Alterations to enteric neural signaling underlie secretory abnormalities of the ileum in experimental colitis in the guinea pig

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1Snyder Institute of Infection, Immunity and Inflammation and Hotchkiss Brain Institute, Department of Physiology and Biophysics, University of Calgary, Calgary, Alberta, Canada; 2Department of Physiology and Internal Medicine, Virginia Commonwealth University, Richmond, Virginia; and 3Department of Anatomy and Neurobiology, University of Vermont, Burlington, Vermont

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Hons IM, Burda JE, Grider JR, Mawe GM, Sharkey KA. Alterations to enteric neural signaling underlie secretory abnormalities of the ileum in experimental colitis in the guinea pig. Am J Physiol Gastrointest Liver Physiol 296: G717–G726, 2009. First published February 12, 2009; doi:10.1152/ajpgi.90472.2008.—Inflammatory bowel diseases (IBD) can involve widespread gastrointestinal dysfunction, even in cases in which inflammation is localized to a single site. The underlying pathophysiology of dysfunction in noninflamed regions is unclear. We examined whether colitis is associated with altered electrogenic ion transport in the ileal mucosa and/or changes in the properties of ileal submucosal neurons. Colitis was induced by administration of trinitrobenzene sulfonic acid (TNBS), and the uninflamed ileum from animals was examined 3, 7, and 28 days later. Electrogenic ion transport was assessed in Ussing chambers. Intracellular microelectrode recordings were used to examine the neurophysiology of the submucosal plexus of the ileum in animals with colitis. Noncholinergic secretion was reduced by 33% in the ileum from animals 7 days after the induction of colitis. The epithelial response to vasoactive intestinal peptide (VIP) was unaltered in animals with colitis, but the response to carbachol was enhanced. Slow excitatory synaptic transmission was dramatically reduced in VIP-expressing, noncholinergic secretomotor neurons. This change was detected as early as 3 days following TNBS treatment. No changes to fast synaptic transmission or the number of VIP neurons were observed. In addition, cholinergic secretomotor neurons fired more action potentials during a given stimulus, and intrinsic primary afferent neurons had broader action potentials in animals with colitis. These findings implicate changes to enteric neural circuits as contributing factors in inflammation-induced secretory dysfunction at sites proximal to a localized inflammatory insult.

METHODS

In animals with ileitis we observed changes to the neural circuitry of the submucosal plexus of the colon that were qualitatively and quantitatively similar to those observed at the site of inflammation (45). Specifically, neurons that display a long afterhyperpolarization (AHP) (AH neurons), which are regarded as intrinsic primary afferent neurons (19), become hyperexcitable (33, 34, 38, 39). Fast synaptic input to synaptic (S) neurons is facilitated owing to the recruitment of additional presynaptic neurotransmitters (38, 39). These changes are reflected in functional alterations in the control of electrogenic secretion in the colon. To date, the enteric neural circuitry of the submucosal plexus of the ileum in animals with colitis has not been examined. However, it has been demonstrated that secretory responses to direct stimulation of the epithelium and to capsaicin are blunted over the first 7 days of ileitis, but these responses returned to normal by 30 days (41).

In the present study we tested the hypothesis that submucosal neurons of the ileum are affected by inflammation of the colon. The potential impacts of changes to submucosal neurons include alterations to secretion and absorption (15, 57, 58), as well as maintenance of the integrity of the epithelial barrier (43). We first examined whether there were secretory abnormalities in the ileum of animals with colitis. We also assessed the proportion of vasoactive intestinal peptide (VIP) neurons in the submucosal plexus and VIP release. We then went on to study the enteric neurophysiology in these animals. Our results indicate that there are marked changes to the noncholinergic secretomotor neurons and that these are accompanied by a reduction in noncholinergic secretion. Moreover, the ileal submucosal plexus is altered in response to colitis in a fundamentally different manner from that seen previously in the colon.

THE INTEGRATIVE NERVE CIRCUITRY OF THE ENTERIC NERVOUS SYSTEM provides regulatory control of the gastrointestinal (GI) tract (16). The output of the ENS is modulated by extrinsic autonomic influences from the sympathetic and parasympathetic nervous systems. When the gut is diseased or damaged, the nervous system attempts to adapt to maintain the essential digestive and defensive functions of the GI tract. However, in chronic disease, gut function is frequently compromised. Inflammatory bowel diseases (IBD), which include Crohn’s disease and ulcerative colitis, are relapsing and remitting chronic intestinal inflammatory conditions. They are the source of considerable morbidity, arising from disturbances of motility and secretion (10, 20, 54). These functional alterations develop throughout the GI tract, even at sites distant from a localized region of active inflammation (2, 28, 47, 51). Abnormalities in function also occur during periods of remission from IBD, when there is resolution of inflammation (14, 27, 55). To understand how inflammation in one region of the gut gives rise to functional disturbances at distant sites, we have examined the enteric neural circuitry in noninflamed regions of the gut.

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In animals with ileitis we observed changes to the neural circuitry of the submucosal plexus of the colon that were qualitatively and quantitatively similar to those observed at the site of inflammation (45). Specifically, neurons that display a long afterhyperpolarization (AHP) (AH neurons), which are regarded as intrinsic primary afferent neurons (19), become hyperexcitable (33, 34, 38, 39). Fast synaptic input to synaptic (S) neurons is facilitated owing to the recruitment of additional presynaptic neurotransmitters (38, 39). These changes are reflected in functional alterations in the control of electrogenic secretion in the colon. To date, the enteric neural circuitry of the submucosal plexus of the ileum in animals with colitis has not been examined. However, it has been demonstrated that secretory responses to direct stimulation of the epithelium and to capsaicin are blunted over the first 7 days of ileitis, but these responses returned to normal by 30 days (41).

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METHODS

Animals. The methods used in this study were approved by the University of Calgary Animal Care Committee and conform to the...
guidelines of the Canadian Council on Animal Care. Male albino guinea pigs (250–350 g, Charles River, Montreal, QC, Canada) were administered intracolonic trinitrobenzene sulfonic acid (TNBS, 0.3–0.5 ml, 20–25 mg/ml in 25% ethanol, Sigma-Aldrich, St. Louis, MO) or saline under halothane anesthetic as described elsewhere (34). Briefly, TNBS or saline were given via enema through a flexible polyethylene catheter inserted rectally 7 cm proximal to the anus. At 3, 7, or 28 days later animals were exsanguinated under deep halothane anesthesia and the distal ileum (10 cm proximal to the ileocecal junction) and distal colon were removed. Body weight change and macroscopic damage score of the colon and ileum were used to assess the severity of colitis as previously described (40).

Measurement of electrogenic ion transport. Segments of ileum 10 cm proximal to the ileocecal junctions were maintained in oxygenated Krebs solution (in mM: 117 NaCl, 4.8 KCl, 2.5 CaCl2, 1.2 MgCl2, 25 NaHCO3, 1.2 Na2HPO4, and 11 d-glucose; aerated with 95% O2-5% CO2) containing nicardipine (3 μM, Sigma-Aldrich) and scopolamine (1 μM, Sigma-Aldrich). The submucosa was dissected from a segment of ileum as previously described (35, 38). Submucosal preparations were transferred to Silastic elastomer-lined recording chamber and superfused with oxygenated Krebs preheated to 33–34°C.

Neurophysiological characterization of submucosal neurons. Neurons were impaled with microelectrodes fabricated from 1-mm outer-diameter borosilicate glass (World Precision Instruments) and filled with 1% biocytin in 1 M KCl. Electrode resistances were 90–150 MΩ. Recordings were made with a Multiclamp 700A amplifier in current clamp mode (Axon Instruments, Molecular Devices, Sunnyvale, CA). Recordings were digitized at 5–50 kHz and stored and analyzed by using PC-based data acquisition and analysis software (pClamp 9.2 suite, Axon Instruments). Following a 10-min stabilization period after impalement, passive and active electrical properties were evaluated, as well as synaptic events evoked by focal stimulation of adjacent ganglia. Only neurons that fired action potentials of greater than 40 mV in amplitude, which overshoot 0 mV and had a resting membrane potential more negative than −40 mV, were included in the electrophysiological analyses.

Excitability was measured by injecting 500-ms depolarizing and hyperpolarizing current pulses to reveal input resistance and the number of action potentials at threshold (rhoeobase), twice rhoeobase, and the maximum number of action potentials each neuron fired during a 500-ms depolarizing step. Synaptic inputs were stimulated by using a concentric bipolar stimulating electrode positioned on interganglionic nerve bundles. Stimulus pulses of 0.5-ms duration and 5–15-V intensity were delivered via a Grass SD9 (Grass Medical Instruments, Quincy, MA) stimulator. For the measurement of fast excitatory postsynaptic potentials (EPSPs), cells were hyperpolarized to −80 mV to inhibit action potential firing. The amplitude of three fast EPSPs were averaged for each neuron under control conditions and in inflamed preparations, and after superfusion with hexamethonium (100 μM, Sigma-Aldrich) to examine noncholinergic neurotransmission. To elicit slow synaptic input, 1-s trains of 20-Hz 5- to 15-V stimulation were applied to interganglionic nerve bundles. Slow EPSPs were evoked from the resting membrane potential.

To examine neurotransmitter release properties, two pairs of fiber tract stimuli were applied 50 ms apart to generate two fast EPSPs. Fast EPSPs were elicited at a −80 mV membrane potential to inhibit action potentials. The paired-pulse ratio (PPR) was determined as the ratio of the maximum amplitude of the second fast EPSP to the maximum amplitude of the first. Paired-pulsed facilitation was determined to occur if the PPR was greater than one. Paired-pulse depression occurred if the PPR was less than one.

Visualization and classification of recorded neurons. At the end of each recording, depolarizing pulses from the intracellular electrode were delivered to the neuron to ensure complete filling of the neuron with biocytin. For post hoc identification of neurons the shape of the ganglion was drawn and the location in the preparation recorded. At the end of each experiment the tissue was fixed in Zamboni’s fixative (0.1 M PBS containing 2% formaldehyde plus 0.2% picric acid) overnight at 4°C. Following fixation the tissue was washed in PBS. For biocytin visualization, the tissue was washed three times in PBS containing 0.1% Triton X-100 (3 × 10 min) followed by incubation with FITC-conjugated avidin for 2 h at room temperature (1:100, Jackson ImmunoResearch, West Grove, PA) (35). Neurons were

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classified as AH or S according to well-established criteria (59). AH neurons must possess the characteristic action potential shape, as well as a prolonged AHP. In addition, AH neurons possess a multiaxial Dogiel type II morphology. S neurons have narrow action potential shapes, receive fast EPSPs, and have a uniaxial Dogiel type I morphology. S neurons can further be divided into choline acetyltransferase (ChAT)-expressing S neurons (S-ChAT) or VIP-expressing S neurons (S-VIP) by electrophysiological criteria. S-VIP neurons receive slow inhibitory postsynaptic potentials (IPSPs), whereas S-ChAT neurons receive no inhibitory input (8). However, to confirm this we also performed immunohistochemistry on filled neurons.

**Immunohistochemistry.** To determine the proportion of VIP-immunoreactive neurons in the ileal submucosal plexus and to examine biocytin-filled preparations, we performed immunohistochemistry with a mouse anti-VIP antibody (1:500, UBC Regulatory Peptide Group, Vancouver, BC, Canada). Submucosal preparations were also double labeled with a neuronal nuclear marker (1:1,000, Fos4, Santa Cruz Biotechnology, Santa Cruz, CA) (46). Segments of ileum, 10 cm from the ileocecal junction, from control and TNBS-treated animals (7 days) were placed in a Sylgard-lined dissecting dish, opened along the mesenteric border, and pinned tautly with the mucosa facing up. Tissue was fixed overnight in Zamboni’s fixative at 4°C. Fixative was removed by three 10-min washes in PBS. Ileal segments were dissected to yield preparations of submucosal plexus. Preparations were incubated in the primary antiserum for 48 h at 4°C. Following incubation in primary antisera, immunoreactivity was detected following incubation of preparations for 2 h at room temperature in donkey anti-mouse IgG-CY3 (1:100; Biocan Scientific, Mississauga, ON, Canada) and anti-rabbit IgG-FITC (1:50; Biocan Scientific). Following washes in PBS, the preparations were mounted in bicarbonate-buffered glycerol at pH 8.6. The whole-mount preparations or preparations containing filled neurons were visualized with a Zeiss Axioplan fluorescence microscope (Zeiss Instruments, Toronto, ON, Canada) and photographed with a Sensys digital camera (Sensys, Photometrics, Tucson, AZ).

**Statistics.** Statistical analyses and data plotting were carried out by use of Prism 3 (Graphpad Software, San Diego, CA). Differences between neurons from normal and inflamed animals were compared by using unpaired two-tailed Student’s t-tests, a one-way ANOVA with a Dunnett post hoc test, or a two-way ANOVA with the Bonferroni posttest to compare values to controls. Linear regression analysis and two-way ANOVA were utilized to compare current-frequency relationships. A P value of <0.05 was considered statistically significant.

**RESULTS**

Macroscopic characterization of the ileum and colon after the induction of colitis. As previously reported (39), guinea pigs treated with TNBS lost weight over the first 3–4 days, whereas control guinea pigs maintained or increased body weight (data not shown). At 3, 7, and 28 days following induction of inflammation significant macroscopic damage was observed in the colon compared with saline-treated controls. The macroscopic damage scores of the colon were as follows: control, 0.33 ± 0.03, n = 45; 3-day colitis, 7.0 ± 0.54, n = 34; 7-day colitis, 6.12 ± 0.43, n = 49; 28-day colitis, 2.37 ± 0.29, n = 20. At all time points following TNBS administration to the colon, the appearance and macroscopic damage score of the ileum from treated animals was indistinguishable from that of normal ileal tissue. Damage scores were as follows: control, 0.23 ± 0.1, n = 25; 3-day colitis, 0.23 ± 0.1, n = 17; 7-day colitis, 0.24 ± 0.1, n = 30; 28-day colitis, 0.23 ± 0.1, n = 12.

Short-circuit current measurements from the ileum of animals with colitis. We investigated whether there were secretory abnormalities in the ileum of animals with colitis in the absence of any morphological alterations in the tissue. Preparations of mucosa-submucosa containing the submucosal plexus from the terminal ileum of inflamed animals were mounted in Ussing chambers to examine electrogenic ion transport. Baseline $I_{SC}$ and conductance values in the ileum of animals treated 7 days previously to induce colitis were similar to those of controls (Table 1). Baseline secretion was significantly reduced 3 days following TNBS treatment, without a change in conductance (Table 1). The maintenance of tissue resistance suggests that epithelial integrity was unaltered in the ileum of animals with colitis. The Na+ channel activator veratridine (10 μM) was applied to the serosal side of the tissue to elicit neurogenic secretion, measured as an increase in $I_{SC}$, as previously described (26). Veratridine caused a rapid biphasic increase in the $I_{SC}$ that was abolished in the presence of TTX (data not shown). The total magnitude of the veratridine-stimulated response was unchanged in animals 7 days after TNBS treatment (control, 64.9 ± 11.3 μA/cm², n = 9; 7-day TNBS, 60.0 ± 8.0 μA/cm², n = 9, P > 0.05). To confirm that the relative contributions of cholinergic and noncholinergic components of neurally mediated responses were unchanged, veratridine stimulation was repeated in the presence of atropine (1 μM) to block the cholinergic component of the $I_{SC}$. Atropine had no effect on baseline secretion from the ileum in any group of animals, but it significantly reduced the veratridine-stimulated response, confirming the inhibition of the cholinergic component of $I_{SC}$ evoked by veratridine. In the ileum of animals treated with TNBS 7 days previously, the magnitude of this reduction in secretion was significantly greater than that observed in controls (Fig. 1). This effect was not observed 3 days following TNBS treatment (Fig. 1).

The major noncholinergic neurotransmitter for mucosal secretion is VIP (31, 48). We hypothesized that the reduced atropine-resistant component of secretion in TNBS-treated animals was due to blunting of VIP signaling. To test this, we added VIP (30 nM) to the serosal side of our preparations in the presence of TTX (300 nM) to block neuronal transmission. VIP responses of the epithelium were virtually identical between TNBS-treated and untreated animals (Fig. 1), indicating that we were not observing a blunted epithelial responsiveness to VIP. Furthermore, the response to the adenylate cyclase activator forskolin (10 μM) was also unchanged between groups (control, $\Delta I_{SC}$ 32.6 ± 3.8 μA/cm², n = 7; 3 days, $\Delta I_{SC}$ 40.7 ± 4.9 μA/cm², n = 9; 7 days, $\Delta I_{SC}$ 31.3 ± 6.9 μA/cm², n = 7, P > 0.05).

Carbachol elicits a biphasic response when added to the serosal chamber, a rapid transient peak, followed by a slowly
developing sustained increase in $I_{SC}$ (48). The fast peak of carbachol-evoked secretion was significantly increased 7 days following TNBS treatment (Fig. 1). There was a trend for the slow component of carbachol secretion to be increased (control, $I_{SC}$ 120.8 ± 13.2 μA/cm², n = 7; 3 days, $I_{SC}$ 166.3 ± 15.5 μA/cm², n = 6; 7 days, $I_{SC}$ 150.8 ± 13.2 μA/cm², n = 7, $P > 0.05$), but this did not reach statistical significance. The area under the curve was also not significantly changed following inflammation.

Quantification of VIP-immunoreactive neurons in the submucosal plexus. Another possible explanation for the reduced noncholinergic secretory response is that the number of VIP neurons in the submucosal plexus was reduced. Therefore, we counted the number of VIP-expressing neurons in the submucosal plexus of the ileum to address this possibility. At 7 days following TNBS treatment, the number of VIP-expressing neurons was unchanged (Fig. 2). Furthermore, the average number of neuronal nuclei was also unchanged in animals with colitis, indicating no generalized loss of ileal submucosal neurons.

Fig. 1. Effect on inflammation on neurally evoked secretion and changes to the epithelial responsiveness to secretagogues. A: noncholinergic secretion elicited by veratridine (10 μM) in the presence of atropine (1 μM) in tissue from control animals, and animals treated 3 and 7 days previously with trinitrobenzene sulfonic acid (TNBS). B: representative traces of veratridine response in control (solid) and 7-day inflamed animals (shaded) in the presence of atropine. Scale bars: 10 μA, 50 s. C: response to vasoactive intestinal peptide (VIP, 30 nM) and the fast peak response to carbachol (CCH, 1 μM) in the presence of tetrodotoxin (TTX, 300 nM). Inset: representative trace of change in short-circuit current ($\Delta I_{SC}$) following CCH stimulation. Scale bars: 20 μA, 50 s. *$P < 0.05$.

Fig. 2. Quantification of the proportion of VIP-expressing neurons in the submucosal plexus of the ileum. A: absolute numbers of neuronal nuclei per ganglion and proportion of VIP positive neurons are unchanged 7 days following TNBS administration. B: representative submucosal VIP staining from the ileum of an inflamed animal. C: neuronal nuclei in the same ganglion. Arrows indicate VIP-expressing neurons, and arrowheads indicate VIP-negative neurons. Scale bars: 50 μm.
Quantification of VIP release from the ileum of animals with colitis. The RIA employed in this study was able to reliably detect VIP peptide released from the ileal mucosa-submucosa preparations of the guinea pig. However, VIP release between individual preparations was highly variable. There were no significant differences in baseline VIP release between control and TNBS-treated animals (control, 59.3 ± 4.9 pg/g wet wt⁻¹·min⁻¹, n = 5; TNBS, 49.6 ± 2.1 pg/g wet wt⁻¹·min⁻¹, n = 5, P > 0.05). Ethanol treatment (0.1% in Krebs) had an inhibitory effect on the rate of VIP release from the tissue that was similar in control and TNBS-treated animals (control, 23.8 ± 8.7% reduction; TNBS, 13.3 ± 9.5% reduction); in contrast, veratridine caused an increase in the rate of VIP release of 38.6 ± 17.4% for controls and 15.3 ± 15.4 for TNBS-treated animals. This trend of reduced VIP secretion from inflamed animals did not reach statistical significance (P > 0.05).

Neurophysiological characteristics of submucosal S neurons. Having identified an abnormality involving the neural control of secretion, in the absence of a corresponding defect in epithelial responsiveness, we investigated the neurophysiological properties of the submucosal plexus. We focused primarily on enteric S neurons, since these have been established as the secretomotor neurons of the guinea pig ileum (18). In S neurons, input resistance, resting membrane potential, rheobase, and the number and width of action potentials were largely unchanged in TNBS-treated animals (Table 2). There was a trend (P = 0.06) toward a reduced rheobase for action potential generation in S neurons 3 days following TNBS treatment (Table 2). The maximum duration a neuron could fire action potentials was significantly prolonged 3 days after the induction of colitis, but it returned to the normal level by 7 days. The duration of firing was 215.7 ± 25.3 ms (n = 43) in controls, which increased to 334.9 ± 48.5 ms (n = 14) at 3 days following TNBS (P < 0.05) and fell to 216.4 ± 38.5 ms (n = 17) at 7 days and 309.2 ± 63.4 ms (n = 9) at 28 days after TNBS treatment. When we examined the two populations of S-ChAT and S-VIP neurons separately, the basal electrical properties were comparable between the two groups with one exception. In S-VIP neurons, the number of action potentials fired at rheobase was increased over that observed in controls compared with 7 days following TNBS treatment (control, 1.3 ± 0.2 action potentials, n = 23 vs. TNBS, 2.5 ± 0.6, n = 13; P < 0.05). This effect was not observed at earlier (3-day) and or later (28-day) time points (data not shown).

As noted above, colitis caused changes in the S neurons in the ileum. For the most part none of these persisted as colitis resolved (28 days). However, at the 28-day time point, a reduction in excitability, as determined by the reduction in the relationship between current injection and action potential generation, was detected (Fig. 3).

Changes in synaptic inputs to S neurons of the ileum in animals with colitis. S neurons receive both fast and slow synaptic inputs (18). All S neurons retained the capacity to receive fast EPSPs after inflammation. The EPSP amplitudes of the pooled population were 19.1 ± 2.8 mV, n = 8 at 3 days, 18.7 ± 2.7 mV, n = 21 at 7 days, and 16.8 ± 4.8 mV, n = 5 at 28 days following TNBS. These were not different from controls (21.4 ± 1.7 mV, n = 34). After administration of hexamethonium, responses were typically abolished in preparations from control and inflamed animals. A subpopulation (control, 41.2%; 3 days, 37.5%; 7 days, 66.7%, 28 days, 40%) of neurons remained which received small hexamethonium-resistant EPSPs. Neither the size of this subpopulation nor the size of the hexamethonium-resistant EPSP differed significantly between tissues from control and TNBS-treated animals (amplitude of hexamethonium resistant EPSP: control, 9.3 ± 1.6 mV, n = 14; 3 days, 7.7 ± 2.9 mV, n = 3; 7 days, 5.6 ± 1.1 mV, n = 13; 28 days, 14.9 ± 11.2 mV, n = 2; P > 0.05). Thus the changes in the neurochemical properties of fast synaptic transmission seen the colon in colitis and in ileitis were not observed in the present study (38, 39, 45). We utilized the paired-pulse protocol described above, to examine vesicular handling in presynaptic neurons of the submucosal plexus. No change in the PPR was detected 3 or 7 days following TNBS treatment (Fig. 4). However, when we examined the

Table 2. Summary of basal and stimulated electrophysiological characteristics of S neurons

<table>
<thead>
<tr>
<th></th>
<th>Rₚ, mV</th>
<th>RMP, mV</th>
<th>Rheobase, pA</th>
<th>APs at Rheobase</th>
<th>APs at 2× Rheobase</th>
<th>APₚₜₜ, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 42–47)</td>
<td>240±17</td>
<td>−50.3±1.5</td>
<td>91.9±11.7</td>
<td>1.9±0.4</td>
<td>5.5±0.9</td>
<td>1.2±0.1</td>
</tr>
<tr>
<td>3-day TNBS (n = 14)</td>
<td>255±28</td>
<td>−47.6±1.1</td>
<td>50.0±5.9</td>
<td>1.6±0.4</td>
<td>7.6±1.7</td>
<td>1.3±0.1</td>
</tr>
<tr>
<td>7-day TNBS (n = 18)</td>
<td>336±58</td>
<td>−48.6±1.7</td>
<td>73.6±12.0</td>
<td>2.7±0.8</td>
<td>5.3±1.0</td>
<td>1.3±0.1</td>
</tr>
<tr>
<td>28-day TNBS (n = 9)</td>
<td>261±61</td>
<td>−54.6±2.7</td>
<td>97.2±32.9</td>
<td>2.0±0.4</td>
<td>5.9±0.9</td>
<td>1.3±0.04</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. Data from S neurons of saline control animals and animals 3, 7, and 28 days following TNBS treatment. Rₚ, input resistance; RMP, resting membrane potential; rheobase, minimum current injection required to elicit an action potential; APs, action potentials; APₚₜₜ, duration (width) of the AP at 50% amplitude.
This pathway (19). We studied the electrical properties of AH synaptic inputs to S neurons, which are the effector neurons in from submucosal S neurons. They provide a major source of stimuli and in turn initiate secretory and vasodilatory responses. AH neurons in the submucosal plexus respond to luminal stimuli and this pathway of neurotransmission at the later time point (28 days following the induction of colitis), the PPR was significantly increased compared with control animals (Fig. 4). The amplitude of the two EPSPs are nearly equal at control synapses (PPR = 0.93 ± 0.1, n = 5). At 28 days, a paired-pulse facilitation was detected (1.37 ± 0.08, n = 3; P < 0.05).

In contrast to the modest changes observed in fast synaptic transmission, slow synaptic transmission to S neurons was altered as early as 3 days after the induction of inflammation, and this persisted to 28 days after the induction of colitis. Data from all S neurons indicated a reduction in the evoked slow EPSP. When data from S-VIP neurons were analyzed separately this reduction was significant at the 7-day time point (Fig. 4). In contrast, there were no changes in the magnitude of the slow EPSP in S-ChAT neurons (data not shown). In the S-VIP cells, the slow EPSP was reduced from a control amplitude of 6.6 ± 1.2 mV, n = 14, to 2.4 ± 0.9 mV, n = 3 at 3 days and 3.05 ± 0.5 mV, n = 4, at 7 days following TNBS (P < 0.05 for both groups). Interestingly, there was a trend for a long-term reduction of slow EPSP amplitudes (28 days, 1.5 mV ± 0, n = 2). The magnitude of the slow IPSP, which is a characteristic property of these neurons, was unchanged at all time points (control, −23.8 ± 1.8 mV, n = 23; 3 days, −28.1 ± 0.8 mV, n = 5; 7 days, −27.2 ± 2.5 mV, n = 11; 28 days, −31.6 ± 1.4 mV, n = 2).

Changes to AH neurons of the ileum in animals with colitis. AH neurons in the submucosal plexus respond to luminal stimuli and in turn initiate secretory and vasodilatory responses from submucosal S neurons. They provide a major source of synaptic inputs to S neurons, which are the effector neurons in this pathway (19). We studied the electrical properties of AH neurons to test the hypothesis that alterations to AH neurons underlie changes to synaptic transmission. Similar to S neurons, basal characteristics of AH neurons were unchanged following TNBS colitis (Table 3). This is in contrast to studies of AH neurons within the TNBS-inflamed colon, which consistently demonstrate increased excitability of AH neurons during and following inflammation (30, 33, 34, 37–39). Examination of the shape of the action potential in AH neurons demonstrated an increased half-width in the 7-day TNBS treatment group (control, 1.63 ± 0.16 ms, n = 7; 7-day TNBS, 2.47 ± 0.23 ms, n = 5; Fig. 5). The area under the curve of the AHP, which follows an intracellular generated action potential in these neurons, was unchanged from −11,850 ± 2,644 mV·ms, n = 5 in controls and −12,320 ± 4,012 mV·ms, n = 6, P > 0.05, 7 days following TNBS treatment.

Table 3. Summary of basal characteristics of AH neurons

<table>
<thead>
<tr>
<th>Time Point</th>
<th>RMP, mV</th>
<th>Rheobase, pA</th>
<th>APs at Rheobase</th>
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<tbody>
<tr>
<td>Control (n = 5)</td>
<td>−49.1±6.0</td>
<td>141.7±51.4</td>
<td>1.6±0.4</td>
</tr>
<tr>
<td>7-day TNBS (n = 6)</td>
<td>−50.9±3.0</td>
<td>198.7±52.5</td>
<td>1±0</td>
</tr>
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</table>

Data from AH neurons from control animals and animals 7 days following TNBS treatment (TNBS). RMP, resting membrane potential; rheobase, minimum current injection required to elicit an action potential; APs at rheobase, number of action potentials generated at rheobase.
form a major population (total neurons) that project to the epithelium and a smaller one comprising two populations: secretomotor neurons (of the neurons of the submucosal plexus are cholinergic, P<0.05) and noncholinergic nerves (12). In the guinea pig ileum, noncholinergic secretion (11). Seven days following TNBS colitis, total neurogenic secretion evoked by veratridine was unchanged from controls. This was surprising in the light of previous observations in which secretion was consistently reduced in animals with colitis (1, 6, 45, 61). Furthermore, in a study of the jejunum of animals with ileitis, secretion evoked by capsaicin and carbachol were also attenuated (41). Baseline secretion was reduced 3 days following TNBS treatment, but all other evoked responses were the same in tissues from these animals. We infer from these observations that when inflammation is distal and sufficiently distant, the induction of inflammatory mediators that are thought to be responsible for these distant effects (41, 45, 61) are either not produced or insufficient to give rise to significant changes.

Despite not seeing changes in the total evoked response, we studied the relative contribution of cholinergic and noncholinergic components of secretion. Under control conditions we observed that ~60% of the total response was cholinergic, which is similar to that observed by Cooke (11). In contrast, in animals 7 days following colitis, the cholinergic fraction was increased by ~10%. There are a number of possible explanations for these data. We examined the effect of carbachol and VIP, as well as forskolin, which directly activates epithelial cells. In the presence of TTX to block neurogenic secretion, exogenous VIP as well as forskolin generated similar increases in $I_{SC}$ between control and tissue from inflamed animals. These findings suggest that the epithelium itself is intact and that the colitis was not altering epithelial responsiveness. Interestingly, carbachol added to the serosal chamber elicited a greater increase in $I_{SC}$ in the presence of TTX, 7 days following inflammation. These data suggest that there is either some degree of upregulation of the secretory capacity of the epithelium to a stimulus that activates intracellular calcium (12, 23) or that there is enhanced expression of cholinergic receptors on the epithelium. Muscarinic M3 receptors are the major subtype of epithelial cell cholinergic receptor mediating secretion (25). Currently it is not clear whether there is altered muscarinic receptor expression in the epithelium in colitis, but cholinergic signaling is altered at the site of inflammation (53). This effect requires a certain duration of inflammation to develop since it was not present at 3 days following TNBS treatment. Importantly, the total secretion was not changed for carbachol, indicating a specific effect of inflammation on the fast component of the phasic carbachol response. Further studies are required to elucidate the mechanism behind the potentiated response of the epithelium to cholinergic stimulation.

Our observation of unchanged responsiveness of the ileal epithelium to exogenous VIP suggested that the reduced noncholinergic secretion following TNBS colitis does not originate from an epithelial defect. Thus it remains most likely that this arises from a defect of VIP release or a reduced population of VIP neurons. We examined the latter and found no reduction in the proportion of VIP neurons in the submucosal plexus after an inflammatory injury. We also detected changes to the electrical properties of enteric neurons that may account for the changes in synaptic behavior in the ENS. The changes we observed in the ileum in animals with colitis differ considerably from those previously observed in the submucosal plexus of the colon in animals with colitis and ileitis (36–38, 45).

Neural control of secretion is altered in the ileum of animals with colitis. Neurogenic secretion is regulated by cholinergic and noncholinergic nerves (12). In the guinea pig ileum, ~50% of the neurons of the submucosal plexus are cholinergic, comprising two populations: secretomotor neurons (~45% of total neurons) that project to the epithelium and a smaller population of AH (intrinsic primary afferent) neurons (~10%) that activate secretion by local release of their transmitters (13, 17, 32). Noncholinergic secretomotor neurons express VIP and form a major population (~45%) of the total submucosal neurons. In the guinea pig ileum, noncholinergic secretion accounts for 45–50% of the total secretion elicited by electrical field stimulation (11).

The alkaloid veratridine was utilized to depolarize enteric submucosal neurons and elicit neurogenic secretion. Veratri-
measuring VIP release; what we measure is the spillover into the medium bathing the tissue.

Neurophysiological properties of the submucosal plexus are altered in the ileum of animals with colitis. We found that the basal characteristics of S neurons from the submucosal plexus of the ileum are largely unchanged following colitis and are in agreement with the basal properties demonstrated by others in the submucosal plexus of the ileum and colon (38, 45). S neurons from the submucosal plexus of the ileum demonstrated a subtly altered electrophysiology during colitis. When data from all S neurons were pooled, we found that neurons fired action potentials for a greater maximum duration 3 days after TNBS treatment. One possible mechanism for this is a change to the inactivation kinetics of the voltage-gated Na$^+$ channels (56), since acutely following inflammation the neurons must be depolarized for a longer time to enter a refractory state. The impact of a longer train of action potentials in secretomotor S neurons would be a greater influx of Ca$^{2+}$ into the presynaptic bouton and potentiated neurotransmitter release. Enteric S neurons of the ileal submucosal plexus express Na$_{1.2}$ and Na$_{1.3}$ $\alpha$-subunits of voltage gated sodium channels (4). TNBS colitis (7) and inflammatory mediators such has prostaglandin E$_2$ (52) can modulate voltage-gated sodium currents in the dorsal root ganglion. The possibility that TTX-sensitive voltage-gated sodium channels expression and kinetics may be altered in S neurons of the submucosal plexus following colitis warrants further investigation.

Excitatory slow synaptic transmission is reduced in the submucosal plexus of the ileum following colitis. S neurons of the submucosal plexus receive fast and slow synaptic inputs from AH neurons and extrinsic nerve endings in the plexus (9, 35, 49). Alterations to the synapses on S neurons are expected to adversely affect gut function by either leading to excessive or reduced activation of these cells. When we examined synaptic transmission to S neurons we saw no change in fast EPSPs received by the neurons following TNBS treatment, which were comparable in amplitude to control preparations in other studies (38). This is in contrast to previous work, which demonstrated an increased amplitude and neurochemical composition of fast EPSPs following TNBS colitis (38, 39). The proportion of cholinergic and noncholinergic fast EPSPs were also unchanged following colitis. We observed a slightly higher percentage of hexamethonium-resistant EPSPs than has been seen by others in the submucosal plexus of the ileum (~27%; Ref. 42). We conclude that fast EPSPs are not affected by inflammation in the submucosal plexus of the ileum following colitis.

Recordings made at the site of colitis revealed that slow EPSPs were unchanged in submucosal S neurons (38). In the ileum of animals with colitis, slow EPSPs in S-VIP neurons were reduced in magnitude, whereas those in S-ChAT neurons remained unchanged. We have not yet determined whether this effect involves pre- and/or postsynaptic mechanisms. However, from previous studies in the submucosal plexus of the colon, this suppressed excitability is not expected to originate from a desensitization of the postsynaptic neuron (38). In addition, it is not expected that changes in synaptic transmission arise in alterations to the connectivity between neurons in the plexus. Using electron microscopy, Krauter et al. (29) did not detect changes in synaptic connections within the myenteric plexus at the site of inflammation. However, the results of Nurgali et al. (44) could be interpreted to indicate that AH neurons lose their Dogiel type II morphology and become uniaxonal. This would suggest a loss of some synaptic connections from AH neurons occurring in the myenteric plexus of the ileum following ileitis. However, we did not observe Dogiel type I neurons with the characteristics of AH neurons in the submucosal plexus of the ileum following colitis. Further supporting a lack of alteration to the network of the submucosal plexus of the ileum was the observation that there the number of VIP neurons in the submucosal plexus remained unchanged following colitis, nor was the absolute number of neurons per submucosal ganglion reduced. We therefore consider that presynaptic alterations may be underlying the changes we have observed. Interestingly, S-VIP neurons, in the face of reduced slow excitation, fired more action potentials when depolarized to rheobase. This is consistent with an adaptation to the loss of synaptic excitation. Taken together, these findings suggest that there are alterations in the presynaptic transmitter expression or release in the ileal submucosal plexus and we intend to investigate this further.

Inflammation induced alterations to the currents underlying the action potential in ileal submucosal AH neurons following colitis. Inflammation profoundly affects the AH neurons of the ENS at the site of inflammation in the case of colitis and at sites distal to inflammation such as the colon following ileitis (33, 34, 38, 39). The basal characteristics of AH neurons from the submucosal plexus of the ileum were largely unchanged following colitis and are in agreement with the basal electrophysiological properties demonstrated by others (38). AH neurons from the submucosal plexus of the ileum did not display the hyperexcitability seen in the colon (30, 34, 38, 39, 45) or of the myenteric plexus of the ileum following ileitis (44). Action potential firing threshold and frequency were unchanged, and size and duration of the AHP were comparable in saline-treated and TNBS-treated animals. However, AH neurons manifested a significant increase in the half-width of the action potential. This indicates a perturbation to the underlying currents that generate the action potential. A lack of change to the magnitude of the fast AHP indicates that the K$^+$ currents were largely unchanged, whereas the maintenance of the AHP suggests that Ca$^{2+}$ currents and mobilization of Ca$^{2+}$ from intracellular stores were also similar. A greater accumulation of Ca$^{2+}$ would generate a larger AHP and vice versa (19). Hence it is likely that Na$^+$ currents were altered in these cells either in terms of absolute channel number or altered kinetics or subunit expression. Indeed, changes to dorsal root ganglion Na$^+$ channel subunit composition and currents have been reported in response to TNBS colitis (7), suggesting that a similar phenomenon may occur in sensory neurons of the submucosal plexus. Regardless of the cause, the broadened action potential may have important physiological consequences. In the central nervous system, action potential broadening is an important form of short-term plasticity (24). If this were to occur in the GI tract, this may lead to increased local releases of transmitters, including acetylcholine. Another possible consequence is an amplification of signaling in the networks of AH neurons, leading to heightened signaling between these neurons.

Persistence of changes in the submucosal plexus of the ileum following colitis. Inflammation-induced alterations to the ENS persist following the resolution of inflammation (30, 39). To examine the possible persistence of changes in the ileal sub-
mucosal plexus we examined enteric neurons 28 days following TNBS treatment. At this time point macroscopic damage to the colon has substantially resolved. The secretory abnormalities seen in the jejunum of animals with ileitis were resolved at this time point (41). However, others have shown that secretion and changes to enteric neurons remained altered at 56 days after TNBS (30, 39). At 28 days, basal electrophysiological characteristics were still largely unchanged from control and from earlier time points of inflammation. These are similar to results found in the submucosal plexus of the colon 56 days following colitis (39). However, at 28 days there was a subtle alteration from controls. The current-frequency relationship showed a depression in the firing frequency to a given depolarizing stimulus. The consequences of this change in the submucosal plexus would be expected to be a decrease in synaptic release to a prolonged depolarization. Depression of neuronal excitability in the ENS following inflammation has not been demonstrated previously. All previous findings indicate that the only alteration to S neurons is facilitation and/or a change in neurochemical coding of synaptic transmission (29, 30, 38, 39, 45).

Studying the PPR revealed that long-term changes also occur to the presynaptic neurons within the submucosal plexus. Submucosal S neurons from control animals and in animals treated with TNBS 3 and 7 previously demonstrated a slight PPR depression. PPR depression is also the normal situation in myenteric neurones from the ileum (50) and colon (29). However, 28 days following TNBS treatment, PPR facilitation in S neurons was observed. Similar results have been shown in the myenteric plexus of the colon 7 days following TNBS colitis (29). This is an intriguing finding because it suggests that vesicle handling and release have been altered in presynaptic neurons, most likely the AH neurons of the ileal submucosal plexus. The argument has been made that this could reflect an increase in vesicle recycling (29), and indeed that may be the case in the submucosal plexus of the ileum following colitis; however, a change in the Ca\(^{2+}\) sensitivity of the vesicular release machinery cannot be excluded.

Summary and perspective. We demonstrated the impact of colitis on the submucosal plexus of the ileum. The effects that we observed are clearly different from the local inflammation-induced changes that occur in the ENS of the colon during colitis (29, 30, 34, 36, 38, 39), as well as during ileitis (45). Inflammation of the colon or of the ileum leads to characteristic increases in excitability of neurons, as well as alterations to synaptic transmission in both the submucosal and myenteric plexuses. However, in the submucosal plexus of the ileum following colitis, hyperexcitability is largely absent, whereas slow synaptic transmission is profoundly altered.

The pathophysiological consequences of alterations to the submucosal plexus of the ileum may be a reduction of nerve-mediated ileal secretion. Secretion plays an important role in the maintenance of epithelial barrier function. A compromised epithelial barrier will potentially permit the migration of pathogenic bacteria across the epithelium, and this in turn could lead to further inflammation. A reduced epithelial barrier may be one mechanism behind the relapsing nature of Crohn’s disease and ulcerative colitis. Our data indicates that inflammation in a distal region of the gut will have wide-ranging consequences on the ENS and gut function. Future studies will address the mechanisms that propagate inflammatory changes throughout the large and small bowel.

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