Identification of functional intramuscular rectal mechanoreceptors in aganglionic rectal smooth muscle from piebald lethal mice

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Spencer NJ, Kerrin A, Zagorodnyuk VP, Hennig GW, Muto M, Brookes SJ, McDonnell O. Identification of functional intramuscular rectal mechanoreceptors in aganglionic rectal smooth muscle from piebald lethal mice. Am J Physiol Gastrointest Liver Physiol 294: G855–G867, 2008. First published January 24, 2008; doi:10.1152/ajpgi.00502.2007.—The mechanosensitive endings of low-threshold, slowly adapting pelvic afferents that innervate the rectum have been previously identified as rectal intraganglionic laminar endings (rIGLEs) that lie within myenteric ganglia. We tested whether the aganglionic rectum of piebald-lethal (s/l) mice lacks rIGLEs and whether this could explain impaired distension-evoked reflexes from this region. Extracellular recordings were made from fine rectal nerves in C57BL/6 wild-type and s/l mice, combined with anterograde labeling. In C57BL/6 mice, graded circumferential stretch applied to the rectum activated graded increases in firing of slowly adapting rectal mechanoreceptors. In s/l mice, graded stretch of the aganglionic rectum activated similar graded increases in rectal afferent firing. Stretch-sensitive afferents responded at low mechanical thresholds and fired more intensely at noxious levels of stretch. They could also be activated by probing their receptive fields with von Frey hairs and by muscle contraction. Anterograde labeling from recorded rectal nerves identified the mechanoreceptors of muscular afferents in the aganglionic rectal smooth muscle. A population of afferents were also recorded in both C57BL/6 and s/l mice that were activated by von Frey hair probing, but not stretch. In summary, the aganglionic rectum is innervated by a population of stretch-sensitive rectal afferent mechanoreceptor which develops and functions in the absence of any enteric ganglia. These results suggest that in patients with Hirschsprung’s disease the inability to activate extrinsic distension reflexes from the aganglionic rectum is unlikely to be due to the absence of stretch-sensitive extrinsic mechanoreceptors.

rectal distension triggers a variety of intrinsic and extrinsic neural reflexes within the gastrointestinal (GI) tract. Extrinsic rectal afferents are potentely activated by rectal distension and are responsible for stimulating many different neural reflexes such as the defecation reflex (1), the peristaltic reflex (9), and for the signaling of noxious (painful) distension stimuli to the central nervous system (2, 10–12). The cell bodies of rectal afferents lie in dorsal root ganglia of the spinal cord, but their peripheral sensory nerve endings innervate and ramify within many different visceral organs that include the lower GI tract (10).

In patients with Hirschsprung’s disease, it is well known that enteric ganglia fail to develop within the rectum. This results in an absence or substantial impairment of extrinsic and intrinsic neural reflexes (7, 8, 14, 25, 26). In fact, manometric diagnosis of rectal aganglionosis in patients with Hirschsprung’s disease is characterized by the inability of the internal anal sphincter (IAS) to relax in response to rectal balloon distension (7, 8, 14, 25, 26). The failure to trigger the inhibitory IAS reflex following distension of the aganglionic region of rectum suggests that it is devoid of sensory and possibly motor neurons. Although cell bodies of enteric neurons, including intrinsic sensory neurons, are missing from the aganglionic rectum, it is not clear whether extrinsic sensory nerves innervate the aganglionic segment. Neuronal tracing studies of lethal spotted mice have identified extrinsic spinal afferent neurons from the dorsal root ganglia (L6–S1) that innervate the aganglionic rectum (21). Also, in human patients with Hirschsprung’s disease, the rectal nerves enter the aganglionic segment (30), but it is not clear whether their nerve endings are normally responsive to mechanical stimuli. Since the rectal nerve provides a major extrinsic innervation to the rectum and has been shown to play a major role in mediating the inhibitory IAS reflex following rectal distension (34), one would assume that an impairment in either rectal afferent or efferent pathways is responsible for an impaired inhibitory IAS reflex in the aganglionic rectum.

Recent studies have identified the mechanosensitive endings of low-threshold, slowly adapting mechanoreceptors in the rectum as rectal intraganglionic laminar endings (rIGLEs) that branch within myenteric ganglia (16). This raises the question whether low-threshold afferents could function in a region of gut that lacks enteric ganglia. Previous studies from our laboratory (27) have shown that distension of an aganglionic rectum fails to trigger extrinsic spinal afferent reflexes, such as the visceromotor reflex. This suggests that a population of extrinsic mechanoreceptors that normally lie in the enteric ganglia of the rectum are essential for the generation of extrinsic rectal distension reflexes. However, as yet, no extrinsic mechanoreceptors have been morphologically identified in the mouse rectum. Rodents have proved invaluable for investigations into extrinsic neural reflexes, since the entire large bowel can be removed from the animal, while preserving the extrinsic nerve supply for in vitro experimentation. These types of in vitro experiments have not been shown to be feasible in larger mammals, such as humans. In fact, a major advance in our understanding of the pathogenesis of human Hirschsprung’s disease came about with the discovery of spontaneously mutant strains of mice, such as the piebald lethal strain...
for a deletion of the EDNRB gene develop rectal aganglionosis that leads to megacolon and death shortly after birth (15, 24, 31, 32). Since this discovery in mice, it has been shown that mutation of the EDNRB gene also results in rectal aganglionosis in a variety of other mammals, including rats (7), horses (18), and humans (22). For this reason, the piebald strain of mouse has become an established model for studies on human Hirschsprung’s disease and the effects of aganglionosis on gut function (24, 31, 32, 34, 38).

In this study, we have used sl/sl mice to specifically test whether stretch-sensitive, low-threshold rectal mechanoreceptors develop and function normally in an aganglionic rectum. The results show that the aganglionic rectum is innervated by a population of slowly adapting rectal mechanoreceptors that respond to low mechanical thresholds, despite the absence of all enteric ganglia. Their endings in the gut wall ramify within the circular and longitudinal muscle layers. These mechanoreceptors of rectal muscular afferents have similar functional properties to the rectal muscular afferents found in wild-type mice. They respond to low physiological levels of circumferential stretch and are activated by spontaneous contractions of the smooth muscle.

**GENERAL METHODS**

**Preparation of tissues.** Piebald homozygous lethal offspring (Ednrb<sup>−/−</sup>/Ednrb<sup>−/−</sup>) were obtained by intercrossing Ednrb<sup>+/−</sup>/Ednrb<sup>+/−</sup> (heterozygotes), as previously described (21), from an in-house colony raised at the University of Nevada School of Medicine. By convention, throughout this study, we will refer to Ednrb<sup>−/−</sup>/Ednrb<sup>−/−</sup> homozygote lethal mice as sl/sl. C57BL/6 mice (20–90 days of age) were used as experimental wild-type control animals throughout all studies in this proposal. The sl/sl mice were euthanized for experimental purposes at 20–30 days of age. All experiments described in this study were approved by the animal ethics committee at the University of Nevada School of Medicine.

The same length segment of rectum (15–20 mm from the anal sphincter) was removed from male sl/sl mice and male wild-type mice. In these preparations, the fine rectal nerves and pelvic ganglia were removed from mice, while extrinsic neural continuity was maintained between the rectal nerves and rectum. A longitudinal incision was made along the mesenteric border of the distal colon that extended into the rectum. This incision was made in such a way as to minimize damage to the fine rectal nerve fibers entering the rectal wall (Fig. 1B). Once removed from the animal, these preparations were pinned as flat sheets (serosal side uppermost) to the base of a Sylgard (Dow Corning)-lined organ bath. In all preparations used in this study, the mucosa, submucosa, and submucous plexus were sharp dissected free from the underlying circular muscle. Therefore, in C57BL/6 mice, the preparations used consisted of circular and longitudinal muscle with the myenteric plexus, whereas in sl/sl mice preparations consisted of only circular and longitudinal muscle, with no enteric ganglia present. For the purposes of this study, the rectum was defined as the region of distal large bowel that received a detectable innervation from the rectal nerve trunks that arose from the pelvic ganglia. Therefore, in mouse, the rectum encompasses a region ~15–20 mm from the anus (Fig. 1B). The fine rectal nerves (see Fig. 1B) were teased into a separate recording chamber, from which afferent recordings were made. No recordings were made from the pelvic nerve, and in fact the pelvic nerve was not retained in any of our in vitro dissections.

**Activation of stretch-sensitive rectal afferents.** The cut edge of the flat sheet preparation was attached to a rakeslike array of micro hooks made of fine dissecting pins (6). The rake was attached via fine cotton to a cantilever pulley system whereby loads of increasing mass could be applied to cause circumferential stretch of the colonic wall; see Brierley et al. (4). Fine branches of the rectal nerve trunks were dissected free of connective tissue and led into a paraffin-filled side chamber, sealed with silicon grease. Extracellular recordings were made via a platinum wire electrode contacting the nerve trunk, with an indifferent electrode on a strand of connective tissue. Increasing weights were applied to the cantilever so that graded increases in circumferential stretch could be generated. In preparations in which more than two distinct single units responded to stretch, these recordings were discarded from analysis. Only recordings where distinct single units could be clearly discriminated were selected for analysis.
**Extracellular nerve recordings.** Recorded extracellular action potentials in rectal afferents were amplified through an isolated bi-amplifier (ISO-80, World Precision Instruments), then digitized with a Powerlab/4sp (AD Instruments) and recorded on an Apple Mac mini personal computer using Chart software (AD Instruments). The organ bath consisted of a recording chamber (~1.5-ml capacity) in which the rectal nerves (Fig. 1B) were maintained in paraffin oil, while the main chamber contained the attached rectum. The preparation chamber (10-ml volume) was constantly perfused at a flow rate of 4 ml/min with oxygenated Krebs solution at 35 ± 1°C.

**Identification of mechanosensitive hot spots.** In wild-type and s/l/s mouse rectum, mechanosensitive endings of extrinsic rectal afferents were labeled by an anterograde tracing technique (29) and previously used to identify vagal (35, 36) and pelvic (16) afferent endings. Local mechanical distortion of the tissue was delivered by fine, calibrated von Frey hairs, with tip diameters of <50 μm. Gentle probing of the serosal surface with a calibrated von Frey hair across the entire preparation was used to identify mechanosensitive hot spots. These represent the mechanotransduction sites of rectal afferent nerve endings. Carbon particles were then attached to the tip of the hair and the von Frey hair reapplied to mark the hot spot (16, 35, 36) on the preparation.

**Mechanical recordings of smooth muscle contractility.** The protocol for mechanical recordings from circular and longitudinal muscle were essentially identical to that performed previously; see Fig. 9B in Ref. 17. In brief, spontaneous mechanical activity of the circular and longitudinal muscle was monitored simultaneously using spring microclips (Micro-serrefines no. 18055-04; Fine Science Tools, Foster City, CA) that were used to pinch a segment of rectum 2–3 mm in length. These clips were connected via fine cotton thread to two independent isotonic force transducers (model TRN001; Kent Scientific, Litchfield, CT). Each microclip was positioned so as to monitor changes in smooth muscle tension in the longitudinal and circumferential axis of the sheet preparation, against a maintained resting load of 500 mg. The mechanical activity of the longitudinal and circular muscle were recorded and analyzed on an Apple iMac computer, running Chart software (version 5.2) (AD Instruments).

**Immunohistochemical staining.** Polyclonal antibodies against the vesicular glutamate transporter, VGluT2 (1:1,000; cat. no. AB5907) raised in the guinea pig were obtained from Chemicon International, Temecula, CA. Tissues were incubated for 48 h in primary antibody, washed in PBS overnight, then for 1 h in secondary antibody (Alexa Fluor 594; 1:100) goat anti-guinea pig IgG (cat. no. A11076) obtained from Molecular Probes (Eugene, OR).

We double-labeled all anterogradely labeled preparations with the polyclonal antibody against smooth muscle myosin II heavy chain (1:200; Biomedical Technologies, Stoughton, MA), which binds to the smooth muscle myosin II heavy chain. Alexa Fluor 647 goat anti-rabbit IgG (1:100; cat. no. A21244) purchased from Molecular Probes was used as secondary antibody to visualize myosin immunoreactivity in s/l/s and wild-type mouse rectum. Immunofluorescence for smooth muscle myosin II heavy chain revealed the orientation of muscle fibers so that the layers could be unambiguously distinguished.

**Analysis of data.** It was important to statistically quantify the distance between anterogradely labeled nerve endings to the nearest carbon-marked hot spots and compare this distance to randomly assigned computer-generated hot spots. A montage image of fluorescent afferent nerve fibers was displayed in Volumetry G6a. (Hennig), and an outline was drawn around every carbon particle and every nerve fiber ending. The XY midpoint of each outline was used as the reference position, and the distance between each identified nerve ending and nearest carbon particle was calculated in micrometers. In addition, the number and density of both nerve fiber endings and carbon particles was determined. To test whether the distribution of nerve fiber endings and carbon particles was random, for each carbon particle, 256 randomly positioned points (computer-generated hot spots) were created in the montage of the preparation that contained the biotinamide-filled nerve fibers. The distance to the carbon particle was calculated and the average distance of the randomly positioned points was determined.

Throughout the **RESULTS** section, n refers to the number of animals on which observations were made. Statistical comparison of data was made by Student’s paired or unpaired t-tests and one- or two-way ANOVA with Newman-Keuls post hoc tests.
All preparations were left to equilibrate at 35°C in the organ bath for 30 min prior to any measurements being made. This includes spontaneous contractions and action potentials. Drugs were perfused through the organ bath for at least 20 min before any measurements of smooth muscle contractility or electrical activity were made. A recording period of 5 min was used to determine the interval between spontaneous action potentials.

**Drugs and solutions.** The following drugs were used: nifedipine, sodium nitroprusside (SNP), and [βAla8]NKA(4–10). All were obtained from Sigma Chemical, St. Louis, MO. All drugs were made up as stock solutions at a concentration of $10^{-2}$ M and dissolved in water, except for nifedipine, which was dissolved in 100% ethanol. Drugs were exposed to the rectum only.

**RESULTS**

**General observations.** The abdominal cavity of s/s mice was usually bloated, and following euthanasia the entire colon was found to be heavily impacted with feces, as has been previously described. The aganglionic region was usually constricted and devoid of fecal pellets (Fig. 1B). Similar bloating and impacted feces were never observed in the large bowel of wild-type mice, regardless of the age of the animal. It was immediately obvious that the smooth muscle was considerably hypertrophied in the aganglionic rectum of s/s mice (28), associated with a decrease in colonic wall compliance (33).

**Afferent recordings.** Extracellular recordings were made from 29 single afferent units in fine rectal nerve trunks innervating the aganglionic rectum of s/s mice (n = 19) and 38 units from wild-type mice (n = 22). When recordings were made from slack preparations of rectum in both types of mice, a population of afferent fibers showed an ongoing discharge of spontaneous action potentials. In wild-type animals, 34 of 38 single units (n = 22) showed spontaneous firing at a mean frequency of $0.7 \pm 0.2$ Hz. In s/s mice, spontaneous action potentials occurred in 13 of 29 units (n = 5 of 19) at a mean firing frequency of $0.5 \pm 0.1$ Hz (13 units, n = 5). These spontaneous firing frequencies were not significantly different ($P > 0.05$; unpaired Student’s t-test). In s/s mice, spontaneous action potentials were recorded from rectal afferents with endings that terminated in the aganglionic segment, which lacked both myenteric and submucosal plexuses (15, 24, 31).

**Responses of the aganglionic and wild-type rectum to circumferential stretch.** Graded circumferential stretch by applying fixed loads to the gut wall evokes graded increases in firing of muscular afferents (4, 5) in the mouse rectum. In wild-type mice in the present study, a sustained discharge of action potentials was generated for the duration of a circumferential stretch stimulus (1–5 g, Fig. 1 and 2). Firing frequencies ranged from $1.1 \pm 0.5$ Hz at 1 g of stretch to a maximum of $3.5 \pm 0.9$ Hz at 4- to 5-g stretch (38 units, n = 22; Fig. 2A). The same stretch stimuli applied to the aganglionic rectum in s/s mice evoked similar firing frequencies (1 g: $0.4 \pm 0.2$ Hz) to a maximum of $2.7 \pm 0.6$ Hz at 5 g of stretch (29 units, n = 19; $P > 0.05$; Fig. 2A). Despite pronounced smooth muscle hypertrophy in the aganglionic rectum (28), there was no significant difference in the mean firing frequencies of muscular afferents activated by graded circumferential stretch (over 1–5 g) between s/s and wild-type mice (Fig. 1; $P > 0.05$; 2-way ANOVA, Newman-Keuls post hoc test). Nevertheless, the responses were not identical. In wild-type mice, the mean threshold stretch required to activate a single muscular afferent was $1.5 \pm 0.2$ g (range: 1–3 g), whereas in s/s mice, it was significantly higher ($2.3 \pm 0.3$ g; range: 1–3 g, $P = 0.01$, unpaired Student’s t-test; n = 19; see Fig. 2C). All stretch-activated rectal afferents innervating the rectum of wild-type and s/s mice behaved as low-threshold, slowly adapting mechano-

**Fig. 3.** Graded increases in circumferential stretch evoked graded increases in firing of rectal afferents in both wild-type and s/s mice. Comparison of typical stretch responses at 1-g (A and B), at 3-g stretch (C and D), and at 5-g stretch (E and F). In all animals tested, rectal muscular afferents responded to low mechanical thresholds with a slowly adapting response to maintained stretch.
receptors (Fig. 3). No rapidly adapting mechanoreceptors were recorded from any rectal afferents.

Rectal muscular afferents in the aganglionic and wild-type rectum respond to low mechanical thresholds. Since the muscular afferents in wild-type and sl/sl mice were highly sensitive to low levels of circumferential stretch, we tested whether these same units would show larger responses to higher levels of stretch. At the same time, we tested whether a separate, distinct population of high-threshold afferents be recruited at higher levels of stretch. We applied 8 g of load to wild-type preparations, where muscular afferents were activated at thresholds of 1–2 g. In these preparations, muscular afferents showed even higher frequencies of firing when 8 g of stretch was applied. However, importantly, no high-threshold afferents were ever selectively recruited by these intense levels of stretch. The mean firing frequency increased from 4.8 ± 1.9 Hz at 5 g to 10.7 ± 4.6 Hz at 8 g (5 units, n = 3; P > 0.05).

Similar results were obtained in sl/sl mice. Firing increased from 3.2 ± 0.6 Hz at 5 g (27 units, n = 17) to 4.1 ± 0.6 Hz at 8 g (P = 0.23; 27 units, n = 17). As with wild-type mice, higher levels of stretch also failed to recruit any distinct high threshold units in sl/sl mice. This suggests that all muscular afferents functionally behave as low-threshold mechanoreceptors, but can show increased firing frequencies with intense stretch. These mechanoreceptors are often known as wide dynamic range mechanoreceptors.

Activation of rectal afferents during spontaneous contractions of the aganglionic and ganglionic rectum. In 6 of 22 wild-type mice studied, the rectum generated spontaneous contractions of the longitudinal and circular muscle, which often occurred simultaneously. The mean interval between contractions was 5.8 ± 0.9 s, half duration of 4.5 ± 0.8 s, and amplitude of 0.6 ± 0.2 mN (n = 6). Rectal afferents increased their firing frequency during the peak of each contraction, to a mean of 16.9 ± 7.1 Hz (n = 6; Fig. 4A). In similar experiments on sl/sl mice, aganglionic smooth muscle also contracted simultaneously (both the circular and longitudinal muscle) with a mean interval of 7.7 ± 0.5 s, half duration of 3.1 ± 0.3 s, and amplitude of 0.9 ± 0.3 mN (Fig. 4B). Similarly, rectal afferents showed bursts of firing during spontaneous contractions, with a mean firing frequency of 13.6 ± 4.3 Hz (n = 17). In some experiments, the longitudinal muscle contracted out of phase with the circular muscle layer; isolated longitudinal muscle contractions were not associated with rectal afferent firing (Fig. 4B).

Mechanosensitivity of rectal mechanoreceptors to von Frey hair probing of the aganglionic and wild-type rectum. The mechanosensitive sites (hot spots) of low-threshold muscular afferents can be readily detected by focal probing of the serosal surface of the rectum with von Frey hairs (n = 22 mice). When circumferential stretch was applied to these same preparations, 19 of 36 single units (53%) were also activated by graded circumferential stretch (1–5 g) and fitted the description of muscular afferents according to Brierley et al. (4, 5). The remaining 47% of hot spots would fit the definition of serosal afferents (see Ref. 5). In sl/sl mice, 28 of the 56 rectal afferents with identified hot spots (50%) also responded to graded circumferential stretch (1–5 g) and therefore represent muscular afferents; the remainder did not respond to stretch (5) and thus comprise serosal afferents. In preparations from wild-type and sl/sl mice in which afferents were identified that only responded to probing, applying up to 8 g of stretch to these tissues still failed to induce action potentials (n = 5). The proportion of muscular afferents identified in this study is higher than the 44% reported by Brierley et al. (5) in wild-type mice.

What proportion of mechanosensitive hot spots represent muscular afferent fibers? In wild-type mice, 36 hot spots were identified by fine mechanical probing of the serosal surface, using von Frey hairs (n = 22 mice). When circumferential stretch was applied to these same preparations, 19 of 36 single units (53%) were also activated by graded circumferential stretch (1–5 g) and fitted the description of muscular afferents according to Brierley et al. (4, 5). The remaining 47% of hot spots would fit the definition of serosal afferents (see Ref. 5). In sl/sl mice, 28 of the 56 rectal afferents with identified hot spots (50%) also responded to graded circumferential stretch (1–5 g) and therefore represent muscular afferents; the remainder did not respond to stretch (5) and thus comprise serosal afferents. In preparations from wild-type and sl/sl mice in which afferents were identified that only responded to probing, applying up to 8 g of stretch to these tissues still failed to induce action potentials (n = 5). The proportion of muscular afferents identified in this study is higher than the 44% reported by Brierley et al. (5) in wild-type mice.

Is the mechanosensitivity of rectal afferents modulated by changes in smooth muscle tension in the ganglionic and aganglionic rectum? Vagal afferents in the stomach (1, 13, 36) and pelvic afferents in the rectum (rGLES) (16, 17) have been shown to act largely as tension receptors; their mechanosensitivity is largely determined by changes in muscle tension, rather than muscle length. We tested whether the stretch-
sensitivity of rectal mechanoreceptors in wild-type and sl/sl mouse rectum would be modified by increasing smooth muscle tension, using an NK2 receptor agonist (17). In seven units from wild-type mice (n = 7), precontracting the colon with an NK2 agonist had no effect on the mechanosensitivity of rectal afferents during stretch evoked by loads of 1–5 g (Fig. 5A; P > 0.05; 2-way ANOVA with Newman-Keuls post hoc test). Similarly, in sl/sl mice, no overall significant change occurred in the mechanosensitivity of eight rectal afferents (n = 7) to 1-to 5-g stretch of the aganglionic rectum (Fig. 5B).

We also tested whether smooth muscle relaxants affected stretch-evoked firing, reasoning that if rectal muscular afferent mechanoreceptors were tension receptors, then decreasing smooth muscle tension should result in decreased mechanosensitivity. In wild-type mice, SNP (2 μM; 22 units, n = 9; Figs. 6 and 7) and nifedipine (2 μM; 11 units, n = 7; Fig. 8) independently yielded the same result; neither evoked a significant change in the mechanosensitivity of rectal afferents. The same results were obtained from sl/sl mice, in which there was no significant differences in mechanosensitivity in SNP (Fig. 6 and 7; 10 units, n = 5) or nifedipine (11 units, n = 7; Fig. 8; P > 0.05; 2-way ANOVA with Newman-Keuls post hoc test). In fact, in some recordings, SNP enhanced mechanosensitivity slightly (Fig. 7), possibly because of the pharmacologically evoked increase in wall compliance.

We also tested whether pharmacologically induced contractions could evoke firing when the length of preparations was maintained constant, in tightly pinned preparations. Under these conditions, addition of the specific smooth muscle NK2 agonist [β-Ala8]-NKA (4–10) 100 nM caused a potent and significant increase in the amplitude and half duration of spontaneous contractions of the circular muscle. In wild-type mice, contraction duration increased from 4.5 ± 0.8 to 14 ± 8.3 s (P < 0.05; n = 6), amplitude increased from 0.6 ± 0.2 to 1.2 ± 0.7 mN (P < 0.05; n = 6). Associated with the augmented contractility was an increased firing frequency of single units from 5 ± 3 Hz to 19.4 ± 4.8 Hz (P < 0.05; n = 3). Similar findings were obtained from sl/sl mice, where application of [β-Ala8]-NKA (4–10) 100 nM increased the mean amplitude (1.4 ± 0.4 to 1.8 ± 0.5 mN), half duration (3.6 ± 1.0 to 5.8 ± 1.9), and interval (5.4 ± 0.5 to 5.9 ± 0.4 s) between spontaneously occurring myogenic contractions (Fig. 5).
5D). Associated with this augmented contractility was an increase in the mean firing frequency of single rectal units from 5.9 ± 1.4 Hz in control conditions to 13.2 ± 4.7 Hz in the presence of \([\text{[Ala8]}\text{-NKA(4–10)}] 100 \text{ nM} (P < 0.05; \text{Fig. 5D})\); there was also no difference in the total number of action potentials evoked to a 5-g maintained stretch, before or after SNP 2 μM to the rectum (P > 0.05; Student’s paired t-test).

**Morphological identification of rectal mechanoreceptors in the aganglionic rectum.** Recently, the mechanotransduction sites of rectal afferents that innervate the guinea pig rectum have been identified as rIGLEs that lie in myenteric ganglia (16). Extrinsic axons that make arrays of varicose branching axons within the muscle layers (intramuscular arrays or IMAs) did not appear to contribute to mechanoreceptor firing (16, 35, 36). Since rectal mechanotransduction persists in the aganglionic rectum, which lacks myenteric ganglia, we were particularly interested in morphologically identifying their mechanotransduction sites.

In wild-type mice, hot spots identified by von Frey hair probing were marked with carbon particles on the tip of the hair. These markers usually had diameters of 300–600 μm. The distance between myenteric ganglia was usually less than 200 μm (Fig. 9), meaning that a single carbon marked hot spot often extended over two or more myenteric ganglia. Not surprisingly, in wild-type mice, we could not unequivocally identify transduction sites of rectal mechanoreceptors in myenteric ganglia, we were particularly interested in morphologically identifying their mechanotransduction sites.

In wild-type mice, hot spots identified by von Frey hair probing were marked with carbon particles on the tip of the hair. These markers usually had diameters of 300–600 μm. The distance between myenteric ganglia was usually less than 200 μm (Fig. 9), meaning that a single carbon marked hot spot often extended over two or more myenteric ganglia. Not surprisingly, in wild-type mice, we could not unequivocally identify transduction sites of rectal mechanoreceptors in myenteric ganglia. However, in the aganglionic rectum of s/lsl mice, the absence of enteric ganglia facilitated morphological identification of rectal mechanoreceptor endings (Fig. 10). In s/lsl mice, anterograde labeling confirmed that no enteric ganglia or intestinofugal nerve cell bodies were present. However, biotinamide-filled IMAs were still observed in the aganglionic rectum of four of six s/lsl mice. IMAs were never found to morphologically correlate to the mechanotransduction sites of rectal muscular afferents or afferents that only responded to probing.

Using montages we quantified the distance between the anterogradely labeled nerve endings and the nearest hot spots marked with carbon particles. In s/lsl mice, the mean distance between a marked hot spot and the closest nerve ending was 343.2 ± 72.3 μm (n = 6). Random sites were then generated within the area containing biotinamide-filled axons. The mean distance between a marked hot spot and the nearest randomly generated site was 1,199.3 ± 243.5 μm (n = 6; P < 0.05; Student’s unpaired t-test). This suggests that the mechanoreceptors of muscular afferents are more closely associated with hot spots than would be expected by chance. Muscular afferent mechanoreceptors were continuous with varicose axon terminals located in the longitudinal muscle layer and three in the circular muscle layer, whereas four could not be resolved. A confocal 3D reconstructed image of a typical muscular afferent mechanoreceptor is shown in Fig. 11.
We have been unable to morphologically identify any mechanotransduction sites of rectal afferents in the serosa itself.

**DISCUSSION**

Recent studies have shown that myenteric ganglia in the rectum contain the transduction sites of extrinsic stretch-sensitive, low-threshold rectal mechanoreceptors, known as rIGLEs (16). In this study, we tested whether the absence of myenteric ganglia in the aganglionic rectum of sl/sl mice would result in a lack of spinal afferents that respond to stretch. The major finding of the present study is that at least two populations of rectal mechanoreceptor innervate the aganglionic segment. One population of afferent responded to low levels of circumferential stretch applied to the aganglionic segment and probing of its serosal surface; this class of afferent has been previously described as a muscular afferent (5). A second population of rectal afferent fiber was identified also in the aganglionic segment, which was activated by fine mechanical probing of the preparation, but did not respond to stretch; these have been described previously as serosal afferents (5). These observations show that structures within myenteric or submucosal ganglia, the submucosa or the mucosa of the rectum are not required for the mechanosensitivity of two populations of rectal spinal afferent, one that responds to stretch and one that does not. Also, we found morphological evidence that the mechanoreceptors of muscular afferents terminated within the rectal smooth muscle, at least within the aganglionic rectum of sl/sl mice.

**Differences in mechanosensitivity of rectal muscular afferents in wild-type and sl/sl mice.** When graded increases in circumferential stretch were applied to the aganglionic segment of s/sl mouse by application of different loads to the gut wall, a graded increase in rectal afferent firing was consistently evoked. Since the smooth muscle layers of the aganglionic...
segment in s/s¹ mice show pronounced smooth muscle hypertrophy (28), we expected major differences in the mechanosensitivity of rectal afferents in this region. Differences were observed, but they were relatively modest. A significantly larger circumferential stretch was required to elicit action potentials in muscular afferents of s/s¹ mice compared with wild-type controls (1.5 g in control vs. 2.3 g in s/s¹ mice). In addition, the threshold to circumferential stretch was signifi-

Fig. 8. Nifedipine, which is known to reduce smooth muscle tension, had no significant effect on the mechanosensitivity of rectal muscular afferents to circumferential stretch evoked by constant loads applied to the rectal wall. A and B: nifedipine 2 µM has no significant effect on the mean firing frequency or total number of action potentials generated by muscular afferents to maintained stretch in wild-type mice (11 units, n = 7). C and D: similar results were obtained in s/s¹, in which there was no significant difference in the mean firing frequencies or total mean number of action potentials to stretch (11 units, n = 7).

Fig. 9. Anterograde labeling of a single rectal nerve trunk in a wild-type mouse. A: a montage of the innervation of the ganglionic rectum. Extrinsic nerve endings ramify extensively within myenteric ganglia. B: an expanded portion from the white box in A to show the density of extrinsic innervation in myenteric ganglia. C and D: intestinofugal nerve cell bodies with short lamellar dendrites lying in myenteric ganglia. E: intramuscular arrays were observed in the circular and longitudinal muscle but could never be spatially correlated to any mechanosensitive hot spots.
significantly different, with 69% of muscular afferents in wild-type mice responding to 1 g stretch, whereas in s/sl mice only 19% responded at this level. However, when measurements were made of the overall firing frequency, there was no significant difference between muscular afferents in either strain of mouse for stretches evoked by 1- to 5-g loads.

Mechanisms of activation of rectal mechanoreceptors. It has been recently shown that vagal afferents in the guinea pig stomach (36) and pelvic afferents in the guinea pig rectum (16, 17) behave largely as tension receptors. That is, their mechanosensitivity is highly sensitive to changes in smooth muscle tension. For example, in the guinea pig rectum application of an NK2 agonist evoked contractions of the circular muscle and under isometric conditions, these contractions were associated with bursts of firing of low-threshold mechanoreceptors, leading to an increase in mean firing frequency (17). In the present study, an NK2 agonist did not significantly modify the stretch mechanosensitivity of rectal afferents. However, when applied under isometric conditions, the evoked contractions (at a fixed length) were associated with burst of mechanoreceptor firing.

The effects of SNP and nifedipine were more difficult to interpret at first sight. Both agents are known to reduce muscle tension, but neither affected responses to evoked stretch. In fact, in many preparations, SNP or nifedipine appeared to increase the mechanosensitivity of rectal muscular afferents (see Fig. 7). We speculate that the net effect of these muscle relaxants may have been to increase the compliance of the tissue. Thus, for any given load, the circumference would have been greater, in the presence of the relaxant, but the overall force (determined by the applied load) would have been the same. Thus tension (force divided by mean cross-sectional area) would have been higher during pharmacological relaxation, consistent with these units acting primarily as tension receptors.

Some of the properties of the spinal muscular afferents recorded in this study differ from intestinofugal afferents in the mouse distal colon that project to sympathetic prevertebral ganglia (19, 20). The intestinofugal afferents were also found to increase firing in response to circumferential stretch, and their mechanosensitivity was similarly unaffected by relaxants.
such as nicardipine or nifedipine (20). However, the intestinofugal afferents actually decreased firing during spontaneous contractions of the mouse distal colon (19), whereas we found that the rectal afferents actually increased firing during muscle contraction. In the study of Miller and Szurszewski (19), it was suggested that the mechanosensory endings of intestinofugal afferents were acting primarily to monitor changes in stretch or length of the colon wall. Tension receptors, such as a Golgi tendon organs, increase firing in response to increased muscle tension from active muscle contraction (23). These afferents are clearly in series with the muscle whose tension they reflect. However, interestingly, the rectal muscular afferent mechanoreceptors we identified in the aganglionic rectum of sl/sl mice did not have a consistent preferential orientation with respect to the muscle fibers. Yet muscular afferents consistently showed an increase in firing in response to spontaneous muscle contractions, consistent with other studies of pelvic afferents innervating the rectum (17). The observation that these endings increased firing during spontaneous or pharmacologically evoked muscle contractions suggests that functionally they are more like tension receptors than length receptors. It is likely that any substantial mechanical deformation of their endings can activate them, as it has been recently proposed in the guinea pig rectum (17).

Identification of the mechanoreceptors of rectal muscular afferents in the aganglionic rectum. The mouse distal colon and rectum receive a rich extrinsic afferent innervation via the lumbar splanchnic/coliconic and pelvic/rectal nerves, respectively (4, 5). Within these two nerve pathways, at least five classes of afferent fiber have been distinguished functionally (4, 5), but not yet morphologically. These five classes include mesenteric, mucosal, muscular/mucosal, serosal, and muscular afferents (5). In this study, we specifically focused on morphologically identifying the peripheral nerve endings of the muscular afferents that are activated by circumferential stretch. The previous classification scheme of spinal afferents in mouse was performed on pelvic and lumbar splanchnic afferent fibers (4, 5), whereas in this study, we recorded from rectal afferents (see Fig. 1B).

When anterograde labeling was applied to rectal nerve trunks from which afferent recordings were made in sl/sl mice, all filled nerve endings were located within the smooth muscle layers. No mechanoreceptors or mechanotransduction sites could be clearly identified in the serosa. In wild-type mice, it was not possible to unequivocally identify mechanotransduction sites because the transduction sites could not be marked with better than 300–600 μm resolution, which was considerably larger than the distance between myenteric ganglia. However, in sl/sl mice, the absence of ganglia led to the certain conclusion that intramuscular endings were responsible for all mechanotransduction sites. Close to marked hot spots, discrete varicose endings within the circular or longitudinal muscle were located, with a statistically significant association, close to identified transduction sites. It is possible that the morphol-
ogy of intramuscular endings may have been distorted by the repeated use of von Frey hairs required to localize and mark the hot spots. The conclusion that intramuscular axons comprise the mechanosensitive transduction sites seems inescapable: there were no other candidate endings in s/l/s mice. In the aganglionic rectum, the filled, rectal mechanoreceptor axons did not show preferential orientation with either the circular or longitudinal muscle cells, unlike IMAs, which always lie parallel to the long axis of either the longitudinal muscle or circular muscle layer. Similarly, rIGLEs also show little preferential orientation within myenteric ganglia (17, 35, 36).

It has been suggested that IMAs within the smooth muscle of the stomach function as mechanosensory endings of vagal afferent nerve fibers. However, to date, there are no physiological studies in the esophagus or stomach that provide direct support for this idea (35, 36). In our study, anterograde labeling of rectal nerves commonly revealed IMAs in the circular muscle layer and occasionally in the longitudinal muscle layer of wild-type mouse colon and in the aganglionic segment of s/l/s mice. However, in both types of mice, mechanosensitive hot spots were never found to morphologically correlate with IMAs. The functional role of IMAs in the mouse rectum is not clear.

Conclusions. The major findings of this study show that despite the congenital absence of all enteric ganglia, the aganglionic rectum of s/l/s mice is innervated by a population of spinal afferents that responds to both circumferential stretch and probing, and another that responds only to probing; the latter have been described previously as serosal afferents (6). The functional role of an afferent that only responds to probing is not clear. Taken together, our results suggest that, in patients with Hirschsprung’s disease, the absence of distension-evoked extrinsic neural reflexes from the aganglionic rectum is unlikely to be due to the absence of functional stretch-sensitive extrinsic afferents in this region of gut. It seems more likely that a deficit in the efferent motor pathway to the aganglionic rectum may explain the inability to activate extrinsic motor reflexes within the aganglionic segment.

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