Development of colonic motility in the neonatal mouse—studies using spatiotemporal maps

Rachael R. Roberts,1 Jessica F. Murphy,2 Heather M. Young,2 and Joel C. Bornstein1

Departments of 1Physiology and 2Anatomy and Cell Biology, University of Melbourne, Parkville, Victoria, Australia

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The enteric nervous system (ENS) controls or regulates most propulsive intestinal motility patterns in adults. The development of the ENS has been investigated at the anatomical, molecular, cellular, and genetic levels in the mouse (18). However, little is known about the functional development of the ENS or about the role of the ENS in gut motility during development.

The colonic migrating motor complex (CMMC) is a pattern of spontaneous anally propagating contractions observed in the mature mouse colon in vitro (see Ref. 25). The generation and modulation of CMMCs has been investigated via intracellular recordings from the circular muscle (10, 22, 24) and from tension recordings from the muscle (21). CMMCs are abolished by blockade of neural activity with tetrodotoxin (TTX) (3, 5–7, 10) or by blocking excitatory neuromuscular transmission (3). Between CMMCs, contractile activity of the circular muscle is suppressed by the release of inhibitory neurotransmitters, including nitric oxide (NO). Inhibition of NO synthesis and hence of the neural release of NO reduces the interval between CMMCs (15, 23), with no effect on amplitude or duration (10). During human fetal development, the gastrointestinal tract is completely colonized by enteric neuron precursors, and the longitudinal muscle, circular muscle, and muscularis mucosae layers and an interstitial cells of Cajal (ICC) network are formed by week 11 (26). Studies of premature infants have shown that, although coordinated motor activity of the gut is not fully developed, intestinal transit still occurs (2). Mice are born before the development of their ENS and ICC is complete, but the gut still propels content. Indeed, passage of meconium before birth can be entirely independent of the ENS. Thus the development of mature motor patterns like the CMMC represents a key point in the transition from embryonic to adult gastrointestinal function.

In this study we used spatiotemporal mapping from video recordings (11, 12) to examine the development of spontaneous motility patterns in the mouse colon in vitro. Spatiotemporal mapping is a powerful method for analyzing motility patterns (12) and is particularly practical for analyzing motility in fetal and newborn tissues, which are small and fragile. We show that neurally mediated spontaneous motility patterns are not observed until several days after birth.

METHODS

Experimental Animals

All studies were performed with colon from wild-type or 

mice (9), both on a C57Bl6 background. Embryonic day (E) 18.5, newborn (postnatal day 0, P0), P4, P6, P10, and adult mice were examined. The day at which a vaginal plug was detected was designated E0.5. Mice were killed by cervical dislocation (adults), CO2 gas as approved by the Animal Experimentation Ethics Committee of The University of Melbourne. The cecum to most distal colon was removed and placed in an oxygenated (95% O2 and 5% CO2) physiological saline (room temperature) of the following composition (in mM): 118 NaCl, 4.6 KCl, 2.5 CaCl2, 1.2 MgSO4, 1 NaH2PO4, 25 NaHCO3, 11 d-glucose. Colonic segments from animals aged P0, P4, P6, P10, and adult measured ~1.5, 1.5–2, 2, 2.5–3, and 5 cm, respectively.

Tissue Preparation

The colon was placed in an organ bath containing 15 ml of saline. The saline was continuously superfused through the organ bath at a flow rate of 6 ml/min. P10 and adult tissues were cannulated as described previously (11) and maintained at intraluminal pressures of

Address for reprint requests and other correspondence: R. R. Roberts, Dept. of Physiology, Univ. of Melbourne, Parkville, Vic 3010, Australia (e-mail: r.roberts1@phgrad.unimelb.edu.au).
0.7 and 1 cmH_2O, respectively. The ability to control intraluminal pressure in the cannulated setup makes it the ideal method of tissue attachment within the organ bath. Tissues from mice aged E18.5–P6 were not cannulated to avoid damage to the colonic wall. These tissues were pinned by attached mesentery to the Sylgard base of the organ bath at the oral and anal ends. A small number (n = 5) of adult and P10 preparations were pinned at each end rather than cannulated, and their motor activity was examined to identify differences between the preparation methods. After cannulation or pinning, tissue was left to equilibrate for 1 h. No substantial differences between pinned and cannulated tissues were observed.

**Video Imaging**

Video images were captured with a Canon DM-MVX150i digital video camera mounted on a dissecting microscope, at ×6.5 optical magnification. Images were captured at a rate of 15 frames/s with a resolution of 640 × 480 pixels and acquired to computer in AVI format. The recordings were processed offline with software developed in-house by using the MATLAB 7.0.4 system (version 1.2.7). This software converted the image of the intestine into a silhouette. A spatiotemporal map of the movements of the intestine was generated by counting the number of vertical pixels for each horizontal pixel in the silhouette (11). The diameter of the intestine at each point was plotted as a color or grayscale and as a function of space (distance along the segment) and time (each video frame corresponds to one time point giving a resolution of 67 ms) on a two-dimensional image.

**Experimental Protocol**

Following a 1-h equilibration, video images were recorded for 45 min in control, drug, and washout conditions. A nonrecording period of 15 min was taken from the addition or washout of drugs into the bath.

**Drugs**

Drugs used in these experiments included tetrodotoxin (TTX 1 μM; Alomone Laboratories, Jerusalem, Israel), nitro-arginine (NOLa, 100 μM; RBI, Natick, MA), hyoscine hydrobromide (1 μM, Sigma-Aldrich, St. Louis, MO). All drugs were initially made up in distilled water to form stock solutions. Final concentrations of drug preparations and unaccompanied in six others. In simultaneous video and extracellular recordings, periods of quiescence (Fig. 1A) were separated at 3.6 ± 0.5 min intervals by spiking (Fig. 1B) of 36.3 ± 7.1 s duration (n = 4). The spiking was abolished by TTX (1 μM; n = 4). In corresponding spatiotemporal maps CMMCs cycled at 3.7 ± 0.3 min, which was consistent with the properties determined by other techniques (3, 5, 7, 10).

**Immunohistochemistry**

The colon was dissected from mice aged P0, P4, P6, and P10 that were pinned flat on balsa wood and left for overnight fixation in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2 at 4°C. The mucosa was then removed by dissection to leave the myenteric plexus and circular muscle layers.

Whole-mount preparations of myenteric plexus plus attached longitudinal and circular muscle layers were stained with primary antibodies [anti-NO synthase (NOS) raised in rabbit, 1:500, Zymed Laboratories; anti-vesicular acetylcholine transporter (VACHT) raised in goat, 1:100, Chemicon]. The preparations were then washed in phosphate buffer and exposed to secondary antisera (biotinylated donkey anti-rabbit, 1:100 followed by Jackson Immunoresearch: streptavidin Alexa red, 1:200, Molecular Probes; donkey anti-goat FITC 1:100, Jackson Immunoresearch). The preparations were viewed on a confocal microscope. To quantify the number of VACHT terminals in the muscle, single optical sections through the circular muscle layer in whole-mount preparations were obtained by using a confocal microscope and a ×63 lens. The number of VACHT terminals per field (22,500 μm²) was counted in a minimum of 12 randomly chosen optical sections within the circular muscle from two animals at each age.

**Genotyping Ret−/− Mice**

Embryonic Ret−/− mice were identified by an absence of NADPH diaphorase staining in the small intestine as described previously (28).

**Electrophysiology**

Extracellular recordings of electrical activity of circular muscle were made using a glass electrode into which a silver wire 0.25 mm in diameter was inserted. The electrode was of dimensions 0.78 mm inner diameter, 1.5 mm outer diameter, and 1.1 cm in length. This electrode was gently placed on the serosa, and a weak negative pressure was applied to attach it to the muscle layers of the segment without stimulation of the muscle. The sensitivity of this method in recording muscular and neural modulation in adult mouse gut has been reported by others (17). The recording tip of the wire was coated with silver chloride and when in position lay flush with the attachment side of the glass tip. The electrode attaches via suction provided by a syringe. The recording was amplified (KS-700, World Precision Instruments), digitized (Biopac MP100), then displayed and analyzed on a personal computer using the Acqknowledge 3.2.4 program (both from Biopac Systems, SDR Clinical Technology, Sydney, Australia).

**Statistical Analysis**

Data are reported as means ± SE and were analyzed by paired t-tests or ANOVAs, as appropriate.

**RESULTS**

**Motility Patterns in the Adult Colon**

Cyclic, anally directed propagating constrictions, which have been termed CMMCs in earlier studies, were observed in empty, cannulated segments of adult colon. CMMCs were represented in spatiotemporal maps as long oblique lines (Fig. 1A). They originated from a common oral location and traveled 92.3 ± 2.5% of the colon segment to an anal termination point, at a mean velocity of 44.1 ± 6.9 mm/min. The velocity changed along the length of the colon in many cases (e.g., middle CMMC, Fig. 1A). Complexes cycled at mean intervals of 4.6 ± 0.9 min, which was consistent with adult CMMC activity previously described (10, 20). After 15-min exposure to NOLa (NOS inhibitor; 100 μM), the interval between CMMCs was significantly reduced (2.2 ± 0.3 min; n = 5; Fig. 1B). The velocity and duration were not significantly different from control. TTX (1 μM) abolished CMMC patterning (n = 8; Fig. 1C). After washout of TTX or NOLa, CMMC cycling did not significantly differ by interval, duration, or velocity (3.7 ± 1.3 min; 34.3 ± 8.2 s; 37.4 ± 14.6 mm/min P > 0.05; n = 5) from control recordings (Fig. 1D). These data are summarized in Table 1 and show that the properties of CMMCs recorded using spatiotemporal mapping are consistent with the properties determined by other techniques (3, 5, 7, 10).

The electrical activity of the smooth muscle of the adult colon was recorded with a centrally located extracellular suction electrode, simultaneous with video imaging in four preparations and unaccompanied in six others. In simultaneous video and extracellular recordings, periods of quiescence (Fig. 1A) were separated at 3.6 ± 0.5-min intervals by spiking (Fig. 1B) of 36.3 ± 7.1 s duration (n = 4). The spiking was abolished by TTX (1 μM; n = 4). In corresponding spatiotemporal maps CMMCs cycled at 3.6 ± 2.1-min intervals.

In extracellular recordings performed without corresponding video images, spiking complexes were observed at 3.5 ±
DEVELOPMENT OF COLONIC MOTILITY IN THE MOUSE

I

Adult

II

Electrode position

Time (s)

10

20

100 mV

III

P10

Control

NOLA (100 \mu M)

TTX (1 \mu M)

Washout
Table 1. Properties of adult, P10, and P6 CMMCs

<table>
<thead>
<tr>
<th>Preparation Technique</th>
<th>Interval, min</th>
<th>Duration, s</th>
<th>Velocity, mm/min</th>
<th>Length Propagated, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td>Control (n = 5)</td>
<td>4.6±0.9</td>
<td>43.4±3.2</td>
<td>44.1±6.9</td>
</tr>
<tr>
<td></td>
<td>NOLA (n = 5)</td>
<td>2.7±0.2*</td>
<td>41.3±4.5</td>
<td>31.8±8.3</td>
</tr>
<tr>
<td></td>
<td>TTX (n = 5)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Washout (n = 5)</td>
<td>3.8±1.3</td>
<td>37.4±14.6</td>
<td>76±21.7</td>
</tr>
<tr>
<td>P10</td>
<td>Control (n = 6)</td>
<td>4.3±0.1</td>
<td>39.9±4.8</td>
<td>46.4±9.4</td>
</tr>
<tr>
<td></td>
<td>NOLA (n = 6)</td>
<td>2.1±0.5*</td>
<td>36.0±2.9</td>
<td>44.7±20.6</td>
</tr>
<tr>
<td></td>
<td>TTX (n = 6)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Washout (n = 6)</td>
<td>5.0±0.9</td>
<td>41.3±7.2</td>
<td>32.1±13.6</td>
</tr>
<tr>
<td>Control (n = 10)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>NOLA (n = 8)</td>
<td>3.8±0.4</td>
<td>44.6±2.6</td>
<td>80.0±9.4</td>
<td>99.6±0.3</td>
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<tr>
<td>NOLA + hyoscine (n = 4)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>TTX (n = 4)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Values are means ± SE; P values are stated as obtained by ANOVA (1-way). CMMCs, colonic migrating motor complexes; P, postnatal day; NOLA, nitro-L-arginine. *P < 0.05.

Table 2. Motility patterns in adult and P10 in pinned and cannulated preparations

<table>
<thead>
<tr>
<th>Preparation Technique</th>
<th>Cannulation</th>
<th>Pinning</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult CMMCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interval, min</td>
<td>4.6±0.9</td>
<td>4.1±0.7</td>
</tr>
<tr>
<td>Duration, s</td>
<td>43.4±3.2</td>
<td>60.96±11.1</td>
</tr>
<tr>
<td>Velocity, mm/min</td>
<td>44.1±6.9</td>
<td>46.09±15.7</td>
</tr>
<tr>
<td>Length propagated, %</td>
<td>92.3±2.5</td>
<td>84.3±2.3</td>
</tr>
<tr>
<td>P10 CMMCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interval, min</td>
<td>4.3±0.1</td>
<td>4.2±0.1</td>
</tr>
<tr>
<td>Duration, s</td>
<td>39.9±4.8</td>
<td>39.5±7.9</td>
</tr>
<tr>
<td>Velocity, mm/min</td>
<td>46.4±9.4</td>
<td>18.1±16.5*</td>
</tr>
<tr>
<td>Length propagated, %</td>
<td>98.6±1.6</td>
<td>96.3±2.3</td>
</tr>
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</table>

Values are means ± SE. *P < 0.05 (2-way t-test).

0.6-min intervals and of duration 30.2 ± 7.5 s (n = 6). Slow-wave activity was not recorded with extracellular electrodes in adult colonic segments.

In the developing gut, orally and anally propagating waves of shallow constrictions, termed “ripples,” were observed (see Late embryonic and P0 mice). In preparations of adult colon, ripples were detected between CMMCs in four of five preparations but were more difficult to resolve than in the developing gut (Fig. 1Aa). Ripples in the adult gut had a contraction frequency of 16.3 ± 6.7 contractions/min (n = 4) and were also present in preparations exposed to TTX.

Motility Patterns at P10

Cannulated preparations of P10 colon (2.5–3 cm in length) showed anally propagating constrictions at 4.3 ± 0.1-min intervals (n = 6; see Fig. 1III). These CMMCs propagated at a speed of 46.4 ± 9.4 mm/min and traveled 98.6 ± 1.6% of the colonic segment. NOLA (100 μM) significantly reduced the interval between CMMC propagations (2.1 ± 0.5 min; P < 0.001 in each case), although there was no significant difference in propagation speed. TTX (1 μM; n = 6) abolished all activity. After washout of NOLA and TTX, the interval between propagations, duration, and velocity were not significantly different from control (5.0 ± 0.9 min; 41.3 ± 7.2 s; 37.4 ± 7.2 mm/min; P > 0.05; n = 6). These properties did not significantly differ from adult preparations (Table 1).

Ripples were observed in regions between propagating CMMCs in all spatiotemporal maps from preparations of P10 colon. They occurred at a frequency of 9.0 ± 0.5 contractions/min (n = 6). This pattern did not significantly differ in the presence of NOLA or TTX.

The CMMCs in preparations of adult and P10 colon attached to the organ bath by pinning did not differ in interval or velocity from those held in place via the cannulae (Table 2). The effect of NOLA on colonic motility was examined in adult cannulated tissues and then reexamined in the same preparations after the cannulae were removed and the colon was pinned in place (n = 2). The behavior of CMMCs in NOLA was comparable under each of these recording conditions.

Motility in Mice in Late Embryonic, P0, P4, and P6 Mice: Ripples

Late embryonic and P0 mice. It was not possible to cannulate preparations of colon from embryonic or P0–P6 mice without causing damage. Hence uncannulated preparations were used to examine colonic motility patterns in embryonic and early postnatal mice.

In newborn mice, CMMC-like activity was not observed, but ripples were seen (see Fig. 3). Ripples consisted of small constrictions initiated at various sites along the segment. These then travelled a short distance both orally and anally before colliding with similar constrictions initiated at neighboring sites, whereupon they mutually annihilated. The anally and orally propagating components of the ripples had very similar velocities (1.6 ± 0.5 mm/min; 1.5 ± 0.3 mm/min, respec-
Table 3. Properties of ripples in P0, P4, and P6 mice in various drug conditions

<table>
<thead>
<tr>
<th>Interval, min</th>
<th>Anally Directed Velocity, mm/min</th>
<th>Orally Directed Velocity, mm/min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P6</td>
<td></td>
</tr>
<tr>
<td>Control (n = 10)</td>
<td>0.2±0.01</td>
<td>14.9±5.0</td>
</tr>
<tr>
<td>NOLA (n = 8)</td>
<td>0.1±0.02</td>
<td>12.5±4.4</td>
</tr>
<tr>
<td>NOLA + hyoscine (n = 4)</td>
<td>0.1±0.01</td>
<td>10.8±6.2</td>
</tr>
<tr>
<td>TTX (n = 4)</td>
<td>0.1±0.02</td>
<td>9.7±6.9</td>
</tr>
<tr>
<td>P value</td>
<td>0.68</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>P4</td>
<td></td>
</tr>
<tr>
<td>Control (n = 9)</td>
<td>0.3±0.04</td>
<td>10.2±1.6</td>
</tr>
<tr>
<td>NOLA (n = 7)</td>
<td>0.2±0.04</td>
<td>9.5±1.8</td>
</tr>
<tr>
<td>Washout (n = 6)</td>
<td>0.2±0.03</td>
<td>10.0±1.73</td>
</tr>
<tr>
<td>TTX (n = 5)</td>
<td>0.2±0.05</td>
<td>12±6.01</td>
</tr>
<tr>
<td>P value</td>
<td>0.54</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>P0</td>
<td></td>
</tr>
<tr>
<td>Control (n = 3)</td>
<td>0.6±0.1</td>
<td>1.6±0.5</td>
</tr>
<tr>
<td>NOLA (n = 3)</td>
<td>0.6±0.2</td>
<td>2.4±0.3</td>
</tr>
<tr>
<td>TTX (n = 3)</td>
<td>0.8±0.1</td>
<td>2.5±0.4</td>
</tr>
<tr>
<td>Washout (n = 3)</td>
<td>0.8±0.2</td>
<td>3.0±0.6</td>
</tr>
<tr>
<td>P value</td>
<td>0.57</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Values are means ± SE. P values are stated as obtained by ANOVA (1-way). There is no significant difference in all conditions, with all P values > 0.05.

**Fig. 2.** Ripples in wild-type and Ret<sup>-/-</sup> mouse colon at E18.5. In grayscale spatiotemporal maps, white depicts maximal constriction, and the level of “grayness” increases proportionally to the level of dilation of the gut diameter. Maximal dilation is black. Time increases in a downward direction as in Fig. 1. This spatiotemporal map shows that in wild-type (A) and Ret<sup>-/-</sup> (B) mouse colon, ripples (arrows) appear as wave-like constrictions with short, oral and anally directed components. CMMCs are not present.

Ripples in P4 colon had significantly greater velocities of the anally and orally directed components (10.2 ± 1.6 mm/min; 12.4 ± 3.4 mm/min; P < 0.001; n = 9) and occurred at significantly reduced cycling intervals (0.3 ± 0.0 min; P < 0.001; n = 9) than the ripples at P0 (Table 2).

P6 mice. Like colonic preparations from P0 and P4 mice, spatiotemporal maps of control preparations of colon (2 cm in length) from P6 mice showed ripples, but no detectable CMMCs (Fig. 3A). However, unlike earlier developmental ages, NOLA (100 μM) induced propagating constrictions (i.e., CMMCs, Fig. 4B) in preparations of colon from P6 mice. These cycled at 3.8 ± 0.4 min intervals and were 44.6 ± 3.6 s in duration (n = 8). This activity was initiated at the oral end of the preparation and propagated 99.6 ± 0.3% of the colonic segment, at a velocity 80.0 ± 9.4 mm/min. This activity was not significantly different in cycling frequency, duration, or percentage of the colonic segment traversed (P > 0.05; n = 8) from CMMC patterning in control adult preparations (Table 1). However, the velocity of these patterns was significantly greater than control adult preparations (P < 0.006).

The CMMCs induced by application of NOLA were eliminated by washout of NOLA (n = 6). NOLA-induced CMMCs were also abolished by the nonselective muscarinic acetylcholine receptor antagonist hyoscine (1 μM; n = 4; Fig. 4C). CMMCs were absent in the presence of TTX (n = 4; Fig. 4D).

The properties of ripples in P6 colon were not significantly altered by NOLA, hyoscine, or TTX (see Table 2). However, ripples did not coincide with CMMC activity in the presence of NOLA. A significant decrease in interval between ripples was seen compared with P4 (0.2 ± 0.01 min; p < 0.003; n = 10). However, the frequency of ripples at this age was still only one-third of the frequency in adult tissues.

**Immunohistochemistry**

Whole-mount preparations of colonic circular muscle and myenteric plexus from P0, P4, P6, and P10 mice were processed for immunohistochemistry by using antisera raised against NOS and the VAChT, which is present in the nerve terminals of cholinergic neurons.

At birth, numerous NOS-immunoreactive (IR) cell bodies were present in myenteric ganglia, and NOS-IR terminals were plentiful in the circular muscle layer (Fig. 5, A1 and A3). VAChT-IR terminals were abundant within myenteric ganglia respectively. Neither NOLA (100 μM) nor TTX (1 μM) significantly altered the properties of the ripples (Fig. 3, E–H; Table 2).
at birth, but there was only a low density of VAChT-IR terminals within the circular muscle (Fig. 5, A2 and A3). The density of NOS-IR (Fig. 5, A–C) and VAChT-IR (Fig. 5, A–D) terminals within the circular muscle increased with developmental age. The density of VAChT-IR terminals in single optical sections (obtained by using a confocal microscope) of the circular muscle was quantified in whole mount preparations from P0, P4, P6, and P10 mice. The density of VAChT-IR terminals in the circular muscle increased substantially with developmental age, and there was a statistically significant increase between P6 and P10 (Fig. 5, D–G; ANOVA P < 0.0001). Unlike VAChT, NOS is cytoplasmic and is present in both the varicose and intervaricose regions of nerve fibers. Because it was often difficult to define individual NOS-IR terminals, the density of NOS terminals in the circular muscle could not be quantified.

**DISCUSSION**

The results of this study indicate that during early postnatal development the spontaneous motor activity of the mouse colon observed in vitro undergoes a qualitative change from an entirely myogenic motor pattern, ripples, to a neurogenic motor pattern, CMMCs. This change occurs between P6 and P10, a time when the density of the cholinergic innervation of the circular muscle increases considerably from that observed at birth.

*Ripples are the dominant motor pattern before and just after birth.* A major finding of this study is that the mouse colon exhibits a spontaneous motor pattern both before and after the development of a neurally mediated pattern of motility, CMMCs. This spontaneous pattern consists of small constrictions that are initiated at multiple sites along the length of the colon and then appear to propagate both orally and anally away from their point of origin. When two such constrictions reach the same point along the colon, they annihilate. We have termed them ripples by analogy with similar constrictions observed using spatiotemporal mapping methods in the guinea pig proximal colon after exposure to TTX (8). We suggest that this pattern is an important motility pattern in the developing mouse colon.

Several lines of evidence indicate that the ripple contractions we observed are entirely independent of neural activity. They are unaffected by blockade of voltage-dependent Na⁺ channels, and hence neural activity, with TTX. They are not modulated by either blockade of NOS or muscarinic receptors. Most importantly, ripples were seen in the colon of E18.5 Ret⁻/⁻ mice, which lack enteric neurons in the small and large

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**Fig. 3.** Ripples in P0 and P4 colon. Ripples in P0 mice appear as waveform constrictions (A). Ripples are not significantly altered in NOLA (100 μM), washout, or TTX (1 μM) conditions (B–D). In preparations of P4 mouse colon, ripples are closely packed and flat in appearance (E). This patterning persists in NOLA (100 μM), washout and TTX (1 μM) conditions (F–H). Refer to Fig. 2 for description of grayscale spatiotemporal maps.

**Fig. 4.** Spatiotemporal maps of P6 wild-type colon. CMMCs are absent from control recordings. In NOLA (100 μM), anally directed constrictions propagate the full length of the colonic segment. These are CMMCs (black arrows). CMMCs are abolished in NOLA (100 μM) + hyoscine (1 μM). Only ripples are seen in TTX (1 μM). Refer to Fig. 2 for description of grayscale spatiotemporal maps.
Fig. 5. A–D. Whole-mount preparations of myenteric plexus and circular muscle from P0, P6, and P10 mice processed for nitric oxide synthase (NOS; A1, A3, B1, B3, C1, C3) and vesicular acetylcholine transporter (VACHT; A2, A3, B2, B3, C2, C3, D–F) immunohistochemistry and imaged using a confocal microscope. A–C: low-magnification, stacked Z series images through the myenteric plexus and the circular muscle layer of P0, P6, and P10 mice. At P0, NOS-immunoreactive (IR) cell bodies are abundant in myenteric ganglia (A1, A3) and NOS-IR fibers are also present in the circular muscle layer (A1, yellow arrows). At P0, VACHT-IR terminals are plentiful within myenteric ganglia (G), but occur only at low density (white arrow) in the circular muscle (A2, A3). Note that in the colon of P0 mice, myenteric ganglia occur close together with only little space between ganglia. In P6 and P10 mice (B–C), there is an increase in the density of both NOS-IR (yellow arrows) and VACHT-IR (white arrows) in the circular muscle. Scale bar: 50 μm. D–F: stacked Z series (confocal microscope) images through the circular muscle layer only of whole-mount preparations of colon from P0 (D), P6 (E), and P10 (F) mice that had been processed for VACHT immunohistochemistry. The density of VACHT-IR terminals increases with developmental age. Scale bar: 10 μm. G: quantification of number (means ± SD) of VACHT-IR nerve terminals per field (22,500 μm²) in P0, P4, P6, and P10 mice. The density of VACHT-IR terminals increases significantly between P6 and P10 (P < 0.0001; ANOVA).
in intestines. These were indistinguishable from those in the colon of wild-type E18.5 mice.

D’Antona et al. (8) suggested that the ripples they saw in the guinea pig proximal colon might have been due to slow waves, the rhythmic depolarizations produced by the activity of ICC adjacent to the myenteric plexus. However, although ICC are present in the proximal colon of the mouse before birth, electrophysiological analysis of the colonic smooth muscle over the 10 days after birth indicate that slow waves are not present at this time (27). Indeed, our extracellular recording data indicate that slow waves are rarely, if ever, seen even in the adult colon. Furthermore, frequency analysis of P10 and adult spatiotemporal maps revealed contractions at frequencies fivefold greater than slow-wave activity previously described in the proximal colon and small intestine of the adult mouse (27). Thus it is highly unlikely that the ripples are due to slow-wave activity. This does not rule out a role for ICC in the generation or propagation of the ripples, but it does suggest that other mechanisms must be considered. One possible explanation is a series of cyclic propagating waves of Ca$^{2+}$-induced Ca$^{2+}$ release from intracellular stores, a mechanism that has already been identified in cardiac muscle (16) and in the gastric pylorus (25). Another possibility is spike propagation, which has been described in the dog rectum (14). This activity propagates in the oral and/or anal directions and is described at a frequency greater than slow-wave activity.

Ripples may have important functions in the mouse colon before and immediately after birth. Meconium reaches the terminal colon before E18.5 and does so even in Ret$^{-/-}$ mice, which lack enteric neurons (1). Thus colonic transit before birth can occur independently of neurons and, as mentioned above, slow waves are also absent from the colon. Ripples are thus the only mechanism identified to date that might account for the neurally independent movement of intestinal content before birth.

Our data indicate that the ripple mechanism itself undergoes some kind of maturation process. Both the frequency and propagation speed of ripples increased markedly between P0 and P4, with further less marked increases seen at P6. They persist at least until P10, when the adult CMMC pattern is fully established. Indeed, our data indicate that they can also be detected in adult colon.

CMMCs develop after birth. The anally propagating contraction complexes observed in this study at P10 and in adults are almost certainly the motility pattern termed “CMMCs” that have been identified with lower resolution methods (see Refs. 20 and 29). The pharmacology, underlying electrical activity in the circular muscle, cycling rates, and propagation speeds of CMMCs reported in the present study are almost identical to previously published descriptions of CMMCs (3, 4, 10). We found that CMMCs can be induced by NOS inhibition around P6 and were fully mature at P10. This latter observation is similar to that of the only previous study of the development of neurogenic motor activity in the distal colon of mice (27) in which intracellular recordings were made from the circular muscle of the proximal colon of P0, P6, P8, P10, and P20 mice. Spontaneous spike complexes that may have corresponded to CMMCs were seen from P8 onward, although whether these propagated and how they were affected by pharmacological agents was not determined. Thus this present study, using spatiotemporal mapping methods that allow the propagation speeds and the lengths over which contractions propagate to be determined, confirms and extends the previous study.

In the present study, CMMCs were not observed in the colon of P4 mice. Thus, although the mouse colon is completely colonized by enteric neuron precursors by E14.5 (13), the neuronal circuitry mediating CMMCs is still not functional by P4. CMMCs were observed in the colon of P6 mice, but only in the presence of NOS inhibitors; the circuitry mediating CMMCs must therefore be in place by P6 (~12 days after the colon is first colonized by neuron precursors) but is not mature. The presence of CMMCs at P10 coincides with the time at which there is a large increase in the density of cholinergic nerve terminals in the circular muscle. The development of CMMCs may therefore require the presence of a minimal density of cholinergic nerve terminals in the circular muscle. However, not all of the components of the neural circuitry mediating CMMCs have been identified, and because considerable neurochemical maturation of enteric neurons (and presumably their synaptic connections) occurs after birth (30), other explanations are also possible.

In summary, this is the first study of the development of spontaneous propagating motility patterns and their regulation in the mouse colon. Although the propulsion of meconium occurs before birth, the only colonic motor patterns observed in late fetal and early postnatal mice were ripples, which were not mediated by neurons. Neurally mediated spontaneous motility patterns (CMMCs) were first observed at P6. This study also demonstrated that spatiotemporal mapping is a valuable method to examine motility patterns during development.

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