 30% ethanol in 300 μL) delivered under anesthesia.

Colitis Paradigms

For the prevention experiments, mice received DSS for 5 days and were then switched to tap water for 2 days. Alternatively, mice and guinea pigs were given a single enema of TNBS. Enemas with either vehicle (1% dimethyl sulfoxide (DMSO) in 0.9% saline; 0.2 mL/mouse) or drug (tegaserod provided by John McRorie from Proctor and Gamble, GR113808 from Sigma-Aldrich; both delivered at 1 mg/Kg) were administered daily for 5-7 days starting 24 h after induction of colitis. These doses were chosen because they were effective in previous studies of the effects of luminal administration of these compounds on visceral sensitivity.[8] In a preliminary study, we found that enema treatment did not affect the histological damage score (HDS) (naïve, 0.6±0.25, n=5; vehicle enema, 1.1±0.3; p=0.13, n=7). In another preliminary study, involving enema administration of a vehicle solution containing 0.5% Evans Blue, we found that the solution delivered spread as far orally as the cecum after 10 minutes. Animals were euthanized on day 6 or 7 for TNBS or DSS studies, respectively. In the recovery paradigm drug treatment began on day 6 and lasted for 10 days, with animals euthanized on day 15. The time courses were chosen to test the effectiveness of the treatments

leading up to the peak of inflammation, or beginning once the peak had been reached. Transcript levels for the 5-HT_4 receptor were not altered in DSS ($p=0.56$) or TNBS ($p=0.9$) colitis.

Assessment of Inflammation

Colitis severity was monitored using the disease activity index (DAI), which includes evaluation of weight loss, stool consistency, and presence of fecal blood.[11] Fecal blood was assessed using Hemoccult Single Slide testing slides from Beckman Coulter (Brea, CA). After euthanasia, tissue was collected and fixed overnight in 4% paraformaldehyde for immunohistochemistry.

Hematoxylin and eosin stained sections from paraffin embedded tissue were used for histological assessment of colitis. A scoring rubric based on histological features of human inflammatory bowel disease (IBD) was developed.[12] This histological damage score (HDS) reflects epithelial damage, altered crypt architecture, infiltration of monocytes and polymorphonuclear cells into the *lamina propria* and epithelium, and evidence of ulcers or erosions. Two slides for each experimental group were scored by an observer blinded to the treatment groups.

Intestinal permeability

On day 7 following enema treatment, mice were orally gavaged with FITC-dextran (150 mg/mL, 60 mg/100 g body weight). Four hours following gavage, mice were anesthetized, the chest and abdomen cleaned with 70% ethanol and blood was drawn via cardiac puncture.[13] Blood was allowed to clot, then spun at 2000 x g for 10 minutes, the supernatant was read on a spectrophotometer at 485/535 nm.

Immunohistochemistry

Immunohistochemical staining of sections from paraffin embedded tissue was performed as previously described.[8] Immunostaining with a rat anti-mouse Ki-67 primary antiserum (1:100; eBioscience, San Diego, CA) was visualized with a goat anti-rat Cy3 antibody (1:600; Jackson ImmunoResearch, West Grove, PA), in sections counterstained with DAPI (1:1,000; Sigma-Aldrich). The data are presented as proportion of Ki-67 positive cells relative to total epithelial cells per crypt.[14] Images were produced on an Olympus AX70 fluorescence microscope and captured using an Optronics MagnaFire digital camera and software.

Motility

Guinea pig distal colon motility was examined using a GastroIntestinal Motility Monitor (GIMM; Catamount Research and Development, St. Albans, VT), and prepared as previously described.[15] The most distal 5-10 cm of colon was placed in the organ bath, and equilibrated for 30 minutes in circulating Kreb's solution (37°C). Five trials, spaced by 5 min rest periods, were performed for each colon.

Murine colonic transit was assessed as previously described.[16] Mice were lightly anesthetized using 3% isoflurane, and a small glass bead was inserted 2 cm into the distal colon. Time from the insertion of the bead to expulsion was recorded. This assay was performed before and after treatment with either vehicle, agonist or antagonist. For each time point, measurements were taken on two consecutive days and the two times averaged. For each mouse, bead expulsion time was normalized to the before treatment value.

Intracellular Recording

Intracellular recordings from guinea pig colonic circular muscle were carried out in a Sylgard-lined recording chamber with a circulating, aerated Kreb's solution (37°C) containing nifedipine (5 μM; Sigma-Aldrich).[17 18] Cells were impaled with glass microelectrodes (70 to 120 MΩ, filled with 2M KCl) under visualization with an inverted microscope at 100X magnification. Junction potentials were evoked by transmural stimuli (0.5ms pulse duration, 0.5Hz, 50V). Voltage recordings were obtained with an Axoclamp-2A amplifier (Axon-instruments, Union City, CA, USA) and analyzed with PowerLab Chart (version 5.01; AD Instruments, Castle Hill, NSW, Australia).

Cell lines culture and treatment for oxidative stress

Human Caco-2 cells, a epithelial colorectal adenocarcinoma cell line (ATCC, UK), were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin and 100 Ag/mL streptomycin. To produce oxidative stress in Caco-2, cells were treated with 200 μM of H_2O_2 in phosphate-buffered saline (PBS) for 30 min[1918] 24 h after the cells were seeded. [17]

The sulforhodamine B (SRB) assay was used to determine cell density, as previously described and validated. [20 19,18] Cells grown in 24 well plates were treated for 1 hr with vehicle, agonist or agonist plus antagonist. Supernatant was aspirated from wells and cells were fixed with cold trichloroacetic acid solution (30% w/v) at 4°C for 1 h. Fixed cells were washed with H_2O and dried, and SRB solution (0.057% w/v) was applied to stain the cellular protein contents. Using spectrophotometry, absorbance was measured at 540 nm with a reference wavelength of 630 nm.

Scratch Assay

Caco-2 cells (provided by Dr. J Turner, University of Chicago) were cultured in 6 well plates to ~90% confluence in DMEM high glucose supplemented with 10% FBS, 1% GlutaMAX, 10 mM Hepes and 100 U/mL Penicillin-Streptomycin (ThermoFisher Grand Island, NY). Once ~90% confluence was reached, three wounds were created using a sterile 200 uL pipet tip dragged perpendicular to a black line drawn on the underside of the plate for reference. Images were captured of each scratch at time points 0 h and 48 h with a Nikon D7100 camera on a Nikon Diaphot inverted microscope at 4X magnification. Only scratches whose edges could be captured in one frame at time point 0 h were included for final analysis. Measurements were taken from edge to edge at time 0 h and compared to measurements from 48 h using ImageJ software.[21] The reported values are the difference between 0 h and 48 h, with higher values representing increased cellular migration. Three separate experiments were conducted with all three conditions.

Bacterial Translocation

After euthanasia, mice were wiped with alcohol, and the spleen and liver were removed. Samples were weighed and homogenized in 1 mL sterile PBS. Between samples the homogenizer was rinsed in sterile PBS, water, then 70% ethanol to prevent cross contamination. Homogenates (200 μL) were plated on Columbia and MacConkey agar plates (Dalynn Biologicals, Calgary, AB) and incubated at 37° C in 5% CO_2.[22] After 48 h, the number of positive plates was determined; contaminated plates were not counted.

Data Analysis

The data are presented as mean ± SEM for *n* of animals or preparations. Statistical analyses were performed using the GraphPad Prism software application (version 6.0c, GraphPad Software, La Jolla, CA). Data sets were examined prior to analysis to ensure the validity of test assumptions such as similar variability and n-values between groups. In cases in which assumptions were met, data sets were compared using unpaired student's t-test, 1-way ANOVA or 2-way ANOVA with Bonferroni's correction, or Fisher's exact test, when appropriate. The assumptions were not met in two of the experiments: a Welch's correction was used in the case of a t-test and a square root transformation was used to compress variance in the case of an ANOVA. These tests are indicated in the figure legends. Statistical significance was defined as one tailed p-values of less than 0.05.

RESULTS

Effects of Epithelial 5-HT_4 Receptor Stimulation on Colitis

5-HT_4 Receptor stimulation attenuates the development of colitis

In DSS-inflamed mice, treatment with tegaserod (1 mg/Kg), beginning 24 hr after DSS was introduced, significantly reduced the clinical (DAI, $p<0.05$) and the histological (HDS, $p<0.001$) damage of the colon compared to vehicle treated DSS inflamed animals (Fig 1A; Supplemental Fig 1). The protective effects of tegaserod were blocked by the 5-HT_4 antagonist, GR113808 (Fig 1A; Supplemental Fig 1; DAI, $p<0.05$; HDS, $p<0.0001$).

In TNBS colitis, treatment with tegaserod significantly reduced the DAI as compared to inflamed controls (Fig 1B; $p<0.05$); however, the HDS was not changed. The protective effect of tegaserod on the DAI was blocked by the 5-HT_4 antagonist (Fig 1B; $p<0.05$).

To further confirm that tegaserod was mediating its protective action via 5-HT_4 receptor activation, experiments were conducted with 5-HT_4 knockout mice and their wild type littermates with DSS colitis. Tegaserod failed to improve the DAI or HDS in mice lacking the 5-HT_4 receptor (Fig. 1C), but it significantly reduced inflammation in the wild type animals (DAI: vehicle, 7.7±0.5; agonist, 5.5±0.5; $p=0.004$; HDS: vehicle, 10.3±0.6; agonist, 8.2±0.3; $p=0.017$ by t-test; n=7-11 per group).

5-HT_4 receptor stimulation accelerates healing from established colitis

To test whether activation of epithelial 5-HT_4 receptors in the distal colon affects the recovery from colonic inflammation, animals were treated with tegaserod after colitis was established (days 6-15; Fig. 2).

5-HT_4 agonist treatment significantly accelerated the recovery from DSS colitis as compared to vehicle treated animals (Fig 2A; $p<0.0001$ at day 15). Furthermore, DSS-inflamed mice treated with tegaserod showed significant improvement in HDS (Fig 2A; $p<0.0001$). These effects were blocked by the 5-HT_4 antagonist (Fig 2A; DAI, $p<0.001$; HDS, $p<0.001$).

5-HT_4 agonist treatment in established TNBS colitis accelerated recovery of the DAI (Fig 2B; $p<0.001$ at day 15) and improved the HDS ($p<0.01$), and these actions were also inhibited by antagonist treatment (Fig 2B; DAI, $p<0.001$; HDS, $p<0.05$).

Intraperitoneal administration of the 5-HT_4 receptor agonist fails to affect colitis

To test whether the effects of enema-administered 5-HT_4 agonist could involve 5-HT_4 receptors at other sites in the GI tract or elsewhere, the agonist was delivered daily by intraperitoneal (IP) injection at the same dose (1 mg/Kg) beginning on day 1. In DSS-inflamed animals, IP administered agonist had no effect on the clinical or histological scores (DAI: vehicle, 5.1±0.6; agonist, 4.5±0.5; $p=0.4$; HDS: vehicle, 10.0±0.9; agonist, 7.8±0.9; $p=0.1$ by t-test; n=7-8 per group).

5-HT_4 receptor mediated protective mechanisms

Epithelial 5-HT_4 receptor activation could mediate the protective effects via a variety of mechanisms, including maintenance or reestablishment of the epithelial barrier through cell proliferation and migration, and also by increasing resistance to epithelial apoptosis induced by oxidative stress. We first assessed epithelial permeability by evaluating FITC-dextran in serum by spectroscopy (arbitrary fluorescence units; AFUs) following gastric gavage. Colitis was associated with a 3-4 fold increase in permeability (control, 3530 ± 125 AFUs, n=17; DSS, 15,690 ± 3222 AFUs, n=22, $P<0.01$). Despite

the fact that the cecum and entire colon is affected in DSS colitis, a tegaserod enema in the distal colon demonstrated a tendency to reduce the permeability to FITC-dextran (9,372 ± 944 AFUs, n=16, P=0.07). Associated with the increase in epithelial permeability there is a degree of bacterial translocation associated with colitis (4/9 animals with colitis had bacterial translocation to the liver or spleen compared to 0/5 control animals). After tegaserod enema there was again a tendency for this to be reduced (1/8 animals with bacterial translocation). These actions prompted us to examine detailed mechanisms of action of 5-HT_4 receptor activation in the epithelium.

The effects of 5-HT_4 receptor activation on proliferation were tested in the DSS recovery paradigm, as this condition yielded the most robust response. Effects of 5-HT_4 receptor stimulation on migration and resistance to oxidative stress were evaluated in Caco-2 cells, which were found to express the 5-HT_4 receptor by rtPCR and immunoblot (Supplemental Fig. 2).

The nuclear protein, Ki-67, is an effective marker of post-mitotic cells.[14] In the colons of DSS-inflamed animals that received daily agonist enemas beginning on day 6, there was a significant increase in the percentage of crypt epithelial cells that were Ki-67 positive at day 15 (Fig. 3; $p<0.05$; Supplemental Fig 3), and this effect was blocked by the 5-HT_4 antagonist (Fig. 3; $p<0.01$; Supplemental Fig 3). The proportion of epithelial cells immunoreactive for Ki-67 was also significantly higher in colons from animals treated with DSS and agonist beginning on day 1 and euthanized on day 7 (vehicle, 0.5±0.04 vs 0.7±0.02; $p<0.001$; n=5 per group).

An important mechanism of epithelial healing is enhanced epithelial cell migration.[23] To assess this we performed a scratch wound healing assay,[24] and saw a

significant increase in the rate of Caco-2 cell migration in cultures treated with tegaserod (1 μM) (Fig. 4A,C; $p<0.001$), and this effect was inhibited in the presence of the antagonist (Fig. 4A,C; $p<0.05$).

Oxidative stress is a feature of colitis,[25] and triggers epithelial apoptosis.[26 27] Therefore, Caco-2 cells were exposed to the free radical donor, H_2O_2 (200 μM), and cell survival in response to 5-HT_4 receptor stimulation was determined using the SRB assay. H_2O_2 caused a significant reduction in cell survival compared to untreated cells (Fig. 4B; $p<0.001$), and cell survival in H_2O_2 treated cultures was significantly improved by tegaserod (10 μM) (Fig. 4B; $p<0.001$). This agonist-mediated protection was blocked by the specific antagonist, GR113808 (10 nM) ($p<0.001$).

Effects of 5-HT_4 Receptor Activation on Propulsive Motility

A central feature of IBD is altered GI motility.[28 29] We have investigated dysmotility in guinea pig TNBS colitis,[17 18 30 31] and have demonstrated that changes in enteric neuronal excitability[31] and purinergic inhibitory neuromuscular transmission[17] contribute to disrupted motility. We therefore treated TNBS-inflamed guinea pigs daily with tegaserod enemas (1 mg/Kg) for 6 days beginning 24 h after TNBS instillation, and evaluated propulsive motility.

Consistent with previous findings [17 18 31 32] the distal colons of TNBS-inflamed animals exhibited a significant reduction in the rate of propulsive motility (Fig. 5A; $p<0.0001$), and an increase in trials in which fecal pellets became obstructed (Fig. 5B; $p<0.0004$). Tegaserod significantly improved the rate of propulsive motility in TNBS inflamed colons ($p<0.0001$), and eliminated the obstructions (Fig. 5A,B). Antagonist treatment blocked the protective effects of tegaserod on the rate of propulsive motility

($p<0.0001$), and on the occurrence of obstructions ($p<0.0001$). It is worth noting that preparations from animals receiving antagonist treatment were more frequently obstructed than vehicle-treated TNBS inflamed preparations ($p=0.0012$).

Since the disruption of propulsive motility in guinea pig TNBS colitis involves an attenuation of inhibitory junction potentials (IJPs), we measured IJP amplitudes in preparations from animals treated with tegaserod. Daily agonist treatment significantly improved the IJP in TNBS inflamed animals (naïve: -19.1 mV ± 1.3, TNBS with vehicle: -9.4 mV ± 0.9, TNBS with agonist: -18.1 mV ± 0.6; $p<0.0001$ by one-way ANOVA).

Colonic motility was also evaluated *in vivo* in mice in the 15 day recovery paradigm using the bead expulsion assay. As was detected in the TNBS inflamed guinea pig colon, mice with DSS colitis exhibited a slowing of colonic transit that was inhibited by tegaserod treatment (Fig. 5C). Furthermore, the effect of tegaserod (1mg/Kg) was blocked by the 5-HT_4 antagonist, GR113808 (1 mg/Kg).

Epithelial 5-HT_4 receptors play a protective physiological role in healthy animals

The findings described above indicate that 5-HT_4 receptor activation decreases the extent of colitis as it develops, and accelerates recovery from colitis once it has been established, raising the possibility that 5-HT_4 receptors could serve as a novel therapeutic target for the treatment of colitis. These results also suggest that 5-HT_4 receptors might play a role in maintaining the integrity of the epithelial layer under physiological conditions.

To test whether 5-HT_4 receptor activity influences epithelial integrity, normal mice were treated for 10 days with the 5-HT_4 antagonist, GR113808 (1 mg/Kg), administered by enema. Daily treatment of mice with the 5-HT_4 antagonist showed a

significant increase in the DAI ($p<0.0001$; Fig. 6A) and HDS ($p<0.0001$; Fig. 6B, Supplemental Fig. 4).

Consistent with the effect of pharmacologically inhibiting the receptor, 5-HT_4 knockout mice exhibited a significantly higher HDS than wild type littermates ($p<0.05$; Fig. 6C)

The results from colitis paradigms described above demonstrate that 5-HT_4 receptor stimulation by agonist administration increases epithelial proliferation, as measured by Ki-67 immunoreactive cells. Therefore, Ki-67 immunoreactivity was evaluated in normal animals treated with GR113808. In animals treated with the antagonist alone, there was a significant reduction in Ki-67 immunoreactivity compared to vehicle-treated controls (Fig. 7A; $p<0.0001$).

Evaluation of bacterial translocation from the gut to either the spleen or the liver has been shown to be an effective assay to assess barrier permeability.[22 33] Antagonist treatment in normal mice led to a significant increase in the proportion of cultures that were positive for bacterial translocation as compared to vehicle-treated animals (Fig. 7B; $p<0.02$).

To test whether endogenous 5-HT_4 receptor activation influences colonic function, propulsive motility was evaluated in distal colons from guinea pigs treated daily for 10 days with the antagonist alone. Treatment with the antagonist did not have a significant effect on the rate of propulsive motility; however, fecal pellet obstruction was observed in 25% of trials in colons from animals receiving antagonist treatment, which was significantly different from the control patterns (Fig. 7C; $p=0.0035$).

DISCUSSION

This study tested the hypothesis that epithelial 5-HT_4 receptor activation attenuates the development of colitis, and improves recovery from active colitis. Our findings support this hypothesis by demonstrating that epithelial 5-HT_4 receptor stimulation reduced disease activity and histological damage in both DSS and TNBS colitis, supporting an anti-inflammatory effect. The epithelial 5-HT_4 receptor stimulation can exert its protective effects through several mechanisms, including increased epithelial proliferation, enhanced epithelial cell migration, and resistance to oxidative stress-induced apoptosis. Furthermore, treatment with the 5-HT_4 agonist attenuated inflammation-induced changes in colonic motor function. Importantly, all of these effects were blocked by the 5-HT_4 antagonist, GR113808, and protection was not detected in 5-HT_4 KO mice. Our findings also indicate that epithelial 5-HT_4 receptors serve an important physiological role in maintaining mucosal integrity since inhibition of 5-HT_4 receptor activity in normal animals leads to inflammation and disrupted motor function. Collectively, these studies contribute new knowledge regarding the protective actions of 5-HT_4 receptor activation, and provide evidence for an anti-inflammatory role of 5-HT_4 in normal physiology.

Prior to the current investigation, it had been established that 5-HT can exert a pro-inflammatory influence in the GI tract.[34] For example, colitis is reduced in mice lacking or deficient in mucosal 5-HT,[35] and it is worsened in SERT knockout mice, which have elevated mucosal 5-HT availability. [36] This effect is likely mediated by activation of 5-HT_7 receptors on dendritic cells in the lamina propria.[4 5] These previous studies examined the global effect of gut-derived 5-HT on inflammation. The current

study specifically examined the role of 5-HT_4 receptor activation in the context of inflammation and we found an anti-inflammatory role of 5-HT signaling in the mucosa, supporting previous work from our labs suggested that activation of these receptors may be protective.[8] It will be interesting, in future studies, to directly compare the relative pro- and anti-inflammatory effects of mucosal 5-HT signaling. Regardless, during colitis, the protective actions of 5-HT_4 stimulation by endogenous 5-HT are dominated by an over-riding influence of pro-inflammatory mediators and mechanisms. On the other hand, this does not preclude the possibility that stimulation of the 5-HT_4 receptor pharmacologically could have a beneficial effect, as has been demonstrated in the current study.

In addition to these protective, anti-inflammatory actions of epithelial 5-HT_4 receptors, there is evidence that 5-HT_4 receptors play a beneficial, neurogenic effect in the muscularis. Activation of 5-HT_4 receptors in primary cultures of enteric neurons promotes neuronal growth and survival, and *in vivo*, agonist treatment promotes neurogenesis in adult mice.[3] This neuro-protective action has been supported by *in vivo* studies demonstrating that recovery of the recto-anal reflex is significantly augmented, through neurogenesis and axon outgrowth, by 5-HT_4 receptor treatment following rectal transection and anastomosis.[10 37 38] Furthermore, 5-HT_4 receptor-mediated enteric neurogenesis occurs in colitis,[39] a condition in which bioavailability of 5-HT is increased.[2] Taken together with the results reported here, it is becoming increasingly clear that the 5-HT_4 receptor exerts protective actions in the inner and outer layers of the gut.

Several mechanisms appear to contribute to the protective effects of epithelial 5-HT_4 receptor stimulation, and these mechanisms are apparently effective for both Th1- (TNBS) and Th2-predominant (DSS) inflammatory responses. One mechanism by which 5-HT_4 receptor stimulation is acting is through enhanced wound healing processes. 5-HT_4 receptor stimulation increased both cell proliferation and epithelial cell migration in a 5-HT_4 antagonist-sensitive manner. Epithelial erosions, ulcers and decreased epithelial barrier integrity are all common features of active colitis, and these conditions would likely be mitigated by enhanced epithelial proliferation and migration.

The anti-inflammatory effects of 5-HT_4 receptor activation may also involve resistance of the epithelium to the harmful effects of oxidative stress. Oxidative stress, and resultant epithelial apoptosis, is a key feature of inflammation and has been demonstrated in both DSS and TNBS colitis.[25] Treatment with a 5-HT_4 agonist protected CaCo-2 cells from apoptosis that was elicited by the free radical donor, H_2O_2, in a 5-HT_4 antagonist-sensitive manner.

Another unexplored mechanism that likely contributes to the protective effect of 5-HT_4 receptor stimulation is secretion of mucus from goblet cells. The mucus layer serves as a protective barrier, and disruption of this barrier with mucolytic agents or deletion of the mucin 2 gene results in colitis.[40 41] Goblet cells express the 5-HT_4 receptor, and 5-HT_4 receptor activation leads to degranulation.[8]

The guinea pig distal colon *ex vivo* model of motility is probably the most extensively characterized animal model of propulsive motility.[15 42 43] We have previously used this assay to investigate changes in motility that are associated with TNBS colitis,[18 44] and we have linked disruptions in motility to inflammation-induced neuroplasticity,

particularly intrinsic primary afferent neuron hyperexcitability[31] and attenuated purinergic neuromuscular transmission.[17] In the current study, treatment of TNBS-inflamed guinea pigs, and DSS-inflamed mice with the 5-HT_4 agonist improved propulsive motility and eliminated obstructive motility patterns. Consistent with our previous report linking disrupted motility to IJP attenuation,[17] the amplitude of the IJP is comparable to that of control animals following 5-HT_4 agonist treatment in TNBS colitis animals.

Data from studies reported here involving various models and paradigms of 5-HT_4 receptor stimulation in colitis indicate that the 5-HT_4 receptor plays a host defense role in inflammatory conditions through a number of actions that support the epithelial barrier and resistance to damage from oxidative stress. These novel findings, and the knowledge that 5-HT released from enterochromaffin cells reaches the lumen,[45 46] led to the question of whether 5-HT_4 receptor activity exerts a protective influence in the mucosal layer under physiological conditions. Treatment of normal mice with a 5-HT_4 receptor antagonist led to increased DAI and HDS scores, bacterial translocation, and reduction in cell proliferation. Furthermore, inhibition of 5-HT_4 receptors in the colonic epithelium of normal guinea pigs resulted in obstructed motility patterns, which is not a feature of the healthy colon. Also, antagonist treated mice exhibited slowed colonic transit. Consistent with these results, there were situations in our colitis paradigms in which antagonist treatment not only blocked the agonist, but led to a condition far worse than the vehicle treated inflamed group. This included the histological damage in the DSS mouse colitis prevention paradigm (Fig. 1) as well as the obstructed motility pattern in guinea pigs with TNBS colitis (Fig. 7). These findings suggest that endogenous 5-HT

may be acting on epithelial 5-HT_4 receptors to dampen physiological inflammation and maintain homeostasis.

It is possible that treatment with the 5-HT_4 antagonist mediates its effect by blocking stimulation of epithelial 5-HT_4 receptors by 5-HT released from enterochromaffin cells. Another possibility is that the antagonist that was used, GR113808, decreased constitutive activity of the epithelial 5-HT_4 receptors. Certain isoforms of the 5-HT_4 receptor have low levels of constitutive activity, which could lead to a steady state activation of the protective pathways stimulated by 5-HT_4 receptor activation with an agonist.[47-49] The antagonist that was used in the current studies, GR113808, can suppress this constitutive activity by acting as an inverse agonist.[50]

Conclusion

Here we report the discovery of a protective and healing action of epithelial 5-HT_4 receptor stimulation in the colon. Translation of these observations could provide a new and safe treatment strategy for the treatment of colitis, and that expand our appreciation of the roles of 5-HT receptor signaling in the GI tract. Treatment in two different models of colitis decreased the extent of inflammation as it was occurring, and accelerated the process of remission once colitis had been established. This beneficial effect likely involves several mechanisms that include enhanced wound healing, resistance to oxidative stress, and improved colonic motor function. Thus these findings demonstrate that luminally restricted, and therefore low risk, administration of 5-HT_4 agonists could be beneficial in the treatment of IBD. The discovery that 5-HT_4 receptors also contribute to the epithelial integrity in healthy animals reveals a newly identified role

for 5-HT signaling in the mucosal layer, and one that physiologically opposes the previously identified pro-inflammatory actions of 5-HT in the colon. Collectively, these findings advance our understanding of colonic physiology and pathophysiology, and provide a new target for the treatment of colonic inflammation.

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References

1. Berger M, Gray JA, Roth BL. The expanded biology of serotonin. Annu Rev Med 2009;**60**:355-66.
2. Mawe GM, Hoffman JM. Serotonin signalling in the gut--functions, dysfunctions and therapeutic targets. Nat Rev Gastroenterol Hepatol 2013;**10**:473-86.
3. Liu MT, Kuan YH, Wang J, et al. 5-HT4 receptor-mediated neuroprotection and neurogenesis in the enteric nervous system of adult mice. J Neurosci 2009;**29**:9683-99.
4. **Li N, Ghia JE**, Wang H, et al. Serotonin activates dendritic cell function in the context of gut inflammation. Am J Pathol 2011;**178**:662-71.
5. Kim JJ, Bridle BW, Ghia JE, et al. Targeted inhibition of serotonin type 7 (5-HT7) receptor function modulates immune responses and reduces the severity of intestinal inflammation. J Immunol 2013;**190**:4795-804.
6. Pan H, Galligan JJ. 5-HT1A and 5-HT4 receptors mediate inhibition and facilitation of fast synaptic transmission in enteric neurons. Am J Physiol 1994;**266**:G230-8.
7. Tonini M, Galligan JJ, North RA. Effects of cisapride on cholinergic neurotransmission and propulsive motility in the guinea pig ileum. Gastroenterology 1989;**96**:1257-64.
8. Hoffman JM, Tyler K, MacEachern SJ, et al. Activation of colonic mucosal 5-HT(4) receptors accelerates propulsive motility and inhibits visceral hypersensitivity. Gastroenterology 2012;**142**:844-54 e4.
9. Fujisawa M, Murata T, Hori M, et al. The 5-HT4 receptor agonist mosapride attenuates NSAID-induced gastric mucosal damage. J Gastroenterol 2010;**45**:179-86.
10. Takaki M, Goto K, Kawahara I. The 5-hydroxytryptamine 4 Receptor Agonist-induced Actions and Enteric Neurogenesis in the Gut. J Neurogastroenterol Motil 2014;**20**:17-30.
11. Cooper HS, Murthy SN, Shah RS, et al. Clinicopathologic study of dextran sulfate sodium experimental murine colitis. Lab Invest 1993;**69**(2):238-49.
12. Villanacci V, Antonelli E, Geboes K, et al. Histological healing in inflammatory bowel disease: a still unfulfilled promise. World J Gastroenterol 2013;**19**:968-78.
13. Savidge TC, Newman P, Pothoulakis C, et al. Enteric glia regulate intestinal barrier function and inflammation via release of S-nitrosoglutathione. Gastroenterology 2007;**132**:1344-58.
14. Joly F, Mayeur C, Messing B, et al. Morphological adaptation with preserved proliferation/transporter content in the colon of patients with short bowel syndrome. Am J Physiol Gastrointest Liver Physiol 2009;**297**:G116-23.
15. Hoffman JM, Brooks EM, Mawe GM. Gastrointestinal Motility Monitor (GIMM). J Vis Exp 2010(46).

16. Nasser Y, Fernandez E, Keenan CM, et al. Role of enteric glia in intestinal physiology: effects of the gliotoxin fluorocitrate on motor and secretory function. Am J Physiol Gastrointest Liver Physiol 2006;**291**:G912-27.
17. Roberts JA, Durnin L, Sharkey KA, et al. Oxidative stress disrupts purinergic neuromuscular transmission in the inflamed colon. J Physiol 2013;**591**:3725-37.
18. Strong DS, Cornbrooks CF, Roberts JA, et al. Purinergic neuromuscular transmission is selectively attenuated in ulcerated regions of inflamed guinea pig distal colon. J Physiol 2010;**588**:847-59.
19. Clement MV, Ponton A, Pervaiz S. Apoptosis induced by hydrogen peroxide is mediated by decreased superoxide anion concentration and reduction of intracellular milieu. FEBS letters 1998;**440**:13-8.
20. Vichai V, Kirtikara K. Sulforhodamine B colorimetric assay for cytotoxicity screening. Nat Protoc 2006;**1**:1112-6.
21. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 2012;**9**:671-5.
22. MacEachern SJ, Patel BA, Keenan CM, et al. Inhibiting Inducible Nitric Oxide Synthase in Enteric Glia Restores Electrogenic Ion Transport in Mice With Colitis. Gastroenterology 2015;**149**:445-55 e3.
23. Heath JP. Epithelial cell migration in the intestine. Cell Biol Int 1996;**20**(2):139-46.
24. Denker SP, Barber DL. Cell migration requires both ion translocation and cytoskeletal anchoring by the Na-H exchanger NHE1. J Cell Biol 2002;**159**:1087-96.
25. Shi XZ, Winston JH, Sarna SK. Differential immune and genetic responses in rat models of Crohn's colitis and ulcerative colitis. Am J Physiol Gastrointest Liver Physiol 2011;**300**:G41-51.
26. Novak EA, Mollen KP. Mitochondrial dysfunction in inflammatory bowel disease. Front Cell Dev Biol 2015;**3**:62.
27. Becker C, Watson AJ, Neurath MF. Complex roles of caspases in the pathogenesis of inflammatory bowel disease. Gastroenterology 2013;**144**:283-93.
28. Reddy SN, Bazzocchi G, Chan S, et al. Colonic motility and transit in health and ulcerative colitis. Gastroenterology 1991;**101**:1289-97.
29. Brierley SM, Linden DR. Neuroplasticity and dysfunction after gastrointestinal inflammation. Nat Rev Gastroenterol Hepatol 2014;**11**:611-27.
30. Linden DR, Sharkey KA, Ho W, et al. Cyclooxygenase-2 contributes to dysmotility and enhanced excitability of myenteric AH neurones in the inflamed guinea pig distal colon. J Physiol 2004;**557**:191-205.
31. Hoffman JM, McKnight ND, Sharkey KA, et al. The relationship between inflammation-induced neuronal excitability and disrupted motor activity in the guinea pig distal colon. Neurogastroenterol Motil 2011;**23**:673-e279.
32. Linden DR, Sharkey KA, Mawe GM. Enhanced excitability of myenteric AH neurones in the inflamed guinea-pig distal colon. J Physiol 2003;**547**:589-601.

33. Deitch EA, Kemper AC, Specian RD, et al. A study of the relationship among survival, gut-origin sepsis, and bacterial translocation in a model of systemic inflammation. J Trauma 1992;**32**:141-7.
34. Gershon MD. Serotonin is a sword and a shield of the bowel: serotonin plays offense and defense. Trans Am Clin Climatol Assoc 2012;**123**:268-80; discussion 80.
35. Ghia JE, Li N, Wang H, et al. Serotonin has a key role in pathogenesis of experimental colitis. Gastroenterology 2009;**137**:1649-60.
36. Bischoff SC, Mailer R, Pabst O, et al. Role of serotonin in intestinal inflammation: knockout of serotonin reuptake transporter exacerbates 2,4,6-trinitrobenzene sulfonic acid colitis in mice. Am J Physiol Gastrointest Liver Physiol 2009;**296**:G685-95.
37. Matsuyoshi H, Kuniyasu H, Okumura M, et al. A 5-HT(4)-receptor activation-induced neural plasticity enhances in vivo reconstructs of enteric nerve circuit insult. Neurogastroenterol Motil 2010;**22**:806-13, e226.
38. Kawahara I, Kuniyasu H, Matsuyoshi H, et al. Comparison of effects of a selective 5-HT reuptake inhibitor versus a 5-HT4 receptor agonist on in vivo neurogenesis at the rectal anastomosis in rats. Am J Physiol Gastrointest Liver Physiol 2012;**302**:G588-97.
39. Belkind-Gerson J, Hotta R, Nagy N, et al. Colitis induces enteric neurogenesis through a 5-HT4-dependent mechanism. Inflamm Bowel Dis 2015;**21**:870-8.
40. Van der Sluis M, De Koning BA, De Bruijn AC, et al. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology 2006;**131**:117-29.
41. Johansson ME, Gustafsson JK, Holmen-Larsson J, et al. Bacteria penetrate the normally impenetrable inner colon mucus layer in both murine colitis models and patients with ulcerative colitis. Gut 2014;**63**:281-91.
42. Costa M, Furness JB. The peristaltic reflex: an analysis of the nerve pathways and their pharmacology. Naunyn Schmiedebergs Arch Pharmacol 1976;**294**:47-60.
43. D'Antona G, Hennig GW, Costa M, et al. Analysis of motor patterns in the isolated guinea-pig large intestine by spatio-temporal maps. Neurogastroenterol Motil 2001;**13**:483-92.
44. Linden DR, Chen JX, Gershon MD, et al. Serotonin availability is increased in mucosa of guinea pigs with TNBS-induced colitis. Am J Physiol Gastrointest Liver Physiol 2003;**285**:G207-16.
45. Bertrand PP. Real-time detection of serotonin release from enterochromaffin cells of the guinea-pig ileum. Neurogastroenterol Motil 2004;**16**:511-4.
46. Patel BA, Bian X, Quaiserova-Mocko V, et al. In vitro continuous amperometric monitoring of 5-hydroxytryptamine release from enterochromaffin cells of the guinea pig ileum. Analyst 2007;**132**:41-7.
47. Claeysen S, Sebben M, Becamel C, et al. Constitutively active mutants of 5-HT4 receptors are they in unique active states? EMBO Rep 2001;**2**:61-7.

48. Blondel O, Gastineau M, Langlois M, et al. The 5-HT4 receptor antagonist ML10375 inhibits the constitutive activity of human 5-HT4(c) receptor. Br J Pharmacol 1998;**125**:595-7.
49. Claeysen S, Sebben M, Becamel C, et al. Pharmacological properties of 5-Hydroxytryptamine(4) receptor antagonists on constitutively active wild-type and mutated receptors. Mol Pharmacol 2000;**58**:136-44.
50. Kamel R, Eftekhari P, Garcia S, et al. A high-affinity monoclonal antibody with functional activity against the 5-hydroxytryptaminergic (5-HT4) receptor. Biochem Pharmacol 2005;**70**:1009-18.

Author names in bold designate shared co-first authors.

Figure Legends

Figure 1. Daily intraluminal treatment with the 5-HT_4 agonist, tegaserod (1mg/Kg) reduced the extent of DSS and TNBS colitis. A. In DSS colitis, tegaserod caused an antagonist-sensitive reduction in the DAI and HDS (vehicle, n=9; agonist, n=10; agonist/antagonist, n=5; for HDS, 2 values were obtained from each animal). B. In TNBS colitis, agonist treatment significantly reduced the DAI, but did not affect the HDS (n=7 animals for all groups, with 2 values per animal for HDS). C. Tegaserod failed to improve DAI or HDS in DSS inflamed 5-HT_4 knockout mice (n=8-9 animals per group, with 2 values per animal for HDS). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 by one-way ANOVA. †p<0.05 by one-way ANOVA with square root transformation.

Figure 2. Daily intraluminal treatment with the 5-HT_4 agonist, tegaserod (1mg/Kg) beginning on day 6, after colitis had been established, accelerated recovery from DSS and TNBS colitis. A. In DSS colitis, the 5-HT_4 receptor agonist significantly improved the DAI by day 12, and at the termination of the experiment on day 15, both the DAI and HDS were significantly improved as compared to the vehicle control and antagonist plus agonist treatment groups. (vehicle, n=21; agonist, n=23; antagonist, n=5 with 2 values per animal obtained for HDS). C. In TNBS colitis, significant improvement in the DAI was detected on Day 9, and at the day 15 time point, both the DAI and HDS were significantly improved (vehicle, n=13; agonist, n=14; antagonist, n=5 with 2 values per animal obtained for HDS). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 by two-way

ANOVA for DAI and one-way ANOVA for HDS. In the DAI graphs, comparisons were made between agonist treatment and the other groups.

Figure 3. Intraluminal treatment with tegaserod increased epithelial proliferation. Graph demonstrating the proportion of crypt epithelial cells immunoreactive for the proliferation marker, Ki-67 (vehicle, n=10; agonist, n=9; antagonist, n=9). * $p<0.05$, **$p<0.01$ by one-way ANOVA.

Figure 4. Treatment of Caco-2 cells with tegaserod increased the rate of cell migration, and caused protection from cell loss due to oxidative stress. A. Closure of scratches in Caco-2 monolayer cultures after 48 h (1 well per treatment group, 3 scratches per well x 3 experiments). B. Survival data from cultures that were treated with normal medium (control) or 200 µM H_2O_2 (n=4 per group). C. Photomicrographs of Caco-2 cultures showing scratches at the 0 and 48 h time points. ** $p<0.05$, **** $p<0.0001$; one-way ANOVA.

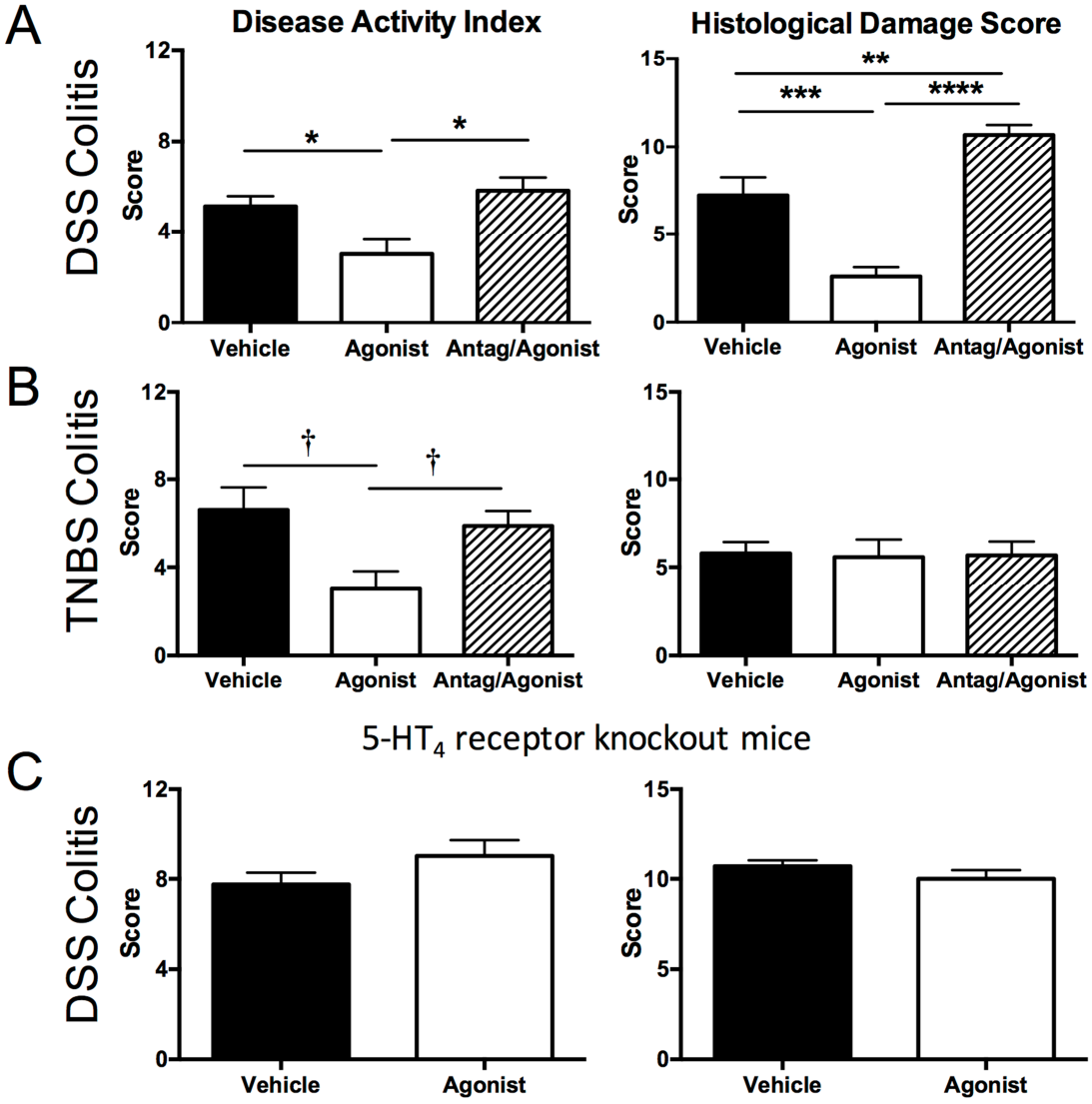
Figure 5. Daily intraluminal treatment with tegaserod (1 mg/Kg) improved distal colon propulsive motility in guinea pigs with TNBS colitis. A. Graph illustrating rate of pellet propulsion along the length of the colon (control, n=6; TNBS/vehicle, n=9; TNBS/agonist, n=6; TNBS/agonist plus antagonist, n=4; ****$p<0.0001$, one-way ANOVA). B. Graph showing the proportion of trials in which the fecal pellet cleared the colon or was obstructed, and did not clear the colon within 5 min (5-6 trials per animal; ***$p<0.001$, ****$p<0.0001$, Fisher's Exact Test). C. Graphs demonstrating results of

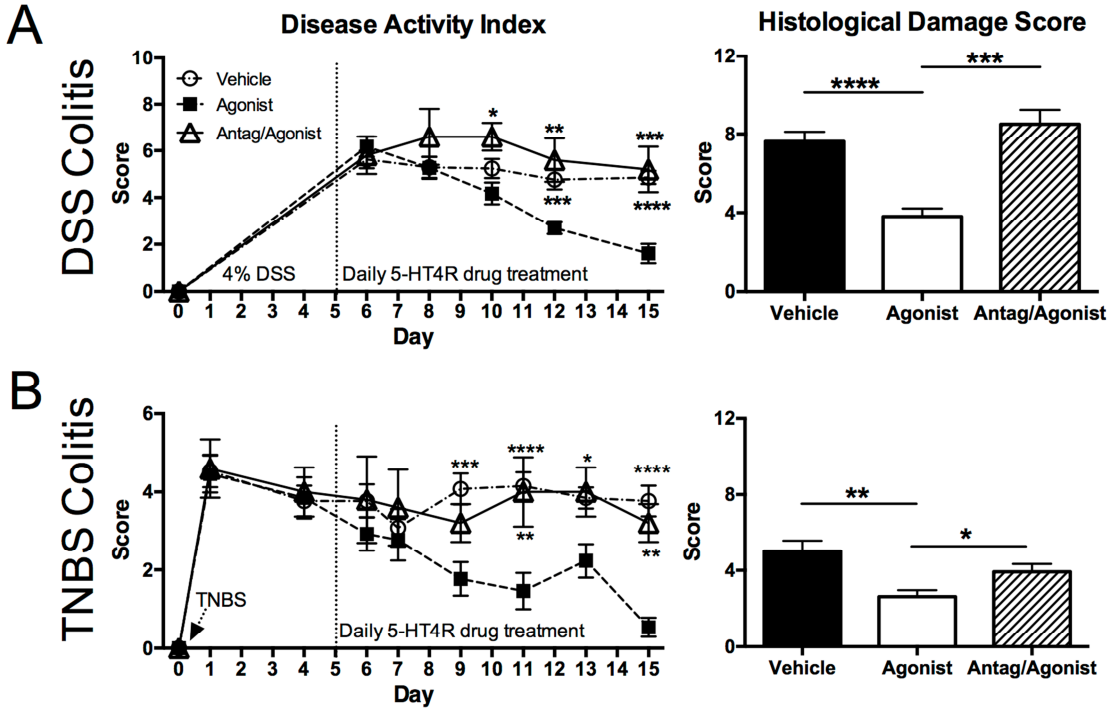
bead expulsion assays from mice with DSS colitis in the 15 day recovery paradigm that were treated *in vivo* by enema (DSS/vehicle, n=5; DSS/agonist, n=4; DSS/agonist plus antagonist, n=5; *p<0.05, paired t-test).

Figure 6. Pharmacological or molecular disruption of 5-HT4 signaling increased inflammatory scores in normal mice. Intraluminal administration of the 5-HT_4 antagonist GR113808 (1 mg/Kg) induced colitis in mice. Antagonist treatment resulted in a significant increase in the DAI that was detected as early as day 3 (A), and an increase in the HDS (B). n=10/group. **p<0.01; ****p<0.0001 by two-way ANOVA; ††††p<0.0001 by t-test with Welch's correction. C. The HDS was significantly higher in 5-HT_4 knockout mice, as compared to wild type littermates (*p<0.05 by t-test; WT, n=4; 5-HT_4 KO, n=8).

Figure 7. Daily intraluminal administration of the 5-HT_4 antagonist GR113808 (1 mg/Kg) disrupted barrier function in normal mice, and motility in guinea pigs, as well as motility in mice. A. The proportion of crypt epithelial cells that were immunoreactive for the proliferation marker, Ki-67 was significantly decreased in animals treated with the antagonist (vehicle, n=8; antagonist, n=10; ****p<0.001 by t-test). B. Proportion of mice in which bacteria were detected in the liver or spleen following 10 days of vehicle or antagonist treatment. Vehicle, n=5; antagonist, n=3; ****p<0.0001, Fisher's Exact Test. C. Proportion of trials in which pellet propulsion was obstructed for at least 5 min. (vehicle, n=31 trials from 4 colons; antagonist, n=30 trials from 5 colons; ****p<0.0001, Fisher's Exact Test). D. Time to bead expulsion at the onset and following daily

antagonist treatment in normal CD-1 mice. Data for each mouse were normalized to data collected at onset of treatment (*$p<0.05$, paired t-test; $n=5$).


A
Disease Activity Index
Histological Damage Score
DSS Colitis
Score
12
8
4
0
15
10
5
*
*
**
***
****
Vehicle
Agonist
Antag/Agonist
B
TNBS Colitis
†
†
5-HT4 receptor knockout mice
C
DSS Colitis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DSS Colitis
Disease Activity Index
Histological Damage Score
Vehicle
Agonist
Antag/Agonist
Score
Day
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Vehicle
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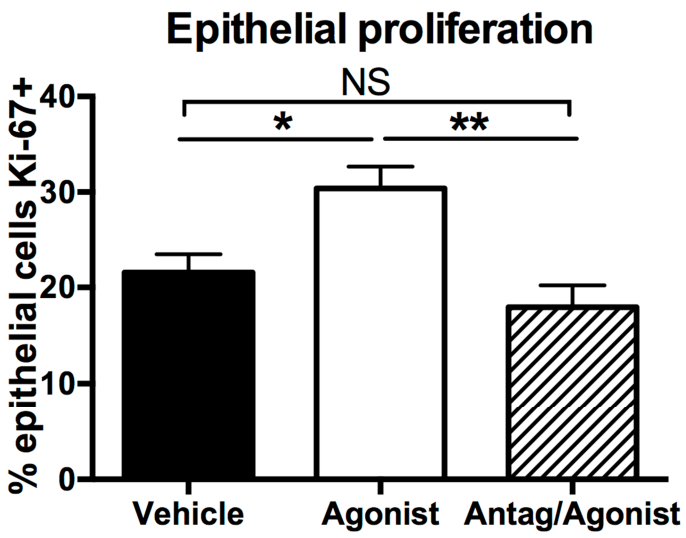
Antag/Agonist
B
TNBS Colitis
TNBS
Daily 5-HT4R drug treatment


Epithelial proliferation
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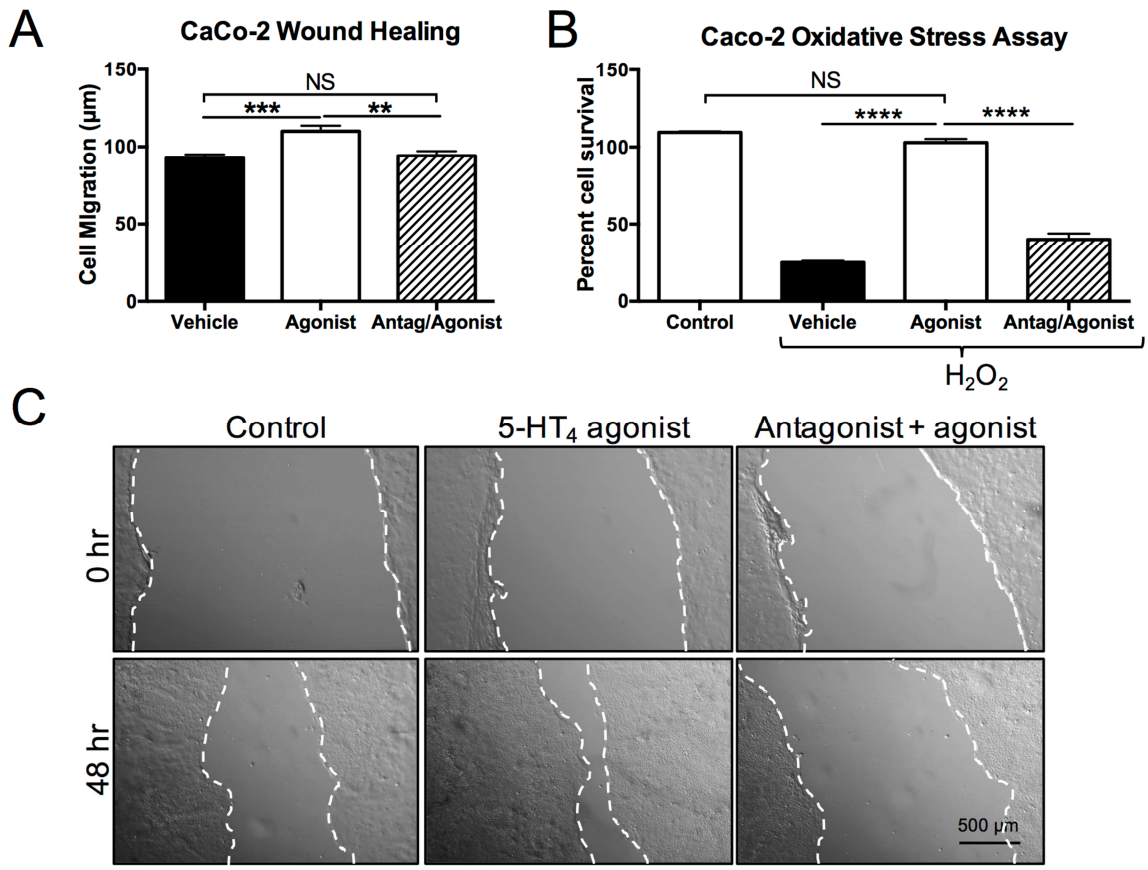
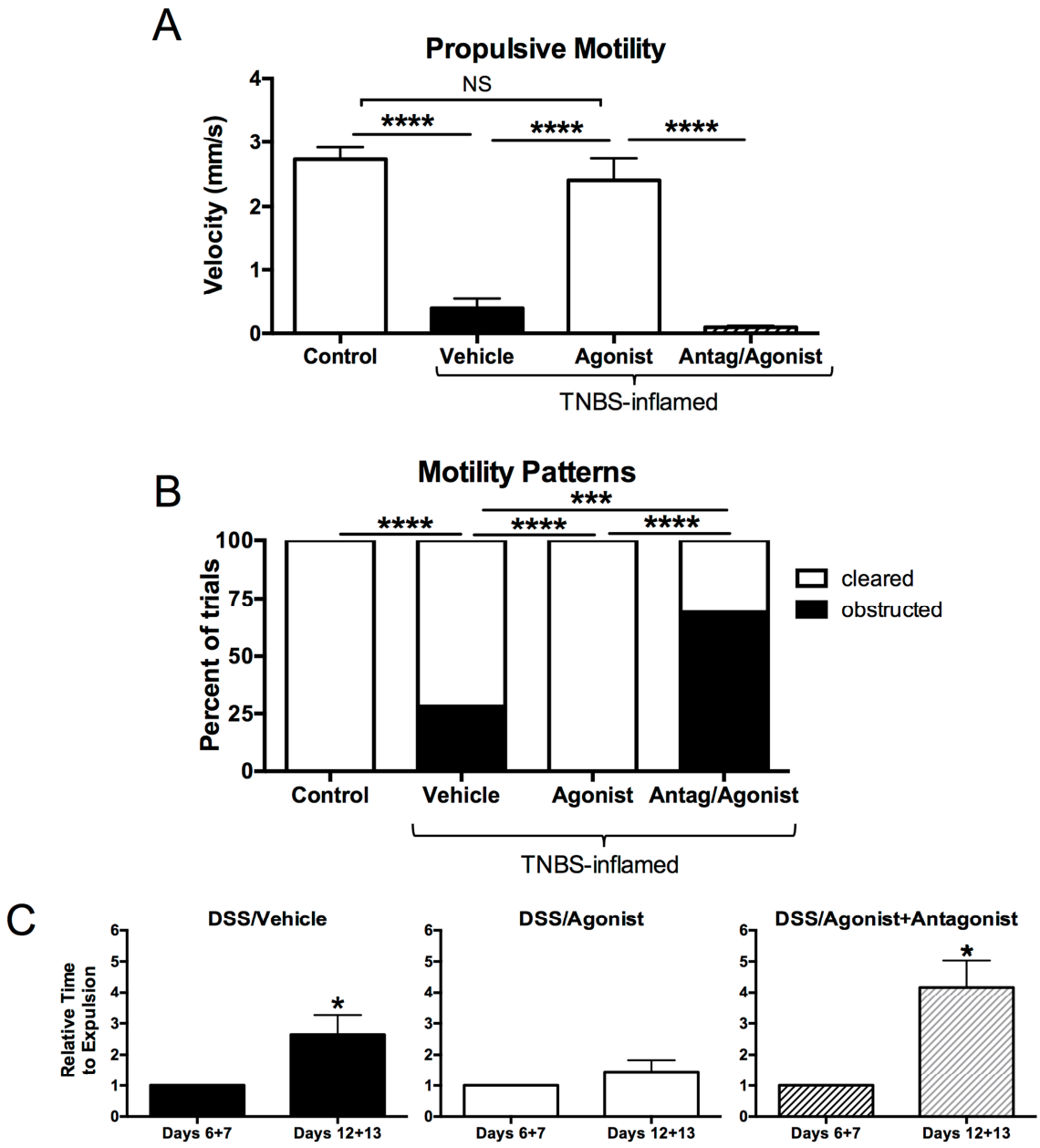
*
**
% epithelial cells Ki-67+
40
30
20
10
0
Vehicle
Agonist
Antag/Agonist


A
CaCo-2 Wound Healing
Cell Migration (µm)
150
100
50
0
NS
***
**
Vehicle
Agonist
Antag/Agonist
B
Caco-2 Oxidative Stress Assay
Percent cell survival
150
100
50
0
NS
****
****
Control
Vehicle
Agonist
Antag/Agonist
H2O2
C
Control
5-HT4 agonist
Antagonist + agonist
0 hr
48 hr
500 µm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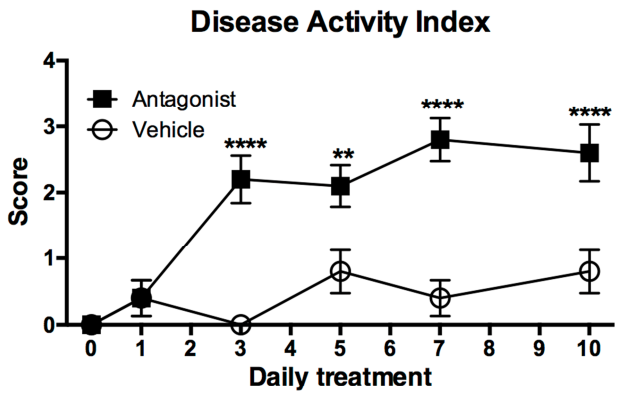
Propulsive Motility
Velocity (mm/s)
NS
****
****
****
Control
Vehicle
Agonist
Antag/Agonist
TNBS-inflamed
B
Motility Patterns
***
****
****
****
Percent of trials
cleared
obstructed
Control
Vehicle
Agonist
Antag/Agonist
TNBS-inflamed
C
DSS/Vehicle
DSS/Agonist
DSS/Agonist+Antagonist
Relative Time to Expulsion
Days 6+7
Days 12+13

A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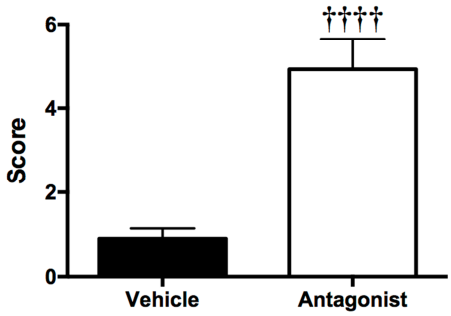
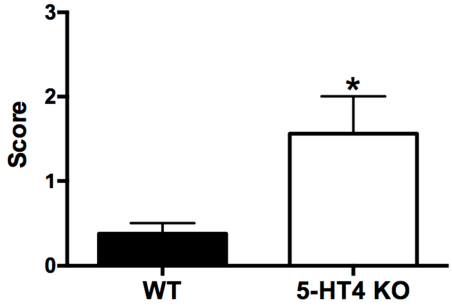
Antagonist
Vehicle
Score
Daily treatment
****
**
****
****

B


Histological Damage Score
Score
Vehicle
Antagonist
††††

C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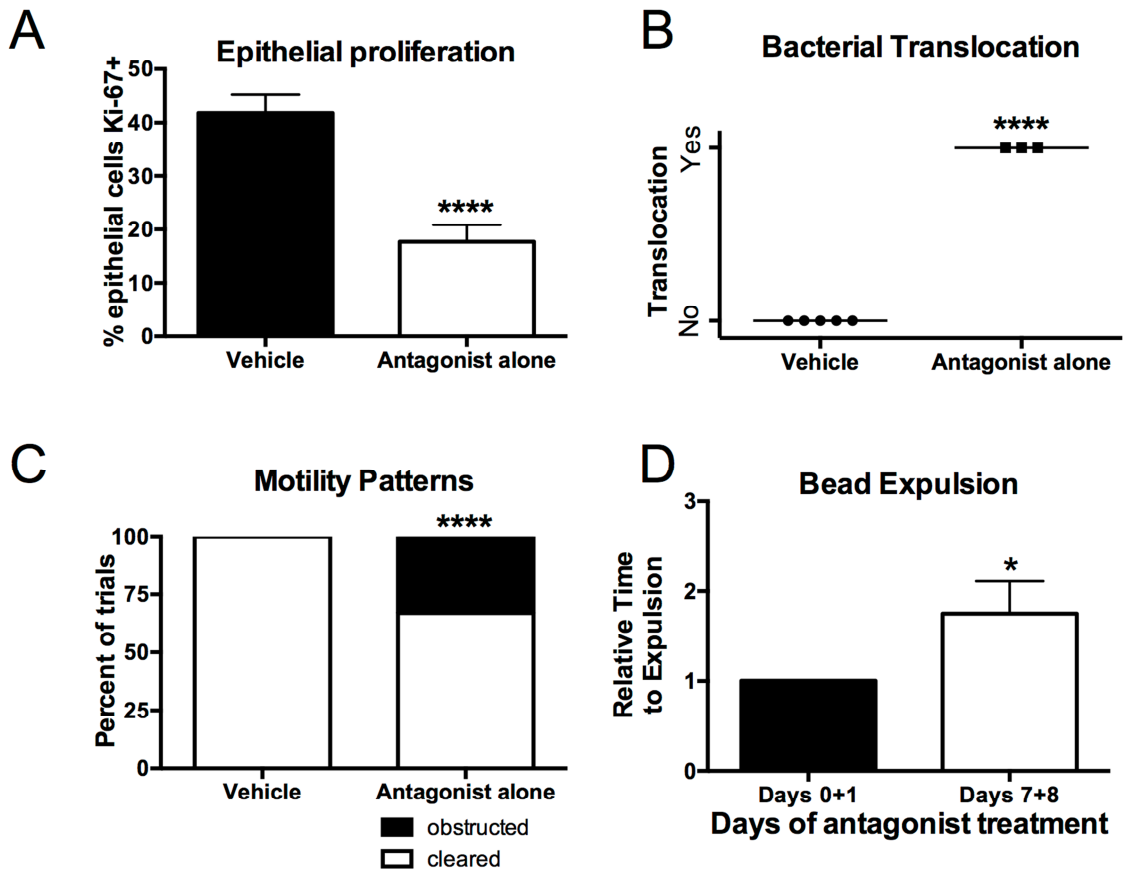
WT vs 5HT4 KO
Score
WT
5-HT4 KO
*

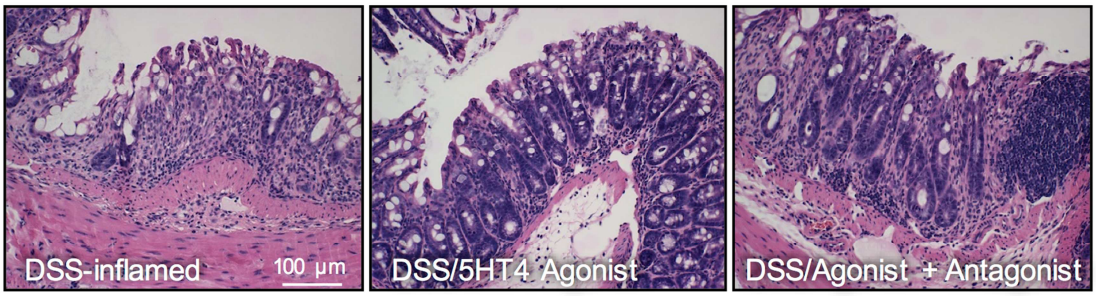

A
Epithelial proliferation
% epithelial cells Ki-67+
50
40
30
20
10
0
****
Vehicle
Antagonist alone
B
Bacterial Translocation
Translocation
Yes
No
****
Vehicle
Antagonist alone
C
Motility Patterns
Percent of trials
100
75
50
25
0
****
Vehicle
Antagonist alone
obstructed
cleared
D
Bead Expulsion
Relative Time to Expulsion
3
2
1
0
*
Days 0+1
Days 7+8
Days of antagonist treatment

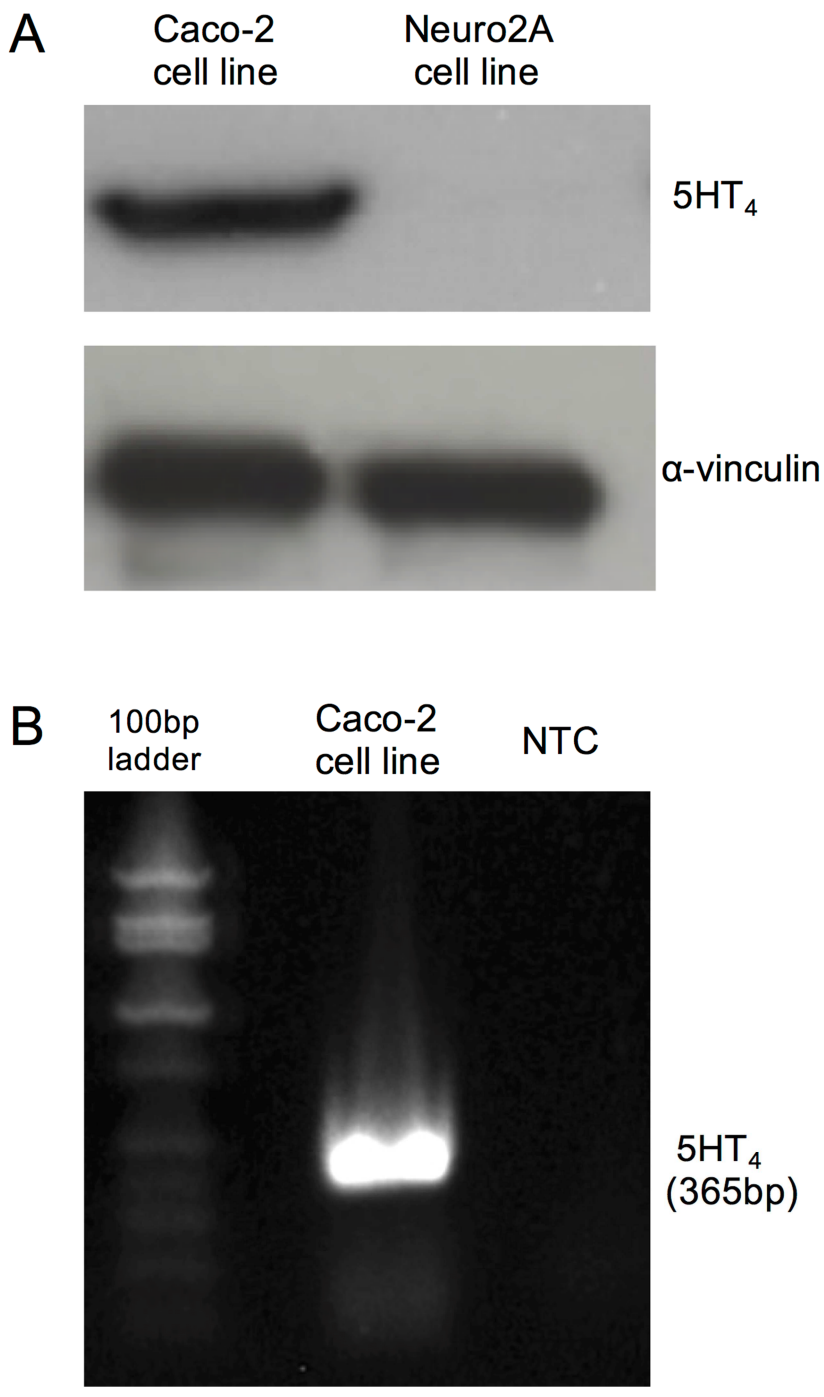
Supplemental figure 1. Daily intraluminal treatment with the 5-HT_4 agonist, tegaserod (1mg/Kg) reduced the extent of DSS colitis, and this protective effect was inhibited by antagonist treatment. Micrographs illustrating typical histological features in colons from the three treatment groups.

Supplemental Figure 2. Evidence for expression of the 5-HT_4 receptor by Caco-2 cells. A. Western blot analysis of $5HT_4$ expression in human CaCo-2 cell line (lane 1) using a human specific $5HT_4$ antibody (1:200, Abcam, Cambridge, UK); the murine Neuro2A neuroblastoma cell line (lane 2), which does not express 5-HT_4, was used as negative control. An internal control for loading was performed using an anti-vinculin antibody (1:50000, Sigma-Aldrich, Saint Louis, MO, USA). B. A *$5HT_4$* transcript is detectable by RT-PCR in RNA extracted from Caco-2 cells supporting the presence of this receptor in this cell line. A low diffuse primer-dimer band was present in both Caco-2 cells and no template control (NTC) lanes (l00bp DNA ladder). Total RNA was isolated from Caco-2 using the RNAeasy Kit (Qiagen) according to the manufacturer's specifications and cDNA was generated from 2 ug of RNA using M-MLV Reverse Transcriptase (Promega) . 5HT4R expression was determined using SYBR GreenER qPCR SuperMix (Invitrogen) with the following primers: forward 5'-GGCCTTCTACATCCCATTTCTCCT-3'; reverse: 5'- CTTCGGTAGCGCTCATCATCACA-3'. The predicted product size is 365 bp.

Supplemental Figure 3. Intraluminal treatment with tegaserod increased epithelial proliferation. Representative photomicrographs demonstrating Ki67 immunoreactivity (red) and DAPI counterstaining (green).

Supplemental figure 4. Intraluminal administration of the 5-HT4 antagonist GR113808 (1 mg/Kg) induced colitis in mice. Micrographs illustrating typical histological features in colons from mice treated with a vehicle or with the antagonist. Indications of inflammation in the antagonist-treated micrograph include signs of edema (*), cellular infiltration (arrows), and increased spacing between the base of the crypts and the muscularis mucosa.


DSS-inflamed
100 µm
DSS/5HT4 Agonist
DSS/Agonist + Antagonist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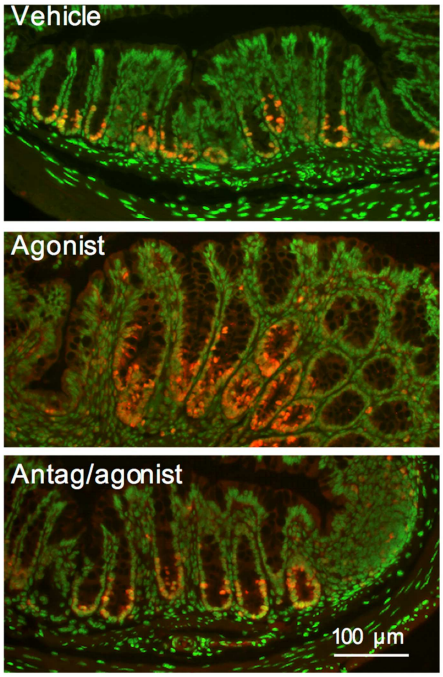
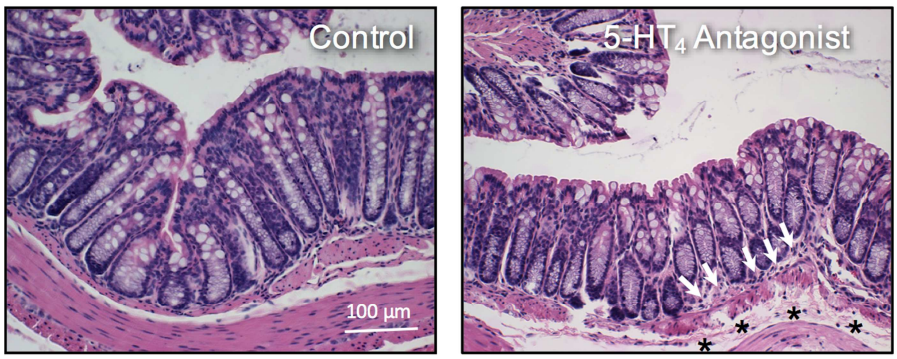
Caco-2
cell line
Neuro2A
cell line
5HT4
α-vinculin
B
100bp
ladder
Caco-2
cell line
NTC
5HT4
(365bp)


Vehicle
Agonist
Antag/agonist
100 µm


Control
100 µm
5-HT4 Antagonist