Role of serotonin in intestinal inflammation: knockout of serotonin reuptake transporter exacerbates 2,4,6-trinitrobenzene sulfonic acid colitis in mice

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Bischoff SC, Mailer R, Pabst O, Weier G, Sedlik W, Li Z, Chen JJ, Murphy DL, Gershon MD. 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 296: G685–G695, 2009. First published December 18, 2008; doi:10.1152/ajpgi.90685.2008.—Serotonin (5-HT) regulates peristaltic and secretory reflexes in the gut. The serotonin reuptake transporter (SERT; SLC6A4), which inactivates 5-HT, is expressed in the intestinal mucosa and the enteric nervous system. Stool water content is increased and colonic motility is irregular in mice with a targeted deletion of SERT. We tested the hypotheses that 5-HT plays a role in regulating intestinal inflammation and that the potentiation of serotonergic signaling that results from SERT deletion is proinflammatory. Rectal installation of 2,4,6-trinitrobenzene sulfonic acid (TNBS) was used to induce an immune-mediated colitis, which was compared in SERT knockout mice and littermate controls. Intestinal myeloperoxidase and histamine levels were significantly increased, whereas the survival rate and state of health were significantly decreased in TNBS-treated mice that lacked SERT. Deletion of SERT thus increases the severity of TNBS colitis. These data suggest that 5-HT and its SERT-mediated termination play roles in intestinal immune/inflammatory responses in mice. Neutrophils; motility; inflammatory bowel disease; epithelial cells

Enterochromaffin (EC) cells transduce mucosal physical and chemical sensory signals from the gut lumen. These cells are located in the intestinal mucosa and release serotonin (5-HT) in response to chemical stimuli and increases in intraluminal pressure (37, 49). 5-HT is a major gastrointestinal (GI) paracrine hormone and an enteric neurotransmitter. 5-HT from EC cells is involved in the initiation of peristaltic and secretory reflexes whereas neuronal 5-HT participates in reflex propagation (21). Following its release from EC cells, 5-HT activates a variety of receptors. 5-HT3 receptors stimulate extrinsic (23) and intrinsic primary afferent neurons (IPANs) (4). The activated extrinsic primary afferents transmit nociceptive signals to the central nervous system (23) whereas myenteric IPANs initiate giant migrations of the bowel (50). 5-HT1P receptors activate submucosal IPANs and 5-HT4 receptors enhance the release of ACh and CGRP from the terminals; stimulated submucosal IPANs initiate peristaltic and secretory reflexes (22). The serotonin reuptake transporter (SERT; SLC6A4) modulates the strength of serotonergic signaling by transmembrane transport of 5-HT, which removes 5-HT from its receptors and subjects 5-HT to catabolism by monoamine oxidase and other enzymes, all of which are intracellular (21).

The SERT molecule is a member of the Na+/Cl− neurotransmitter transporter family and is expressed by epithelial cells and neurons in the gut (12, 25). Prior studies of transgenic mice that lack SERT [SERT knockout (KO)] mice have shown that despite an absence of enteric transcripts encoding SERT, the 5-HT content of the intestinal mucosa in SERT KO mice is not significantly different from that of their wild-type (WT) littermates (12). The deletion of SERT, however, leads to compensatory changes in the bowel. These changes include a fourfold decrease in the expression of the 5-HT3B receptor subunit. This decrease changes the 5-HT3A-to-5-HT3B ratio and has the effect of reducing the sensitivity of 5-HT3 receptors while increasing the rapidity of their desensitization (36). These alterations protect the SERT KO bowel from overstimulation of 5-HT3 receptors by the increased availability of extracellular 5-HT. The high-affinity SERT-mediated reuptake of 5-HT in the gut is also partially replaced by nonscavenger transport mediated by the dopamine transporter and polyspecific organic cation transporters, OCT-1 and OCT-3, respectively, which have a lower affinity for 5-HT than SERT but a high capacity (12). These compensatory mechanisms probably explain why SERT KO mice do not manifest severe alterations of development or behavior; moreover, the defects in gut function that SERT KO mice exhibit are relatively minor. These defects include an increase in stool water content and an abnormal pattern of colonic motility in which excessively rapid or slow transit alternate (12). Experiments in which SERT was inhibited with paroxetine have confirmed these observations and indicated that SERT inhibition also increases the sensitivity of colonic nociception (13).

In animals in which GI inflammation is induced by rectal installation of 2,4,6-trinitrobenzene sulfonic acid (TNBS), the numbers of EC cells and tissue levels of 5-HT have been reported to increase and transcription of SERT to decrease (34, 35, 44). TNBS treatment of mice initially causes an acute toxic colitis (for 1–2 days) as a result of epithelial damage by the ethanol-containing TNBS solution. This reaction is followed by a hyperinflammatory reaction to TNBS as a hapten. The levels of intestinal IFN-γ and IL-12, but also of TNF-α, IL-1β, IL-6,
PGE$_2$, and leukotriene B$_4$ are all increased during TNBS colitis, suggesting a Th1-type inflammation (17, 41). Interestingly, IFN-γ and TNF-α, the two major cytokines in acute inflammatory bowel disease (IBD), decrease SERT expression in vitro in human epithelial Caco-2-cells (18). Data are thus consistent with the ideas that loss of SERT transcription can either cause intestinal inflammation or be a result of it.

Changes similar to those seen in mice with TNBS-induced colitis have been reported in other animal models of inflammation and have also been seen in humans with irritable bowel syndrome (IBS) or IBD. SERT transcription, for example, is reduced in a mouse model of postinfectious bowel dysfunction (57) and in a mouse model of enteric infection (45). Although SERT transcription has been reported to be reduced in some patients with IBS and in those with ulcerative colitis (14), the IBS-related change in SERT expression was not found in another set of subjects with IBS (9). This apparent discrepancy suggests that the pathogenesis of IBS, which is defined only as a symptom complex, may differ in different sets of subjects. Although SERT promoter polymorphisms have been described in some patients with IBS (10, 60), a meta-analysis suggests that SERT polymorphisms are not associated with IBS (55). Recently, manometric studies have revealed that pharmacological SERT inhibition in healthy human subjects increases colonic contractility and compliance while suppressing the tonic response of the colon to a meal (53).

The present study was designed to test the hypotheses that serotonergic signaling and its termination by SERT play important roles in intestinal inflammation. The study plan was based on the idea that the differences between SERT KO mice and their WT littermates, which under normal conditions are relatively minor, might become severe when animals are subjected to the stress of intestinal inflammation. If 5-HT secretion from EC cells were to increase as a result of inflammation, then amplification of responses to released 5-HT due to impaired 5-HT inactivation in SERT KO mice would be predicted to intensify the damage due to inflammation within the gut and systemic reactions, such as anaphylaxis, might even be anticipated.

MATERIALS AND METHODS

Animals. All experiments were carried out with C57BL/6J mice derived from animals generated in the Laboratory of Clinical Science at the National Institute of Mental Health, Bethesda, MD. SERT KO mice lacking both SERT genes were produced by homologous recombination of the second exon of the SERT gene with a phosphoglycerate kinase-neo gene cassette preventing functional SERT protein expression (3). Control animals consisted of homozygous (wild-type; SERT WT) littermates of the SERT KO mice. At 14–18 wk of age, the animals were transferred to Columbia University, where experiments were carried out. Genotypes were confirmed by using a reverse transcription polymerase chain reaction (RT-PCR) protocol as described elsewhere (12). The animal care committee of Columbia University (IACUC: protocol AAAA1043) approved all experiments. Animals were housed under standard conditions (12:12-h light-dark cycle; water and food ad libitum, 23–24°C).

TNBS colitis model. Rectal administration of TNBS was used, according to a standardized protocol, to evoke colitis in two groups of adult animals, which differed in age and in whether prior epidermal sensitization was used (Fig. 1). The protocol was adapted from a technique originally employed to induce colitis in rats (40) and modified successfully for use in mice (39). The TNBS stock solution (1 M, dissolved in water, Research Organics, Fluka, Sigma) was diluted in a mixture of 30% vol/vol ethanol and 70% vol/vol PBS; the final concentrations of TNBS were 150 mg/ml for epidermal administration of 2,4,6-trinitrobenzene sulfonic acid (TNBS), followed by a rectal challenge 7 days later with TNBS or ethanol control buffer. Old mice (>40 wk old, set 2) were challenged rectally with TNBS without previous sensitization. All animals were killed 6 days (±1 day) after rectal challenge. Mice were analyzed at the onset of the protocol (day −7 or day 0) and at its end (day 6 ± 1 day). A global assessment of the animals’ condition was carried out at days −7, −3, 0, 3, and 6. Set 2: 2 sets of animals (old age, young adults) were examined. Mice were divided into 4 groups according to their genotype [serotonin reuptake transporter (SERT) knockout (KO) or wild type (WT)] and the type of rectal challenge (TNBS or ethanol buffer control). Spontaneous death rates of old and young mice did not differ significantly ($\chi^2$ test, $P > 0.05$).
2 is a mouse with a dull coat; 3 adds a curved back or rectal prolapse; 4 adds reduced movement; 5 adds severe illness without body movement, necessitating that animals be euthanized immediately; 6 is applied when mice die spontaneously. Scoring was carried out at the onset of the protocol (day −7 if skin sensitization was performed, day 0 if not) and repeated at least every 3–4 days. Body weight, stool water content, and colon motility were also measured at the beginning of the experiments (day −7 with skin sensitization, day 0 without) and prior to euthanizing the animals (day 6 ± 1 day). Stool water content was calculated by using the formula 1 − (dry weight/wet weight) and expressed in percent. Wet weight of stool pellets was measured immediately after their collection; dry weight was measured after overnight incubation of the same pellets in an oven at 60°C. To estimate colonic motility, the time was measured that was required to expel a glass bead (3 mm in diameter) pushed into the rectum with a polished glass rod to a distance of 2 cm (12, 47).

Scoring of inflammation. After euthanasia of the animals, the whole colon was removed for macroscopic, microscopic, and biochemical analysis of inflammation. For macroscopic scoring of colonic samples (34), the severity of adhesions, presence of diarrhea, thickness of the bowel wall, and severity of mucosal damage were quantified. These parameters were summarized and the specimens were categorized as 0 (no visible inflammation), 1 (hyperemia without ulcerations), and 2 (hyperemia with ulcerations plus stenosis). Additionally, the length and the weight-length relation of the colon (a measure of colon thickness) were recorded.

Histological and immunocytochemical analyses of the colon were used to complete the evaluation of inflammation. For this purpose, the whole colon (N = 8 mice) or two sets (paraffin and frozen samples) of segments of proximal, intermediate, and distal colon, (N = 32 mice) were processed. The segments of colon were fixed for 2 h with 4% buffered formaldehyde (from paraformaldehyde), washed, and embedded in paraffin (for histology) or frozen TissueTek (for immunocytochemistry). Sections were cut from paraffin blocks at 4 µm; frozen sections were cut at 10 µm. Whole colon was washed, opened along the mesenteric border, and prepared as spiral "Swiss rolls"; the tissue strips were rolled with the proximal end located at the centre of the roll and the luminal side facing outward (47). The rolls were sectioned in a Cryostat-microtome at 10 µm, air-dried, and fixed for 10 min in ice-cold acetone. A graded scale was applied to hematoxylin and eosin (H&E)-stained sections to assess histological changes (6). Parameters rated included the severity of mucosal destruction (0, normal; 1, mild; 2, moderate; 3, extensive), the extent of cellular infiltration (0, absent; 1, mild; 2, pronounced), the thickening of the muscularis externa (0, absent; 1, mild; 2, moderate; 3, extensive), the presence or absence of crypt abscesses (0, absent; 1, present), and the presence or absence of goblet cell mucus (0, absent; 1, present). These parameters were poored to score inflammation as 0 (normal), 1 (mild), 2 (moderate), or 3 (extensive) (13). The numbers of neutrophils, eosinophils, and lymphocytes were also counted in the lamina propria and submucosa and expressed as a percent of the total cells counted in corresponding paraffin sections stained with H&E. At least 200 cells were quantified in each section by an observer who was blinded to the prior treatment of the animals.

To study immunofluorescence, cryosections were rehydrated in TBS (0.1 M Tris pH 7.5, 0.15 M NaCl) supplemented with 0.1% Tween 20 (TBST) and transferred to a vertical flow washing chamber (Thermo Life Sciences). Sections were preincubated twice on TBST containing 5% rat serum and subsequently incubated with avidin blocking reagent and biotin blocking reagent (0.001% avidin and 0.001% biotin solution in PBS) for 15 min each. Sections were incubated in a mixture of appropriately diluted (typically 1 g/ml) biotinylated or fluorescent dye-coupled antibodies in 2.5% serum-TBST for 1.5 h and washed three times with TBST. When required, sections were subsequently incubated with a streptavidin conjugate in 2.5% serum in TBST for 1 h. Sections were washed three times with TBST and stained twice for 2 min with 1 g/ml DAPI-TBST to visualize nuclei. Sections were washed three times with TBST and mounted with Mowiol (19). For analysis of distribution and cellular composition of lymphoid aggregations, composite images were automatically assembled using a motorized Axiosver 200M microscope (Carl Zeiss) with an auto focus module and KS300 software (Carl Zeiss). For immunocytochemistry, sections were blocked for 2 × 10 min with rabbit serum 1:20 and stained with rabbit antiserum (diluted in serum 1:40, overnight incubation at 4°C) at an appropriate concentration (6).

Antibodies and conjugates. Rabbit antibodies to histamine (anti-histamine, final dilution 1:200, from Incstar, Stillwater, MN), 5-HT (anti-5-HT, final dilution 1:5,000, Sigma-Aldrich, St. Louis, MO), and major basic protein (MBP; anti-MBP, final dilution 1:1,500, kindly provided by Dr. Jamie J. Lee, Mayo Clinic, Scottsdale, AZ) were used as primary antibodies, followed by incubation with biotinylated goat-anti-rabbit IgG (1:200, Zymed, San Francisco, CA). The monoclonal antibodies anti-CD11b-bio (Caltag/Pharmingen), anti-Ly5.2-bio/anti-CD45 (Cymbus Biotechnology), anti-IgA-bio (Bio-source) were purchased. Anti-CDS (clone KM CD8), anti-CD3 (clone 17A2), and anti-B220 (clone TIB146) antibodies were provided by Elisabeth Kremmer, GSF München, Germany, and labeled with FITC, Cy3, or Cy5 as recommended by the manufacturer (Amersham). Biotinylated primary or secondary antibodies were visualized with streptavidin coupled to Alexa 488 (Molecular Probes) or Cy3 or Cy5 (Jackson Laboratories).

Determination of tissue mediators. Samples of colon that were derived from young animals (<30 wk old) and not used for microscopic analysis were collected, pooled, weighed, and stored at −80°C for measurement of tissue mediators. For analysis, tissues were thawed, transferred into a petri dish containing 1 ml ice-cold hexadecyltrimethylammonium bromide (HTAB) buffer, minced with a scalpel, and transferred to a test tube by adding another 0.5 ml of HTAB buffer to clean the petri dish. Tissues were homogenized for 3 × 30 s (Ultra-Turrax T8, IKA, Staufen, Germany). Following homogenization, the homogenizer was rinsed with 166 µl of ice-cold HTAB buffer to avoid loss of material. A 2-ml suspension was finally obtained and aliquoted for measurement of mediators.

5-HT levels were determined by radioimmunoassay according to the manufacturer’s instructions (DLD Diagnostics, Hamburg, Germany). Because the bowel swells during inflammation (34), the resulting gain in water distorts measurements of weight; therefore, results were expressed in picomoles of 5-HT per centimeter of tissue. Spiking the initial samples by addition of defined quantities of 5-HT revealed that measurement of 5-HT was not affected by the HTAB buffer used for extraction or by the homogenization procedure (relative error < ±20% of expected value). In contrast, protein precipitation, centrifugation of the samples after homogenization (13,000 g, 10 min), and ultrasound treatment significantly reduced the recovery of 5-HT and were therefore omitted. The limit of 5-HT detected by the assay was 50 ng/ml, which corresponds to ~3 pmol/cm of tissue.

Myeloperoxidase (MPO) activity of tissue was measured by spectrophotometry (40). Homogenates were frozen, thawed, and sonicated 3 × 5 min. After centrifugation (40,000 g) for 15 min at 4°C, supernatants were diluted (1:10) with distilled water. Aliquots (50 µl) were transferred to a 96-well plate and mixed with 3,3’,5,5’-tetramethylbenzidine (50 µl) for 30 min at room temperature. Changes in absorbance at 450 nm were measured after addition of 3 M phosphoric acid (100 µl). MPO activity in experimental samples, expressed as MPO units per gram of tissue, was determined by comparison to a standard MPO activity curve, which was prepared from purified human MPO (purchased from Calbiochem) (26). One unit is defined as the amount of MPO enzyme that can metabolize 1 µmol of H2O2 per min at 25°C. The assay permitted MPO to be detected in the range of 400 to 3,000 ng/ml, which corresponds to 7.6–56.8 units per gram of colon. Samples were spiked with purified MPO to determine the rates of MPO recovery from tissue.
Histamine was extracted from tissue (11) and levels were measured by radioimmunoassay (RIA) using a commercial kit (Coulter-Immunotech, Hamburg, Germany) according to the manufacturer’s instructions. Suspensions of homogenized tissue were frozen, thawed, and sonicated 3 × 5 min. The sonicates were then diluted 1:10 with 3% trichloroacetic acid and heated for 5 min in boiling water. Precipitated proteins were pelleted by centrifugation (2,500 rpm for 15 min); the resulting supernatants were diluted 1:5 with distilled water and neutralized with 1.0 M NaHCO₃, and aliquots (100 µl) were taken for RIA of histamine. Spiking experiments indicated that a range of ±20% of added histamine was recovered. Data were expressed as nanomoles of histamine per gram of tissue.

Statistics. Biochemical measurements were performed at least in duplicate. Quantitative histological analyses were carried out by a person who was blind to the identity of the specimens. The data were usually presented as means ± SD if not indicated otherwise for n animals. For comparison of means of continuous data derived from >2 groups we used univariate ANOVA; individual pairs of groups were further analyzed by Bonferroni’s post hoc test, provided that ANOVA yielded a P < 0.05. Qualitative data were analyzed by χ² test. Statistical analyses were carried out with computer assistance (SPSS software, release 12.0, SPSS Chicago, IL).

RESULTS

Mortality and morbidity in SERT KO mice challenged with TNBS. A total of 50 mice with a C57BL6/6J background, 20 SERT KO and 30 WT, were analyzed. Of these animals, 32 TNBS. A total of 50 mice with a C57BL6/6J background, 20 (SPSS software, release 12.0, SPSS Chicago, IL).

Fig. 2. Mortality and morbidity of SERT KO and WT mice challenged rectally with TNBS or buffer control. A: premature death: animals dying before completion of the protocol. The differences between the 4 groups were statistically significant (χ² test, P = 0.018). B: global assessment of the general health (mean scores ± SD) of animals that survived and completed the protocol (6 ± 1 days after rectal challenge). Scores ranged from 1 (normal) to 4 (severe illness). Differences between groups were significant (ANOVA; P < 0.05). Bonferroni’s post hoc test was used to compare individual means. TNBS KO, SERT KO mice challenged rectally with TNBS (n = 13); Ethanol KO, SERT KO mice challenged rectally with ethanol buffer (n = 7); TNBS WT, WT mice challenged rectally with TNBS (n = 19); Ethanol WT, WT mice challenged rectally with ethanol buffer (n = 11). The numbers of mice are reduced in B, because animals that did not complete the protocol were excluded.

Body weight and stool water content were measured the start of the TNBS experimental colitis protocol and, for animals that completed the protocol, again at its end. The thickness of the colon wall of surviving mice was also measured at the end of the protocol. Because none of the control animals challenged with ethanol buffer had visible inflammation, their macroscopic scores were all 0. In contrast, visible inflammation was present in every mouse treated with TNBS; therefore their macroscopic scores were either 1 or 2. Ulcerations and stenosis (score 2) were present in 4 of 7 KO mice (57%) and 5 of 15 WT mice (33%) treated with TNBS. The most severe decrease in body weight (~20% within a few days) was observed in the TNBS KO group (Fig. 3A). A much less pronounced loss of body weight occurred in the TNBS WT group of mice and
almost no loss of weight was seen in the two control groups of mice exposed only to ethanol buffer (Fig. 3B). Stool water content, which is used as a marker for diarrhea, was significantly greater at the end of the protocol in TNBS KO mice than their ethanol-treated controls; however, the stool water content at the end of the protocol of TNBS WT animals was not statistically different from that of ethanol WT mice (Fig. 3B).

Similarly, colon thickness was also statistically greater in TNBS KO mice than in their ethanol-treated counterparts, but the colon thickness measured in TNBS WT mice did not differ statistically from that of ethanol-treated WT mice (Fig. 3C).

**Rectal motility in SERT KO mice challenged with TNBS.**

Rectal motility has been reported to be altered in SERT KO mice (12). In some animals motility was found to be more rapid than in WT controls, suggesting diarrhea, whereas in other SERT KO animals it was much slower, suggesting constipation. Rapid motility was associated with an increase in stool water content. When individual SERT KO mice were followed as a function of time, the speed of rectal motility was found to alternate. In the present study, which was designed to investigate the effect of SERT KO on experimental intestinal inflammation, stool water content and rectal motility were measured only at a single point in time; therefore, no attempt was made to capture the time-linked variability that SERT KO mice exhibit in these parameters. Stool water content (~60%) was not observed to differ statistically in any of the four animal groups of mice prior to administration of TNBS; however, stool water increased by almost 20% in SERT KO mice but not in WT mice following rectal challenge with TNBS (Fig. 3B).

Before TNBS administration, rectal motility tended to be faster in SERT KO mice (7.6 ± 5.8 min) than in WT mice (11.7 ± 7.1 min). After TNBS administration, the relative rates of motility in the two groups reversed (11.9 ± 7.5 min in SERT KO mice; 8.2 ± 6.9 min in WT mice). Differences between groups, however, did not reach levels of statistical significance because the variability between animals was too great; moreover, changes could not clearly be related to TNBS administration, since similar tendencies were observed in the control groups of mice challenged only with ethanol buffer (data not shown).

**Assessment of colonic inflammation by microscopic analyses.**

Macroscopic examination of the colon (Fig. 4, A and D) and estimates of its thickness (Fig. 3C) clearly revealed that the inflammation induced by TNBS was more pronounced in SERT KO than in WT mice. To characterize the type of inflammation in detail, both conventional histology (H&E staining) and immunocytochemistry were used to analyze the colon. Histological analysis revealed that the submucosa and, less so, the mucosa of the TNBS-exposed colon were markedly infiltrated with polymorphonuclear leukocytes (Fig. 3, E and F). This cellular infiltration caused the thickness of the wall of the colon to increase, especially in the middle segment of the colon, where TNBS had been administered. The inflammatory reaction was most severe in the colon of TNBS KO mice, but it was also visible in TNBS WT mice. Analysis of two TNBS KO mice that had to be euthanized before the protocol was completed suggested that the most pronounced inflammation occurred in animals that died prematurely (data not shown).

Most of the cells that infiltrate the TNBS-inflamed intestine expressed the immunoreactivities of leucocytes in a myeloid lineage, including CD45 (Fig. 4G) and CD11b (Fig. 4H). They did not express the lymphoid markers, CD3 (Fig. 4I) or B220 (Fig. 4, G and J). Relatively few cells of the infiltrates expressed mast cell (histamine; Fig. 4J) or eosinophil (MBP; Fig. 4L) markers. The morphology of the infiltrating cells (Fig. 4F), their expression of myeloid but not lymphoid markers, and the relative paucity of cells expressing MBP suggest that most of...
the inflammatory effector cells that infiltrate the TNBS-inflamed intestine are polymorphonuclear leukocytes. Although more mast cells and eosinophils were observed in the TNBS-inflamed gut of SERT KO mice than in that of WT controls, these increases were not statistically significant. EC cells (Fig. 4K) in the mucosal epithelium were visualized with antibodies to 5-HT. A distinction was drawn between weakly and strongly 5-HT-immunoreactive EC cells; the number of highly 5-HT-immunoreactive cells was clearly greater in WT animals than in SERT KO animals (P < 0.002). The number of strongly 5-HT-immunoreactive EC cells tended to be lower in both WT and SERT KO animals challenged with TNBS than in control animals challenged with ethanol buffer. 5-HT immunoreactivity was present in neurites of the myenteric plexus in WT mice but almost absent in KO mice. Following challenge of WT mice with TNBS, the number of 5-HT-immunoreactive myenteric neurites appeared to be greater than in mice challenged with ethanol buffer control; however, these changes did not reach statistical significance (data not shown).

Quantitative analysis of sections stained with H&E confirmed that colonic inflammation was most pronounced in SERT KO mice (Fig. 5). Histological scoring of tissue inflammation 0 (normal), 1 (mild), 2 (moderate), and 3 (severe) revealed significant differences. Only marginal changes were seen in animals challenged with ethanol buffer control, whereas most of the animals challenged rectally with TNBS showed microscopic evidence of inflammation. [Only marginal changes were seen in animals challenged with ethanol buffer control (KO 2× score “0”, 1×“1”, 1×“2”; WT 4×“0”, 1×“1”, 1×“2”), whereas most of the animals challenged rectally with TNBS showed microscopic evidence of inflammation (KO 1×“1”, 2×“2”, 2×“3”; WT 1×“0”, 4×“2”)] Epithelial destruction was observed in both SERT KO and WT mice following induction of TNBS colitis. The submucosa of TNBS

Fig. 4. Colonic inflammation in SERT KO and WT mice following rectal administration of TNBS. A and D: macroscopic appearance of the colon in a control mouse (A) and a SERT KO mouse (D). B and E: low-power microscopy of “Swiss rolls” of whole colon preparations stained with hematoxylin and eosin. Note that the inflammatory cell infiltrate of the mucosa and particularly the submucosa is more extensive in the SERT KO mouse (E) than in the WT control mouse (B). C and F: most of the infiltrating cells are polymorphonuclear leukocytes, which appear to be more activated in SERT KO (F) than in control mice (C). G–I: 3-color immunofluorescence. DNA is displayed in blue. Most infiltrating cells are CD45 immunoreactive (red fluorescence; pan-leukocyte marker, G) and CD11b immunoreactive (green; myelocyte marker, H) but do not display the immunoreactivities of CD3 (red; T lymphocytes, I), B220 (green; B lymphocytes, G and I), or IgA (red; H). J–L: immunocytochemistry with antibodies to histamine (J), serotonin (K), and anti-major basic protein (L) sera identifies mast cells, enterochromaffin cells (EC cells) and eosinophils within the mucosa.
KO mice was thicker than that of TNBS WT mice. Correspondingly, the submucosa of TNBS KO mice was far more infiltrated by cells than that of TNBS WT mice. As noted above (see Fig. 4), most of the infiltrating cells were neutrophils, the percentage of which increased after challenge with TNBS from 6 to 28% in WT mice and from 9 to 56% in KO mice. Similar, but less pronounced, changes were seen in the mucosa. A statistically significant TNBS-induced increase in infiltration of the lamina propria by cells and an increase in the proportion of neutrophils in the mucosa and submucosa was observed only in SERT KO mice. Numerical increases were also seen in these parameters in WT mice but in these animals the increases did not reach statistical significance (Fig. 5).

Measurement of MPO, histamine, and 5-HT in colonic tissue. Rectal challenge with TNBS caused significant increases in tissue levels of MPO (Fig. 6A) and histamine (Fig. 6B) to occur in SERT KO but not in WT mice. The increase in MPO confirms the TNBS-induced infiltration of the bowel wall of SERT KO mice with polymorphonuclear leukocytes, whereas the increase in histamine suggests that a significant infiltration by mast cells also occurs in these animals. In contrast, there was no change in the 5-HT content of the bowel as a result of TNBS challenge, either in WT or SERT KO mice (Fig. 6C).

**DISCUSSION**

Prior studies of the relationship of SERT to intestinal inflammation have focused on the ability of the inflammatory process to reduce the expression of SERT and thus to affect...
serotonergic regulation of intestinal motility and secretion (9, 14, 34, 35, 44). The present study utilized SERT expression as a tool with which to investigate the putative role played by serotonergic signaling in inflammation. We thus tested the hypothesis that endogenous 5-HT contributes to the pathogenesis of intestinal inflammation. Inflammation was compared in SERT KO mice, in which termination of the action of 5-HT is defective, to that in their WT littermates, in which 5-HT inactivation is, by definition, normal. The relative inability of SERT KO mice to terminate the action of 5-HT enhances serotonergic signaling in these animals (5) but also activates a variety of partially protective compensatory mechanisms (12, 36). As a result of these compensations, differences between unstressed SERT KO mice and their WT littermates are relatively minor (12). The present study confirmed that this is so in the absence of inflammation; however, we anticipated that the stress of inflammation would release 5-HT and overwhelm the protective compensations in SERT KO mice, such as decreased sensitivity and rapid desensitization of 5-HT receptors as well as a low-affinity, nonspecific uptake of 5-HT by backup transporters (12, 36). If enteric 5-HT is involved in intestinal inflammation, therefore, intestinal inflammatory responses would be expected to be significantly exacerbated by the potentiation of serotonergic signaling that occurs when SERT is inactive (5).

Our observations strongly support the hypothesis that endogenous 5-HT plays a role in intestinal inflammatory responses. The difference between the consequences of inflammation that rectal challenge with TNBS evokes in the colon in SERT KO mice and that it evokes in wild-type littermates was dramatic. The mortality of TNBS-induced colitis in WT mice was more than doubled in SERT KO mice; moreover, severe symptoms, including rectal prolapse, a curved back, and reduced movement in the cage, were regularly seen in the SERT KO animals that survived TNBS-induced colitis until the end of the protocol. Such symptoms were also observed occasionally in WT mice with TNBS-induced colitis but were far less severe than in the SERT KO animals and were never seen in control mice that received enemas only of ethanol, regardless of whether the mice were SERT KO or WT. Multiple assessments of the structure and function of the bowel, including colon thickness, stool water content, and histological changes of the mucosa, confirmed that the local manifestations of inflammation induced by TNBS were more severe in the SERT KO than WT colon.

The TNBS-induced inflammatory infiltrate of the colonic mucosa, both in SERT KO and in WT mice consisted predominantly of polymorphonuclear leukocytes; however, mast cells and eosinophils were also present, particularly in SERT KO animals. In keeping with the cellular analyses of the TNBS-associated mucosal infiltrates, biochemical measurements showed that tissue concentrations of MPO activity, a polymorphonuclear leukocyte marker, and histamine, a mast cell marker, were increased as a result of TNBS-induced colitis in SERT KO mice. These increases did not occur in WT animals, indicating again that TNBS-induced colitis was potentiated in SERT KO mice. The increased level of histamine is consistent with previous studies that have found increases in mast cells in TNBS colitis (34). It is also possible that the polymorphonuclear leukocytes of the inflammatory infiltrates contribute to the increase in histamine because there is evidence that these cells, as well as mast cells, can be a source of histamine (58).

The preexisting deletion of SERT compromises 5-HT inactivation and thus was expected to increase the strength of serotonergic signaling. Inflammation in TNBS-induced colitis might be generated independently of 5-HT but cause 5-HT release. If so, then the potentiated serotonergic signaling could, as a secondary effect, exacerbate the local and/or systemic consequences of inflammation. In contrast, if serotonergic signaling were to contribute to the generation of the inflammatory process itself, the deletion of SERT would be expected to enhance, not only the adverse consequences of inflammation, but also the inflammatory response itself. These alternatives are not necessarily mutually exclusive. Certainly, as noted...
above, the consequences of TNBS-induced colitis were more severe in SERT KO than in WT mice; death, global assessment of health, and weight loss were all adversely affected by the deletion of SERT. These observations are consistent with the possibility that SERT KO enhances toxic actions of free 5-HT, which are known to include decreased mucosal blood flow with induction of gastric lesions (46), increased vascular permeability (2, 38, 54), anaphylaxis (20), ischemia in nerve (28) and muscle (27), bronchoconstriction (29), and pulmonary hyper-tension (16). The observations that death and tissue damage increase when the intestine is inflamed in SERT KO mice, however, do not distinguish whether the toxicity of 5-HT released during inflammation is enhanced in SERT KO mice or whether the inflammatory response proper is stronger. In contrast, the observation that infiltration of the bowel by polymorphonuclear leukocytes, eosinophils, and mast cells is intensifi ed in SERT KO mice suggests that intestinal inflammation is augmented in these animals. There is reason to anticipate that intestinal inflammation would be made more severe by the potentiation of serotonergic signaling that occurs SERT KO mice. 5-HT is an important inflammatory mediator (32, 42, 43). 5-HT, for example, initiates delayed-type hypersensitivity reactions through the recruitment and activation of CD4+ T cells (1). This is both an effect mediated through the action of 5-HT on the vasculature and a direct response mediated by the multiple 5-HT receptors (5-HT1A, 5-HT2A, 5-HT3A, 5-HT7) that subpopulations of lymphocytes express (1, 32, 56, 59, 61).

5-HT also affects macrophages (62) and regulates their expression of matrix metalloproteinase (42). 5-HT, moreover, is chemotactic for eosinophils (7), stimulates the secretion of IL-16 (lymphocyte chemoattractant factor) from CD8+ lymphocytes (31), and attracts mast cells to sites of tissue damage by promoting their adhesion and migration (30). The chemotactic effects of 5-HT may underlie the increase in mast cells and eosinophils in the cell infiltrates that accompany TNBS-induced colitis in SERT KO mice. The consequences of inflammation in SERT KO mice may thus be more severe than in WT animals because the intensity of the inflammation engendered by TNBS is greater in animals in which serotonergic signaling to the immune system is enhanced.

Both the 5-HT content of the mucosa and the numbers of EC cells have previously been reported to be increased in the guinea pig colon as a result of TNBS-induced colitis (44). We did not observe such an increase in the mucosal concentration 5-HT in animals subjected to TNBS-induced colitis, either in SERT KO or WT animals; moreover, challenge with TNBS did not significantly change the proportion of epithelial cells that were 5-HT immunoreactive. In contrast to TNBS-induced colitis in the guinea pig, TNBS-induced colitis in the mouse colon has not previously been reported to be associated with a change in the number of EC cells (35). In fact, in the present study, TNBS-induced colitis caused the number of strongly 5-HT-immunoreactive to fall, particularly in SERT KO animals. Although a slight numerical increase in the number of 5-HT-immunoreactive EC cells was found, the variability was great and the increases failed to reach statistical significance. The decrease in strongly 5-HT-immunoreactive EC cells is consistent with the idea that EC cells release 5-HT during TNBS-induced inflammation. EC cells, however, contain a very high concentration of 5-HT and, because they express TpH1, they are capable of replacing the 5-HT they release through new biosynthesis. Major changes in serotonergic signaling due to EC cell secretion thus could occur yet not be reflected in the mucosal content of 5-HT, which is also similar in SERT KO and WT mice, despite the compromise of the recapture of secreted 5-HT in SERT KO animals. Much less 5-HT is stored in enteric neurons than in EC cells. This difference probably explains why the maintenance of neuronal 5-HT was found to be more dependent on 5-HT reuptake than that of EC cells; thus the 5-HT immunoreactivity of enteric neurites was found to be low in SERT KO mice. Despite this evident dependence on reuptake, neuronal 5-HT immunoreactivity was unchanged TNBS-induced colitis.

Serotonergic signaling and thus SERT may be involved in the pathophysiology of IBD and IBS (21, 51, 52). Although there is no evidence that the function of SERT is completely lost either in IBD or IBS, there is evidence that mucosal SERT expression is decreased in IBD and in at least some patients with IBS as well as in the inflamed bowel of patients with celiac disease and those with GI infections (14, 15, 51, 52). SERT expression is also decreased in mice as a result of TNBS-induced colitis (35) and the inflammatory cytokines TNF-α and IFN-γ decrease the expression of SERT in an intestinal cell line (18). A polymorphism, called 5-HTTLPR, has been described in the promoter region of the human gene SLC6A4, which encodes SERT (8, 24, 33). The short allele of the HTTLPR polymorphism might be associated with decreased SERT expression in vitro and with affective disorders (24). Although some reports have suggested that the HTTLPR polymorphism might be associated with IBS, a meta-analysis of these studies indicates that there is no linkage between the two (55). The effect of the HTTLPR polymorphism on the function of SERT in the bowel, however, might be relatively minor because there is evidence that alternative noncoding exons support SERT transcription in the gut and the brain (48). It is thus unclear that enteric transcription of SERT is significantly affected by the HTTLPR polymorphism.

Whether or not a congenital decrease in SERT expression predisposes an individual to IBD or IBS, the present experiments suggest that if SERT expression in the gut were to decrease secondarily for whatever reason, it would be likely to exacerbate an intestinal inflammatory response. Given that inflammation can reduce mucosal SERT expression, it is possible that SERT expression decreases after intestinal inflammation is initiated and that the resulting amplification of serotonergic signaling exerts an autocatalytic effect on the inflammatory response. This feedforward effect of serotonergic signaling may contribute both to the maintenance of inflammation as well as to the discomfort and abnormal motility associated with it. Certainly, the present experiments suggest that the nonselective transporters and changes in expression and sensitivity of 5-HT receptors that enable animals to survive with only minor deficits in motility cannot substitute for SERT when the bowel is undergoing inflammation. A better understanding of the receptors and transduction pathways that mediate the effects of 5-HT on inflammation might thus provide new therapies for IBS and/or IBD.

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