Persistent alterations to enteric neural signaling in the guinea pig colon following the resolution of colitis

Alan E. Lomax, Jennifer R. O’Hara, Niall P. Hyland, Gary M. Mawe, and Keith A. Sharkey

Persistent alterations to enteric neural signaling in the guinea pig colon following the resolution of colitis. *Am J Physiol Gastrointest Liver Physiol* 292: G482–G491, 2007. First published September 28, 2006; doi:10.1152/ajpgi.00355.2006.—Functional changes induced by inflammation persist following recovery from the inflammatory response, but the mechanisms underlying these changes are not well understood. Our aim was to investigate whether the excitability and synaptic properties of submucosal neurons remained altered 8 wk post-trinitrobenzene sulfonic acid (TNBS) treatment and to determine whether these changes were accompanied by alterations in secretory function in submucosal preparations voltage clamped in Ussing chambers. Mucosal serotonin (5-HT) release measurements and 5-HT reuptake transporter (SERT) immunohistochemistry were also performed. Eight weeks after TNBS treatment, colonic inflammation resolved, as assessed macroscopically and by myeloperoxidase assay. However, fast excitatory postsynaptic potential (fEPSP) amplitude was significantly increased in submucosal S neurons from previously inflamed colons relative to those in control tissue. In addition, fEPSPs from previously inflamed colons had a hexamethonium-insensitive component that was not evident in age-matched controls. AH neurons were hyperexcitable, had shorter action potential durations, and decreased afterhyperpolarization 8 wk following TNBS administration. Neuronally mediated colonic secretory function was significantly reduced after TNBS treatment, although epithelial cell signaling, as measured by responsiveness to both forskolin and bethanecol in the presence of tetrodotoxin, was comparable with control tissue. 5-HT levels and SERT immunoreactivity were comparable to controls 8 wk after the induction of inflammation, but there was an increase in glucagon-like peptide 2-immunoreactive L cells. In conclusion, sustained alterations in enteric neural signaling occur following the resolution of colitis, which are accompanied by functional changes in the absence of active inflammation.

submucosal plexus; electrolyte transport; enterochromaffin cells; serotonin; glucagon-like peptide 2

INFLAMMATION of the gastrointestinal (GI) tract, as occurs during inflammatory bowel disease (IBD) and acute bacterial enteritis, markedly alters GI function (9, 15, 40, 42). In addition, persistent alterations in GI function are commonly observed after the resolution of intestinal inflammation. These include modified patterns of motility, abnormal secretion, and changes in visceral sensation. Clinically, this spectrum of changes frequently occurs after the resolution of self-limiting enteropathogenic infections or during periods of remission in patients with chronic intestinal inflammatory conditions such as Crohn’s disease, ulcerative colitis, and infectious enteritis (12, 20, 43). Data obtained with biopsies from patients with IBD and animal models of IBD have consistently suggested a role for inflammatory effects on neurons in the generation of symptoms associated with IBD (6, 9, 10, 18, 31, 34); whether the persistent alterations observed are due to neural mechanisms is as yet unknown.

The question of whether prior inflammation can alter GI function and visceral sensation following the resolution of inflammation is an important one. In a rat model of hapten-induced colitis and a mouse model of nematode infection, alterations in gut function and visceral sensation persisted beyond the time course of GI inflammation (4, 25, 48). Furthermore, prior inflammation or infection can affect the number of enteric neurons and enterochromaffin (EC) cells in the previously inflamed region. A sustained reduction in enteric neurons and an increase in EC cell numbers postinflammation have been detected (27, 46). In addition to serotonin (5-HT)-containing EC cells, there are at least 14 other subpopulations of enteroendocrine cells. Glucagon-like peptide (GLP)-2 and peptide YY (PYY)-containing L cells, along with 5-HT-containing EC cells, are the most prominent enteroendocrine cell populations in the large intestine. GLP-2 and PYY have been reported to be involved in GI motility, secretion, and/or cellular proliferation. Previous studies (23, 44) have demonstrated that PYY tissue and plasma levels are reduced in patients with ulcerative colitis. Likewise, tissue levels of GLP-2 (11) and circulating levels of GLP-2 (47) were altered in an animal model of colitis and patients with IBD, respectively. However, these cells have not been examined under postinflammatory conditions. Therefore, it is possible that persistent alterations in neuronal and enteroendocrine cell signaling following a bout of inflammation may contribute to the long-term functional consequences of prior inflammation.

The aim of the present study was to determine whether trinitrobenzene sulfonic acid (TNBS)-induced colitis in guinea pigs leads to alterations in enteric neurophysiology and mucosal 5-HT signaling as well as functional changes in secretory responses that persist beyond the resolution of inflammation. This model was chosen because our laboratories have previously characterized the effects of TNBS-induced colitis on neural signaling and mucosal enteroendocrine signaling (26,
enabling us to compare acute and persistent effects of inflammation on enteric neuroendocrine function.

METHODS

All methods used in this study were approved by the University of Calgary Animal Care Council and were carried out in accordance with guidelines of the Canadian Council on Animal Care. Animals were housed in a temperature-controlled room maintained on a normal 12:12-h light-dark cycle and were allowed access to food and water ad libitum. To induce inflammation in the distal colon, adult male guinea pigs (Charles River Laboratories, Montreal, QC, Canada) were anesthetized with halothane (induced at 4% and maintained on 1.5% in oxygen), and 0.5 ml TNBS (25 mg/ml) in 25% ethanol was delivered into the lumen of the colon through a polyethylene catheter 7 cm proximal to the anus. Animals were then allowed to recover for 8 wk following TNBS administration, during which time their weight changes were monitored. Age-matched naive guinea pigs were used as controls and were housed under identical conditions until tissue collection. At the time of tissue collection, animals were deeply anesthetized with halothane and exsanguinated. The distal colon was collected at the time of death, examined to attain a macroscopic damage score (35), and subsequently used for the assays described below.

Neurophysiological characterization. The distal colon was removed, and the oral end was marked and placed in Krebs solution [composed of (in mM) 117 NaCl, 4.8 KCl, 2.5 CaCl2, 1.2 MgCl2, 25 NaHCO3, 1.2 NaH2PO4, and 11 d-glucose and aerated with 95% O2-5% CO2] containing nicardipine (3 μM) to hyperpolarize to −80 mV to prevent APs and to minimize changes in the electrochemical driving force, and the average amplitude of at least three fast EPSPs at −80 mV was recorded for each neuron under control conditions and following superfusion with hexamethonium. One-second trains of pulses at 20 Hz were applied to internodal strands to determine whether the impaired neurons received slow synaptic input. Slow EPSP amplitudes were measured from slow EPSPs evoked at the resting membrane potential of each neuron.

Ussing chamber experiments. Distal colons were immediately placed in fresh Krebs solution of the same composition used for neurophysiological recordings above with the exception of the addition of nicardipine and nicardipine. Mucosal preparations (with an intact submucosal plexus) were obtained by opening the colon along the mesenteric border and removing the muscularis externae (comprising the circular and longitudinal smooth muscle layers plus the myenteric plexus) by blunt dissection. Preparations were subsequently mounted in Ussing chambers (exposed mucosal area of 0.6 cm2) containing 10 ml of oxygenated (95% O2-5% CO2) Krebs solution maintained at 37°C.

Tissues were voltage clamped at 0 mV using an automatic voltage clamp (DVC 4000, World Precision Instruments), and changes in the short-circuit current (Isc) required to maintain the 0-mV potential were continuously monitored using DataTrax software (World Precision Instruments). All pharmacological agents were added to the basolateral (submucosal) reservoir; n values reflect the numbers of animals that contributed to each different group.

The alkaloid veratridine (30 μM), which activates voltage-gated Na+ channels, was used to stimulate secretomotor neurons in these experiments (19). Veratridine-evoked changes in Isc reached their peak within 15 min; the peak change in Isc was recorded. Agonist-induced Cl− secretion in GI epithelia occurs via Ca2+-dependent or cAMP-dependent signaling pathways (2). Thus, we examined the effects of bethanechol (BCh; 10 μM), a muscarinic receptor agonist that evokes Ca2+-dependent secretion, and forskolin (10 μM), which stimulates cAMP-dependent secretion, on Isc in control and TNBS-treated animals in either the presence or absence of TTX. This allowed us to determine whether there was any differential sensitivity, neuronal versus epithelial, exhibited to either agonist in TNBS-treated compared with control colons.

Immunohistochemistry. Colonic segments to be used for immunohistochemistry were opened along the mesenteric border, stapled flat with the mucosa up, and fixed overnight at 4°C in Zamboni’s fixative (2% paraformaldehyde and 0.2% picric acid; pH 7.4). Samples were then transferred to 20% sucrose in PBS overnight at 4°C. Transverse segments from each animal were embedded, with the mucosa oriented in the same direction, in OCT compound (Miles, Elkhart, IN). Sections of the colon (10 μm) were cut on a cryostat, thaw mounted onto poly-d-lysine-coated slides, and stored at −20°C until use.

Changes in enteroendocrine cell populations and 5-HT-selective reuptake transporter (SERT) expression were examined in the colon of animals treated with TNBS 8 wk previously and in age-matched naive controls. Tissue sections were washed with PBS containing 0.1% Triton X-100 (3 x 10 min), followed by incubation with rabbit anti-5-HT (1:5,000, Incstar), rabbit anti-GLP-2 (1:500, Biogenesis), or rabbit anti-PYY (1:1,000, Dr. John Walsh, Center for Ulcer Research and Education) for 48 h at 4°C. Sections were washed again with PBS containing 0.1% Triton X-100 (3 x 10 min) and incubated with CY3-conjugated donkey anti-rabbit IgG (1:100, Jackson Immuno-Research) secondary antibody for 2 h at room temperature. Sections were subsequently washed in PBS (3 x 5 min). Stained sections were coverslipped with bicinearbonate-buffered glycerol and examined with a Zeiss Axioskop fluorescence microscope. Photomicrographs were taken using a digital imaging system consisting of a digital camera (Sensys, Photometrics, Tucson, AZ) and image analysis software (V for Windows, Digital Optics, Auckland, New Zealand). All micro-

AJP-Gastrointest Liver Physiol • VOL 292 • FEBRUARY 2007 • www.ajpgi.org
graphs of SERT immunofluorescence were taken at the same exposure time and magnification (×40).

To quantify the number of enteroendocrine cells, the number of cells was expressed as a function of length of the colon. Five sections per animal were included, and the mean number of enteroendocrine cells from four random areas (250 μm in length) in each section was calculated. Controls consisting of liquid phase preabsorption of primary antisera with cognate peptides or 5-HT (10 nmol/ml diluted antisera) and omission of the primary antisera have been previously shown to abolish any immunoreactivity (37).

Measurements of mucosal 5-HT release in the colon. The colon was opened along the mesentery and cut into two segments (1 × 0.5 cm). Segments were pinned flat; mucosal side up, in a Sylgard-coated six-well dish containing 37°C HEPES solution [containing (in mM) 110 NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂·6H₂O, 60 sucrose, 5 glucose, and 20 HEPES]. Following a 15-min incubation period, the bathing solution was replaced by 3 ml of warm, fresh HEPES solution. To mechanically stimulate the mucosa, segments of the colon were gently stroked in the circumferential direction with a rounded glass probe (diameter ~2 mm) at a rate of 8 strokes/min for a total of 15 min (26). The mucosal stimulation was analogous to the passage of stool pellets through the lumen of the colon. Basal levels of 5-HT release were determined by leaving preparations undisturbed in the bathing solution for 15 min. 5-HT released into the bathing solution was measured with an enzyme immunoassay kit used according to the manufacturer’s instructions (Beckman Coulter, Fullerton, CA).

MPO assay. MPO, an enzyme found in cells of myeloid origin, is used as a marker of neutrophil infiltration. MPO activity was determined as previously described (35). Briefly, segments of the distal colon were removed, opened along the mesenteric border, blotted dry, and weighed before being snap frozen on dry ice. Changes in absorbance at 450 nm over a 2-min period were determined using a Multiskan Ascent plate reader (Thermo Labsystems). Values are expressed as units of MPO activity per gram of tissue sample, where one unit of MPO is defined as that which degrades 1 μmol of hydrogen peroxide per minute.

Drugs. Veratridine, BCh, forskolin, and hexamethonium were purchased from Sigma (St Louis, MO). TTX was purchased from Tocris (Ellisville, MO).

Statistical analysis. Peak changes in the Isc response relative to baseline were recorded, converted to μA/cm², and are quoted throughout as means ± SE. Comparisons between three or more groups were done with one-way ANOVA using the Bonferroni’s test. Comparisons between two groups were analyzed with a two-way Student’s unpaired t-test. Nonparametric values were compared using the Fisher’s exact test or the Mann-Whitney test. The intensity of SERT immunofluorescence was measured using the Scion Image program, and statistical comparisons were conducted with Graphpad Prism software (GraphPad Software, San Diego, CA). Summary data were plotted using Graphpad Prism software (version 3.03). P values of <0.05 were considered statistically significant.

RESULTS

As previously described (26), guinea pigs treated with TNBS lost weight over the first 2 days following injection and gained significantly less weight than control animals over the first week (Fig. 1A). From the second week through to death, however, the weight gain of these animals was indistinguishable from controls (Fig. 1B). Eight weeks following TNBS administration, neither MPO activities nor macroscopic damage scores were elevated compared with controls (Fig. 1, C and D).

Fig. 1. Weight changes, macroscopic damage scores, and MPO levels in previously inflamed animals and age-matched controls. Animals treated with trinitrobenzene sulfonic acid (TNBS) gained significantly less weight in the first week following TNBS instillation than age-matched controls (*P < 0.05, n = 16 animals/group; A). Following the first week, in previously inflamed animals, there were no significant differences in weight gain for the next 7 wk (B). Macroscopic damage scores (C) and MPO activities (D) of previously inflamed animals were indistinguishable from controls.
Enteric neurophysiology. In total, 7 AH neurons and 19 S neurons from animals previously exposed to TNBS were electrophysiologically characterized. Data from TNBS-treated animals were compared with data from 30 S neurons and 14 AH neurons from control animals. Many electrophysiological parameters in enteric neurons were unaltered by prior inflammation with TNBS; details for S neurons and AH neurons are shown in Tables 1 and 2 respectively. However, several marked changes were observed following the resolution of TNBS-induced colitis, including aspects of AH neuron excitability and synaptic potentials recorded in S neurons.

Fast EPSPs recorded at −80 mV were significantly larger in postinflammatory S neurons compared with untreated controls (Fig. 2A). Similar to previous findings during active TNBS-induced colitis, this increase in fast EPSP amplitude was accompanied by a change in fast EPSP pharmacology. In age-matched controls, fast EPSPs were abolished by the nicotinic receptor antagonist hexamethonium (100 μM). The hexamethonium-resistant component of the evoked fast EPSP was significantly larger in previously inflamed animals compared with controls (Fig. 2B), indicating a change in neurotransmitter pharmacology following the resolution of colitis.

AH neurophysiology in previously inflamed animals also differed from controls. AP discharge during intracellular depolarizations to twice rheobase and maximal depolarizations were significantly elevated in previously inflamed AH neurons (Fig. 3A). AH neuron excitability is determined by a number of currents, including a Ca2+-activated K+ current (gKCa), which underlies a sustained afterhyperpolarizing potential following AP discharge. In previously inflamed animals, the peak amplitude of the afterhyperpolarizing potential was markedly reduced (Fig. 3B). A possible mechanism for this reduction is suggested by the significant reduction in the AP duration at half-maximal amplitude in these animals (Fig. 3C), which may reflect reduced calcium influx and subsequent activation of the gKCa that underlies afterhyperpolarizing potentials in these neurons (16).

It is worth noting that the characteristics of previously inflamed animals could not be directly compared with age-matched controls, due to a complete lack of successful impalements of AH neurons in these animals. Immunohistochemical analysis of a marker [calbindin (32)] of AH neurons in the guinea pig submucosal plexus did not identify any age-related changes of AH neurons from control animals. Many electrophysiological parameters in enteric neurons were unaltered by prior inflammation, with controls (Fig. 2A), naive control, top trace). To confirm the neurogenic nature of this response, TTX was added 15 min after veratridine and abolished veratridine-stimulated ISc changes (Fig. 4A, bottom trace). To further confirm a neuronal site of action for veratridine, we pretreated the control (Fig. 4A, bottom trace) and TNBS-treated (data not shown) colon with TTX, which abolished veratridine-induced secretion. No significant differences were detected in ongoing neuronal tone, measured as the sensitivity of basal ISc to TTX, between TNBS-treated and control colons (control: −13.0 ± 3.5 μA/cm², n = 14; and TNBS: −28.8 ± 10.8 μA/cm², n = 9). The veratridine-induced change in ISc was significantly reduced in previously inflamed colons compared with controls (P < 0.001; Fig. 4B).

Ca2+ and CAMP-stimulated ISc. Asfaha et al. (1) reported that previously inflamed colitis rats have a reduced ISc response to nerve stimulation that lasts over 12 wk after TNBS instillation. The authors concluded that this hyposecretion was due in large part to a reduced responsiveness of the colonic mucosa to secretagogues and therefore may be a result of altered epithelial cell signaling rather than a change in neuronal function per se. Therefore, we assessed secretory responses to BCh and forskolin in the presence and absence of TTX.
Both BCh and forskolin caused a sustained increase in $I_{SC}$ in control and TNBS-treated animals. DMSO vehicle controls did not significantly alter basal $I_{SC}$ in either control or TNBS-treated preparations (data not shown). No significant differences were observed for BCh-induced $I_{SC}$ responses in control or TNBS-treated colons in either the presence or absence of TTX (Fig. 5A). However, in previously inflamed animals, the forskolin response was significantly increased relative to controls, and this response was reduced to the control level in the presence of TTX, suggesting that submucosal neurons in TNBS-treated animals display increased sensitivity to cAMP stimulation (Fig. 5B; $P < 0.01$).

Enteroendocrine cell numbers. 5-HT-, GLP-2-, and PYY-immunoreactive enteroendocrine cells were quantified in transverse sections of the colon from control and previously inflamed guinea pigs and expressed as the mean numbers of cells per millimeter of the muscularis mucosa. Eight weeks following intraluminal administration of TNBS into the colon, the numbers of 5-HT-positive EC cells were not significantly different from age-matched controls (Fig. 6A; control: $n = 6$, and TNBS: $n = 5$). In contrast, the numbers of GLP-2-immunoreactive L cells were significantly greater in the colon of TNBS-treated guinea pigs compared with controls (Fig. 6B; $P < 0.05$; control: $n = 7$, and TNBS: $n = 5$). The increase in L cell numbers appeared to be limited to GLP-2-positive cells as there were no significant differences in the numbers of PYY-immunoreactive L cells in the colon of control versus TNBS-treated guinea pigs (Fig. 6C; control: $n = 5$, and TNBS: $n = 5$).

5-HT release. To determine if there was a functional change in the amount of 5-HT secreted from EC cells, mucosal 5-HT release was measured in isolated segments of the colon under basal and stimulated conditions. The amount of 5-HT released in response to mechanical stimulation of the mucosa was significantly increased compared with basal release in the colon of both control and TNBS-treated animals (Fig. 7; $P < 0.05$; control: mechanical, $n = 7$, and basal, $n = 6$; and TNBS: mechanical, $n = 7$, and basal, $n = 6$). There were no significant differences in the amount of 5-HT released from the colon of control versus TNBS-treated animals under basal or mechanically stimulated conditions.

SERT. The SERT terminates the action of 5-HT by transporting the amine into epithelial cells, where it can be metab-olized (7). To determine if mucosal SERT expression was altered 8 wk after the administration of TNBS, SERT protein was evaluated by immunohistochemistry. In the colons of control animals, SERT immunoreactivity was observed throughout epithelial cells (Fig. 8A) as well as in the myenteric plexus (data not shown). The intensity of staining was highest in epithelial cells lining the lumen of the gut, with less staining observed deeper in the crypts. Mucosal SERT immunoreactivity was similar in the colon of TNBS-treated animals compared with controls (Fig. 8A). The intensity of SERT immunofluorescence was quantitatively compared in control and TNBS-treated animals, and the mean intensity was not significantly different between the two groups (Fig. 8B).

DISCUSSION

The goal of this investigation was to test the hypothesis that TNBS-induced colitis- alterations in enteric neurophysiology and mucosal enteroendocrine cell signaling persist beyond the resolution of inflammation (56 days following TNBS administration) and are accompanied by changes in secretory function. We chose the guinea pig colon because previous studies (26, 29, 33) have thoroughly characterized the effects of TNBS-induced colitis on neuroendocrine signaling in the colon during active inflammation 6 days following TNBS administration. Our present findings indicate that many of the features of neuromplasticity observed 6 days following TNBS administration persist for at least 8 wk and are accompanied by alterations in neuronal control of colonic electrolyte transport. In contrast, the marked changes in EC cell 5-HT signaling observed 6 days following TNBS administration are no longer evident at this time point following recovery from inflammation.

Enteric neurophysiology. TNBS-induced colitis in guinea pigs alters enteric neural circuits both during active inflammation and following the resolution of inflammation. Previously inflamed S neurons had significantly larger fast EPSP amplitudes due to an increase in noncholinergic neurotransmission. This finding is very similar to that observed in S neurons during TNBS-induced colitis (33). During active colitis, the increase in noncholinergic fast neurotransmission was due to increased release or decreased catabolism/reuptake of ATP and 5-HT. One synaptic change observed during inflammation that...
did not persist at the 56-day time point was slow EPSP amplitude. The amplitude of this metabotropic synaptic potential was significantly larger 6 days after TNBS treatment (33) but returned to control levels following the resolution of inflammation.

AH neurons in previously inflamed animals exhibited features of hyperexcitability similar to those detected during active TNBS-induced colitis. The number of APs discharged during a depolarizing current pulse was increased, accompanied by decreases in the duration of the AP and the amplitude of the afterhyperpolarizing potential. The mechanisms responsible for these changes in excitability are not yet understood, but they could involve a persistent alteration in channel expression and/or a continuous release of inflammatory mediators due to a low-grade inflammation. In addition, we have recently demonstrated alterations in electrical and synaptic properties of submucosal neurons in the noninflamed colon of animals with TNBS-induced ileitis. These changes were accompanied by significantly increased PGE₂ tissue levels, despite the lack of overt inflammation in the colon (38). Furthermore, similar changes to colonic myenteric AH neurons occurred during the acute phase of TNBS-induced colitis, and these changes were attenuated in the presence of cyclooxygenase inhibitors (28). Therefore, increased prostaglandin levels may underlie some of the changes in neuronal properties observed in the present study.

**Colonic electrolyte transport.** As the submucosal plexus and its reflex pathways tightly regulate fluid and electrolyte trans-
port, it is possible that the altered neurophysiological properties observed at the 8-wk time point in this study may be associated with abnormal secretory function. Eight weeks postinflammation veratridine-evoked increases in $I_{sc}$ were significantly reduced in TNBS-treated animals. The responses to veratridine were TTX sensitive, indicating a neuronal mechanism of action for this alkaloid. This indicates altered submucosal secretomotor nerve function in previously inflamed animals. A similar hyporesponsiveness of the colonic mucosa to nerve stimulation was observed in the rat colon 9 wk following TNBS treatment (1). However, this decrease in EFS-induced secretion was accompanied by a decrease in TTX-insensitive responses to IBMX and carbachol. These data would suggest that persistent changes 9 wk post-TNBS treatment results in epithelial hyporesponsiveness and subsequent blunting of EFS-induced secretion in the rat colon (1). Therefore, to determine whether the change in veratridine-induced secretion in our guinea pig model of long-term colitis was as a result of altered neuronal function or epithelial dysfunction, we measured both secretory responses to BCh and forskolin in the presence and absence of TTX.

In the guinea pig colon, BCh responses were TTX insensitive and were not significantly altered in previously inflamed animals, suggesting that epithelial muscarinic receptors and Ca$^{2+}$ signaling are unaltered in TNBS-treated guinea pigs. In contrast, forskolin responses were augmented in the TNBS-treated colon, and this response was significantly decreased by pretreatment with TTX. In control tissues, forskolin responses were insensitive to TTX pretreatment. Collectively, our data suggest that 8 wk postrecovery from TNBS, colonic submucosal neurons display increased sensitivity to forskolin and therefore intracellular cAMP increases. Forskolin stimulation of enteric neurons increases fast EPSP amplitude (17) and decreases the magnitude of afterhyperpolarizing potentials in AH neurons (45). Although we did not measure cAMP levels in the submucosal plexus, the observed increased sensitivity to cAMP stimulation might contribute to the increased fast EPSP amplitude observed in S neurons and decreased afterhyperpolarizing potential amplitude in the guinea pig submucosal plexus 8 wk post-TNBS treatment.

Fig. 5. Comparison of cAMP- and Ca$^{2+}$-dependent secretion in postinflammatory colons and age-matched controls. Peak changes in $I_{sc}$ relative to baseline for bethanechol (BCh; 10 μM, $n = 5–7$; A) and forskolin (10 μM, $n = 6–7$; B) were measured, converted to μA/cm², and pooled. Peak changes in $I_{sc}$ in response to BCh were not significantly altered in the previously inflamed colon by TTX pretreatment in naïve preparations (A). However, forskolin responses in the previously inflamed colon were significantly increased and sensitive to TTX pretreatment compared with naïve controls (B). Data were analyzed using one-way ANOVA with Bonferroni’s post test (**P < 0.01).

Fig. 6. Quantitative comparison of enteroendocrine cell numbers in the colon of guinea pigs 8 wk following administration of TNBS versus naïve controls. A: numbers of serotonin (5-HT)-immunoreactive enterochromaffin (EC) cells were similar in the colon of TNBS-treated animals compared with the colon of naïve controls. B: numbers of glucagon-like peptide (GLP)-2-immunoreactive L cells were significantly increased in the colon of guinea pigs treated with TNBS compared with naïve controls. *P < 0.05 compared with control (means ± SE) by an unpaired, two-tailed Student’s t-test (naïve controls: $n = 7$, and TNBS: $n = 5$). C: in contrast, L cells immunoreactive for peptide YY (PYY) were not significantly different in the colon of previously inflamed animals versus naïve controls.
Our data indicate that at 8 wk, previously inflamed guinea pig colonic epithelia are functioning normally, suggesting that reduced veratridine responsiveness involves a change in neural reflex pathways. This may be due to increased sympathetic innervation of submucosal neurons 8 wk post-TNBS treatment and the subsequent blunting of veratridine-induced secretion. However, as submucosal neurons appear to be hyperexcitable 8 wk postinflammation, the significant decrease in veratridine-induced secretion may also reflect either neurotransmitter depletion of submucosal neurons or neurotransmitter receptor desensitization as a result of increased neuronal activity in the TNBS-treated colon. Although a previous study (27) has suggested that there is significant myenteric neuronal loss up to 56 days in the previously inflamed guinea pig colon, this does not appear to be the case in the submucosal plexus as neither total submucosal plexus neuron numbers (protein gene product 9.5 immunoreactive; data not shown) nor AH neuron numbers (calbindin immunoreactive) were altered in TNBS-treated guinea pigs compared with age-matched controls. A postinflammatory change in neuronal function rather than epithelial function is further supported by our observation that TTX-sensitive forskolin responses are significantly increased in TNBS-treated tissues.

**Enteroendocrine cell signaling.** In the acute model of guinea pig colitis, marked changes in 5-HT-immunoreactive EC cells, SERT expression, and mucosal release of 5-HT were observed (26). In addition, 5-HT, acting via 5-HT3 receptors, contributes to the increased fast EPSP amplitude in S-type submucosal neurons (33). As enteroendocrine cells function as mucosal sensory transducers that are important in afferent signaling within the GI tract, we quantified both the numbers of EC cells and release of 5-HT from EC cells, which release 5-HT in response to various luminal stimuli. 5-HT released from EC cells can activate nerve terminals of extrinsic and intrinsic primary afferent nerves involved in gut sensation, motility, and secretion (5, 21, 22). Colitis leads to changes to enteroendocrine cell populations during active inflammation in human patients and in animal models of IBD (8, 13, 26, 39). In addition, prolonged EC cell hyperplasia occurs in *Trichinella spiralis*-infected mice as well as in patients following the resolution of Campylobacter enteritis (43, 46). In contrast, it has been recently reported that EC cell numbers were comparable in ileal and colonic tissues taken from Crohn’s disease patients in remission with and without IBS-like symptoms compared with healthy controls (36). However, mRNA expression levels of tryptophan hydroxylase-1 were significantly higher in the colon of Crohn’s disease patients with IBS-like symptoms, suggesting increased 5-HT synthesis in these patients (36). In the present study, the numbers of 5-HT-containing EC cells in the colon of animals 8 wk following the induction of TNBS-induced colitis were comparable with controls. We did not observe any changes in mucosal 5-HT release or in the expression of SERT. The mucosal 5-HT signaling system does not appear to be affected during postinflammatory conditions following a bout of TNBS-induced colitis, whereas this system undergoes significant changes during the more acute phase of inflammation (26, 37).

In contrast, the numbers of GLP-2-containing L cells were significantly increased 8 wk following administration of TNBS into the colon. GLP-2 has a number of important physiological effects in the GI tract, including potent trophic effects on the intestinal mucosa, enhanced barrier function, and nutrient absorption (14). Furthermore, the administration of GLP-2 has also been reported to be protective in a murine model of

![Fig. 7. Mechanical stimulation of the mucosa significantly increased the amount of 5-HT release from both naïve controls and TNBS-treated animals compared with basal release. *P < 0.05 compared with basal release (means ± SE) by a paired, two-tailed Student’s t-test (naïve controls: basal, n = 6, and mechanical, n = 7; and TNBS: basal, n = 6, and mechanical, n = 7). However, under basal or mechanically stimulated conditions, amounts of colonic 5-HT release did not differ between previously inflamed guinea pigs and naïve controls.](image-url)
dextran sodium sulfate-induced colitis (11). Preliminary data have suggested that GLP-2-immunoreactive L cells are increased in the colon of guinea pigs with acute colitis (unpublished observation). Levels of the bioactive form of GLP-2 have also been reported to be significantly elevated in human patients with IBD (47). Thus, it is a possibility that inflammation leads to increased numbers of GLP-2-containing L cells, and this is associated with elevated levels of GLP-2. This may serve as an adaptive response to injury and inflammation, which persists following a bout of colitis, to facilitate the healing and regeneration of the colon.

Conclusions. A growing body of literature implicates the resetting of neuroimmune communication following a prior inflammatory episode in the development of postinflammatory GI symptoms (3, 41). The findings reported here demonstrate that prior inflammation can have profound effects on neural signaling in the enteric nervous system that persist beyond the resolution of inflammation. Interestingly, EC cell signaling appears to have recovered to normal following the resolution of inflammation after an initial increase during the acute phase of colitis (26, 37). A recent study (46) in mice has shown that intestinal infection with Trichinella spiralis resulted in an increase in EC cells in the small intestine that persisted beyond the initial inflammation. These data suggest that the nature of the immune response that causes GI inflammation may be a critical determinant of which cell types exhibit persistent alterations in their properties. In conclusion, this is the first evidence of sustained alterations in enteric neural signaling following transient GI inflammation and illustrates how functional changes persist in the absence of active inflammation. Future studies will further examine the mechanisms and functional consequences of such changes.

GRANTS

This work was supported by an operating grant from the Crohn’s and Colitis Foundation of Canada (CCFC) (to K. A. Sharkey and G. M. Mawe) and by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-62267 (to G. M. Mawe). K. A. Sharkey is an Alberta Heritage Foundation for Medical Research (AHFMR) Medical Scientist and the CCFC Chair in IBD Research at the University of Calgary, Alberta, Canada. The authors are recipients of Canadian Association of Gastroenterology/AstraZeneca/Canadian Institutes of Health Research Postdoctoral Fellowships, and J. R. O’Hara is an AHFMR Graduate Student.

REFERENCES