Structural differences in the enteric neural network in murine colon: impact on electrophysiology

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Since the original description of the enteric nervous system in the small bowel by Auerbach (1), it has been believed that the enteric ganglia form a regular network over the entire length of the gut wall. Distribution of subgroups of enteric neurons is known to vary along the longitudinal axis of the gut as demonstrated, for example, for nitric oxide (NO) synthase (NOS)-containing neurons (25, 26), but it is assumed that there is a homogeneous distribution of the neural network within a short gut segment. The shape of the small and large intestine in mammals resembles an elongated hollow organ. In contrast to simple tubes, however, the gut has to generate the force needed to ensure intraluminal transport (6, 14). Accordingly, a sophisticated neural and muscular network (6, 8, 9, 17, 24) has emerged in the course of evolution (2, 7, 10, 11, 17, 26, 27).

Staining techniques and optical magnification are generally used to describe the anatomy of this network at the microscopic level. In the small intestine, the neurons of the myenteric plexus, which lie between the inner circular muscle layer and the outer longitudinal muscle layer and which provide input to both layers, are evenly distributed around the circumference of the gut. Examining and magnifying any one spot in a gut region are therefore expected to provide representative findings.

Several observations indicate that NO is a major inhibitory neurotransmitter in the murine large bowel. NADPH diaphorase staining, which is widely used to visualize nitricergic neurons (2, 8, 17, 18, 23, 26), has been demonstrated in the colon (9). NADPH diaphorase staining as well as the more specific NOS immunoreactivity are present in myenteric plexus preparations in the murine cecum (2, 31). It is known that NADPH diaphorase staining in some enteric tissues does not stain enteric neurons in the same pattern as does NOS immunoreactivity, thus resulting in an un-
derestimation of the neuronal population, although for the colon, there seems to be a one-to-one correlation (2, 32). Stimulation of intrinsic NO-dependent nerves induced mechanical relaxation of murine cecum longitudinal muscle (31) and murine proximal colon circular muscle (24). Intracellular electrical recordings of the circular muscle cells of murine colon have demonstrated functional neural input of inhibitory neurotransmitters such as NO (14, 24).

On the basis of our own observations that inhibitory junction potentials (IJPs) sometimes differ in the murine proximal colon, we performed a circumference-preserving three-dimensional (3D) preparation for NADPH diaphorase and macroscopically discovered a ganglion-free area, which was of interest because it might be the explanation for the above-mentioned observations. The aim of the present study was therefore to characterize the observed hypoganglionic areas by using morphological and electrophysiological methods.

MATERIALS AND METHODS

Tissue preparation for electrophysiological experiments. Mice of three different genetic backgrounds (BALBc, C III H, and C57/black 6) or Wistar rats of either sex were anesthetized by phenoxybital sodium and killed by cervical dislocation in accordance with the recommendations of the animal ethics committee at the Technical University of Munich. The colon was exposed through an abdominal midline incision. The complete large bowel was removed and placed in oxygenated Krebs solution. The size of the large bowel, and hence the size of the segments of the large bowel, varied according to the weight of the animal, the temperature of the surrounding media, and the volume of the bowel contents. The need to cut short gut segments for the electrophysiological examination made it necessary to reliably identify locations along the large bowel by using fixed topographic points. The following fixed points, which did not change during the tissue preparation, were selected: the ileocecal junction, the ascending colon characterized by haustra (Fig. 1), and the aboral dissection limit at the level of the pelvic diaphragm. The haustra started at a distance of 3–8 mm and ended at a distance of 12–18 mm from the ileocecal junction. Before dissection of the mucosa, the appropriate impalement locations were marked. The colon was then opened along the mesenteric border, cleaned of remaining fecal material, and pinned out in a Sylgard-lined (Dow Cornig, Midland, MI) dissecting dish containing oxygenated Krebs solution. The mucosa and submucosa were removed, resulting in sheets of tissue consisting of circular and longitudinal muscle layers, together with the attached myenteric plexus. For the cecum, the cecal tenia was not used for the electrophysiological experiments.

Intracellular electrical recording. Sheets or strips of muscle (cecum and proximal or distal colon) were pinned by using ~150–200 micropins (15- and 25-μm thick), obtained from wolfram wire, to the Sylgard-based electrophysiological chamber, with the exposed circular muscle layer uppermost. The chamber was perfused (5 ml/min; Kwik Pump, World Precision Instruments, Sarasota, FL) with prewarmed (37°C) oxygenated (95% O2:5% CO2) Krebs solution of the following composition (in mM): 120.35 NaCl, 5.9 KCl, 2.5 MgCl2, 1.2 NaH2PO4, 15.5 NaHCO3, 2.5 CaCl2, and 11.5 glucose, pH 7.4. Muscle strips were allowed to equilibrate for 90–120 min before the experiments were started. Atropine, guanethidine, and nifedipine (all 1 μM) were present throughout all experiments. Capillary glass microelectrodes (borosilicate glass capillaries, 1.0 mm outer diameter × 0.58 mm inner diameter; Clark Electromedical Instruments) were made by using a microelectrode puller (model P-97, 3-mm wide filament; Sutter Instrument, Novato, CA), were filled with KCl (3 M), and had resistances in the range of 80 to 120 MΩ. Neurons were stimulated (15 V; 0.3 ms duration; single pulses; 5 Hz or 10 Hz) via platinum electrodes arranged perpendicularly to the circular muscle layer and were connected to a Grass S11 stimulator via a stimulus isolation unit (model SIU59; Grass Instruments, Quincy, MA). These responses were recorded against a ground Ag-AgCl electrode placed in the bath medium. Evoked electrical events were amplified (model DUO 733 microelectrode amplifier; World Precision Instruments) and digitalized with an analog-to-digital converter (model SCB 68 interface; National Instruments, Austin, TX). Permanent recordings of membrane potentials were made on a personal computer using the LABVIEW 5.0 program (National Instruments).

IJPs, elicited by stimulation of intrinsic inhibitory neurons, usually contained a transient (fast) and, if present, a sustained (slow) component. The amplitudes of the fast and slow components were measured in millivolts compared with the resting membrane potential (RMP) before application of the electrical stimulus.

Whole mount preparation for NADPH diaphorase, methylene blue, and cuprolinic blue staining. The large bowel was removed as described in the electrophysiological experiments. Segments of cecum and of the proximal and distal colon were opened along the mesenteric border, washed, and pinned in a dissecting dish containing Krebs solution with nifedipine, atropine, and guanethidine (all 1 μM). The mucosa and submucosa were removed, and the muscle layers were pinned out while being stretched to ~125% of the normal size.

NADPH diaphorase staining was performed as follows. The tissue was fixed for 2 h in 4% paraformaldehyde at room temperature. NADPH diaphorase activity was rendered visible by incubating the tissues in 0.05 M Tris-HCl buffer (pH 8.0) containing 0.1 mM β-NADPH (reduced form), 0.05 mM nitroblue tetrazolium, and 0.3% Triton X-100, at 37°C for 2 h. Washing of the whole mount preparations in 0.05 M Tris-HCl buffer terminated the reaction. After several washings with 0.05 M Tris-HCl, the whole mounts were placed on...
glass slides, air-dried, and coverslipped with DePex (Serva, Heidelberg, Germany).

Methylene blue staining is a nonspecific but effective and duration-dependent staining method for interstitial cells of Cajal (ICCs) and neural structures (21). After the removal of the mucosa, tissues were washed with fresh oxygenated Krebs solution and then incubated in Krebs solution containing 10 mM methylene blue at 37°C bubbled with 95% O2–5% CO2 (vol/vol) for 30–180 min in the dark with intermittent visual checking of the staining intensity. After staining, the tissues were immediately placed in 8% ammonium molybdate for 2–3 h, as described by Ehrlich (20). Then the tissues were placed on glass slides, air-dried, and coverslipped with DePex.

Cuprolinic blue staining is used as a marker for total neuron counting (12). The tissue was fixed for 1 h in Carnoy solution. The staining solution [0.3% cuprolinic acid in 0.025 M sodium acetate buffer (pH 5.6) with 1 M MgCl2] was applied for 60 min at room temperature, and the tissue was then rinsed in distilled water and placed in a sodium acetate buffer (pH 5.6) containing 1 M MgCl2. Finally, the tissue was mounted in water, placed in ethanol and then xylene, and mounted in DePex.

Circumference-preserving 3D preparation for NADPH diaphorase, methylene blue, and cuprolinic blue staining. Mice of different genetic backgrounds (BALBc, C III H, or C57/ black 6), Wistar rats, or guinea pigs of either sex were anesthetized and killed as described in the electrophysiology section. The whole large bowel with adjacent terminal ileum was removed through an abdominal midline incision and placed in Krebs buffer (37°C) containing nifedipine, atropine, guanethidine (all 1 μM). The lumina of the terminal ileum and distal colon were cannulated and cleaned of fecal material by using repeated gentle washes with Krebs-Ringer solution at room temperature. The whole organ was then carefully filled with liquid paraffin (Fluka liquid paraffin) from the oral end at a pressure of 2 cm H2O and ligated with a suture at both ends. Some preparations were filled with Sylgard instead of paraffin.

The NADPH diaphorase, methylene blue, and cuprolinic blue staining procedures were performed as described above. Quantitation of neuron density in the NADPH diaphorase-stained and cuprolinic blue staining procedures. Tissues were photographed by using an Axioplan (Karl Zeiss, Jena, Germany) microscope with a digital camera (Olympus Camedia C-3030 Zoom, with a C3040-ADU adapter; Olympus, Japan) at a microscope with a digital camera (Olympus Camedia C-3030 Zoom, with a C3040-ADU adapter; Olympus, Japan) at a magnification of ×20. Squares of identical size corresponding to 1.4 mm2 on the tissue were marked for each section of the large bowel [cecum, proximal colon (oral, middle, aboral) with heterogeneous neuron density] and distal colon. The total number of ganglia and neurons was counted by using GrDigit software (www.nick-gd-chat.ru) in each square and expressed as the number of ganglia or neurons per square millimeter. The number of neurons per ganglion was calculated. The total number of neurons per 1-mm long segment was estimated by multiplying the number of neurons per square millimeter by the extent of the gut circumference at the respective gut location.

Identification of ICCs in KitW-loxPtransgenic mice. Identification of ICCs was carried out by using the tyrosine kinase receptor Kit as a marker (13, 15). KitW-loxP-ICCs heterozygous mice (donated by Jean–Jacques Pariser) were maintained and bred at the animal facility of the Faculty of Medicine, Free University of Brussels, Belgium. KitW-loxP-transgenic mice carry the Escherichia coli lacZ gene inserted in place of the first exon of the Kit gene. A viral (SV40) nuclear localization signal has been fused to lacZ, and the resulting β-galactosidase fusion protein is enzymatically active and remains associated with the nuclear membrane (4). The presence of a functional allele of Kit allows the KitW-loxP transgenic mice to develop normally, whereas the lacZ reporter gene allows identification of the Kit-expressing cells either by the β-galactosidase histochemical reaction (X-gal, 5-bromo-4-chloro-3-indoyl-β-D-galactoside) or by immunohistochemistry with antibodies raised to the bacterial β-galactosidase (3, 28). Four adult KitW-loxP transgenic mice and four control littersmates (+/+ ) were used. The colon was harvested as described in Tissue preparation for electrophysiological experiments and washed with Krebs-Ringer solution to remove residual fecal material from the anal to the oral end. The colon was opened at the mesenteric attachment, and the mucosa and submucosa were removed. The remaining muscle plexus preparation was fixed for 60 min in 0.1 M PBS, pH 7.4, containing 2% (vol/vol) paraformaldehyde and 2% (vol/vol) glutaraldehyde. X-gal histochemistry was performed as described previously (28) by overnight incubation at 32°C in PBS containing 1 mM X-gal, 2 mM potassium ferricyanide, and 4 mM magnesium chloride. The preparation was rinsed in PBS, incubated in X-gal, and then in distilled water and was mounted in an aqueous mounting medium.

Drugs. Ammonium molybdate, atropine, guanethidine, N(G,N)-nitro-l-arginine (l-NNA), nifedipine, Triton X-100, TTX, Tris (base), the Krebs-Ringer buffer salts, and potassium ferricyanide were obtained from Sigma. β-NADPH (reduced form), nitroblue tetrazolium, and methylene blue were obtained from Neopharma (Aschau, Germany). Paraformaldehyde and glutaraldehyde were obtained from Agar Scientific (Sansted, Essex, UK), and X-gal from Boehringer-Mannheim (Mannheim, Germany).

Data presentation and statistical analysis. All data are expressed as means ± SE. The significance of differences among groups was determined by using the paired Student’s t-test. For multiple comparisons, adequate Bonferroni correction was performed. A P value < 0.05 was considered significant. Values given with n refer to the number of experiments performed in tissues from different animals.

RESULTS

Electrophysiology. In basal conditions in the presence of nifedipine, atropine, and guanethidine (all 1 μM), circular smooth muscle cells of murine large bowel displayed stable RMPs (−56.7 ± 6.9 mV, n = 13 in the cecum; −46.1 ± 2.3 mV, n = 7 in the proximal colon; −46.4 ± 3.8 mV, n = 11 in the distal colon; cecum RMP is significantly different from colon RMP, P < 0.05). Stimulation of enteric inhibitory neurons via electrical field stimulation (EFS) gave rise to TTX-sensitive IJP s varying in size and form along the longitudinal axis of the colon.

In the cecum (midway between the ileocecal junction and the apex of the cecum), EFS (single stimuli) elicited monophasic IJPs of short duration (918 ± 200 ms, n = 8) with an amplitude of 16.8 ± 3.6 mV. Varying stimulus parameters including 10 pulses at 5 Hz or 20-s long continuous stimulation at 5 Hz did not elicit additional components of IJPs in the cecum. In the oral, middle, and aboral proximal colon and in the distal colon, single stimuli of EFS produced IJPs that consisted of two components. The initial “fast component” was characterized by a rapid hyperpolarization and did not vary within the proximal colon (duration:
A second “slow” component (duration: 7.0 ± 1.4 s in the oral and 3.1 ± 0.6 s in the distal colon; P < 0.05; n = 10), elicited by EFS, showed major differences between the proximal and distal colon and varied even within short distances in the proximal colon. The slow component was characterized by a distinct and gradual recovery to RMP and had an amplitude of 13.6 ± 2.0 mV in the oral, 10.3 ± 3.0 mV in middle, 7.3 ± 1.7 mV in the aboral proximal colon, and 3.8 ± 1.5 mV in the distal colon. The size of the slow component differed significantly at all locations (P < 0.01, n = 10). Both components of the IJP were blocked by 3 μM TTX (n = 5). Larger, slow components of IJPs were obtained in the proximal and distal colon with multiple stimuli (10 pulses at 5 Hz). In these conditions, EFS revealed a clear, slow component in the distal colon, which was still significantly smaller than in the proximal colon (8.8 ± 1.6 mV in the distal colon vs. 15.3 ± 2.9 mV in the proximal colon; P < 0.01, n = 4).

The NOS inhibitor L-NNA (100 μM) produced a significant depolarization (7.3 ± 3.8 mV, n = 9, P < 0.01) of smooth muscle cells in the murine proximal colon, which was of permanent nature. Maximal effects of L-NNA occurred within 10 min of exposure. When single stimuli or 10 pulses (5 Hz, 0.3 ms, 15 V) were tested, L-NNA completely abolished the sustained hyperpolarization (slow component of IJPs) (16.3 ± 3.2 vs. 2.1 ± 1.7 and 8.5 ± 2.3 vs. 1.4 ± 0.6 mV; n = 6; P < 0.05 in the proximal and distal colon) (Fig. 2B). L-NNA did not change the form and size of IJPs in the murine cecum (16.8 ± 3.6 vs. 18.8 ± 4.5 mV fast component, NS; the slow component is absent in the cecum) (Fig. 2B), demonstrating an absence of functional NO-dependent neural input.

**NADPH diaphorase histochemistry.** Neuronal NOS (nNOS)-positive neurons were found throughout the large bowel. There were usually several positive cells in each myenteric ganglion. The results of NADPH diaphorase histochemistry were similar in whole mount preparations and in the circumference-preserving 3D preparation. The number of nNOS-positive cells per ganglion was highest in the proximal colon and decreased in the oral direction (to the cecum) and distally (Table 1). In the aboral third of the proximal colon, ~30 mm aboral from the ileocolonic junction, NADPH diaphorase staining reveals two areas that

![Image](http://ajpgi.physiology.org/)

**Table 1. Distribution of nNOS-positive neurons in the murine large bowel**

<table>
<thead>
<tr>
<th></th>
<th>Cecum</th>
<th>Proximal Colon</th>
<th>Proximal Colon</th>
<th>Proximal Colon</th>
<th>Distal Colon</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Oral</td>
<td>Middle</td>
<td>Neuron-rich</td>
<td>Hypoganglionic</td>
<td>Neuron-rich</td>
</tr>
<tr>
<td>Gut circumference, mm</td>
<td>34.5</td>
<td>21.1</td>
<td>19.6</td>
<td>16.6</td>
<td>3</td>
</tr>
<tr>
<td>Ganglia per mm²</td>
<td>2.8 ± 1.5</td>
<td>9.3 ± 2.4</td>
<td>12.1 ± 1.9</td>
<td>16.2 ± 3.5</td>
<td>4.7 ± 1.5</td>
</tr>
<tr>
<td>Neurons per mm²</td>
<td>26.2 ± 12.6</td>
<td>167.8 ± 44.5</td>
<td>218.6 ± 16.5</td>
<td>292.4 ± 26.4</td>
<td>29.4 ± 18.7</td>
</tr>
<tr>
<td>Neurons per gut segment</td>
<td>6.6 ± 3.1</td>
<td>8.7 ± 1.5</td>
<td>11.9 ± 1.4</td>
<td>12.6 ± 1.1</td>
<td>4.4 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>905.6 ± 434.0</td>
<td>3,532.7 ± 936.2</td>
<td>4,293.5 ± 324.2</td>
<td>4,185.2 ± 472.0</td>
<td>1,894.5 ± 214.3</td>
</tr>
</tbody>
</table>

Data shown are means ± SD for the numbers of ganglia and neurons counted in 6 preparations. Note that in the aboral proximal colon, the heterogeneous neural distribution made it necessary to distinguish between neuron-rich and hypoganglionic areas, each of which made up different parts of the extent of the gut circumference. Neurons were calculated per gut segment 1 mm in length (last row). nNOS, neuronal nitric oxide synthase.

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contain few neuronal ganglia with an average of just 4.4 ± 2.8 neurons per ganglion. One of these is located at the mesenteric attachment, 4 mm wide and 13–15 mm long, and the other antimesenterically, 3–5 mm wide and 10–14 mm long (see Figs. 1 and 3). These two hypoganglionic regions are surrounded by a dense clustering of enteric ganglia, each containing a high number (12.6 ± 1.1) of neurons per ganglion (Table 1 and Fig. 4). The nerve cell density in this surrounding clustering of ganglia was not significantly different from the cell density in the gut segment oral to the hypoganglionic regions, where the network is still regularly distributed around the circumference (NS, n = 6) (Fig. 3, Table 1). When estimating the total number of neurons per segment, the heterogeneous segment consisting of the two hypoganglionic areas within its circumference shows lower total numbers than the segment with a homogeneous neuronal network just oral of the heterogeneous segment. Hence, the number of nNOS-positive neurons was maximal in the oral proximal colon and decreased on both sides, orally and aborally. The number of nerve fibers within the circular muscle layer was measured in transected tissue of the hypoganglionic and the ganglionic sections. The nerve fibers were counted and are given in number per millimeter of tissue length. By counting the nerve fibers, we could not find a significant difference in this number comparing the ganglionic and the hypoganglionic area (ganglionic area: 57 ± 9.1; hypoganglionic area: 54 ± 14.6; n = 5; NS).

The finding of the hypoganglionic regions in the proximal colon was consistent in mice of different genetic backgrounds (BALBc, C III H, or C57 black 6) and was also demonstrated in rat large bowel (Wistar rats). In guinea pigs, however, only one hypoganglionic region is present at the mesenteric attachment.

**Cross sections of murine proximal colon.** Cross sections of murine proximal colon at the location of the hypoganglionic region demonstrated that the longitudinal muscle layer was lacking in the hypoganglionic region (Fig. 5).

**Methylene blue and cuprolinic blue histochemistry.** Methylene blue vital staining (Fig. 6) and cuprolinic blue staining of a 3D preparation and of standard whole mounts confirmed the location and size of the hypoganglionic regions, the maximum nerve cell density in the proximal colon, and the nerve cell density gradient decreasing on both sides in the oral and anal directions. Generally, additional neurons were stained in all regions of the large bowel, so that 51–54% of the total number of cuprolinic blue-stained neurons were NADPH-diaphorase positive (Tables 1 and 2).

**Identification of ICCs by using X-gal histochemistry in KitW-lacZ/ mice.** Kit-expressing ICCs were oriented parallel to both muscle layers of the colon. The X-gal staining density was higher in the proximal
### DISCUSSION

On the basis of our own observations that IJPs sometimes differ in the murine proximal colon, we performed a circumference-preserving 3D preparation for NADPH diaphorase and macroscopically discovered a ganglion-free area. Starting from this initial observation, we now present a study in which we report regional differences in the network of the enteric nervous system in the murine large bowel with marked regional hypoganglionic. These anatomical differences were characterized by using different staining techniques, and the hypoganglionic region, which is also associated with functional differences in inhibitory NANC innervation, is characterized by electrophysiological methods.

The 3D preparation and the flat whole mount preparation both provide evidence that the murine proximal colon consistently contains two hypoganglionic regions. This finding was not limited to mice of a single genetic background and was also present in rats. The longitudinal muscle layer and the innervating structures, such as the associated ICCs and most neural ganglia, are polarized on two sides of the circumference, representing an exception to the tube-like structure of the gut. This hypoganglionic area is anatomically clearly distinct from whole aganglionic segments described in models for Hirschsprung's disease, such as the lethal spotted mouse (22). Moreover, cross sections of the proximal colon and X-gal staining of ICCs in KitW-tacZ/ transgenic mice revealed that the longitudinal muscle layer and the associated intramuscular ICCs (5, 28), which are closely associated with and oriented parallel to this muscle layer, are lacking in these hypoganglionic regions. The impact on function of these morphological findings is still unclear, but because the irregularly distributed longitudinal muscle results in flat pouches in the proximal colon, the morphology could be linked to a reservoir function, which could also decrease fluid propulsion.

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**Figure 4.** Numbers of NADPH diaphorase-positive neurons at different locations in the large bowel. A: density of neurons was highest in the clustered areas (a) adjoining the hypoganglionic areas (b) in the aboral proximal colon. B: when calculated per gut segment, the total number of neurons in the segments with heterogeneous neuronal distribution is similar to the segment just orally with homogeneous neuronal distribution.

![Graph showing neuronal density across colon segments](http://ajpgi.physiology.org/)

<table>
<thead>
<tr>
<th>Segment</th>
<th>Neurons per mm²</th>
<th>Neurons per segment</th>
</tr>
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<tbody>
<tr>
<td>Caecum</td>
<td>600 ± 100</td>
<td>12,000 ± 2,000</td>
</tr>
<tr>
<td>Proximal Colon</td>
<td>800 ± 200</td>
<td>18,000 ± 3,000</td>
</tr>
<tr>
<td>Distal Colon</td>
<td>1,000 ± 150</td>
<td>30,000 ± 4,500</td>
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**Figure 6.** Electrophysiology of the hypoganglionic region. In a separate set of experiments, the function of the hypoganglionic region was investigated before and after separation from its surrounding tissue within the same segment of proximal colon. The resting potential of ganglionic vs. hypoganglionic region showed no significant difference: (-46.1 ± 2.3 vs. -45.4 ± 1.7 mV; n = 7; NS). First, after identification of the hypoganglionic area (Fig. 7), EFS of intrinsic inhibitory neurons elicited similar IJPs in the hypoganglionic area and the surrounding ganglion-rich area (24.6 ± 6.6 vs. 22.6 ± 4.3 and 12.1 ± 2.1 vs. 13.1 ± 2.1 mV fast and slow component of IJP, respectively, n = 6, NS). After microscopic sharp separation of the hypoganglionic region from its surrounding tissue, however, the normal inhibitory neurotransmission to the hypoganglionic area was disrupted (Fig. 7) (1.6 ± 1.4 and 2.6 ± 1.7 mV for the fast and slow component of IJP amplitude in the hypoganglionic area vs. 16.5 ± 1.9 and 23.7 ± 2.7 mV for the fast and slow component of IJP amplitude in the neuron-rich area, respectively, P < 0.01, n = 6). In contrast, a similar microscopic separation of the neuron-rich surrounding tissue with identical sheet size had no influence on the inhibitory neurotransmission (22.6 ± 4.3 and 13.1 ± 2.1 mV fast and slow component of IJP, respectively, n = 6). These findings indicate that the hypoganglionic area receives functional neural input from the neuron-rich surrounding tissue in a radial pattern.

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**Figure 7.** Morphology of the hypoganglionic area in the proximal colon. A: example of the hypoganglionic area with the surrounding ganglion-rich area. B: comparison of the distribution of Kit-positive cells oriented parallel to the longitudinal muscle layer in the hypoganglionic area and the associated ICCs (5, 28), which are closely associated with and polarized on two sides of the circumferential section of the proximal colon. C: comparison of the distribution of Kit-positive cells oriented parallel to the longitudinal muscle layer in the hypoganglionic area and the associated ICCs (5, 28), which are closely associated with and polarized on two sides of the circumferential section of the proximal colon.

**DISCUSSION**

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Fig. 5. Overview (A), details (B and C) and further magnification (D and E) of a cross section of proximal murine colon, including the hypoganglionic regions in the myenteric plexus (MP) by using hematoxylin-eosin staining. D: lack of myenteric ganglia in positions located opposite to each other is associated with an absence of the longitudinal muscle layer (L). In these areas, the circular muscle (C) is covered only by the serosal layer. E: around these hypoganglionic areas, the gut wall contains normal circular (C) and longitudinal (L) muscle, as well as clustered ganglia in the myenteric plexus (MP). The asterisk marks the location of the mesenteric attachment.

Fig. 6. A and B: whole mount flat preparations of the proximal colon of Wlac-Z/ mice after X-gal staining. Kit-positive cells oriented parallel to the longitudinal muscle layers were lacking in the hypoganglionic area, thus confirming that the region lacking the longitudinal muscle layer also lacks the Kit-positive interstitial cells associated with this muscle layer. C and D: whole mount flat preparations of murine proximal colon with vital methylene-blue staining, showing the structural organization of the ICC network.
It is certainly surprising that to our knowledge these hypoganglionic areas have not been described previously. One possible explanation might be the preparation techniques used. For magnification of the gut wall using optical lenses, the general practice has been to cut the gut into pieces to mount the samples on glass. A serious limitation of this technique is that anatomical features at the edges of the preparation cuts are lost. A circumference-preserving 3D preparation technique in which the gut is stained and visualized without affecting the 3D anatomy provides a more complete picture of the gut as a hollow organ than flat whole mount preparations, although it can only be used with histochemical and not with immunocytochemical staining techniques.

The morphology and functioning of intrinsic inhibitory nerves display congruent behavior not only along the length of the large intestine but also in the hypoganglionic regions. Within the hypoganglionic region, only small neural fibers run parallel to the circular smooth muscle cells, ensuring normal innervation of the muscle region. After macroscopic identification of the hypoganglionic area, EFS of intrinsic inhibitory neurons elicited similar IJPs for the fast and slow IJPs in the hypoganglionic area compared with the surrounding ganglion-rich area. The electrical responses reported here are in agreement with the electrical responses previously described (24) in murine proximal colon by others (30). Interestingly, cutting the circumference of the bowel wall at the borders of the hypoganglionic area by microincision disrupted inhibitory neurotransmission exclusively to the hypoganglionic area, while leaving the inhibitory neurotransmission of the ganglion-rich area unchanged. Hence, the functional inhibitory innervation is organized on a segmental level and runs from the neuron-rich areas supposedly by using the tertiary plexus to reach the muscle layer of the hypoganglionic region.

In addition to the changes in electrophysiology in the hypoganglionic region, the present study characterizes regional differences in inhibitory neurotransmission from the cecum to the distal colon for the circular smooth muscle. Although there is no L-NNA-dependent slow IJP in the cecum or the distal colon, there is a prominent L-NNA dependent inhibitory neurotransmission in the proximal colon, and within the proximal colon there is a gradient in this L-NNA-dependent neurotransmission from oral to aboral. Interestingly, for the cecum, despite a few, but clearly present, nitricergic neurons, functional NO-dependent inhibitory input to the muscle was completely absent in our experiments. The cecum results partially contrast with...
the findings of Young et al. (31), who reported NO-dependent relaxation of cecum longitudinal muscle, although with a different experimental design by using a muscle strip-contraction setup. Although this difference might be due to the differences in the observed muscle (circular vs. longitudinal), it might also be due to stimulus differences. In the colon, the electrical recording proved to be sensitive enough to demonstrate clear differences in functional NO-dependent neuronal supply to the muscle. The NO-dependent functional innervation was prominent in the oral third of the proximal colon and decreased in the distal direction. This functional gradient was associated with a decrease in neural density, specifically in the density of NADPH diaphorase-positive neurons. In addition to the nerve cell density suggested here, other causes might contribute to the functional gradient reported here, such as increased sensitivity of the muscle cells to NO (16) or enhanced synthesis or release of NO from myenteric neurons (19, 25).

The hypoganglionic area in the proximal colon described here should not be mistaken for the aganglionic colon of lethal (ls/ls) mice described recently (29). Interestingly, in the lethal aganglionic mice, the IJPs were completely absent, whereas in the hypoganglionic region described here, electrophysiological responses remain unchanged until the hypoganglionic region is separated from the surrounding tissue. When the hypoganglionic region is separated, the electrophysiological responses are nearly absent and therefore comparable to the responses in lethal aganglionic mice (29).

In summary, the proximal colon displays clear morphological and functional irregularities. This part of the colon represents a clear exception to the generally accepted view that the bowel has a homogeneous holonomic and functional irregularities. This part of the colon represents a clear exception to the generally accepted view that the bowel has a homogeneous holonomic and functional irregularities. This part of the colon represents a clear exception to the generally accepted view that the bowel has a homogeneous holonomic and functional irregularities. This part of the colon represents a clear exception to the generally accepted view that the bowel has a homogeneous holonomic and functional irregularities. This part of the colon represents a clear exception to the generally accepted view that the bowel has a homogeneous holonomic and functional irregularities. This part of the colon represents a clear exception to the generally accepted view that the bowel has a homogeneous holonomic and functional irregularities. This part of the colon represents a clear exception to the generally accepted view that the bowel has a homogeneous holonomic and functional irregularities. This part of the colon represents a clear exception to the generally accepted view that the bowel has a homogeneous holonomic and functional irregularities. This part of the colon represents a clear exception to the generally accepted view that the bowel has a homogeneous holonomic and functional irregularities.


DISCLOSURES

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