Interstitial cells of Cajal and electrical activity in ganglionic and aganglionic colons of mice

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Ward, Sean M., Michael D. Gershon, Kathleen Keef, Yulia R. Bayguinov, Cheryl Nelson, and Kenton M. Sanders. Interstitial cells of Cajal and electrical activity in ganglionic and aganglionic colons of mice. Am J Physiol Gastrointest Liver Physiol 283: G445–G456, 2002. First published March 20, 2002; 10.1152/ajpgi.00475.2001.—An antibody directed against Kit protein was used to investigate the distribution of interstitial cells of Cajal (ICC) within the murine colon. The ICC density was greatest in the proximal colon and decreased along its length. The distribution of the different classes of ICC in the aganglionic colons of lethal spotted (ls/ls) mice was found to be similar in age-matched wild-type controls. There were marked differences in the electrical activities of the colons from ls/ls mutants compared with wild-type controls. In ls/ls aganglionic colons, the circular muscle was electrically quiescent compared with the spontaneous spiking electrical activity of wild-type tissues. In ls/ls aganglionic colons, postjunctional neural responses were greatly affected. Inhibitory junction potentials were absent or excitatory junction potentials inhibited by atropine were observed. In conclusion, the distribution of ICC in the aganglionic regions of the colons from ls/ls mutants appeared similar to that of wild-type controls. The electrical activity and neural responses of the circular layer are significantly different in aganglionic segments of ls/ls mutants.

Hirschsprung’s disease; lethal spotted; enteric nerves; megacolon; gastrointestinal tract.

THE COLONIZATION OF THE gastrointestinal (GI) tract by enteric neural precursors depends on activation of endothelin β-receptor (EDNRB) and Ret receptors by their respective natural ligands, endothelin-3 (ET-3) and the interaction of Ret with glial cell-derived neurotrophic factor (GDNF) and GFR-α1 to initiate signal transduction (5). Mutations that abolish expression of either ligand or receptor produce intestinal aganglionosis in both mice and humans (1, 4, 9, 16, 19, 20, 23). Mice that are homozygous for the lethal spotted (ls) allele display a loss of function mutation in the ET-3 gene and have no enteric neurons in the terminal regions of the large intestine (20). These animals have a similar phenotype to Hirschsprung’s disease (HD) in humans and are a useful model for the examination of the consequences of colonic aganglionosis (12, 18).

Kit-like immunoreactivity has been used to identify networks of interstitial cells of Cajal (ICC) in a variety of species (22). ICC are critical in generating electrical slow waves (11, 26, 32, 33) and mediating neurotransmission to smooth muscle in the GI tract (2, 31, 34). In the human colon, ICC are located along the boundary of the submucosa with the circular muscle (IC-SM), within the muscular layers (IC-IM), and around the myenteric plexus (IC-MY; 6, 7, 25). In the aganglionic segments of patients with HD, it has been reported that the distribution of ICC is scarce within the tunica muscularis (42) and that their networks are greatly disrupted (30). It has been suggested (30) that the lack of ICC in the aganglionic regions of patients with HD may explain the abnormal spontaneous electrical activity in this region of the gut and provide new insight into the ontogeny of ICC. However, in a later report (10), no differences from control tissues were found in the distribution of ICC in the aganglionic segments of the descending colons in six cases of HD (2 with short segment aganglionosis, 3 with extensive aganglionosis, and 1 with total aganglionosis). This later study (10) concluded that regional differences in the distribution of ICC within the human colon might account for the apparent abnormalities in ICC observed in previous studies.

The importance of the Kit signaling pathway for the development and maintenance of the ICC phenotype has been established (11, 26, 33). ICC require signaling of the Kit receptor via its ligand, stem cell factor (SCF). SCF is expressed in a subpopulation of enteric nerves (45), but the importance of this source of Kit ligand for ICC development and maintenance has been called into question by studies (15, 35, 41, 43) finding that ICC develop even when enteric neurons are absent.

In the present study, we have sought to determine whether the loss of neurons or changes in the enteric microenvironment in the aganglionic bowel of ls/ls mutants...
mice affect ICC populations and the spontaneous electrical activity of colonic muscles. The distribution of ICC along the entire length of the colon was examined in age-matched wild-type and ls/ls mutant mice. Kit immunoreactivity was used as a reporter for ICC. We also compared the distribution of ICC in ganglionic and aganglionic regions at the same level of the colon in wild-type and ls/ls mice to determine whether loss of enteric ganglia affects ICC development. Finally, we also recorded the spontaneous electrical activities and responses to enteric motor nerve stimulation in the proximal and distal regions of wild-type and ls/ls colons.

MATERIALS AND METHODS

Animals. Lethal spotted homozygotes (ls/ls; a spontaneous mutation in C57BL strain) and age-matched wild-type controls (same C57BL/6 background strain as the ls/ls mice), CD1 or BALB/c mice between the ages of 20 and 30 days postpartum, were anesthetized by carbon dioxide suffocation and exanguinated by cervical dislocation followed by decapitation. CD-1 and BALB/c mice were used to generalize our observations across several stains and to ensure that any observed defects were not specific to the C57BL/6J strain. Data obtained from CD-1 and BALB/c mice were only used for comparison and not included in the final data analysis or figures. The use and treatment of animals were approved by the University of Nevada Institutional Animal Use and Care Committee.

The entire colon from 1 mm below the ileocecal sphincter to the anus was removed and placed in Krebs-Ringer buffer (KRB). The bowel was opened along the mesenteric border, and the luminal contents were washed out with KRB. The mucosa was left intact when cryostat sections were to be cut; however, when electrophysiological recordings and whole mount immunohistochemical studies were to be carried out, the mucosa was removed by sharp dissection, revealing the underlying tunica muscularis.

Morphological studies. Immunohistochemical studies were carried out with tissues dehydrated in graded sucrose solutions, embedded in Tissue-Tek (Miles), and frozen in liquid nitrogen. Cryostat sections were cut at 10-μm thickness, fixed in acetone for 5 min, washed with PBS (0.1 M; 4 times for 15 min each), and preincubated with goat nonimmune serum for 1 h (10% in 0.1 M PBS) before being incubated with a monoclonal antibody against Kit protein (ACK-2; Gibco-BRL, Gaithersburg, MD; 5 μg/ml in PBS, 0.1 M) at 4°C overnight. Immunoreactivity was detected with FITC-conjugated secondary antibody [goat anti-rat (1:100) for 1 h at room temperature; Vector Laboratories, Burlingame, CA]. Control tissues were prepared in a similar manner, omitting ACK-2 or secondary antibodies from the incubation solution.

Whole mount preparations were labeled with the ACK-2 antibody as described above. Tissues were placed in KRB containing nifedipine (1 μM for 15 min) and stretched to 110% of resting length and width by pinning the tissue to the base of a Sylgard elastomer dish (Dow Corning, Midland, MI). The mucosa was removed by sharp dissection, and the tunica muscularis was fixed in acetone for 10 min at 4°C. After fixation, tissues were washed in PBS for 1 h (4 times for 15 min each) before demonstrating Kit immunoreactivity as described above.

For immunohistochemical studies, labeled tissues were examined using a Bio-Rad MRC 600 confocal microscope, with an excitation wavelength appropriate for FITC (496 nm). Confocal micrographs were obtained every centimeter along the length of the colon from the most proximal region adjacent to the ileocecal sphincter to the internal anal sphincter (IAS). Digital composites are of Z-series scans of 1-μm optical sections through a depth of 10 μm for cryostat sections and 15–20 μm for whole mounts. Final images were constructed with Bio-Rad Comos software.

ICC within the circular muscle layer (IC-IM) were quantified from cryostat cross sections cut in transverse section to the circular muscle layer. Ten digital images (10 images of 1 μm each) were collected from each region of six wild-type and five ls/ls mutant animals using a ×40 objective and composites made using Bio-Rad software. Cross-sectional profiles of IC-IM were counted within the area of the image (289 × 193 μm) and the number of cells extrapolated to the number per square millimeter within the circular muscle layer.

Reduced NADP (NADPH)-diaphorase histochemistry was carried out with whole mounts fixed with formaldehyde, freshly prepared from paraformaldehyde, as described previously (36, 44). Briefly, terminal segments of colon (4–5 × 5-mm segments from the anal end) were removed from ls/ls and wild-type control animals. The luminal contents were washed out, and the mucosa was removed by sharp dissection. In most cases, half of the colonic wall (mesenteric to anti-mesenteric border) was examined for NADPH-diaphorase activity and the other half used for electrophysiological studies. In some preparations, the absence of enteric nerves was also confirmed using the same preparation on which electrophysiological studies were performed. Muscles were subsequently incubated with nifedipine (1 μM) for 15 min at room temperature and stretched to 110% of their relaxed state before fixation with formaldehyde (4% wt/vol, from paraformaldehyde, in PBS (0.1 M, pH 7.4) for 2 h at 4°C. After fixation, muscles were washed with PBS for 30 min (3 times for 10 min each), and NADPH-diaphorase activity was demonstrated by incubation with PBS (pH 7.4) containing 1.2 mM β-NADPH and 0.3 mM nitrotetrazolium blue (Sigma, St. Louis, MO) for 30 min at 37°C. After staining, whole mount preparations were washed with PBS and mounted in Aquamount (Lerner Laboratories, Pittsburgh, PA) before examination by means of bright-field microscopy using a Leitz Diaplan microscope (Wetzlar, Germany).

Physiological experiments. After removing the mucosa, strips of proximal and distal colons (~1 cm in circumference × 0.5 mm in length) were cut and pinned to the Sylgard (Dow Corning) elastomer-lined floor of a recording chamber with the mucosal side of the circular muscle facing upward. The proximal colons were taken 1–2 cm from the ileocecal sphincter and characterized by the presence of haustra in the lamina propria and mucosal lining; the distal colon-rectum region was characterized as the terminal 2–4 mm of tissue. Circular muscle cells were impaled with glass microelectrodes filled with 3 M KCl; electrode resistances were 50–80 MΩ. Transmembrane potentials were measured by using a high-input impedance amplifier (WPI Duo 773, Sarasota, FL). Outputs were displayed on an oscilloscope. Electrical signals were recorded on videotape (model 420M, Vetter, Rebersburg, PA). Responses to nerve stimulation were elicited by square-wave pulses of electrical field stimulation (EFS; Grass S48, Quincy, MA; 1–20 Hz for 1 s; 0.5 ms in duration at supramaximal voltage) delivered via parallel platinum electrodes placed on either side of the muscle strips. Data are expressed as means ± SE. Differences in the data were evaluated by Student’s t-test. Differences between means were considered to be significant at P < 0.05. The number of cells from which recordings were made is denoted by n. The number of animals from which n was obtained is also provided. For morphological studies, 12 wild-type control...
animals and 11 ls/ls animals were used; for electrophysiological experiments, 7 wild-type control and 6 ls/ls animals were used.

**Solutions and drugs** Muscles were maintained in KRB (37.5 ± 0.5°C; pH 7.3–7.4) containing (in mM) 137.4 Na⁺, 5.9 K⁺, 2.5 Ca²⁺, 1.2 Mg, 134 Cl⁻, 15.5 HCO₃⁻, 1.2 H₂PO₄⁻, and 11.5 dextrose and bubbled with 97% O₂-3% CO₂. Atropine sulfate, guanethidine sulfate, and N⁴-nitro-L-arginine (l-NNA) or N⁴-nitro-l-arginine methyl ester (l-NAME; Sigma) were dissolved in distilled water at 10⁻¹-10⁻² M and diluted in KRB to the stated final concentrations.

**RESULTS**

After relaxation was induced with nifedipine and muscles were stretched to 110% of their original length, the average length of the colons of 20- to 30-day-old mice was 8.1 ± 0.3 cm (n = 14).

**Lack of nitric oxide synthase enteric neurons in aganglionic colon of ls/ls mice.** Tissues from ls/ls animals used for immunohistochemistry and electrical recordings were tested for the presence of nitric oxide synthase (NOS)-containing enteric neurons. NADPH-diaphorase staining, a histochemical test for NOS-containing nerves in the myenteric plexus (36, 44), was used as a rapid assay to determine whether a given segment of muscle was aganglionic. Adjacent sections of tissue (0.5 × 0.5 mm) from wild-type (n = 7 animals) and ls/ls animals (n = 6 animals) were used for electrical recordings and NADPH-diaphorase histochemistry. In tissues from wild-type mice, NADPH-diaphorase activity was found in a subpopulation of myenteric neurons in regions 1 to 4 mm from the IAS. NADPH-diaphorase-containing fibers were found to surround myenteric neurons that did or did not possess NADPH-diaphorase activity. Nerve fibers surrounding ganglia and traveling within nerve fascicles between ganglia exhibited numerous NADPH-diaphorase-containing varicosities (Fig. 1A). Myenteric neurons, including those containing NADPH-diaphorase, were totally lacking in the same region of the colons of ls/ls mice. NADPH-diaphorase-containing nerve trunks were observed traversing through the aganglionic region in an oral-to-aboral direction and circumferentially around the terminal colon, however, these fascicles were large in diameter and not varicose (Fig. 1B).

Enteric neurons were observed in the distal colons of ls/ls mutant mice orad to the site of circular muscle constriction at distances of ~10 mm from the IAS. **Morphological appearance of ICC in murine colon.** An antibody (ACK-2) to Kit protein was used to ascertain the locations of ICC within the tunica muscularis of the murine colon of wild-type controls. ICC were found at several levels, and variations in cell morphology were most evident in the proximal colon (Fig. 2; n = 6 animals). IC-SM were observed, and at the light microscopy level, these cells were structurally similar to IC-IM (Fig. 2, B and E). Z-series reconstructions of the circular layer revealed that IC-SM and IC-IM formed an extensive three-dimensional network in the proximal colon. IC-SM and IC-IM in the colon possessed a prominent nucleic region and were oriantated along the longitudinal axis of the circular smooth muscle cells. The long axis of IC-SM and IC-IM was ~300 µm in length, and numerous (4–10) lateral branching processes extended from the main axis of the ICC and contacted the cell bodies or processes of adjacent ICC.

In the myenteric plexus region, the profiles of IC-MY...
were flattened and their nuclear region was quite prominent (Fig. 2, C, E, and F). Multiple fine processes extended from the perinuclear region of IC-MY and formed contacts with primary or secondary processes of adjacent IC-MY. Thus these cells were organized into a flat anastomosing network, which was 1–2 cells thick. IC-MY possessed a flattened profile and a prominent nuclear region (arrows). In the longitudinal muscle layer, ICC formed an interconnecting network of cells that ran parallel to smooth muscle fibers of this muscle layer (D; arrows). In cryostat sections (E and F; arrows), the locations of each distinct class of ICC could be observed. CM, circular muscle layer; LM, longitudinal muscle layer; SMB, submucosal surface of the circular muscle layer; MG, myenteric ganglion. Scale bar in D applies to A–D; scale bar in F applies to E and F.

Distribution of ICC along the colon. ICC were distributed along the entire length of the colon, from the most proximal region to the IAS. The density of ICC varied considerably, being greatest in the most proximal regions (0–to 3-cm region) and reduced in number toward the distal colon and IAS. Because of the high density of ICC in the most proximal regions of the colon, numbers could not be accurately calculated using whole mount preparations. However, cryostat sections through the wall of specific regions revealed the number of cells and their exact locations. In the proximal colon (2 cm from the ileocecal sphincter), the number of IC-IM within the circular muscle layer averaged $1,768 \pm 190$ cells/mm$^2$. Toward the distal regions of the colon (4–8 cm), ICC decreased from $1,246 \pm 57$ cells/mm$^2$ at 4 cm along the colon to $581 \pm 61$ cells/mm$^2$ at 8 cm along the length of the colon (Fig. 3).

The decrease in numbers of ICC along the murine colon appeared to be due to a reduction in specific populations of ICC. Approximately midway along the colon (4 cm), the numbers of IC-MY decreased in density (although exact numbers could not be determined in cryostat sections), and at the rectoanal region of the terminal colon these cells were greatly reduced in number. In this region of the colon, IC-IM, which were predominately located within the circular muscle layer, were spindle shaped with occasional lateral processes that contacted neighboring IC-IM. IC-IM within the circular muscle of the distal colon (6–8 cm from the proximal) also appeared to decrease in numbers and occasionally occurred in bundles running through the circular muscle layer. Occasional IC-IM were also observed within the longitudinal muscle layer (Fig. 3).

Distribution of ICC in colon of ls/ls mutant. We investigated the distribution of ICC along the length of ls/ls colon ($n = 5$) to determine whether there were differences from wild-type mice in the density or mor-
phology of ICC in the aganglionic regions of the large intestine or whether apparent differences were due to the normal variation in ICC along the length of the colon. The distribution of ICC along the colon of ls/ls mice was also compared with that of age-matched control animals. The distribution of the different classes of ICC in ls/ls mice was similar to that of control animals along the complete length of colon, including the aganglionic terminal region (Fig. 4). In the most proximal part (2 cm from the ileocecal sphincter), the number of IC-IM averaged 1,680 ± 145 cells/mm² within the circular muscle layer. This number decreased to 1,190 ± 65 cells/mm² at a distance of 4 cm along the colon and to 630 ± 45 cells/mm² at 8 cm along the colon (P > 0.05 for all values compared with similar regions in wild-type animals). IC-IM within the circular layer were occasionally observed to exist in bundles running through the circular muscle layer (Fig. 4, C and D). Thus the presence of enteric neurons in the terminal regions of the distal colon does not appear to be essential for the phenotypic development of ICC in this region of the GI tract. Furthermore, the partial distension of the large intestine and the associated hypertrophy and hyperplasia of the circular and longitudinal muscle layers of the colon wall, proximal to the site of aganglionosis, which occur in the ls/ls colon (8, 24), did not produce a significant change in the distribution of ICC in this segment of the colon.

Fig. 3. The distribution and morphological appearance of specific classes of ICC varied along the length of the murine colon. A–D: whole mount preparations of the tunica muscularis taken every 2 cm along the length of the colon. In the proximal colon (2 cm; A), the density of IC-SM, intramuscular ICC (IC-IM; arrows), and IC-MY was greatest but became more dissipated in an oral-to-aboral direction. In the terminal colon, the anastomosing networks of IC-MY were greatly reduced in density (C and D). Cryostat sections (E–H) taken at different sites along the colon also confirmed the dissipation of ICC networks. IC-SM and IC-IM (arrows) and IC-MY (*) also appeared reduced in number along the length of the colon when examined with cryostat cross sections through the tunica muscularis. Scale bar in D applies to A–D; scale bar in H applies to E–H.
Electrical activity of proximal colons of wild-type and ls/ls mutants. Intracellular electrical recordings were obtained from the proximal and distal colons of both wild-type and ls/ls animals. Resting membrane potentials averaged \(-49 \pm 2\) mV (n = 20 cells from 10 animals) in the circular muscle layer of the proximal colons of wild-type mice. Spontaneous spike complexes with an average amplitude of \(35.5 \pm 2.8\) mV and a frequency of \(2.9 \pm 0.2\) cycles/min occurred in the circular layer. An average of \(11 \pm 2\) action potentials was associated with each complex (Fig. 5A). In the proximal colons of ls/ls mice, resting membrane potentials averaged \(-48 \pm 2\) mV (n = 36 cells from 8 animals, Fig. 5B). These data were not significantly different from those obtained in control animals (P > 0.05). Spontaneous electrical activity in the proximal colons of ls/ls mice consisted of spike complexes with an average amplitude of \(32 \pm 4\) mV and a frequency of \(2.6 \pm 0.6\) cycles/min. These findings were not significantly different from those of control animals (Fig. 5A) and are similar to the electrical activity of proximal colonic circular muscle previously reported (13).

EFS of circular muscles (single pulse, 0.5-ms duration) from the proximal colon of wild-type animals elicited a TTX-sensitive hyperpolarization that averaged \(15.8 \pm 1.7\) mV in amplitude and \(3.2 \pm 0.5\) s in duration (Fig. 5C). EFS of the circular muscle layer of the proximal colons of ls/ls mutants also hyperpolarized the membrane potential; responses averaged \(11.1 \pm 1.2\) mV in amplitude and \(2.4 \pm 0.6\) s in duration.

Fig. 4. The density and distribution of specific classes of ICC in the colons of ls/ls mutant animals did not differ from wild-type control animals. A–D: the distribution of ICC in digital reconstructions of the tunica muscularis of whole mount preparations taken at different sites along the length of the colon of a ls/ls mutant animal. E–H: cryostat sections through the tunica muscularis at different distances (2–8 cm) in an oral-to-aboral direction of a ls/ls mutant animal. ICC populations (IC-IM, arrows; IC-MY, *) decreased in a proximal-to-distal manner similar to that of wild-type control animals. Scale bar in D applies to A–D; scale bar in H applies to E–H.
These data are not statistically different from each other ($n = 36$ cells from 6 animals; $P > 0.05$).

**Electrical activity of distal colons of wild-type and ls/ls mice.** Spike complexes, such as those seen in the proximal colon, were not observed in the circular muscle layer of the distal colons of controls. Resting membrane potentials of circular muscle cells in the distal colons of control animals averaged $-48 \pm 0.4$ mV ($n = 30$ cells from 10 animals). Small fluctuations in membrane potential were seen, and spontaneous action potentials were superimposed on the resting potential. Action potentials averaged $38 \pm 4.5$ mV in amplitude and occurred intermittently at a frequency of $20.5 \pm 2.8$ cycles/min (Fig. 6).

EFS of circular muscles (single pulse, 10 Hz for 1 s, 0.5-ms duration) from the distal colons of control animals produced a TTX-sensitive hyperpolarization of the membrane potential. The amplitude and duration of the hyperpolarization were frequency dependent. Single pulses of EFS (0.5-ms duration) evoked membrane hyperpolarizations that averaged $17.5 \pm 1.5$ mV in amplitude and $1 \pm 0.05$ s in duration ($n = 25$ cells from 7 animals). EFS (10 Hz for 1 s; 0.5-ms duration) produced an average membrane hyperpolarization of $23 \pm 2.3$ mV and $2.8 \pm 0.3$ s in duration ($n = 20$ cells from 7 animals; $P < 0.05$ vs. 1 pulse).

The resting membrane potential of the circular muscle layer in the distal colon of ls/ls mice averaged $-50.9 \pm 1.4$ mV ($n = 48$ cells from 6 animals); this value was not significant compared with tissues from wild-type animals. Spontaneous action potentials were absent from the majority of circular muscle cells of the distal colon (5 of 8 mice; Fig. 7A). In two animals, small oscillations of the membrane potential were observed that averaged $6.5 \pm 1$ mV in amplitude with a frequency of $26 \pm 2.4$ cycles/min (Fig. 7, B and C). In one animal, spontaneous spiking activity was seen; spikes averaged $20 \pm 3$ mV in amplitude and 36 cycles/min in frequency. These events occurred in bursts of $\sim 1$ min.

Fig. 6. Intracellular recordings of the circular muscle layer from the distal colon of a control animal. Spontaneous electrical activity consisted of action potentials that were irregular in both frequency and amplitude. A–C: EFS (1–10 Hz for 1 s; 0.5-ms in duration; arrows) produced a hyperpolarization in membrane potential or IJP. The amplitude and duration of the IJP was frequency dependent. On cessation of the EFS, electrical activity often consisted of an increased frequency in action potentials or rebound excitation.
Interspersed with the spikes were small oscillations of the membrane potential that occurred with a frequency of $40 \pm 2$ cycles/min (Fig. 7D). EFS of the circular muscle layer of the terminal colon in *ls/ls* mutants elicited a mixture of postjunctional responses. In the majority of preparations (70.3% of a total of 27 recordings), no response to EFS (1–20 Hz for 1 s; 0.5-ms pulse duration) was observed (Fig. 8A). In one preparation (3.7%), inhibitory junction potentials (11 mV in amplitude and 3 s in duration) were recorded in response to EFS (0.5 ms, single pulse). In 26% of the tissues, excitatory junction potentials, averaging $18.4 \pm 3.4$ mV in amplitude and $0.7 \pm 0.2$ s in duration, were recorded in response to similar EFS stimuli. EFS produced a graded excitatory response in the electrical and mechanical activities, being greatest with higher stimulus parameters. Also associated with EFS was a graded depolarization in the resting membrane potential that was most prominent at 20 Hz (Fig. 8, B and C). Each spike produced a small oscillation in mechanical excitability.

Fig. 7. Typical intracellular recordings from the circular muscle layer of the distal colons of *ls/ls* mutants. Electrical activity was recorded from aganglionic regions of terminal colons (confirmed by histochemistry as shown in Fig. 1). The majority of recordings from the circular muscle layers of *ls/ls* mutants displayed little or no spontaneous electrical activity (62.5%; A). In 2 animals (25%), small oscillations in membrane potential averaging $6.5 \pm 1$ mV in amplitude were recorded (B and C), and in 1 animal (12.5%) spontaneous action potentials occurred in bursts with a frequency of 1/min (D).

Fig. 8. Postjunctional neural responses recorded from the circular muscle layer of the distal colons of *ls/ls* mutant animals. In the majority of circular muscle cells (70.3%), no changes in membrane potential were recorded in response to EFS (A; 1–20 Hz for 1 s; 0.5-ms duration; arrows indicate point of EFS, downward deflections are EFS artifacts). In 1 preparation, small IJPs were recorded, and in 26% of the tissues excitatory junction potentials were recorded that displayed a frequency dependent increase in the electrical (B) and mechanical excitability (C) of the tissue. B and C are simultaneous electrical and mechanical recordings from the same tissue. The excitatory junction potential in the distal colons of *ls/ls* mutant animals was inhibited by atropine, suggesting the activation of muscarinic receptors (D).
activity that was superimposed on the EFS-evoked contractions (Fig. 8B). These events were completely inhibited by atropine (1 μM; Fig. 8D), suggesting that they were cholinergically mediated and due to the activation of muscarinic receptors.

DISCUSSION

ICC, identified on the basis of their Kit-like immunoreactivity, were heterogeneous in morphology and differently distributed in different regions of the murine colon. The greatest density of ICC was observed in the most proximal colon. Subpopulations of ICC were located along the submucosal surface of the circular muscle layer (IC-SM), within the circular and longitudinal muscle layers (IC-IM), and around the myenteric plexus (IC-MY). The densities of ICC subpopulations were not homogenous along the colon, but decreased proximodistally. In a previous report (29), Kit-immunoreactive cells were found beneath the serosa, around the myenteric plexus in the outer but not the inner half of the circular muscle layer, and associated with the plexus of nerve fibers found at the interface between the submucosa and the circular muscle. In contrast, in the current study, ICC were observed throughout the entire circular and longitudinal muscle layers; that is, they were not restricted to the outer half of the circular muscle and they were observed in the longitudinal layer of smooth muscle. Regional differences in the distribution of ICC throughout the human colon have also been reported (6, 25). In humans, Kit-immunoreactive cells are mainly distributed in the circular and longitudinal muscle layers. In human circular muscle, the number of Kit-immunoreactive cells has been reported (7, 25) to be greater in the distal than in the proximal colon; moreover, no distinct concentrations of Kit-immunoreactive cells were observed around the myenteric plexus.

The Ls/ls mouse lacks neurons in the terminal portion of the intestine (14) because of a point mutation in the ET-3 gene that prevents the conversion of big ET-3 to the active ET-3 peptide (1). Stimulation of EDNRB by ET-3 inhibits the differentiation of migrating neural crest-derived precursors into postmitotic neurons, which are thought not to be migratory (40). The effect of deprivation of this stimulus is to cause neurons to differentiate prematurely at the expense of migrating precursors. The early differentiation of neurons also depletes the pool of proliferating neuronal progenitor cells. As a result, the last portion of the bowel to receive émigrés from the neural crest becomes aganglionic. The vagal pool of crest-derived cells fades out proximally, and the sacral precursors cease migrating short of the bowel itself, forming ectopic pelvic ganglia. The absence of crest-derived precursors in the terminal gut of Ls/ls mice thus provides a good test of the ability of enteric neurons to influence the development, distribution, and function of ICC.

The density and distribution of ICC in the aganglionic region of the colon of Ls/ls animals were similar to those of ICC at the same level of the colon of age-matched wild-type control animals. Enteric neurons, therefore, are not necessary for the development of ICC in the terminal region of the colon. The distribution of ICC has been investigated (30, 42) in the terminal aganglionic bowel in HD. Vanderwinden et al. (30) reported that ICC are scarce in the aganglionic gut and claimed that their networks are greatly disrupted, whereas Yamataka et al. (42) reported that only a few Kit-immunopositive cells were found in the bowel segments from 12 patients with HD compared with many Kit-positive cells in the ganglionic bowel segments from 14 control patients. However, Horisawa et al. (10), who used confocal microscopy to investigate ICC in whole mount preparations of human colon, found that the density and distribution of ICC in the aganglionic colon segments of six patients with HD were not different from those of the normal human colon. Horisawa and co-workers (10) attributed the disparity between results obtained by different investigators to regional differences in the density of ICC in the colon. An apparent decrease in the numbers of ICC might mistakenly be attributed to aganglionsis if identical regions were not used as controls; therefore, in the present study, the regional distribution of ICC in the murine colon was carefully assessed before conclusions were drawn about the numbers and distribution of ICC in the aganglionic region of the Ls/ls colon. It is thus clear that the density of ICC in the aganglionic gut of Ls/ls mice is not distinguishable from that in the equivalent portion of the bowel of control animals.

The tyrosine kinase receptor, Kit, is essential for the development of ICC within the GI tract (see Ref. 22 for review). Kit is a transmembrane protein with an extracellular receptor region and an intracellular tyrosine kinase domain. Binding of SCF, the natural ligand for Kit, activates the tyrosine kinase, causing Kit to autophosphorylate and generate high-affinity binding sites in its kinase insert sequence, binding and activating secondary signaling molecules (38). Membrane-bound SCF is the most biologically active form of SCF (17); therefore, cells requiring Kit signaling must form close associations with SCF-presenting cells in their immediate vicinity. Loss-of-function mutations that affect either Kit or membrane-bound SCF compromise the development of specific populations of ICC in various regions of the GI tract (2, 11, 32, 33). Specifically, Kit and SCF-deficient mice lose IC-MY in the small intestine and exhibit an associated loss of slow wave activity. They also lose IC-IM in the gastric fundus, which causes excitatory and inhibitory enteric neurotransmission to the smooth muscle to become abnormal (2, 31, 34). Cells that express SCF in the GI tracts of transgenic mice that express lacZ under the control of the SCF promoter can be identified by histochemically demonstrating β-galactosidase activity (28). Examination of these mice reveals that a subpopulation of myenteric neurons expresses SCF. This observation led to the suggestion that the SCF expressed by enteric neurons could be the source of the SCF that promotes the development of and then maintains ICC in the bowel (28). The development of ICC and slow
waves does not appear to require enteric nerves to supply SCF. ICC have been found to develop independently of enteric neurons in chorioallantoic membrane grafts of chick hindgut explanted before the colonization of the hindgut by precursors from the neural crest (15). ICC have also been found (43) to develop under similar conditions in aneuronal segments of fetal mouse intestine. In this case (43), the fetal hindgut that was transplanted under the renal capsule of adult host animals before the donor bowel contains neurons or precursors that can survive in the adult environment of the renal capsule. mRNA encoding Kit and SCF as well as Kit-immunoreactive cells have been reported (41) to be present in the aganglionic gut of ls/ls and c-ret knockout mice. These data are in good agreement with the observation that specific populations of ICC are also present in aganglionic proximal regions of the GI tracts of mice that lack GDNF, the ligand for Ret; moreover, slow wave activity is associated with the development of these ICC (35). ICC have been found (41) to develop in cultures of ls/ls terminal colon, which contains no neural crest-derived precursor cells, and when crest- and noncrest-derived cells are immunoselected from the fetal mouse gut and cultured separately, ICC develop only from the noncrest-derived cells. SCF immunoreactivity, furthermore, has been found in cells that coexpress neuronal and smooth muscle markers. Mammalian ICC, therefore, are not derived from the neural crest nor are they developmentally dependent on neurons. Similarly, the development of enteric motor neurons is not dependent on the presence of ICC (2, 31), although the two cell types may develop in association with one another when both are present in the developing gut. An examination of the time dependence of Kit expression in fetal mice revealed that mRNA encoding Kit is present in the bowel as early as embryonic day 11, although Kit-immunoreactive ICC cannot be detected until embryonic day 12 in the foregut and embryonic day 14 in the hindgut; moreover, the development of ICC lags behind that of enteric neurons and smooth muscle cells (41).

Changes in the distribution or density or irregularities in the ultrastructure of organelles of ICC are current morphological criteria used to assess an alteration in ICC function in various motility disorders. Even if ICC are present in typical numbers and normally distributed in the bowel they may not function normally. When particular sphincters or regions of the gut do not function properly, as in infantile hypertrophic pyloric stenosis, idiopathic megarectum, and megacolon or Hirschsprung's stenosis of the rectoanal region of the GI tract, hypertrophy and hyperplasia frequently occur in the tunica muscularis of both humans and animals (14, 37). The hypertrophic muscle may undergo a structural and biochemical remodeling, which is associated with an increased force output in the portion of the bowel immediately orad to the aganglionic segment (8, 24). Such subtle changes in the tunica muscularis may not alter the development of ICC but may change their function; however, this possibility has yet to be demonstrated. Although the distribution of Kit-immunoreactive ICC in the terminal aganglionic regions of ls/ls mutant mice was similar to that of wild-type controls, the loss of spontaneous electrical activity and responses to nerve stimulation in the terminal colon of the majority of ls/ls mice suggests that ICC function may be compromised in this enteric nervous system-free tissue.

Electrical activity has previously been recorded with suction electrodes placed on the serosal surface of the aganglionic region of the colon of piebald lethal mice. These mice lack the gene encoding EDNRB, for which ET-3 is the natural ligand. Spontaneous electrical discharges were recorded from the distal bowel at a site 5 mm from the anal end (39). The aganglionic bowel appeared to be abnormally contracted. Peristaltic waves of contraction of the circular muscle layer that originated orad to the aganglionic site appeared to propagate in oral and aboral directions but did not enter the contracted region of the piebald mouse gut (39).

The loss of a dominant inhibitory junction potential in response to EFS in ls/ls aganglionic tissues is likely to be the defect that leads to a failure of circular muscle relaxation in this region of the gut. This relaxation appears to be essential for normal sphincter opening and defecation (3, 21). In the majority of ls/ls mice, there was a complete absence of postjunctional neural responses to EFS. In a small subset (2) of animals, however, a cholinergic excitatory junction potential, blocked by the muscarinic antagonist atropine, was detected. Mechanical recordings from the IAS of ls/ls mutant animals revealed that the predominant response to nerve stimulation was contractile, whereas a relaxation followed by an aftercontraction was typically seen in wild-type control mice (3, 21). The presence of excitatory junction potentials in some tissues suggests that there is an excitatory cholinergic innervation of the muscle in an at least some aganglionic colonic segments. This innervation may derive from an extrinsic source originating in the sacral parasympathetic nucleus or from descending excitatory motor neurons in the hypoganglionic region proximal to the aganglionic section of colon (18). Activation of these nerves in the aganglionic region of the gut could lead to contraction rather than relaxation of the terminal colons of ls/ls mutant mice (Ref. 3 and the current study) and may contribute to the abnormal contractions seen in the colons of piebald lethal mice (39).

In conclusion, ICC were observed throughout the murine colon. We observed a gradient in the distribution of ICC along the colon but, despite the aganglionosis of the ls/ls terminal colon, no differences between ls/ls mice and their wild-type controls were observed in the density of ICC in this region of the gut. Loss of spontaneous electrical activity and postjunctional neural responses are likely to be a consequence of the loss of enteric nerves, rather than of defects in ICC networks. By analogy, it is similarly plausible that the loss of the coordinated neural activity associated with the absence of ganglia and a deficient inhibitory innervation of muscle, rather than an abnormality of ICC, underlies the intestinal pseudo- obstruction of HD.
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