Activation of neural circuitry and Ca\textsuperscript{2+} waves in longitudinal and circular muscle during CMMCs and the consequences of rectal aganglionosis in mice

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Spencer NJ, Bayguinov P, Hennig GW, Park KJ, Lee H-T, Sanders KM, Smith TK. Activation of neural circuitry and Ca\textsuperscript{2+} waves in longitudinal and circular muscle during CMMCs and the consequences of rectal aganglionosis in mice. Am J Physiol Gastrointest Liver Physiol 292: G546–G555, 2007. First published October 12, 2006; doi:10.1152/ajpgi.00352.2006.—In mammals that develop rectal aganglionosis, the aganglionic segment still exhibits spontaneous phasic contractions that contribute to dysmotility and pseudoobstruction in this region. However, almost nothing is known about the mechanisms that generate these myogenic contractions or the effects of aganglionosis on the generation of Ca\textsuperscript{2+} waves that underlie contractions of the longitudinal muscle (LM) and circular muscle (CM). In a mouse model of Hirschsprung’s disease [endothelin type B receptor-deficient (Ednrb\textsuperscript{-/-}/Ednrbs\textsuperscript{-/-}) mice], the Ca\textsuperscript{2+} indicator fluo-4 was used to simultaneously monitor the temporal activation and spread of intercellular Ca\textsuperscript{2+} waves in the LM and CM during spontaneous colonic motor activities. During the intervals between colonic migrating motor complexes (CMMCs) in control mice, Ca\textsuperscript{2+} waves discharged asynchronously between the LM and CM. However, in these same mice, during CMMCs, a burst of discrete Ca\textsuperscript{2+} waves fired simultaneously in both muscle layers, where the propagation velocity of Ca\textsuperscript{2+} waves significantly increased, as did the rate of initiation and number of collisions between Ca\textsuperscript{2+} waves. Hexamethonium (300 \mu M) or atropine (1 \mu M) prevented synchronized firing of Ca\textsuperscript{2+} waves. In the aganglionic distal colon of Ednrb\textsuperscript{-/-}/Ednrbs\textsuperscript{-/-} mice, not only were CMMCs absent, but Ca\textsuperscript{2+} waves between the two muscle layers fired asynchronously, despite increased propagation velocity. The generation of CMMCs in control mice involves synchronized firing of enteric motor nerves to both the LM and CM, explaining the synchronized firing of discrete Ca\textsuperscript{2+} waves between the two muscle layers. Aganglionosis results in a sporadic and sustained asynchrony in Ca\textsuperscript{2+} wave firing between the LM and CM and an absence of CMMCs.

In newborn infants with Hirschsprung’s disease, the aganglionic rectum remains tonically constricted and generates a pseudoobstruction to the normal propulsion of colonic content. It is this pseudoobstruction that is believed to contribute to chronic constipation and the onset of megacolon. Interestingly, despite the absence of enteric nerves, the aganglionic rectum is still able to generate irregular phasic contractions of the muscle. These irregular myogenic contractions likely contribute to dysmotility in this region. However, nothing is known about the mechanisms that generate contractions of the aganglionic distal colon. An understanding of these mechanisms is particularly important since many patients with short-segment aganglionosis suffer years of chronic constipation with intermittent success in treatment.

A major step forward in the understanding of human Hirschsprung’s disease came about from the discovery of spontaneously mutated strains of mice, such as the piebald lethal mouse (17, 31–34). Piebald homozygote lethal offspring [endothelin type B receptor (EDNRB)-deficient (Ednrb\textsuperscript{-/-}/Ednrbs\textsuperscript{-/-}) mice] develop congenital rectal aganglionosis that leads to megacolon and death shortly after birth. Ednrb\textsuperscript{-/-}/Ednrbs\textsuperscript{-/-} mice are now known to have a mutation in the Ednrb gene, and, at a similar time as to when this mutation was first identified in Ednrb\textsuperscript{-/-}/Ednrbs\textsuperscript{-/-} mice, other investigators realized that a subset of human patients with Hirschsprung’s disease also carry mutations in EDNRB (19). It is for this reason that the piebald mouse strain has proved an invaluable model for studies on human Hirschsprung’s disease and is increasingly becoming the focus of much attention.

Normal motor activity recorded from the isolated whole colon in wild-type mice has been shown in a variety of laboratories to consist of regularly occurring colonic migrating motor complexes (CMMCs; for a review, see Ref. 23). Wood et al. (34) first described these events as “...migrating contractile complexes” that are clearly neural in origin and occur about every 1–2 min. It was suggested that these were the motor correlates of extracellularly recorded spike bursts and that they “...may be the electromechanical basis for organized propulsion of intraluminal fecal pellets.” More recently, these events have been described simply as CMMCs (1, 5–7, 9, 23–25). The mechanisms that generate CMMCs involve complex intrinsic neural circuitry that is only recently being unraveled (23, 25). To date, however, all of the electrophysiological knowledge gathered on CMMCs in mice has been from the circular muscle (CM) layer (7, 25). Unfortunately, there have been no electrophysiological recordings made from the longitudinal muscle (LM) layer of the mouse colon, and this has led to a major weakness in our understanding of CMMC generation in wild-type mice, let alone mutant strains of mice that develop rectal aganglionosis.

In this study, we used Ca\textsuperscript{2+}-imaging techniques to determine 1) the mechanisms by which the two muscle layers of the mouse colon are activated during CMMCs and 2) how the properties of Ca\textsuperscript{2+} waves underlying phasic contractions are modified in the aganglionic distal colon of Ednrb\textsuperscript{-/-}/Ednrbs\textsuperscript{-/-} mutant mice, where CMMCs are absent. In brief, we showed that during the intervals between CMMCs in wild-type mice, Ca\textsuperscript{2+} waves discharge largely asynchronously between the LM and CM.

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and CM. However, during CMMCds, discreet Ca\textsuperscript{2+} waves switch to a highly synchronized firing state between both muscle layers that involves synaptic activation of myenteric interneurons and excitatory motor neurons to both muscle layers. In the aganglionic distal colon of Ednr\textsuperscript{b-/-Ednr\textsuperscript{b-/-}} mice, Ca\textsuperscript{2+} waves propagate faster in the hypertrophic muscular but discharge sporadically and show consistently poor temporal synchronization between both muscle layers.

**METHODS**

Preparation of tissues. Ednr\textsuperscript{b-/-Ednr\textsuperscript{b-/-}} homozygote lethal offspring of the piebald mouse strain and their experimental controls (C57BL/6 mice) of either sex (20–90 days old) were euthanized humanely by inhalation anesthetic (Nembutal) followed by cervical dislocation, in accordance with University of Nevada Animal Ethics Committee guidelines. All Ednr\textsuperscript{b-/-Ednr\textsuperscript{b-/-}} homozygote offspring were bred in the animal facility at the University of Nevada by intercrossing Ednr\textsuperscript{+/Ednr\textsuperscript{b-/-}} and Ednr\textsuperscript{b-/-Ednr\textsuperscript{b-/-}} heterozygote siblings (19). The original Ednr\textsuperscript{+/Ednr\textsuperscript{b-/-}} heterozygote breeder pair was obtained from Jackson Laboratories. The use and treatment of all animals was approved by the Institutional Animal Use and Care Committee of the University of Nevada. Following euthanasia, the entire colon was removed, and a midline incision was made along the mesenteric border along the distal half (3 cm) of the colon was ganglionic in C57BL/6 mice and aganglionic in Ednr\textsuperscript{b-/-Ednr\textsuperscript{b-/-}} mutant mice.

Simultaneous Ca\textsuperscript{2+} imaging of the LM and CM. The uppermost muscle layers (CM and LM) were incubated with fluo-4 solution (see composition in Drugs and solutions) for 20 min at room temperature (3). Following the loading procedure, the preparation was perfused with warmed KRB (37°C) for 20 min to allow for deesterification. Two different setups were used to capture Ca\textsuperscript{2+} activity. The first setup consisted of an Olympus BX50WI microscope (Olympus, Melville, NY) fitted with epifluorescence. Water-immersion lenses (×20 and ×40, Olympus UMPlanF) were used. A video-rate iCCD camera (30 frames/s IC-300B, Photon Technology, Monmouth Junction, NJ) recorded fluorescence activity. The second setup consisted of an Eclipse E600FN microscope (Nikon, Melville, NY) with a Lambda DG-5 light source (Sutter Instruments, Novato, CA). Several lenses were used to assess muscle activity at different magnifications (×10, ×20, and ×40, Nikon Plan Fluor). Image sequences were captured using a Cascade 512B camera (Roper Scientific, Trenton, NJ) and MetaMorph 6.26 software (Universal Imaging, Molecular Devices, Downingtown, PA) at a rate of 15.6 -30 frames/s. Image sequences were visualized and analyzed using custom written software (Volumetry 1.3, GWH).

Ca\textsuperscript{2+} imaging. To better portray Ca\textsuperscript{2+} activity over long recording periods, spatiotemporal (ST) maps were constructed. Ca\textsuperscript{2+} fluorescence was averaged along the length of smooth muscle cells across the field of view for each row in the image. This resulted in a single column of averaged pixels. This procedure was repeated for each frame, and the resulting columns of averaged pixels were placed side each other (time starting on the left and continuing to the right). The white vertical lines visible in ST maps (e.g., Figs. 2 and 5) indicate that a particular row of smooth muscle cells were active. If activity spread across the field of view, this would appear as an angled white line in ST maps.

ST maps of the average fluorescence intensity were constructed for both the LM and CM layers. ST maps of Ca\textsuperscript{2+} wave activity were constructed by averaging fluorescent intensity in the field of view of the CM and from the field of view of the LM orthogonal to the direction of their muscle fibers. Thus, activity represents Ca\textsuperscript{2+} wave activity in the slow conducting axes. That is, waves appear to propagate along the bowel in the CM and around the bowel in the LM. Frequency, the interval and velocity of Ca\textsuperscript{2+} waves that propagated through the LM or CM syncitia were calculated. Closest-peak analysis was used to determine if Ca\textsuperscript{2+} transients were coordinated between the LM and CM. After motion stabilization, the average Ca\textsuperscript{2+} induced fluorescence was extracted from individual LM and CM muscle fibers that were in close proximity to each other. Peaks of Ca\textsuperscript{2+}-induced fluorescence in both traces were located. The time at which a peak occurred in a reference trace (LM or CM) was compared with the time of the closest peak (up to ±3 s) in the other trace, and the time difference was calculated. In the results, the “initiation rate” of Ca\textsuperscript{2+} waves refers the rate at which Ca\textsuperscript{2+} waves were generated only from within the field of view, whereas measurements of mean frequency of Ca\textsuperscript{2+} waves refers to the mean frequency at which Ca\textsuperscript{2+} waves propagate across the field of view that were generated either within or outside the field of view. For statistical comparisons of data, Students’ paired t-tests were used, where P values of <0.05 were considered statistically significant. In the results, n values refer to the numbers of animals on which observations were made.

Protocol for hematoxylin-eosin staining. Isolated whole preparations of the mouse colon were cut into several segments (labeled as the ileum, cecum, proximal, midcolonic, or distal colon) for cross sections and placed in labeled cassettes. Cassettes were mounted on a tissue processor (Sakura VIP 5, Torrance, CA) where the tissues are then infiltrated with paraffin. Upon the completion of processing, tissues were embedded in paraffin and sectioned at 4 μm thickness. Slides were air dried overnight and placed in staining racks, where they were stained with hematoxylin and eosin on a staining machine (Sakura DRS-601). Once stained, slides were placed on a coverslipper (Sakura VIP 5) and permanently mounted. Slides were then given a microscopic quality check to make sure proper trimming, embedding, sectioning, and staining had been achieved. The same procedure was used to stain both C57BL/6 wild-type and piebald mutant mouse colon preparations. To measure the thickness of the LM and CM walls, a bright-field Leitz Diaplan microscope and a Leica LEI-750 camera, running metamorph software (version 3.0. Metamorph imaging software), were used with lens objectives of ×10 and ×20 magnification.

Drugs and solutions. The composition of KRB was (in mM) 120.35 NaCl, 5.9 KCl, 15.5 NaHCO\textsubscript{3}, 1.2 NaH\textsubscript{2}PO\textsubscript{4}, 1.2 MgSO\textsubscript{4}, 2.5 CaCl\textsubscript{2}, and 11.5 glucose. Atropine, hexamethonium bromide, and nicardipine were all obtained from Sigma Chemical (St. Louis, MO). For Ca\textsuperscript{2+} imaging, a solution consisting of 25 μg fluo-4 (FluoroproCureTM-AM, Molecular Probes, Eugene, OR), 0.02% DMSO, and 0.01% nonotox detergent Cremophor EL was used.

**RESULTS**

Spontaneous intercellular Ca\textsuperscript{2+} waves in the LM and CM during intervals between CMMCds. During the intervals between CMMCds in C57BL/6 control mice, spontaneous intercellular Ca\textsuperscript{2+} waves were commonly observed in both the LM and CM layers (n = 8), an example of which is shown in Fig. 1. B and C. Ca\textsuperscript{2+} waves occurred more frequently in the LM (1.3 ± 0.3 waves/s) than in the CM (0.5 ± 0.3 waves/s, P < 0.05, n = 8 preparations). Ca\textsuperscript{2+} waves fired asynchronously between each muscle layer, i.e., discreet intercellular Ca\textsuperscript{2+}
waves occurred independently in both muscle layers 66% of the time, where each wave propagated anisotropically through each muscle layer. We found no evidence to suggest that the generation of any Ca\(^{2+}\)/H\(^{100}\) waves in either the LM or CM electrotonically spread into the neighboring muscle layer to activate a Ca\(^{2+}\)/H\(^{100}\) wave in both layers simultaneously, as has been reported previously. Ca\(^{2+}\)/H\(^{100}\) waves propagated comparatively slower orthogonal to CM (3.8 ± 0.2 mm/s, n = 8) or LM (6.6 ± 0.5 mm/s, n = 8) fibers (Table 1) and considerably more rapidly along or parallel to the muscle fibers (see Fig. 1, B and C). Thus, Ca\(^{2+}\) waves in each muscle layer were readily distinguishable from one another since their fast and slow conducting axes through the LM were orthogonal to those Ca\(^{2+}\) waves in the CM and vice versa (Fig. 1, B and C). Spontaneous Ca\(^{2+}\) waves in either muscle layer originated and terminated in discreet zones of muscle (100- to 400-μm width; see Fig. 1, B and C), suggesting that these zones may represent morphological distinct muscle bundles or regions of muscle activated by terminals of motor neurons innervating a particular region of muscle.
Characteristics of Ca\textsuperscript{2+} waves in the LM and CM during CMMCs. We were particularly interested to determine how the firing pattern, onset, and propagation velocity of Ca\textsuperscript{2+} waves in the LM and CM would change when CMMCs propagated along the colon. CMMCs occurred every 1–4 min and were associated with a distinct discharge of higher-frequency Ca\textsuperscript{2+} waves in both muscle layers that lasted for a mean duration of 17.7 ± 3.4 s (n = 8). During CMMCs, Ca\textsuperscript{2+} waves in the CM

Table 1. Ca\textsuperscript{2+} waves in the LM and CM between and during CMMCs

<table>
<thead>
<tr>
<th></th>
<th>Between CMMCs</th>
<th>During CMMCs</th>
<th>P Value</th>
<th>No. of Waves</th>
</tr>
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<tbody>
<tr>
<td>Frequency, Hz</td>
<td></td>
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<tr>
<td>LM</td>
<td>1.3±0.3</td>
<td>1.6±0.02</td>
<td>&lt;0.05</td>
<td>57</td>
</tr>
<tr>
<td>CM</td>
<td>0.5±0.3</td>
<td>1.7±0.01</td>
<td>&lt;0.05</td>
<td>57</td>
</tr>
<tr>
<td>Orthogonal velocity of discreet Ca\textsuperscript{2+} waves, mm/s</td>
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<tr>
<td>LM</td>
<td>6.6±0.5</td>
<td>9.9±0.1</td>
<td>&lt;0.05</td>
<td>57</td>
</tr>
<tr>
<td>CM</td>
<td>3.8±0.2</td>
<td>5.9±0.4</td>
<td>&lt;0.01</td>
<td>116</td>
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<tr>
<td>Orthogonal distance traveled by discreet Ca\textsuperscript{2+} waves, μm</td>
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</tr>
<tr>
<td>CM</td>
<td>316.3±18.2</td>
<td>572±42.2</td>
<td>&lt;0.01</td>
<td>57</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8 animals/group. LM, longitudinal muscle; CM, circular muscle; CMMCs, colonic migrating motor complexes.

Fig. 2. Changes in properties of Ca\textsuperscript{2+} waves in LM and CM before and during a CMMC. Spatiotemporal (ST) maps were constructed for both the CM (A and D) and LM (B and E). In this example, Ca\textsuperscript{2+} waves in the CM (A) were sporadic, often being initiated and terminating in similar zones along the bowel, whereas in the LM (B) Ca\textsuperscript{2+} waves discharged in a regular tonic fashion. Closest-peak analysis comparing the time at which Ca\textsuperscript{2+} waves occurred in the LM layer to the closest Ca\textsuperscript{2+} wave in the CM layer showed little coordination of firing between the two muscle layers (maximum change in time: 3 s; C). During the CMMC, the frequency of the ongoing discharge of Ca\textsuperscript{2+} waves in the CM increased (D) and the LM became active for an extended period (E). During the burst of Ca\textsuperscript{2+} waves associated with the CMMC, closest-peak analysis showed that the LM and CM fired near simultaneously (change in time: −0 s; F). An example of LM and CM firing during the CMMC is shown in G. Two Ca\textsuperscript{2+} waves were initiated in the CM (*), each propagating in both directions. At 133 ms, a Ca\textsuperscript{2+} wave appeared in the LM at the bottom of the field of view and propagated upward. After 200 ms, the two CM Ca\textsuperscript{2+} waves collided in the middle of the field of view and annihilated each other. H: examples of the pattern of propagation of Ca\textsuperscript{2+} waves in the CM layer during the CMMC (taken between dotted lines in D). Note that there were often numerous sites of initiation (*) and collisions (•) and that the propagation velocity varied considerably from wave to wave, giving the appearance of the “zigzag”-type pattern seen.
and LM discharged at a significantly higher frequency than during the period between CMMCs in both the LM (control: 1.3 ± 0.3 Hz and during CMMCs: 1.6 ± 0.02 Hz) and CM (control: 0.5 ± 0.3 Hz and during CMMCs: 1.7 ± 0.01 Hz, P < 0.05, n = 8; see Fig. 2 and Table 1). One of the most notable differences was that the temporal relationship between Ca\(^{2+}\) waves between the two muscles became highly synchronized compared with during the period between CMMCs (Fig. 2). In fact, during CMMCs, the onset of each Ca\(^{2+}\) wave in the CM had a direct temporal correlation with a Ca\(^{2+}\) wave in the LM 96 ± 1% of the time (Fig. 2, C and F). There was no consistent order to the firing of Ca\(^{2+}\) waves in CM and LM layers, as 40 ± 7% of the time both layers fired at the same time, whereas 29 ± 7% of the time the LM fired before the CM, and 31 ± 7% of the time the CM fired before the LM (n = 8). During CMMCs, there was a significant increase in both the distance Ca\(^{2+}\) waves propagated across the smooth muscle syncitium (Table 1) and their propagation velocity orthogonal to CM fibers (Table 1 and Figs. 2 and 3). In the CM layer, Ca\(^{2+}\) waves during the CMMC had a mean propagation velocity of 5.9 ± 0.4 mm/s (n = 8), whereas in the LM Ca\(^{2+}\) waves had a mean propagation velocity of 9.9 ± 0.1 mm/s (n = 8). These values were significantly faster than the mean propagation velocities obtained before CMMCs (i.e., CM: 3.8 ± 0.2 mm/s, n = 8; and LM: 6.6 ± 0.5 mm/s, n = 8, P < 0.05).

When individual Ca\(^{2+}\) wave events occurring during the CMMC were analyzed in the CM, they were found to have variable propagation velocities and numbers of initiation sites and collisions (Fig. 2H). This “zigzag” appearance (as in Fig. 2H) was due to an increase in both numbers of initiation sites (Figs. 2H and 3D) and numbers of collisions between Ca\(^{2+}\) waves.

Pharmacological sensitivity of Ca\(^{2+}\) waves in the LM and CM. The generation of CMMCs in the mouse colon has been shown to involve a discharge of cholinergic excitatory junction potentials (EJPs) in the CM layer (7, 23, 25). In light of this, we tested whether atropine would prevent the generation of Ca\(^{2+}\) waves in both the LM and CM during CMMCs. Atropine (1 \(\mu\)M) or hexamethonium (300 \(\mu\)M) consistently abolished Ca\(^{2+}\) waves in both muscle layers (n = 4). Ca\(^{2+}\) waves were also blocked by nicardipine (1–2 \(\mu\)M), an L-type Ca\(^{2+}\) channel antagonist, suggesting that they are caused by an influx of Ca\(^{2+}\) as an action potential propagates through the smooth muscle syncitium.

Properties of Ca\(^{2+}\) waves in the aganglionic distal colon of Ednrbs\(^{-/-}\)/Ednrbs\(^{-/-}\) mice. It was of particular interest to determine whether the properties of Ca\(^{2+}\) waves that underlie the phasic contractions in the aganglionic distal colon of Ednrbs\(^{-/-}\) mice.

**Fig. 3.** Changes in properties of Ca\(^{2+}\) waves in the CM during CMMCs and in rectal aganglionosis. A: during CMMCs, the distance traveled by Ca\(^{2+}\) waves orthogonal to CM fibers significantly increased compared with spontaneous waves between CMMCs. However, in the aganglionic segment of endothelin receptor type B-deficient (Ednrbs\(^{-/-}\)/Ednrbs\(^{-/-}\)) mice, the mean distance of propagation was not significantly different from control. B: during CMMCs, the velocity of Ca\(^{2+}\) wave propagation in the CM also significantly increased in C57BL/6 control mice. Interestingly, the propagation velocity in aganglionic muscles was significantly higher than even during CMMCs in wild-type mice. C: there was a dramatic increase in numbers of collisions between Ca\(^{2+}\) waves during CMMCs. Very few collisions occurred between CMMCs or in the aganglionic segment. D: the rate at which Ca\(^{2+}\) waves were initiated in the CM dramatically increased during CMMCs. Note that we only measured waves that originated and terminated within the field of view. A wave could propagate away from its site of initiation in opposite directions; therefore, one-half of the wave could terminate outside the field of view. To avoid this, the distance a wave traveled was measured from its site of initiation to its point of termination; therefore, the measured propagation distances are likely to represent the minimum distance a wave could travel. Some waves may have traveled twice this distance (*P < 0.01). Data were extracted from control (n = 5), CMMC (n = 4) and Ednrbs\(^{-/-}\)/Ednrbs\(^{-/-}\) (n = 8) mice.
Ednrb<sup>−/−</sup> mice would be different from C57BL/6 controls. In both LM and CM layers, spontaneous Ca<sup>2+</sup> waves were routinely recorded from the aganglionic distal colon of Ednrb<sup>−/−</sup>/Ednrb<sup>−/−</sup> mice (n = 8). The mean propagation velocity of Ca<sup>2+</sup> waves in the CM was found to be significantly higher in the aganglionic distal colon compared with C57BL/6 mice (P < 0.05; n = 8; Fig. 3B). The mean frequency of Ca<sup>2+</sup> wave discharges in the LM and CM was found to be 0.57 ± 0.05 and 1.72 ± 0.11 Hz (211 and 301 waves, respectively, n = 8). Interestingly, the number of collisions between Ca<sup>2+</sup> waves was low in the aganglionic distal colon (Fig. 3C). In the CM but not LM, it was possible to ascertain the number of collisions between Ca<sup>2+</sup> waves. Only 11 detectable collisions occurred from 301 waves (n = 8 animals). It was not possible to reliably determine how many collisions occurred in the LM layer due to the removal of part of the LM (see METHODS).

The most notable difference in the characteristics of Ca<sup>2+</sup> waves in the aganglionic distal colon was the reduced temporal synchrony between the two muscle layers (Fig. 4, A and B). In 78% of the discreet Ca<sup>2+</sup> waves that fired in the CM, it was not possible to temporally correlate the onset of any discreet Ca<sup>2+</sup> wave with another Ca<sup>2+</sup> wave in the LM (within a 100-ms time window, n = 8; Fig. 4, A and B). In 22% of the Ca<sup>2+</sup> waves identified in the CM, a Ca<sup>2+</sup> wave could be also identified in the LM (within a 100-ms window of the onset of the Ca<sup>2+</sup> wave in the CM).

Since it is known that smooth muscle develops significant hypertrophy in response to partial bowel obstruction and...

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**Fig. 4.** Asynchronous firing of Ca<sup>2+</sup> waves between the LM and CM in the aganglionic rectum of Ednrb<sup>−/−</sup>/Ednrb<sup>−/−</sup> mutant mice. A and B: ST maps of Ca<sup>2+</sup> wave activity imaged simultaneously in LM and CM layers. Time is represented from the top to bottom, whereas the distance of Ca<sup>2+</sup> wave propagation is represented from the left to right. It can be seen that Ca<sup>2+</sup> waves in the two muscle layers occurred independently and rarely discharged simultaneously. C: sequence of Ca<sup>2+</sup> waves in the LM. In the top left image (0 ms), a single Ca<sup>2+</sup> wave was initiated, which then spread in the direction of the arrow to the top middle image (67 ms). At 133 ms, the top right image showed a second Ca<sup>2+</sup> wave in the LM that was propagating toward the first wave initiated at 0 ms. At 267 ms (bottom middle image), the two waves collided. During these Ca<sup>2+</sup> waves in the LM, there was no accompanying Ca<sup>2+</sup> wave in the underlying CM layer. D: a single propagating Ca<sup>2+</sup> wave in the CM. At 67 ms (top middle image), the CM Ca<sup>2+</sup> wave was initiated, which propagated as one continuous wave from the right side of the image to the left (see the direction of the arrow). Eventually, at 333 ms (bottom right image), the wave propagated outside the field of view. No evidence of any accompanying LM Ca<sup>2+</sup> wave was shown when the Ca<sup>2+</sup> wave in the CM was active.
megacolon, we were particularly interested in whether the differences in the characteristics of Ca$^{2+}$ waves in the aganglionic distal colon could be due to increases in muscle thickness. When whole colons were obtained from $Ednrb^{-/-}/Ednrb^{-/-}$ lethal and $Ednrb^{+/Ednrb^{-/-}}$ heterozygote mice, sections of each region were stained for hematoxylin and eosin. After sections had been stained, it was found the most pronounced increase in smooth muscle hypertrophy was actually in the aganglionic region itself (Fig. 5). Both the LM and CM layers were twofold thicker in the aganglionic distal colon (measured 3–4 mm from the anus) than in C57BL/6 controls (Fig. 5). In the CM layer, the mean muscle wall thickness increased from 77.7 ± 9.7 to 139.7 ± 16.7 μm in $Ednrb^{-/-}/Ednrb^{-/-}$ (n = 4, P < 0.05), whereas in the LM layer the muscle wall thickness increased from 29.0 ± 4.3 to 52.6 ± 6.2 μm (n = 4, P < 0.05; Fig. 5).

DISCUSSION

In this study, we used Ca$^{2+}$ imaging of intact whole preparations of the murine colon to determine how the temporal activation and spread of Ca$^{2+}$ waves in LM and CM fibers changed during CMMCs in wild-type mice and, second, to determine how the properties of Ca$^{2+}$ waves were modified in the aganglionic rectum of $Ednrb^{-/-}/Ednrb^{-/-}$ mutant mice that do not exhibit CMMCs. A number of findings arose from this study. Namely, during CMMCs in wild-type mice, a discharge of discreet Ca$^{2+}$ waves occurs simultaneously in both LM and CM layers. This temporal synchronization of Ca$^{2+}$ waves between the two muscle layers involves the synaptic activation of many myenteric interneurons and cholinergic excitatory motor neurons. Second, during CMMCs, the orthogonal propagation velocity and distance traveled by discreet Ca$^{2+}$ waves

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**Fig. 5. Hypertrophy induced in the LM and CM of $Ednrb^{+/Ednrb^{-/-}}$ and $Ednrb^{-/-}/Ednrb^{-/-}$ mutant mice.** A–C: photomicrographs of sections of the far distal colon (3 mm from the rectum) stained for hematoxylin and eosin. A: normal LM and CM layers in C57BL/6 mice. B and C: sections of the colon from $Ednrb^{+/Ednrb^{-/-}}$ heterozygote and $Ednrb^{-/-}/Ednrb^{-/-}$ homozygote lethal mice, respectively. It can be seen that both piebald mice exhibited a similar degree of hypertrophy in both the LM and CM. D: cumulative graphical representation of the relative changes in muscle wall thickness in both the CM and LM. The most prominent increase in hypertrophy was detected in the aganglionic rather than ganglionic region of the colon (from n = 4 animals).
increases, as do the numbers of initiation sites and collisions between Ca\textsuperscript{2+} waves. However, in the aganglionic rectum of Ednrb\textsuperscript{-/-}/Ednrb\textsuperscript{-/-} mice, although the propagation velocity of Ca\textsuperscript{2+} waves was significantly increased, Ca\textsuperscript{2+} waves between the two muscle layers showed consistently poor temporal synchronization.

**Dependence of intercellular Ca\textsuperscript{2+} waves on action potentials.** Ca\textsuperscript{2+} waves appear to arise from Ca\textsuperscript{2+} influx during a smooth muscle action potential since, like muscle action potentials, they are blocked by L-type Ca\textsuperscript{2+} channel antagonists (13, 15, 26, 29, 30). Therefore, a Ca\textsuperscript{2+} wave is likely due to the sequential rise in Ca\textsuperscript{2+} within each muscle cell produced by an action potential as it propagates through the smooth muscle syncitium rather than a spread of Ca\textsuperscript{2+} from muscle cell to muscle cell. In the present study, we found that the velocity of Ca\textsuperscript{2+} waves orthogonal to the muscle fibers was similar for both the LM and CM. These values were similar to those we reported previously for the LM of the guinea pig ileum (30), the LM and CM of the guinea pig colon (29), and the LM of the murine colon (13). They were also consistent with the velocities of propagating action potentials in the LM of the guinea pig colon measured orthogonal to the muscle fibers with two intracellular microelectrodes (15). Previously, we had estimated the velocity of Ca\textsuperscript{2+} waves parallel to muscle fibers to be 8–10 times faster than perpendicular to the direction of the fibers (29, 30).

**Multiple initiation sites and multiple collisions during CMMCs.** It is particularly noteworthy that when Ca\textsuperscript{2+} waves discharge during CMMCs, they appeared to make a zigzag appearance across the CM. This was because they were found to be not a single propagating wave but rather composed of multiple discreet Ca\textsuperscript{2+} waves. This zigzag appearance is caused by an increase in both the numbers of sites at which Ca\textsuperscript{2+} waves were initiated and due to annihilating collisions between Ca\textsuperscript{2+} waves (13, 29, 30). This suggests that the synchronized EJPs that occur in the muscle during the CMMC (25) are initiating action potentials at many discreet sites across the muscle at about the same time. The resulting action potentials (Ca\textsuperscript{2+} waves) can only propagate orthogonally a short distance before colliding with another Ca\textsuperscript{2+} wave. Our recent electrophysiological study (26) using two microelectrodes support these conclusions since we have shown that action potentials could readily propagate over 100 μm across the LM, but they were rarely coordinated over a distance of 1 mm across the LM of the guinea pig colon. As we have previously suggested (13, 30), collisions prevent any one Ca\textsuperscript{2+} wave initiation site from controlling large regions of muscle. It may seem contradictory that individual Ca\textsuperscript{2+} waves travel further during the CMMC yet are subject to more collisions. This maybe due to the fact that they also travel faster; therefore, each wave can propagate over a larger area of muscle before it collides with another wave.

**Ca\textsuperscript{2+} waves and EJPs during CMMCs.** One of the major findings of the present study was that during CMMCs, Ca\textsuperscript{2+} waves propagated over larger distances orthogonal to the muscle fibers and at a higher velocity across the smooth muscle syncitium. It is known that during CMMCs, there is a repetitive discharge of cholinergic EJPs in the CM layer that become temporally synchronized over large regions of the CM layer (up to 15 mm in the longitudinal axis and across the entire width of the colon) (25). It is likely that if action potential threshold is reached during the EJP, then a considerably larger area of smooth muscle will have its resting membrane potential at or near action potential threshold that may result in Ca\textsuperscript{2+} waves spreading over larger distances at a higher velocity. Also, the release of ACh during the CMMC is likely to change the conductance properties of the smooth muscle syncitium, which is likely to promote the faster propagation of action potentials. It is particularly noteworthy that intracellular electrophysiological recordings from the CM during the CMMC demonstrates that the frequency of cholinergic EJPs is ~1.9 Hz (7, 23, 25), which is remarkably similar to the frequency of Ca\textsuperscript{2+} waves in both muscle layers during the CMMC. This similarity in EJP and Ca\textsuperscript{2+} wave frequency, together with the observation that Ca\textsuperscript{2+} waves were blocked by atropine, supports the idea that the discharge of suprathreshold cholinergic EJPs in both muscles may be the underlying electrical event responsible for the initiation of Ca\textsuperscript{2+} waves. Indeed, in the small intestine of other laboratory animals such as the guinea pig, the MMC in the small intestine has also been recorded (10, 11) and shown to be abolished by atropine or hexamethonium (11), suggesting that in other regions of the intestine, MMC...
generation is also critically dependent on the release of ACh from cholinergic motor neurons. Since single cholinergic motor neurons project for relatively short distances along the intestine (2, 4, 8), it seems highly likely that many myenteric interneurons and motor neurons are activated simultaneously to generate a single EJP in gastrointestinal smooth muscle (25).

There have not been any electrical recordings made from the LM layer of the mouse colon. We have shown previously in the guinea pig distal colon that the LM and CM both received a synchronized discharge of cholinergic EJPs in response to maintained circumferential stretch that was due to the synchronous activation of excitatory motor neurons to both the LM and CM (27). It is likely that a similar arrangement of enteric circuitry may be responsible for the simultaneous discharge of activity in both muscle layers in the mouse colon (Fig. 6) (for a review, see Ref. 22).

The concurrent activation of the LM and CM during CMMCs corresponds well to that observed during peristalsis. Recently, in the guinea pig distal colon, we showed that during propulsion, the LM contracts immediately behind a fecal pellet in vitro (20). By contracting behind a pellet, the LM adds to propulsion by pulling the gut over the rear of the pellet. Also, the coactivation of both muscle layers in the small and large intestine (3, 12, 20, 21) creates a contracting muscle mass behind the pellet that is much greater than if there was no longitudinal contraction. This muscle mass provides a zone of contraction that can exert considerable force behind a pellet while minimizing the stress on the gut wall (18). Interestingly, it has been shown in the esophagus, that the LM and CM only contract out of phase with each other during pathological conditions (16).

Properties of Ca\(^{2+}\) waves in the aganglionic distal colon of Ednr\(^{-/-}\)/Ednr\(^{b-/-}\) mice. We found that despite the absence of enteric nerves, the aganglionic smooth muscle was readily able to generate myogenic contractions (19) and Ca\(^{2+}\) waves in both the LM and CM. The mechanisms underlying these contractions have been elusive. In this study, the major finding in the aganglionic segment was that the two muscle layers fired almost exclusively out of phase with one another, likely due to the absence of interneurons and motor neurons to synchronize their firing. Surprisingly, the propagation velocity of Ca\(^{2+}\) waves was significantly faster in the hypertrophic region. It is known that the conduction of action potentials is increased in thicker nerve axons (28). A similar phenomenon may also account for these results in the hypertrophic aganglionic segment.

Conclusions. We have shown that during CMMCs in wild-type mice, a rapid discharge of Ca\(^{2+}\) waves occurs simultaneously in both smooth muscle layers. Since no evidence of electrotonic conduction was found between the two muscle layers, we suggest that the underlying mechanism must involve synchronized firing of myenteric interneurons and excitatory motor neurons to both muscle layers that results in simultaneous EJPs and action potentials. The net response is likely to lead to simultaneous shortening of both muscle layers during the propulsion of a fecal pellet. The absence of enteric ganglia in the distal colon of Ednr\(^{-/-}\)/Ednr\(^{b-/-}\) mice leads to sporadic and largely asynchronous firing of Ca\(^{2+}\) waves in both muscle layers.

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REFERENCES