Colitis is associated with a loss of intestinofugal neurons

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Linden DR. Colitis is associated with a loss of intestinofugal neurons. Am J Physiol Gastrointest Liver Physiol 303: G1096–G1104, 2012. First published September 20, 2012; doi:10.1152/ajpgi.00176.2012.—Intestinofugal neurons sense and receive information regarding mechanical distension of the bowel and transmit this information to postganglionic sympathetic neurons in the prevertebral ganglia. Previous studies have demonstrated that trinitrobenzene sulfonic acid (TNBS)-induced colitis is associated with a loss of myenteric neurons that occurs within the first 12 h following the inflammatory insult. The purpose of this study was to test the hypothesis that intestinofugal neurons are among the myenteric neurons lost during TNBS-induced colitis. The retrograde tracing dye Fast Blue was used to label intestinofugal neurons, and immunohistochemical staining for the RNA-binding proteins HuC/D was used to count all myenteric neurons. Ongoing synaptic input to neurons in the guinea pig inferior mesenteric ganglion (IMG) was recorded via conventional intracellular electrophysiology. In control preparations, intestinofugal neurons account for 0.25% of myenteric neurons. In the distal colon of TNBS-treated animals, the proportion of intestinofugal neurons was reduced to 0.05% (an 80% reduction) within the region of inflammation where 20–50% of myenteric neurons were lost. Neither intestinofugal neurons specifically nor myenteric neurons were reduced in more proximal uninflamed regions. There is a reduction in the frequency of ongoing synaptic potentials in visceromotor neurons of the IMG at 12 and 24 h and 6 and 56 days after TNBS. Collectively, the results of this study suggest that intestinofugal neurons are among the myenteric neurons lost during inflammation and may be selectively targeted. Because intestinofugal neurons are a major driver of sympathetic output to the gut, the loss of intestinofugal neurons may have a profound pathophysiological significance.

MATERIALS AND METHODS

Animals. All methods used in this study were approved by the Mayo Clinic Animal Care and Use Committee. Adult Hartley guinea pigs (Charles River, Kingston, NY) of either sex, weighing 175–300 g, were housed in metal cages with soft bedding. The animals had access to food and water ad libitum and were maintained at 23–24°C on a 12:12-h light-dark cycle.

Surgical procedure. To identify intestinofugal neurons in the distal colon, the retrograde tracer dye Fast Blue (EMS Chemie) was injected in the inferior mesenteric ganglia (IMG) of guinea pigs using a surgical procedure. Animals were anesthetized with pentobarbital sodium (18–28 mg/kg ip), a laparotomy was performed, and the IMG was visualized by reflecting abdominal cavity organs on gauze moistened with sterile saline (0.9%). With the aid of a dissecting microscope, both the superior and inferior lobes of each ganglion were injected with 1–3 μl of Fast Blue (25 mg/ml in sterile H2O) using a 10-μl Hamilton syringe and a 30-gauge needle. The injection site was swabbed with a saline-soaked sterile cotton swab to remove excess label. The organs were replaced to their original positions, and the muscle and skin layers were closed separately with intermittent sutures. Each animal was allowed to recover from the surgical procedure for 5 days before induction of inflammation. This time period is sufficient to allow the tracer dye to reach the cell soma of intestinofugal neurons in the colon before induction of inflammation (9, 23). Guinea pigs typically lost 10–15% of their original weight following the surgery but regained that weight and continued with normal weight gain by day 4. One animal that continued to lose weight after 3 days was assumed to have prolonged ileus from the surgical procedure and was removed from the study. This occurred before assignment in a trinitrobenzene sulfonic acid (TNBS)/control treatment group.

Induction of inflammation. To generate inflammation in the distal colon, guinea pigs were anesthetized with isoflurane, and 0.5 ml of TNBS (25 mg/ml) in 30% ethanol was delivered in the lumen of the colon through a polyethylene catheter inserted rectally 7 cm proximal to the anus. Control animals were age and sex matched to inflamed animals and received 0.5 ml of saline. This control is deemed sufficient, since the vehicle (30% ethanol) does not cause a reduction in the number of myenteric neurons (14). In three animals that were not injected with Fast Blue, a single 0.5-ml enema of 3% acetic acid delivery of the inflammatory insult (3). The loss of myenteric neurons is indiscriminate since several major classes, identified by neurochemical coding, experience similar losses (14). Perhaps because of redundancy offered by relatively large populations of neurons that regulate motility and secretion, there seems to be little physiological consequences that can be directly attributed to the loss of these neurons (3, 14, 15, 25). Because intestinofugal neurons account for 0.25% of all myenteric neurons in the distal colon, any decrease in this population would be expected to have significant effects on the coordination of motor activities in the lower and upper regions of the gastrointestinal tract. The aim of this study was to determine if hapten-induced colitis in the guinea pig leads to a loss of intestinofugal neurons.
in distilled water was delivered to cause localized colitis. Animals were maintained in a controlled environment for 24 h after TNBS, saline, or acetic acid administration. At the time of tissue collection, animals were killed by CO₂ inhalation. The severity of colitis was assessed by macroscopic colonic damage scoring as described previously (13).

**Immunohistochemistry of whole mount preparations.** The distal colon, identified as the part of the colon between the hypogastric flexure and the anus, was removed and placed in icd PBS (0.1 M, pH 7.4). Because the majority of intestinofugal neurons are located near the mesenteric attachment (8, 9, 23), the distal colon was opened along the antimesenteric border and pinned flat by stretching the tissue to its limit. These preparations were fixed for 2–16 h at 4°C in phosphate buffer (0.1 M, pH 7.4) containing 4% paraformaldehyde. Following three washes with PBS, the mucosa, submucosa, and circular muscle of the colon were removed with forceps, exposing the myenteric plexus on the longitudinal smooth muscle.

Dissected preparations were divided into eight equal segments that were ~5–6 cm in length. Each segment was washed with PBS and incubated for 2 h at room temperature with PBS containing 0.5% Triton X-100 and 4% normal goat serum. This solution was removed, and the sections were incubated overnight at room temperature in PBS containing 4% normal goat serum, 0.5% Triton X-100, and a 1:200 dilution of mouse anti-HuC/HuD antiserum (Molecular Probes, Eugene, OR). After three 15-min washes with PBS, the tissues were incubated with a 1:300 dilution of indocarbocyanine (Cy3)-conjugated goat anti-mouse antiserum (Jackson Immunoresearch, West Grove, PA) in PBS for 2 h. Samples underwent three 15-min washes with PBS and a quick dip in water and were mounted on Superfrost precleaned slides (Fisher Scientific; Pittsburg, PA) and cover slipped with AFI antifadent (Citifluor, London, UK).

**Image analyses.** Tissues mounted on slides were examined on an Olympus BX51WI fluorescence photomicroscope. Filter sets included the following: for Cy3, excitation 545/30 nm, emission 610/75 nm; for Fast Blue, 350/50 nm excitation, 460/50 nm emission. Images were captured with an Optronics MagnaFire digital camera attached to the microscope (Goleta, CA). Images were cropped in Adobe Photoshop with minimal alteration (minor adjustments to brightness and contrast) for construction of figure sets.

In each 5- to 6-cm segment of colon, either 30 or 50 ganglia were randomly selected to be counted while under fluorescence conditions to detect Hu immunoreactivity by an observer who was blinded to the experimental conditions. Each ganglion was viewed using filter sets for Cy3 at a magnification of ×200, and Hu-immunoreactive cell bodies were counted. The filter set was changed to view Fast Blue, and the labeled neurons were counted. If the ganglion contained a Fast Blue-labeled cell, photomicrographs were taken of the ganglion using a ×10 objective and filter sets for both Cy3 and Fast Blue. The observer noted the shape of the labeled cell as either lamellar, round, broadly filamentous, slender filamentous (according to criteria presented in RESULTS), or unknown. Photomicrographs of Fast Blue-labeled cells filled sufficiently to distinguish morphological characteristics were analyzed using Adobe Photoshop to measure the size of the cell. Cells were assumed to be elliptical in shape, so using a line tool with automatic measurement of length in pixels, both the major axis (longest diameter) and minor axis (diameter perpendicular to the major axis at the center) of the labeled cells were measured and converted to micrometers. Eccentricity, a measure of deviation of an ellipse from a circle (circle = 0; parabola = 1), was calculated according to the formula \( \sqrt{1-\frac{(M^2)^2}{(m^2)^2}} \). Where \( M \) and \( m \) are the lengths (in μm) for the major and minor axis, respectively.

**Electrophysiology.** After death, a 4- to 5-cm segment of the distal colon along with the colonic mesentery, abdominal aorta, and ileal arteries were removed and placed in icd Krebs solution (in mM: 120 NaCl, 5.9 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 15.5 NaHCO₃, 1.2 NaH₂PO₄, and 11.4 glucose; aerated with 97% O₂-3% CO₂) for further dissection. The IMG was visualized by cutting the inferior mesenteric artery at the aorta and removing the aorta and vena cava. The hypogastric nerves were sectioned superior to the pelvic ganglia, and the intermesenteric nerve was sectioned just inferior to the superior mesenteric ganglion. The preparation was transferred to a two-chambered recording dish that was continuously perfused with 37°C aerated Krebs solution. The colonic segment was placed in one chamber and catheterized on the anal end with tubing attached to a calibrated in-line pressure transducer (WPI, Sarasota, FL) and a 3-ml syringe. The colonic nerves and inferior mesenteric artery were draped over the divider between the two chambers and kept moist with a small piece of absorbent paper. In the second chamber, the IMG was visualized at ×30 with a Wild dissecting microscope, individual neurons were randomly impaled with glass microelectrodes that were filled to the shoulder with 1% (wt/vol) neurobiotin (Vector Laboratories, Burlingame, CA) in 1.0 M KCl, and the remainder was filled with 3.0 M KCl and had resistances in the range of 50–150 MΩ. Visceromotor neurons were identified as cells that received ongoing synaptic input that increased during colonic distension (1.5 ml air) and responded with tonic firing action potentials in response to a depolarizing current pulse. The frequency of ongoing synaptic potentials caused by intestinofugal neurons at the resting membrane potential was determined by counting the number of potentials that occurred within a 2- to 10-s interval when the colonic segment was at a resting pressure of 1–2 cmH₂O. The voltage difference between resting membrane potential and the peak of 20 random ongoing fast synaptic potentials were selected from each recording to calculate the mean amplitude.

**Statistical analyses.** All statistical analyses were completed using GraphPad Prism software using tests described in RESULTS. For all tests, \( P \) values <0.05 were considered significant.

**RESULTS**

Hapten-induced colitis is associated with a loss of intestinofugal neurons. Following recovery from the surgical procedure to label intestinofugal neurons (5 days postsurgery), a single enema of TNBS in ethanol, administered 7 cm proximal to the anus, caused focal inflammatory damage to the distal colon by 24 h. The gross damage score of animals that received TNBS (7.3 ± 0.8; \( n = 4 \)) was significantly higher than animals that received an enema of saline (0.67 ± 0.05; \( n = 5 \); \( P < 0.01 \), \( t \)-test) and was the same as previously reported (14). In the TNBS-treated animals, ulceration, hyperemia, and edema were always present in segment number 2, which was located between 5 and 12 cm proximal to the anus in fixed, stretched tissue. In two of the four animals, ulcers extended more proximally into segment 3 (10–18 cm proximal to the anus in fixed, stretched tissue). In three of the four animals, hyperemia extended between segments 1 and 3 (the anus to 18 cm proximal to the anus in fixed, stretched tissue). In all animals, edema extended into segment 4 (15–24 cm proximal to the anus in fixed, stretched tissue). In all animals, segments 5–8 (~23 cm proximal to the anus, in fixed, stretched tissue, extending to the hypogastric flexure) showed no signs of inflammation (ulceration, hyperemia, or edema).

Injection of Fast Blue into the IMG effectively labeled intestinofugal neurons in the colon (Fig. 1) similar to previous descriptions (9, 16, 23). Within each of eight equal segments that spanned the length of the distal colon, in both inflamed (\( n = 4 \)) and control tissue preparations (\( n = 5 \)), either 30 or 50 ganglia were randomly selected to count labeled intestinofugal neurons. From a total of 2,510 observed ganglia that contained a total of 229,975 neurons, 570 Fast Blue-labeled neurons were
counted in this study. The total number of Fast Blue-labeled cells, HuC/D-immunoreactive cells, and ganglia observed in each distinct preparation are presented in Table 1.

To determine whether there were inflammation-induced changes in the population of intestinofugal neurons, Fast Blue-labeled cells were quantified in two ways. First, the numbers of labeled cells were expressed as a proportion of the total number of cells within each segment (Fig. 2). In saline-treated animals, there were similar proportions of total neurons (Hu-immunoreactive) that were labeled with Fast Blue, ~0.25%, along the length of the distal colon. In contrast, tissue within segments 2 and 3 from TNBS-treated animals (4 ± 1%, n = 4; 5 ± 3%, n = 4; 12 ± 1%, n = 4, respectively) compared with control animals (20 ± 2%, n = 5; 20 ± 2%, n = 5; 22 ± 3%, n = 5, respectively) (P < 0.05; 2-way ANOVA with Bonferroni posttest).

The mean number of neurons (Hu-immunoreactive) per ganglion within each of the eight segments was also calculated for each animal (Fig. 4). In control preparations, the mean number of neurons per ganglion, which was 95, did not vary between segments except the most distal segment, which contained significantly more neurons per ganglion (109 ± 3; n = 5) than the other segments (for example, segment 2 contained 94 ± 3 neurons/ganglion; n = 5) (P < 0.05; 2-way ANOVA with Bonferroni posttest). In animals treated with TNBS, there was a significant reduction in the number of neurons per ganglion in each of segments 1 through 4 compared with similar regions in control preparations (P < 0.05; 2-way ANOVA with Bonferroni posttest). For example, seg-

Fig. 1. Fast Blue injected in the inferior mesenteric ganglion-labeled intestinofugal neurons in the distal colon. Micrographs in A–H are representative of the raw data of Fast Blue label used to analyze intestinofugal neurons in the present study. Micrographs in panels A’–H’ are matching fields of view with Hu immunoreactivity to illustrate the data used to count myenteric neurons. A: Fast Blue label in an intestinofugal neuron with a lamellar morphology. B and C: Fast Blue label in intestinofugal neurons with a round or square morphology. D and E: Fast Blue label in intestinofugal neurons with a slender filamentous morphology. F and G: Fast Blue label in intestinofugal neurons with a broad filamentous morphology. H: Fast Blue label in an intestinofugal neuron that was not filled sufficiently to define the morphology. I: the distribution of major and minor axis lengths of 174 neurons from control preparations in histograms with 5-μm bin widths. The mean and SE values of the neurons are illustrated. J: the distribution of the no. of intestinofugal neurons/ganglion for 1,390 ganglia observed in control preparations. This distribution is significantly different from the expected Poisson distribution given the mean occurrence of 0.2532 labeled cells/ganglion (P < 0.05, Chi square).
ment 3 in control preparations contained a mean of 95 ± 3 neurons/ganglion (n = 5), whereas the same segment in inflamed preparations contained 77 ± 3 neurons/ganglion (n = 4). The loss of neurons (~20–25% in segments 2 and 3) is consistent with previous findings (14).

The morphology of intestinofugal neurons was unaltered by colitis. Although the cell bodies of all of the labeled neurons were clearly evident, in many cells, there was little to no visible staining of the neural processes (Fig. 1H), making the determination of the morphological class of all cells difficult. Of the 570 counted intestinofugal neurons, 298 cells were labeled clearly to perform morphological analysis. Labeled cells within the proximal segments (5–8) of the distal colon tended to have more complete Fast Blue staining, since 122 of the 162 counted cells (75%) were filled sufficiently to define a morphological class. Likewise, in segments 5–8 of inflamed preparations, 106 of the 161 counted cells (66%) had neural processes filled. In the four distal segments (1–4), 52 of 190 counted cells (27%) in control preparations and 18 of 57 counted cells (32%) in inflamed preparations were filled sufficiently to examine the morphology.

Cells were subjectively classified into one of the following four categories: “lamellar” (Fig. 1A), “round” or “square” cell with smooth soma and multiple long processes (Fig. 1, B and C), “slender filamentous” with major irregularly shaped yet slender neurites oriented in the direction of the major axis (Fig. 1, D and E), or “broad filamentous” with several prominent neurites in the direction of the major axis but also one to four neurites in the direction of the minor axis (Fig. 1, F and G).

Table 1. Raw counts of Fast Blue-labeled and HuC/D-immunoreactive cells in the distal colon of the guinea pig in control and inflamed preparations

<table>
<thead>
<tr>
<th>Animal</th>
<th>Segment No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
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<tbody>
<tr>
<td>C1</td>
<td>Fast Blue</td>
<td>9</td>
<td>6</td>
<td>5</td>
<td>9</td>
<td>8</td>
<td>6</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>HuC/D</td>
<td>3,328</td>
<td>3,271</td>
<td>3,484</td>
<td>3,905</td>
<td>2,724</td>
<td>2,634</td>
<td>2,616</td>
<td>2,745</td>
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<tr>
<td></td>
<td>Ganglia</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
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<td>30</td>
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<td>30</td>
</tr>
<tr>
<td>C1</td>
<td>Fast Blue</td>
<td>9</td>
<td>8</td>
<td>7</td>
<td>9</td>
<td>7</td>
<td>8</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>C2</td>
<td>HuC/D</td>
<td>3,333</td>
<td>3,279</td>
<td>3,279</td>
<td>3,685</td>
<td>2,947</td>
<td>2,705</td>
<td>2,814</td>
<td>2,678</td>
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<tr>
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<td>30</td>
<td>30</td>
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<td>30</td>
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<tr>
<td>C3</td>
<td>Fast Blue</td>
<td>9</td>
<td>7</td>
<td>7</td>
<td>11</td>
<td>10</td>
<td>9</td>
<td>6</td>
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</tr>
<tr>
<td>C4</td>
<td>HuC/D</td>
<td>5,740</td>
<td>4,671</td>
<td>5,144</td>
<td>5,030</td>
<td>5,075</td>
<td>4,638</td>
<td>4,704</td>
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<tr>
<td></td>
<td>Ganglia</td>
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<td>50</td>
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<td>50</td>
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<tr>
<td>C5</td>
<td>Fast Blue</td>
<td>14</td>
<td>13</td>
<td>19</td>
<td>12</td>
<td>16</td>
<td>15</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>HuC/D</td>
<td>2,666</td>
<td>2,101</td>
<td>2,103</td>
<td>2,321</td>
<td>2,928</td>
<td>2,751</td>
<td>2,948</td>
<td>2,906</td>
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<tr>
<td>T2</td>
<td>Fast Blue</td>
<td>7</td>
<td>1</td>
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<td>10</td>
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<tr>
<td>T3</td>
<td>HuC/D</td>
<td>2,758</td>
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<td>2,414</td>
<td>2,506</td>
<td>2,696</td>
<td>2,543</td>
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<tr>
<td>T4</td>
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</table>
| Data are the total counts of Fast Blue-labeled cells and HuC/D-immunoreactive cells as well as the no. of ganglia observed for each distinct preparation. No statistical analyses were performed on these raw counts.

The distribution and morphology of these neurons are delineated in Table 2. There were no differences in the proportions of these classes of neurons (P > 0.05; Chi square) nor in the major or minor axis lengths or eccentricity (P > 0.05; 2-way ANOVA) between the distal and proximal segments of the colon in control preparations. There were no apparent boundaries in the distributions of major and minor axis lengths (distribution of neurons from control preparations illustrated in Fig. 1I) or eccentricity (data not shown) that could be used to objectively classify the morphology category used in this study. Of the Fast Blue-labeled cells that remained in the inflamed region and that were sufficiently filled (n = 5), all of the four morphology categories were observed, and the morphological characteristics and frequency distribution were similar (although not statistically analyzed due to a lack of power) to both the noninflamed more proximal segments of the same preparations and the distal segments of control-treated animals.
Previous studies have reported that intestinofugal neurons are distributed nonrandomly within the myenteric plexus, with the majority being close to the mesenteric border (9, 23). The nonrandom distribution of intestinofugal neurons was confirmed in the present study. Of the 1,390 ganglia observed in control preparations, there were 52 ganglia that contained two or more retrogradely labeled neurons. One observed ganglion with 39 neurons contained five labeled cells. Using the observed mean occurrence of 0.2532 labeled cells/ganglion, an expected Poisson (random) distribution of labeled neurons per ganglion was calculated and differed significantly from the observed distribution (Fig. 1J) (P < 0.05; Chi square).

Acetic acid-induced colitis does not alter the number of myenteric neurons per ganglion. Previous studies have demonstrated that the vehicle used to deliver TNBS in an enema (30% ethanol) does not cause a reduction in the number of myenteric neurons compared with naïve animals (14) despite the presence of hyperemia and edema. Perhaps the difference between TNBS and vehicle treatment is due to a milder inflammation associated with ethanol. Another model of colitis, that which occurs following a single enema of 3% acetic acid (TNBS) administration (filled bars; n = 4). While the proportion of intestinofugal neurons was equal between segments in control preparations, there was a significant decrease in the proportion of intestinofugal neurons in segments 2 and 3 in inflamed preparations. These regions correspond to areas of the highest levels of inflammatory damage (see text). In segments 5–8, there were no differences between control and inflamed preparations. (*P < 0.05 compared with control preparation; 2-way ANOVA with Bonferroni posttest).

TNBS (72 ± 3; n = 4) (ANOVA with Newman-Keuls multiple-comparisons test). Unlike TNBS-treated animals (14), there was little evidence for granulocyte infiltration of myenteric ganglia in acetic acid-treated animals (Fig. 5).

Hapten-induced colitis is associated with a reduction in the frequency of ongoing synaptic potentials in IMG neurons. Intestinofugal neurons provide a rich innervation of postganglionic sympathetic neurons in PVG, and activity of these neurons can be observed experimentally during intracellular electrophysiological recordings of PVG neurons (6). There were time-dependent changes in the frequency of ongoing synaptic potentials in IMG visceromotor neurons following induction of colitis with TNBS (Fig. 6). The frequency was significantly lower in neurons recorded from animals 12 h (4 ± 1 Hz), 24 h (5 ± 1 Hz), 6 days (5 ± 1 Hz), and 56 days (5 ± 2 Hz) after TNBS administration compared with neurons recorded from control animals (11 ± 1 Hz; P < 0.05, ANOVA with Newman-Keuls multiple-comparisons test). There was no difference in frequency between neurons recorded from guinea pigs 6 h (11 ± 1 Hz) after TNBS and neurons from control preparations (P > 0.05, ANOVA with Newman-Keuls multiple-comparisons test). In contrast to the TNBS model of colitis, the frequency of ongoing synaptic potentials in visceromotor neurons was unchanged 24 h following acetic acid administration (9 ± 1 Hz) compared with visceromotor neurons from control animals (9 ± 1 Hz; *P > 0.05, t-test) (Fig. 6). There were no differences in the mean amplitude of ongoing synaptic potentials or the resting membrane potential or membrane resistance of the postsynaptic neurons between groups (Table 3).
loss of a specific class of myenteric neurons (14). Here I show that the physiologically significant class of intestinofugal neurons is among the myenteric neurons lost during colitis and may be selectively targeted for removal.

Intestinofugal neurons are a unique class of neuron in which the soma is located in the intestinal musculature and the axon projects outside the gut (26). They are slowly adapting mechanosensory neurons (28) that respond to circular muscle stretch but not tension (1, 19) and thus function as volume sensors. Intestinofugal neurons receive cholinergic synaptic input encoding mechanosensory information from other myenteric neurons and thus act as second-order afferent neurons, but at least a subclass of these cells functions as primary afferent neurons, since some responses to stretch persist following blockade of synaptic transmission (4, 19, 24).

Despite their physiological significance, intestinofugal neurons are relatively rare in the myenteric plexus. The current study found that the mean proportion of myenteric neurons that were intestinofugal neurons was $\sim 0.25\%$ or roughly one out of every 400 myenteric neurons. This finding is consistent with previous reports (9). The number and sizes of intestinofugal neurons in the present study were similar to previous reports (8, 9, 23), with the exception that the size and number of round cells, assumed to be Dogiel type II cells, were smaller than one previous report (8). This difference may be accounted for by the differences in the retrograde tracing dye (DiI vs. Fast Blue) or the criteria used to define the soma-neurite boundary. Statistical analysis of the distribution of labeled neurons showed that, while the majority of intestinofugal neurons existed singly, they also tended to cluster more than would be expected by random distribution. Intestinofugal neurons tend to be located closer to the mesenteric attachment (9, 23), further supporting the notion that their distribution is not random. The observation that the more proximal regions of colon contained more cells with filled processes may reflect the fact that the colonic nerves that innervate these regions are longer and it may take more time to fill these cells. Labeled cells tend to lose their dye completely after 10 days (9). It is possible that, at the 6-day time point when the present obser-

![Graph illustrating the mean ± SE no. of myenteric neurons (Hu-immunoreactive) in each of 8 evenly distributed segments of distal colon between the rectum and hypogastric flexure of control preparations (open bars; $n = 5$) and preparations 24 h after TNBS administration (filled bars; $n = 4$). The most distal segment of colon contained slightly more myenteric neurons/ganglion than the other segments in control preparations. There was a significant decrease in the no. of neurons in segments 1 through 4 in inflamed preparations compared with the same regions in control preparations. In segments 5–8, there were no differences between control and inflamed preparations. (*$P < 0.05$ compared with control preparation; 2-way ANOVA with Bonferroni posttest).

**DISCUSSION**

Several studies have identified a loss of myenteric neurons during hapten-induced colitis in rats, mice, and guinea pigs (3, 14, 21, 22). Although the mechanism of cell loss has yet to be fully elucidated, it appears to correspond with the infiltration of the myenteric ganglia with leukocytes, either neutrophils (3, 14) or eosinophils (22), and involve apoptosis (3). The loss of neurons is limited as only between 20 and 50% of neurons are lost (3, 14, 21, 22) but does not seem to be associated with the
vations were made, the intestinofugal neurons with shorter axons in the more distal colon were already losing the dye.

The population of intestinofugal neurons decreased dramatically in the region of inflammation caused by a single enema of TNBS. The loss of retrograde label was likely not due to the trafficking of the dye because all labeling would have been complete by the time the colon was inflamed (9). While it is possible that inflammation caused a change in the rate with which intestinofugal neurons removed Fast Blue, the concurrent loss of Hu-immunoreactive neurons suggests that a loss of these neurons was a more likely explanation. The loss of intestinofugal neurons was part of a broader loss of myenteric neurons. However, the proportional loss of intestinofugal neurons (80%) was greater than the proportional loss of all myenteric neurons (20%), which suggests that intestinofugal neurons may be targeted for removal rather than just a part of a larger more indiscriminate loss of neurons. While this may be due to the proximity of these neurons to the mesenteric attachment (9, 23), a previous study has demonstrated data that support neuronal loss is equally distributed among ganglia regardless of proximity to the mesenteric border (14). There tended to be a greater loss of myenteric neurons and intestinofugal neurons in the most inflamed (ulcerated) segments and less loss in regions where the inflammation was characterized only by the presence of edema, suggesting immune processes were likely involved in the loss of myenteric neurons. This is consistent with previous findings that losses of myenteric neurons are associated with, or dependent on, leukocyte infiltration (3, 14, 22). In the current study, we found that another model of colitis that develops after an enema of dilute acetic acid caused similar damage but was not associated with neutrophil infiltration of the myenteric ganglia, or a loss of myenteric neurons. These data provide further support for the hypothesis that loss of myenteric neurons was dependent on leukocyte infiltration of the myenteric plexus. Although it was not directly tested, it is presumed that intestinofugal neurons remained intact in the acetic acid model of colitis.

Electrophysiological recordings provide strong support for the loss of intestinofugal neurons during TNBS colitis. First, the time course of the reduction in frequency of synaptic potentials observed in the present study matches the time course of loss of myenteric neurons (14). While the proportional loss of intestinofugal neurons (80%) was greater than the proportional loss of all myenteric neurons (20%), which suggests that intestinofugal neurons may be targeted for removal rather than just a part of a larger more indiscriminate loss of neurons. While this may be due to the proximity of these neurons to the mesenteric attachment (9, 23), a previous study has demonstrated data that support neuronal loss is equally distributed among ganglia regardless of proximity to the mesenteric border (14). There tended to be a greater loss of myenteric neurons and intestinofugal neurons in the most inflamed (ulcerated) segments and less loss in regions where the inflammation was characterized only by the presence of edema, suggesting immune processes were likely involved in the loss of myenteric neurons. This is consistent with previous findings that losses of myenteric neurons are associated with, or dependent on, leukocyte infiltration (3, 14, 22). In the current study, we found that another model of colitis that develops after an enema of dilute acetic acid caused similar damage but was not associated with neutrophil infiltration of the myenteric ganglia, or a loss of myenteric neurons. These data provide further support for the hypothesis that loss of myenteric neurons was dependent on leukocyte infiltration of the myenteric plexus. Although it was not directly tested, it is presumed that intestinofugal neurons remained intact in the acetic acid model of colitis.

Electrophysiological recordings provide strong support for the loss of intestinofugal neurons during TNBS colitis. First, the time course of the reduction in frequency of synaptic potentials observed in the present study matches the time course of loss of myenteric neurons (14). Second, acetic acid-induced colitis, which did not exhibit a reduction in myenteric neurons, also did not exhibit a reduction in the frequency of synaptic potentials. Interestingly, the 80% loss of intestinofugal neurons within the segment of the colon that corresponds to that used in the electrophysiology experiments did not translate to an 80% reduction in ongoing synaptic potential frequency but rather a 50% reduction in frequency. It therefore seems possible that the remaining intestinofugal neurons may have individually increased firing frequencies compared with noninflamed controls. It is also possible that intes-
visceromotor neurons from control and inflamed preparations

Mean F-EPSP amplitude, mV

Comparison of the mean amplitude of ongoing fast synaptic potentials and passive electrical properties of visceromotor neurons from control and TNBS-treated preparations. There is a significant decrease in the frequency of F-EPSPs starting at 12 h post-TNBS and persisting to 56 days. *P < 0.05 compared with control ANOVA with Newman-Keuls multiple-comparison test. The no. of neurons included in each group is illustrated at the base of each bar.

A loss of intestinofugal neurons has significant physiological effects. Because colitis is associated with a decrease in the number of intestinofugal neurons in the inflamed region of the guinea pig distal colon (13, 25), there may be prolonged periods of distension that would normally signal to the more proximal bowel via intestinofugal neurons to reduce motor activity (11). If there is a reduction of synaptic inputs from these intestinofugal neurons, more proximal regions may contribute to an overloading of the inflamed segment, further increasing pressure behind the damaged site. In addition to sympathetic control of gastrointestinal motor function, the PVG has significant control over intestinal secretory function. Sympathetic neurons that innervate vasoactive intestinal peptide-ergic secretomotor neurons of the submucosal plexus in the small intestine (5) receive intestinofugal input (10). Disinhibition of intestinal secretion, known as paralytic secretion, is observed when the PVG are removed (29). A loss of synaptic input to these neurons in the PVG would have a functional consequence of increasing intestinal net water secretion, a phenomenon also observed during colitis (2, 20). Inflammation is associated with an increased excitability of postganglionic sympathetic neurons (7, 12). It is possible that hyperexcitability may be a homeostatic response to a decreased intestinofugal input. The causal relationship between these two associated phenomena remains to be identified.

In conclusion, this study shows that there was a reduction in the number of intestinofugal neurons in the inflamed region of the guinea pig distal colon following an enema of TNBS. This loss was part of a larger loss of myenteric neurons, but intestinofugal neurons may be selected for removal. Because these neurons form the afferent limb of intestino-intestinal reflexes that coordinate motor activity over long distances along the gastrointestinal tract, the loss of these neurons may have profound physiological consequences.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

D.R.L. conception and design of research; performed experiments; analyzed data; interpreted results of experiments; prepared figures; drafted manuscript; edited and revised manuscript; approved final version of manuscript.

Table 3. Comparison of the mean amplitude of ongoing fast synaptic potentials and passive electrical properties of visceromotor IMG neurons from control and inflamed preparations

<table>
<thead>
<tr>
<th></th>
<th>Control (26)</th>
<th>6h TNBS (8)</th>
<th>12h TNBS (6)</th>
<th>24h TNBS (37)</th>
<th>6d TNBS (36)</th>
<th>56d TNBS (7)</th>
<th>Control (6)</th>
<th>24 h AA (11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean F-EPSP amplitude, mV</td>
<td>3.4 ± 0.3</td>
<td>3.8 ± 0.6</td>
<td>3.9 ± 0.7</td>
<td>3.8 ± 0.2</td>
<td>3.3 ± 0.2</td>
<td>3.0 ± 0.2</td>
<td>3.3 ± 0.7</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>Resting membrane potential, mV</td>
<td>-58 ± 1</td>
<td>-58 ± 2</td>
<td>-55 ± 2</td>
<td>-58 ± 1</td>
<td>-55 ± 1</td>
<td>-55 ± 3</td>
<td>-56 ± 3</td>
<td>-55 ± 3</td>
</tr>
<tr>
<td>Membrane input resistance, MΩ</td>
<td>84 ± 9</td>
<td>98 ± 25</td>
<td>63 ± 2</td>
<td>79 ± 4</td>
<td>72 ± 6</td>
<td>105 ± 31</td>
<td>83 ± 14</td>
<td>95 ± 14</td>
</tr>
</tbody>
</table>

Data are means ± SE for the no. of cells in parentheses. There were no differences in the mean amplitude of ongoing fast synaptic potentials (F-EPSP), the resting membrane potentials, or the membrane resistance of visceromotor neurons between control and inflamed tissue. Neurons from TNBS-treated preparations were compared with controls using ANOVA. Neurons from acetic acid (AA)-treated preparations were compared with controls using t-test (all P > 0.05).
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