Anatomic modifications in the enteric nervous system of piebald mice and physiological consequences to colonic motor activity

Seungil Ro,* Sung Jin Hwang,* Melodie Muto, William Keith Jewett, and Nick J. Spencer

Department of Physiology and Cell Biology, University of Nevada School of Medicine, Reno, Nevada

Submitted 6 September 2005; accepted in final form 1 December 2005

Ro, Seungil, Sung Jin Hwang, Melodie Muto, William Keith Jewett, and Nick J. Spencer. Anatomic modifications in the enteric nervous system of piebald mice and physiological consequences to colonic motor activity. Am J Physiol Gastrointest Liver Physiol 290: G710–G718, 2006. First published December 8, 2005; doi:10.1152/ajpgi.00420.2005.—It has been assumed that in piebald lethal mice that develop megacolon, impaired colonic motor activity is restricted to the aganglionic distal colon. Peristaltic mechanical recordings, immunohistochemistry, and quantitative PCR were used to investigate whether regions of the colon, other than the aganglionic segment, may also show anatomical modifications and dysfunctional colonic motor activity. Contrary to expectations, colonic migrating motor complexes (MMCs) were absent along the whole colon of piebald lethal homozygote mice and severely impaired in heterozygote siblings. Aganglionosis was detected not only in the distal colon of piebald homozygote lethal mice (mean length: 20.4 ± 2.1 mm) but also surprisingly in their heterozygote siblings (mean length: 12.4 ± 1.1 mm). Unlike homozygote lethal mice, piebald heterozygotes showed no signs of megacolon. Interestingly, mRNA expression for Ednrb was also dramatically reduced (by 71–99%) throughout the entire small and large bowel in both homozygote lethal and heterozygous littermates (by 67–87%). Histochemical staining confirmed a significant reduction in myenteric ganglia along the whole colon. In summary, the piebald mutation in homozygote lethal and heterozygote siblings is associated with dramatic reductions in myenteric ganglia throughout the entire colon and not limited to the distal colon as originally thought. Functionally, this results in an absence or severe impairment of colonic MMC activity in both piebald homozygote lethal and heterozygote siblings, respectively. The observation that piebald heterozygotes have an aganglionic distal colon (mean length: 12 mm) but live a normal murine life span without megacolon suggests that aganglionosis >12 mm and the complete absence of colonic MMCs may be required before any symptoms of megacolon arise.

migrating motor complex; endothelin B receptor

PIEBALD LETHAL MICE undergo a spontaneous mutation of the endothelin B receptor gene (ednrb). This mutation results in failure of the development of at least two neural crest-derived cell lineages, epidermal melanocytes and intrinsic myenteric ganglia. Consequently, homozygous lethal piebald mice inherit congenital megacolon because of the absence of enteric ganglia (aganglionosis) in the distal colon (10, 14, 15, 17). In fact, defective signaling through EDNRB results in aganglionosis in mice, rats, horses, and humans, largely because of the failure of the neural crest to invade the distal colon (18). The development of aganglionic megacolon in piebald lethal mice is strikingly similar to the aganglionosis detected in human Hirschsprung’s disease in humans. It is for this reason that the piebald lethal mouse provides an ideal model for studies on Hirschsprung’s disease in humans.

The mechanisms by which piebald lethal mice develop megacolon is not fully understood. Wood (15) has suggested that piebald homozygous lethal mice develop megacolon largely because of “the lack of intrinsically active inhibitory neurons” in the aganglionic region (15–17). It is clear that in wild-type control mice the circular muscle (CM) layer is normally maintained in a state of tonic neurogenic inhibition, and in aganglionic regions of colon without enteric inhibitory motor neurons there is no tonic inhibition of the musculature, causing the CM to remain contracted. Consequently, these piebald lethal homozygotes fail to expel pellets from the rectum, leading to megacolon (2, 14, 15). Indeed, it has been shown that the CM layer of wild-type preparations of mouse colon are also maintained in a state of tonic neurogenic inhibition during the intervals between propulsive colonic migrating motor complexes (MMCs; see Refs. 5, 8, 11, 12, and 14).

Piebald homozygous lethal mice are readily identified from their heterozygote littermates based solely on differences in coat color (10, 14). Homozygous lethal mice are completely white, with only a small black spot usually located on their forehead. These mice typically die within 4–6 wk after birth, due largely to severe megacolon and/or imperforate anus (2, 10, 14, 15). In contrast, the coat color of piebald heterozygotes shows a mixture of black and white spotting, and these mice appear to live for a similar duration as other wild-type strains of mouse (10, 14). In fact, previous studies have used piebald heterozygote littermates as experimental controls, with the assumption that they are phenotypically normal. Furthermore, it has also been assumed that, in piebald homozygote lethal mice, only the aganglionic distal colon is anatomically and functionally impaired by the piebald mutation. In this study, we investigated the possibility that the entire colon of the piebald lethal mouse may be modified by the piebald mutation and not simply restricted to the distal aganglionic segment. Furthermore, we have investigated whether piebald heterozygote siblings do, in fact, represent a control phenotype or whether they also show an impaired myenteric plexus and dysfunctional colonic motor activity, similar to their homozygote lethal siblings.

Our results suggest that, contrary to expectation, the expression and development of myenteric ganglia is significantly reduced throughout the entire colon of both piebald heterozygote and homozygote lethal mice and not just restricted to the...
distal aganglionic colon of piebald lethal mice, as previously thought. Functionally, piebald lethal homozygote mice do not show any coordinated colonic MMCs, and their heterozygote siblings have severely impaired colonic motor activity. These results are discussed in relation to how piebald homozygote lethal mice develop megacolon and die soon after birth, but their heterozygote siblings do not develop megacolon and live normally.

**MATERIALS AND METHODS**

**Mouse strains.** C57BL/6J controls and piebald homozygote mice (ednrbl~b~/ednrbl~b~) were obtained from the Jackson Laboratory. The gene Piebald-lethal, Sl, was first detected by Jackson Laboratories in 1959. It was identified in the F2 generation of a cross between C3H/HeJ and C57BL/6J strains. For this reason, the C57BL/6J strain is routinely used as an experimental control for more recent studies on C3H/HeJ and C57BL/6J strains. For this reason, the C57BL/6J strain was used as wild-type controls, as performed previously by Jackson Laboratories.

**RNA isolation and RT-PCR.** Total RNA was extracted from the jejenum, ileum, cecum, proximal colon, midcolon, and distal colon of 4-wk-old C57BL/6J, ednrbl~b~/ednrbl~b~, and ednrbl~b~/ednrbl~b~/ mice. The tissue was stripped free of mucosa and submucous plexus, and TRIzol (Invitrogen) was used to isolate total RNA according to the manufacturer’s instructions. First-strand cDNA was synthesized using 200 units SuperScript II (Invitrogen), dNTP (10 mM each), and 500 ng/μl oligo(dT) primers at 42°C for 50 min in the presence of 2 μg total RNA in a 40-μl reaction volume. cDNA was then purified using Amplitaq Gold PCR Master Mix (Applied Biosystems), 1 mM of the primers in a 25-μl reaction mixture, and 500 ng/μl oligo(dT) primers at 42°C for 50 min in the presence of 2 μg total RNA in a 40-μl reaction volume. cDNA was then purified using the QIAquick PCR Purification Kit (Qiagen). Gene-specific oligonucleotide primers were designed and synthesized for RT-PCR and Q-PCR (Table 1). The PCR was performed using 12.5 μl of 2× AmpliTaq Gold PCR Master Mix (Applied Biosystems), 1 μl of the synthesized cDNA, and 10 μM of the primers in a 25-μl reaction volume on the GeneAmp PCR system 2700 (Applied Biosystems). A two-step PCR method (95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 1 min) was used. After PCR, 2 μl of the RT-PCR product were analyzed on a 1.5% agarose gel. The fragments amplified by RT-PCR were gel eluted using the QIAquick PCR Purification Kit (Qiagen) and analyzed by direct sequencing.

**Quantitative PCR.** The same cDNAs and gene-specific primers as used for RT-PCR were also used for quantitative PCR (Q-PCR). Real-time Q-PCR was performed using Syber green chemistry on an ABI Prism 7700 sequence detector (PE Biosystems). Regression analysis of the mean values of eight multiplex RT-PCRs for the log10 diluted cDNA was used to generate standard curves. Unknown amounts of mRNAs were plotted relative to the standard curve for each set of primers and graphical plotted using Microsoft Excel. This gave transcriptional quantitation of each gene relative to the endogenous glyceraldehyde-3-phosphate dehydrogenase standard after log transformation of the corresponding raw data. The data were plotted and graphed using SigmaPlot. Significant differences of each gene among C57BL/6J, ednrbl~b~/ednrbl~b~, and ednrbl~b~/ednrbl~b~/ mice were analyzed using one-way ANOVA with Newman-Keul’s post hoc test. The PCR products were also analyzed on a 1.5% agarose gel. We compared the quantitative molecular expression of ednrbl in the various mouse strains with the C57BL/6J strain as wild-type controls, as performed previously by Jackson Laboratories.

**DNA sequencing and analysis.** DNA sequencing was performed at Nevada Genomic Center (University of Nevada, Reno, NV). The DNA sequences were analyzed using Vector NTI Suite version 6.0 (InforMax) and by the BLAST program (1).

**Immunohistochemistry.** Counts for total neuronal density were performed by calculating the total number of myenteric neurons per square millimeter in colonic preparations that were stained by the general polyclonal neuronal marker PGP 9.5 (catalog no. 7863-0507; Biogenesis, Poole, UK). Preparations stained with PGP 9.5 were first fixed for 10 min in 4% paraformaldehyde solution and then washed three times with 10-min washes. Primary antibody was incubated for 5 h and then washed (3 times, 10-min washes). Alexa Fluor 488 chicken (anti-rabbit IgG; catalog no. A-21441) was used as a secondary antibody (Molecular Probes, Eugene, OR) at a concentration of 1:1,000 for 1 h.

Histochemical staining for NADPH diaphorase was performed using previously described protocols (6). Staining for NADPH diaphorase was performed using whole mount preparations of the colon that were fixed for 4 h in a 4% paraformaldehyde solution and then washed four times (30 min each wash) using a PBS solution.

**Recordings of CM mechanical activity.** Mechanical recordings were made from intact whole preparations of the mouse colon. The entire colon was mounted in an organ bath using the protocol we have previously described (5). In brief, the mechanical activity of the CM was monitored at four sites (proximal, mid, middistal, and distal) along the full length of the colon using isometric tension transducers (Kent Scientific, Litchfield, CT). These were connected by small stainless steel clips (Micro-serrefines no. 18055-04; Fine Science

Table 1. Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>No.</th>
<th>Primers</th>
<th>Sequence (5'–3')</th>
<th>Tm</th>
<th>Gene</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GAPDH2</td>
<td>TGCTGACACCAAGCAAGTGC</td>
<td>53</td>
<td>GAPDH</td>
<td>NM_001001303</td>
</tr>
<tr>
<td>2</td>
<td>GAPDH2r</td>
<td>GTCATCCACACATCTCCAC</td>
<td>56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>ETa1</td>
<td>CCGAGGACCTATCACTTCGTC</td>
<td>59</td>
<td>EDNRA</td>
<td>NM_010332</td>
</tr>
<tr>
<td>4</td>
<td>ETa1r</td>
<td>GCATTAGGCAACAGAGTCAGAG</td>
<td>59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>ETb1</td>
<td>GAAGTTGCTGCAAGTATATCAC</td>
<td>60</td>
<td>EDNRB</td>
<td>NM_007904</td>
</tr>
<tr>
<td>6</td>
<td>ETb1r</td>
<td>CACCTTACACAATCTCAGCTCAATAG</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>PGP2</td>
<td>GTGATCAGTCCTGATGACTCACC</td>
<td>59</td>
<td>PGP 9.5</td>
<td>NM_011670</td>
</tr>
<tr>
<td>8</td>
<td>PGP2r</td>
<td>TGGACTATCCTGGCTTATCACCC</td>
<td>58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>SMH2</td>
<td>CTTGTCATGTTGTTGTTGATG</td>
<td>59</td>
<td>SM-MHC</td>
<td>NM_13607</td>
</tr>
<tr>
<td>10</td>
<td>SMH1r</td>
<td>GAGTTGCTGACAGCTTCTTTGC</td>
<td>59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Kit2</td>
<td>GGAATGAGACCAAGACCTTCC</td>
<td>58</td>
<td>c-Kit</td>
<td>NM_021099</td>
</tr>
<tr>
<td>12</td>
<td>Kit2r</td>
<td>TATGATCTCTGGTGTGTCG</td>
<td>58</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EDNRA, endothelin receptor type A; EDNRB, endothelin receptor type B; PGP 9.5, ubiquitin carboxy-terminal hydrolase L1; SM-MHC, myosin heavy polypeptide 11, smooth muscle; c-Kit, kit oncogene; Tm, melting temperature.

*AJP-Gastrointest Liver Physiol* • VOL 290 • APRIL 2006 • www.ajpgi.org
Tools, Foster City, CA) attached to the serosal surface. Mechanical recordings were made simultaneously at four sites along the isolated whole colon. These recording sites were measured at distances of 10, 25, 45, and 60 mm from the anal sphincter. Initial tensions of the CM were routinely set to 500 mg and were readjusted back to this level when the resting tension decreased. Preparations were bathed in oxygenated Krebs solution at 35–37°C. The Krebs solution was replaced approximately every 30 min.

**Measurements and statistical analysis.** For statistical comparison between the three groups of mice (wild type, piebald heterozygote, and homozygote lethal), a one-way ANOVA with Newman-Keul’s post hoc tests were used. A minimum significance level of \( P < 0.05 \) was used throughout. The use of “n” in the results refers to the number of animals on which observations were made, and data are presented as means ± SE. Measurements of amplitude, half-width, and interval between smooth muscle contractions were all made using Acqknowledge 3.2.6 (BIOPAC Systems, Santa Barbara, CA). Measurements of the numbers of myenteric ganglia and the numbers of myenteric neurons per square millimeter were made from the proximal colon (10 mm from the cecocolonic junction), midcolon (40 mm from the cecocolonic junction), and distal colon (25 mm from the anal sphincter).

**Drugs and solutions.** The composition of the modified Krebs’ solution was (in mM): 120.35 NaCl, 5.9 KCl, 15.5 NaHCO₃, 1.2 NaH₂PO₄, 1.2 MgSO₄, 2.5 CaCl₂, and 11.5 glucose. TTX was purchased from Sigma Chemical (St. Louis, MO).

**RESULTS**

**General observations.** When the abdominal cavity of piebald homozygote lethal mice was incised, it was immediately apparent that the colon was severely distended and impacted with multiple pellets, consistent with the observations of Webster (14). In contrast, piebald heterozygotes or C57BL/6J controls never showed any obvious signs of megacolon and/or imperforate anus and appeared to live normally, the majority for at least 1–2 yr (colony age). No obvious signs of distress or discomfort were apparent in heterozygotes (\( n = 17 \)), whereas the opposite was true of homozygote lethal siblings (\( n = 13 \)). It was common to observe piebald homozygote lethal mice with hunched backs and spiked fur, classic signs of pain and discomfort. Because heterozygote littermates lived normally without any signs of megacolon, we sought to investigate whether there were any differences in their functional colonic motor activity, expression of ednrB, and the development of myenteric ganglia when compared with homozygote lethal mice and C57BL/6J controls.

**Changes in the density of myenteric ganglia in colons of C57BL/6J control and piebald mice.** In control C57BL/6J mice, the mean number of myenteric ganglia in the proximal colon was \( 35.8 ± 6.9 \) ganglia/mm² (range: 20–56 ganglia/mm²; \( n = 12 \); Fig. 1A). Interestingly, however, in the same region of piebald heterozygotes and piebald homozygote lethal mice, it was found that there was approximately one-half the number of myenteric ganglia in both heterozygote (\( 15.4 ± 2.6 \) ganglia/mm²; range: 6–20 ganglia/mm²; \( n = 12 \)) and homozygote (\( 22.8 ± 2.3 \) ganglia/mm²; range: 16–30 ganglia/mm²; \( n = 10 \)) lethal siblings when compared with C57BL/6J controls (Fig. 1A). There was a slight but significant increase in the number of myenteric ganglia in the proximal colon of piebald lethal homozygotes compared with heterozygote siblings (\( P < 0.05 \); Fig. 1A). Perhaps the most notable difference between C57BL/6J controls compared with piebald heterozygotes and homozygote lethal mice was that the numbers of myenteric ganglia per square millimeter and the total myenteric density (neurons/mm²) were not significantly different between the proximal and distal colon of C57BL/6J mice, piebald heterozygotes, and piebald lethal homozygotes. The numbers of myenteric ganglia remained relatively constant in C57BL/6J controls. However, in the proximal colon of heterozygotes and homozygote lethals, the numbers of ganglia/mm² (A) and numbers of myenteric neurons/mm² (B) steadily decreased in number toward the distal colon. Statistically significant difference between groups of data.

In C57BL/6J control mice, no aganglionic region was ever detected in the far distal colon (\( n = 8 \)). However, most surprisingly, we found that the distal colon of all piebald heterozygote mice showed a distinct aganglionic region, an example of which is shown in Fig. 2. The mean length of aganglionosis in the distal colon of heterozygote mice showed some variability between animals, measuring between 8.3 and 16.1 mm (mean length: 12.4 ± 1.1 mm; \( n = 10 \); Fig. 2). As previously shown (10, 14), the distal colon of piebald homozygote lethal mice showed an extensive aganglionic distal colon, the length of which was approximately two times as long as that found in heterozygote littermates. This length of agangli-
Onosis varied between homozygote lethal mice from 12.3 to 27 mm in from the anal sphincter (mean: 20.4 ± 2.1 mm; n = 10).

Colonic motor activity in control C57BL/6J, piebald heterozygote, and homozygote lethal mice. Because it was found that there were approximately one-half the number of myenteric ganglia in the proximal and midcolon of piebald heterozygote and homozygote lethal mice, we were particularly interested in whether colonic MMC activity would be impaired in these regions.

When the whole colon was removed from control C57BL/6J mice and mechanical recordings were made simultaneously from four sites along the full length of colon (proximal, mid, middistal, and distal colon), rhythmic coordinated colonic MMCs were consistently observed (Fig. 3), as previously described from this strain (5, 8, 11, 12). The mean interval between colonic MMCs in control C57BL/6J mice was 3.62 ± 0.37 min (n = 12) and had a mean peak contraction amplitude of 31.22 ± 6.7 mN in the proximal colon, 18.3 ± 3.6 mN in the midcolon, and 4.9 ± 2.9 mN in the distal colon (n = 12). Colonic MMCs were always abolished by TTX (n = 4), and an increase in resting tension of the CM occurred in the proximal colon (mean: 3.6 ± 0.6 mN; n = 5), midcolon (mean: 7.3 ± 4.6 mN, n = 5), and distal colon (0.09 ± 0.02 mN; n = 5; Fig. 3).

Fig. 2. A: whole mount micrograph showing the mid to distal aganglionic colon of a piebald heterozygote mouse stained for NADPH diaphorase. B-D show enlarged regions of the distal colon. A sparse network of myenteric ganglia was observed in the mid to distal region (D), accompanied by some inhibitory motor nerves. C shows the transition zone between aganglionic and ganglionic regions, whereas B shows the aganglionic distal 10 mm region.

Fig. 3. Normal colonic migrating motor complexes (MMCs) recorded from a C57BL/6J control mouse colon in vitro. Colonic MMCs occur approximately every 4 min and propagate along the full length of ganglionated colon. TTX abolishes colonic MMCs and increases the resting tension in all regions of colon.
In contrast, when the same experiments were performed in piebald heterozygote mice, coordinated colonic MMCs were rarely recorded along the colon (Fig. 4). In fact, in 12 of 15 animals, intermittent colonic MMCs were found to occur in the proximal colon and occasionally these propagated in the midcolon (Fig. 4). However, intermittent colonic MMCs never propagated in the aganglionic distal colon, an example of which is shown in Fig. 4. In 2 of these 15 animals, a weak colonic MMC contraction could be recorded 10 mm from the anal sphincter, whereas in one animal no colonic MMCs were ever recorded. Interestingly, in these heterozygote mice, where disrupted colonic MMCs did occur, the amplitude and half-duration of these events in the proximal colon and midcolon were not significantly different from those in the colon of C57BL/6J controls ($P > 0.05$; $n = 11$). In all heterozygotes, despite the absence of regular coordinated colonic MMCs from the proximal to distal colon, spontaneous myogenic contractions always occurred in the aganglionic distal region (Fig. 4). Spontaneous myogenic contractions in the aganglionic distal region had a mean interval of $25.2 \pm 3.5$ s ($n = 5$) and half-duration of $7.1 \pm 0.8$ s ($n = 5$), which was completely unchanged after the application of TTX (1 mM; $n = 5$).

Is tonic neurogenic inhibition of the CM active in piebald mouse colon? Previously, the CM layer of wild-type mouse colon (including the C57BL/6J strain; see Ref. 5) has been shown to be maintained under a level of tonic neurogenic inhibition (5, 8, 11, 12, 15). Therefore, we were interested in whether the CM of the mouse colon in these piebald heterozygotes was also maintained under tonic neurogenic inhibition in both the ganglionic and aganglionic regions. To test this, TTX was applied to the whole colon. TTX (1 $\mu$m) caused an increase in resting tension in the proximal colon (mean: $1.8 \pm 0.6$ mN; $n = 5$) and midcolon (mean: $1.6 \pm 0.52$ mN; $n = 5$) but was without effect on this activity ($n = 5$; see Fig. 4). TTX (1 $\mu$m) caused no increase in resting tension in the CM of either the proximal, mid, or distal colon ($n = 5$; Fig. 5), suggesting the tonic neurogenic inhibition was either minimal or absent in the musculature.

Expression of cell-specific makers in the small and large intestines. We used Q-PCR to determine whether the level of expression of endothelin A receptor (edhra) and edhrb genes throughout the entire length of the small and large bowel in both piebald heterozygote and homozygote lethal mice. Also, we used PGP 9.5 (as a marker for enteric neurons) to determine whether the level of neural expression was consistent with the anatomic findings of reduced numbers of myenteric ganglia throughout the large bowel of piebald mice. Smooth muscle myosin heavy chain (SM-MHC) and c-kit expression were used to determine whether the level of expression of smooth muscle and interstitial cells of Cajal (ICC, intestinal pacemaker cells) were modified throughout the intestines of these mice. RT-PCR was performed on RNA isolated from the jejunum, ileum, cecum, proximal colon, midcolon, and distal colon of C57BL/6J, homozygote lethal siblings would also show a similar impairment of colonic motility. Interestingly, in homozygote lethal mice, colonic MMCs were never recorded at any site along the full length of colon ($n = 12$), an example of which is shown in Fig. 5. Despite the absence of colonic MMCs in these preparations, homozygote lethal mice always showed spontaneous phasic contractions from the proximal (ganglionic) to far distal (aganglionic) colon (Fig. 5). In the proximal colon, spontaneous myogenic contractions occurred with a mean interval of $34.8 \pm 6.6$ s ($n = 10$) and a mean half-duration of $10.7 \pm 2.5$ s ($n = 10$), which was not significantly different from the interval or duration of spontaneous myogenic contractions that occurred in the distal aganglionic region (interval: $34.2 \pm 1.2$ s; $P > 0.05$; $n = 8$ and half-duration: $8.2 \pm 0.4$ s; $n = 8$; $P > 0.05$). None of the spontaneous mechanical activity in the piebald lethal homozygote mice appeared to be mediated by enteric nerves, since TTX was without effect on this activity ($n = 5$; see Fig. 5). Also, TTX (1 $\mu$m) caused an increase in resting tension in the CM of either the proximal, mid, or distal colon ($n = 5$; Fig. 5), suggesting the tonic neurogenic inhibition was either minimal or absent in the musculature.

![Fig. 4. Impaired colonic MMCs in a piebald heterozygote mouse colon in vitro. Colonic MMCs occurred in the proximal colon and propagated to the midcolon but failed to invade the aganglionic distal colon. In the distal colon, rhythmic spontaneous myogenic contractions occurred out of phase with the colonic MMC. TTX (1 $\mu$m) abolished colonic MMCs in the proximal and midcolon.](http://ajpgi.physiology.org/)

<http://ajpgi.physiology.org/>
ednrb<sup>S-1</sup>/ednrb<sup>S</sup>, and ednrb<sup>S-1</sup>/ednrb<sup>S-1</sup> mice. All PCR products were sequenced and analyzed. All matched 100% with the reference cDNA sequences (GenBank accession nos. shown in Table 1). As expected, ednra was abundantly detected in all tissues from the three animals, whereas ednrb was detected in the control and heterozygous mice but not in homozygous mice (Fig. 6). Qualitative RT-PCR performed on the tissues revealed that the ednrb expression levels in both the control and heterozygous mice vary in these tissues (Fig. 7B). Both control and heterozygous mice showed that the cecum expressed ednrb mRNA about eight times higher than in other regions of the small or large bowel. The normalized and calculated expression level of each tissue between both the control and the heterozygous mice is shown in Table 2. The average percentage of expression is 6.6% (Table 2). Interestingly, expression levels of ednrb were also differentially expressed in these tissues (Fig. 7A). This gene was upregulated in heterozygous and homozygous mice. The homozygous mice expressed ednra at higher levels than in the cecum, proximal colon, midcolon, and distal colon of heterozygotes, suggesting that ednra may...
compensate for the loss of \textit{ednrb} expression in homozygous mice.

PGP 9.5, a marker for neuronal cells, was also differentially expressed between the different mice (Fig. 7C). Perhaps the most surprising finding was the dramatic significant reduction in expression of PGP 9.5 mRNA in heterozygous and homozygous lethal mice compared with C57BL/6J controls ($P < 0.05$). In both heterozygous and homozygous lethal mice, there was a significant and progressive decline in the level of PGP 9.5 expression from the proximal to midcolon. In the aganglionic segment, PGP 9.5 expression was essentially absent in both piebald heterozygote and homozygote lethals. This supports the conclusions of previous investigators that showed the development of enteric neurons and migration of the neural crest is indeed closely linked to the expression of EDNRB. In contrast to the expression of PGP 9.5, the expression of SM-MHC, a marker for smooth muscle tissue, was upregulated in heterozygous and homozygous mice (Fig. 7D). Like \textit{ednra}, the homozygous lethal mice showed a higher level of expression than the heterozygous in the cecum, proximal colon, midcolon, and distal colon. Interestingly, mRNA expression for \textit{c-kit} (a marker for ICC) was also upregulated in both the
heterozygous and homozygous lethal mice (Fig. 7E), where the expression profile is similar to that of SM-MHC, suggesting both smooth muscle cells and ICC are developmentally regulated by EDNRB expression.

DISCUSSION

The development of megacolon in mammals has been directly associated with the absence of enteric ganglia (aganglionosis) in the distal colon. Although this is one of the likely factors contributing to the development of megacolon, little consideration has been given to the notion that the organization and density of intrinsic ganglia oral to the region of aganglionosis may also be impaired or reduced, resulting in dysfunctional intestinal motor activity throughout the entire length of bowel and not simply limited to the distal colon. In support of this notion, our findings in the current study suggest that, in piebald mice, the development of the myenteric plexus is severely impaired not only in the distal colon but, in fact, all throughout the entire large bowel in both piebald homozygote lethal and heterozygote siblings. This reduction in the number of myenteric ganglia was found to correlate with a major functional impairment in colonic MMC activity recorded from both piebald lethal homoyzgous and heterozygote animals.

Do piebald heterozygote littermates represent a control gastrointestinal phenotype? One of the findings of the current study is that, contrary to previous thought, the gastrointestinal tract of piebald heterozygotes does not represent a wild-type control phenotype but rather represents a phenotype that more closely resembles the piebald lethal homozygote mouse. This conclusion was supported by the findings that 1) mRNA encoding PGP 9.5 and histochemical staining for enteric nerves revealed that there was a substantial reduction in the number of myenteric ganglia throughout the entire colon compared with control C57BL/6J mice, 2) there was an aganglionic region in the distal colon of heterozygotes that was found to be approximately one-half the length (~12 mm) of that found in the distal colon of piebald lethal homozygotes (~20 mm), and 3) colonic MMC activity was substantially impaired in heterozygote mice but not as severely disrupted as in piebald lethal homozygotes. Curiously, despite a significant reduction in the number of myenteric ganglia throughout the entire colon of piebald heterozygotes, and the presence of an aganglionic region in the distal colon, these mice live for a normal murine life span that far exceeds their lethal homozygote siblings (which typically die from megacolon within the first 4–6 wk after birth). In our experiments, mRNA encoding EDNRB was reduced by 92–97% throughout the entire small and large bowel of piebald heterozygotes compared with C57BL/6J controls. Functionally, this level of expression was not found to be associated with imminent death resulting from megacolon, as normally occurs in homozygote lethal mice where EDNRB expression is absent and the degree of aganglionosis is greater (~20 mm rather than ~12 mm).

Changes in mRNA expression and anatomy of the enteric nervous system in piebald mice. Q-PCR measurements revealed that compared with the control C57BL/6J strain, mRNA encoding PGP 9.5 was reduced by >70% in the gastrointestinal tract of both piebald homozygote lethal and heterozygote mice. This level of expression was very similar from the upper small bowel to the proximal colon of both heterozygotes and homozygote lethals. Interestingly, in homozygote lethal mice, the level of expression of PGP 9.5 in the midcolon and distal colon was significantly further reduced compared with heterozygotes. These molecular findings were strongly supported by our immunohistochemical staining for enteric ganglia that revealed a similar number of ganglia per square millimeter between the ileum and proximal colon, but at the mid and distal colon the number of ganglia per square millimeter was significantly further reduced in the homozygote lethals compared with the heterozygotes.

Why are colonic MMCs absent in ganglionic regions of piebald lethal mouse colon? Although it is not surprising that neurally mediated colonic MMCs were absent in the aganglionic region of piebald homozygote lethal colon, it is perhaps surprising that no colonic MMCs were ever recorded in the ganglionic regions of the proximal and midcolon. It seems unlikely that this could be attributed to the nature of the recording protocol or apparatus used, since the exact recording procedure that was used for the piebald homozygotes was also used for the control C57BL/6J and heterozygote mice, where colonic MMCs were consistently recorded (5). Therefore, we suggest that the absence of any coordinated colonic MMC in piebald lethal mouse colon is likely the result of impaired intrinsic neural circuitry all along the full length of colon and not restricted to the aganglionic region, as previously thought. Interestingly, in previous studies on piebald lethal mouse colon, it was found that MMCs “...occurred spontaneously in ganglionated regions of the large intestine of both normal and mutant mice, but never propagated into the aganglionic segment of the abnormal bowel” (2). We are unable to explain the discrepancies between the two studies at this stage. What is clear is that the colonic MMC is a migrating neural phenomenon (13) that is critically dependent on functional enteric ganglia (3, 4, 7). However, the exact classes of enteric neuron in the mouse colon that are required for colonic MMCs are not fully understood (9).

Recently, we described the intrinsic neural circuitry that underlies the generation and propagation of colonic MMCs in control C57BL/6J mice (13). One major conclusion of that study was that ascending interneuronal pathways were critical for the activation of cholinergic motor neurons and the MMC contraction (13). We speculate that, in piebald homozygote lethal mice, the complete absence of myenteric ganglia in the distal ~20 mm of colon is likely to prevent the normal projections of ascending interneurons from this aganglionic...
region to more oral ganglionic regions in the mid and even proximal colon. These ascending interneuronal pathways are critical for the normal generation of colonic MMCs (13).

Is tonic neurogenic inhibition present in piebald homozygote and heterozygote mouse colon? In the elegant experiments of Wood et al. (15–17), it was found that the CM layer of wild-type mouse colon is normally maintained in a state of tonic neurogenic inhibition during the intervals between colonic MMCs. Interestingly, they found that, in the aganglionic region of piebald lethal mice, the musculature was considerably more excitable because of the absence of enteric ganglia and hence lack of tonic neurogenic inhibition. Our findings are consistent with Wood’s (15) suggestions in that nerve conduction blockade caused no change in resting tension of the aganglionic distal colon in neither the homozygotic lethal nor heterozygote mice and that this aganglionic region showed a considerable level of spontaneous activity. Interestingly, TTX did cause an increase in resting tension of the proximal and midcolonic of piebald heterozygote mice, suggesting that tonic neurogenic inhibition does appear to be present in the ganglionic regions of piebald heterozygotes but is absent in the distal aganglionic colon of homozygote lethal or heterozygote animals.

Colonic MMCs are impaired in heterozygote piebald mice. In the distal colon of piebald heterozygotes, the length of aganglionosis showed some variability between animals (range: 8.3–16.1 mm from the anal sphincter). When mechanical recordings were made from full-length preparations of piebald heterozygote colon, it was found that, in the majority (12 of 15 animals), intermittent colonic MMC activity was recorded from the proximal to midcolon, but these failed to propagate in the aganglionic distal colon (see Fig. 4). It is noteworthy that the aganglionic region of heterozygote mice always showed rhythmic spontaneous myogenic contractions that occurred at a similar frequency as those in the aganglionic colon of piebald lethal homozygote mice. The nature of these events remains elusive. This mechanical activity was resistant to TTX, confirming its myogenic origin. One possibility is that ICC are the source for this spontaneous activity and rhythmic contractions, because the expression of c-kit was not reduced in the aganglionic region of distal colon. In fact, a trend was found that c-kit expression was increased in both the ganglionic and aganglionic regions of heterozygote and heterozygote mice (Fig. 7E).

Contrary to previous thought, the findings of the current study show that the piebald mutation is associated with a dramatic reduction in the number of myenteric ganglia along the entire mouse colon, not only in piebald lethal homozygotes but also in heterozygote siblings. Functionally, this results in a complete absence of coordinated colonic MMCs in homogygote lethal mice and a substantial impairment of colonic MMC activity in heterozygote siblings. The finding that piebald heterozygotes have an aganglionic distal colon (mean length: 12 mm) but do not show any symptoms of megacolon suggests that, in the distal colon, aganglionosis >12 mm in length and the complete absence of colonic MMCs may be required before any signs of megacolon arise.

GRANTS

This study was supported National Institute of Research Resources Center of Biological Research Excellence Grant P2O RR-018751 (to N. J. Spencer).

REFERENCES