RESEARCH ARTICLE | Neurogastroenterology and Motility

Alterations of colonic function in the Winnie mouse model of spontaneous chronic colitis

Ainsley M. Robinson,1 Ahmed A. Rahman,1 Simona E. Carbone,1 Sarron Randall-Demllo,2 Rhiannon Filippone,3 @ Joel C. Bornstein,3 Rajaraman Eri,2 and Kulmira Nurgali1

1College of Health and Biomedicine, Victoria University, Melbourne, Victoria, Australia; 2University of Tasmania, School of Health Sciences, Launceston, Tasmania, Australia; and 3Department of Physiology, Melbourne University, Melbourne, Victoria, Australia

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Inflammatory bowel disease (IBD) is characterized by inflammation of the gastrointestinal (GI) tract and has symptoms including chronic diarrhea, vomiting, abdominal pain, and bloating (24). Experimental animal models provide a useful tool for elucidating the etiology and pathophysiological mechanisms of a disease. However, IBD is multifactorial, so it has been difficult to establish a model that closely resembles the pathological symptoms, clinical manifestations, and complexity of the human disease (21).

Most experimental models of IBD produce acute colitis via chemicals or nematodes including trinitrobenzenesulfonic acid (TNBS), dextran sodium sulfate (DSS), or Trichinella spiralis (T. spiralis; 7). In comparison, there are limited animal models of spontaneous chronic intestinal inflammation, reinforcing the significance of the recently developed Winnie mouse model of colitis which is raised from a C57BL/6 background and closely resembles the clinical symptoms of IBD. In this model, chronic intestinal inflammation results from a primary intestinal epithelial defect conferred by a missense mutation, rather than a deletion, in the Muc2 mucin gene (23, 36). Disruption of Muc2 biosynthesis initiates alterations to the mucus layers, heightens intestinal permeability, and increases vulnerability to luminal antigens (36). Furthermore, the mutation of Muc2 in Winnie mice is comparable to variations in human IBD, where there is a decrease in Muc2 production and secretion in active ulcerative colitis (36, 93) and reduced expression of Muc2 in Crohn’s disease (8). Winnie mice develop colitis in the distal region of the colon as indicated by crypt elongation, neutrophilic infiltrates, goblet cell loss, crypt abscesses, limited mucus secretion, and focal epithelial erosions with an ulcerative colitis-like phenotype (36). Inflammation is evident by 6 wk of age and advances over time, resulting in severe colitis by 16 wk (23, 55). The enteric nervous system (ENS) provides the intrinsic innervation of the GI tract, chiefly regulating, monitoring, and coordinating all gut functions (30). Evidence that the ENS is highly representative of human IBD.
enteric and submucosal plexuses (plexitis), which is predictive of disease recurrence (25, 84). Thus damage to the ENS is prognostic of disease progression and may play a role in IBD recurrence. Previous studies, mostly using either chemicals or nematodes in rodents to induce inflammation, have demonstrated multiple effects of intestinal inflammation on enteric neurons including an indiscriminate loss of neurons (48, 49, 67, 77, 78), changes in their neurochemical coding (77, 78, 97), hyperexcitability, and altered synaptic transmission (46, 47, 51, 62, 63). In both human patients and animal models, disturbed gut functions persist well beyond the period of acute inflammation (57). Thus damage to and death of enteric neurons and changes in their functional properties underlie persistent alterations in motility, secretion, and GI sensation caused by intestinal inflammation. Additionally, it has been reported that not only enteric neurons but also the extrinsic autonomic nerves innervating the GI tract are affected by inflammation (50, 89). Other studies have described alterations in colonic motility considered to result from inflammation-induced changes in neuromuscular transmission (3, 89). Nonetheless, the clinical relevance of results from these models is limited since the mode of evoking inflammation involves application of an aggravating chemical substance (91).

There is currently only one study of the intrinsic and extrinsic innervation of the Winnie mouse model of colitis (71). In this study, reductions in the density of sensory, cholinergic, and noradrenergic fibers innervating the myenteric plexus, muscle, and mucosa correlated with immunological and morphological changes in the muscular and mucosal layers of Winnie mouse distal colon. However, functions of the colon, such as transit and motility, were not investigated. Here we provide the first analysis of intestinal transit and isolated whole colonic motility in the chronically inflamed Winnie mouse colon and investigate mechanisms underlying changes in motility, including altered neuromuscular transmission and structural and functional changes in smooth muscles. Our findings crucially translate to human studies where investigation of colonic motility in IBD patients is limited to specific sections of the colon attained from muscle resected during operations (45, 94).

MATERIALS AND METHODS

Ethical Approval

All procedures performed within this study were approved by the Victoria University Animal Experimentation Ethics Committee and were conducted according to the guidelines of the Australian National Health and Medical Research Council.

Animals

Male and female Winnie mice (12 wk old; 19–29 g; n = 24) were obtained from Monash Animal Services (MAS, Melbourne, Australia). Since the Winnie strain was developed from C57BL/6 background, male and female C57BL/6 mice (12 wk old; 26–30 g; n = 17), obtained from MAS, were used as controls. All animals were housed in a temperature-controlled environment with 12-h day-night cycles and free access to food and water. Mice were humanely euthanized by cervical dislocation. The colon was dissected, placed into physiological saline [containing (in mM) 118 NaCl, 4.6 KCl, 2.5 CaCl2, 1.2 MgSO4, 1 NaH2PO4, 25 NaHCO3, 11 d-glucose, bubbled with 95% O2 and 5% CO2] at room temperature, and used for in vitro motility, smooth muscle contractile activity, immunohistochemistry, and intracellular electrophysiology experiments.

Assessment of Inflammation

To assess the level of colonic inflammation in Winnie mice, fecal lipocalin (Lcn)-2 and gross morphological damage in sections of the colon were measured. Lcn-2 was quantified by ELISA, as previously described (16). Fecal samples were collected from C57BL/6 and Winnie mice twice a week for 4 wk. Briefly, fecal samples were reconstituted in PBS-0.1% Tween 20 (100 mg/ml) and vortexed (20 min) to form a homogenous fecal suspension. Samples were then centrifuged for 10 min at 12,000 rpm and 4°C. Lcn-2 levels were estimated in the supernatants using DuoSet murine Lcn-2 ELISA kit (R&D Systems, Minneapolis, MN). For histology, tissues were embedded in paraffin, sectioned at 5 μm, deparaffinized, cleared, and rehydrated in graded ethanol concentrations. Hematoxylin and eosin (H&E) staining was conducted as previously described (71). Histological scoring was completed blindly and included the following: aberrant crypt architecture (score range 0–3), increased crypt length (0–3), goblet cell depletion (0–3), crypt abscesses (0–3), leukocyte infiltration (0–3), and epithelial damage and ulceration (0–3) (average of 8 areas of 500 μm2 per animal).

Gastrointestinal Transit (Radiographic Study)

GI transit was analyzed using a noninvasive radiological method. Briefly, 0.3 ml of suspended barium sulfate (X-OPAQUE-HD, 2.5 g/ml) was administered to C57BL/6 and Winnie mice via oral gavage following 5 days of acclimatization. Subsequently, serial X-rays were taken using a HiRay Plus Porta610HF X-ray apparatus (JOC, Kanagawa, Japan; 50 kV, 0.3 mA, exposure time 60 ms). X-rays were captured using Fujifilm cassettes (24 × 30 cm) immediately after administration of barium sulfate (0 min), every 10 min for the first hour, then every 20 min through to 250 min. Images were developed via a Fujifilm FCR Capsula XLII and analyzed using eFilm 4.0.2 software. Transit speed was calculated as the time (min) taken for the leading edge of the contrast to travel from the stomach to the cecum (orocoeal transit time) and from the cecum to the anus (colonic transit time).

In Vitro Analysis of Isolated Whole Colon Motility

Segments of the distal colon immediately oral to the pelvic brim were dissected in a Sylgard-lined petri dish containing physiological saline. The L-type Ca2+ channel blocker nicardipine (2 μM) was...
added to limit the muscle contractions. Tissues were opened along the mesenteric border and pinned down flat, and the mucosa and submucosa were removed by sharp dissection. A full circumference, 1.5-cm-long segment of tissue was isolated and repinned circular muscle layer uppermost in a Sylgard-lined recording chamber using gold-plated tungsten pins (50 μm). The recording chamber was fixed to a stage mounted on a Zeiss Axiocam-200 inverted microscope and superfused with warmed physiological saline (rate 3 ml/min) so that the final bath temperature was ~35°C. Preparations were left to equilibrate for 120 min before commencing recordings (14, 15).

Circular smooth muscle cells were impaled with borosilicate glass capillary electrodes (1-mm outside diameter, 0.58-mm inside diameter; Harvard Apparatus) filled with 5% carboxyfluorescein and 1 M KCl in 20 mM Tris buffer solution (pH 7.0). Electrode resistance was confirmed by viewing carboxyfluorescein labeling in situ. For each preparation (n), the electrophysiological properties of four to eight cells were averaged for each test condition and then compared with other preparations.

Measurement of Contractile Force

Experiments were performed using standard organ bath techniques. Freshly excised distal colon was cut into 3-mm rings, cleaned of connective tissue and fat, and placed in a custom-built organ bath containing physiological saline (oxygenated with 95% O₂ and 5% CO₂) maintained at 35°C and pH 7.4. The colonic rings were then mounted between two small metal hooks attached to force displacement transducers (Zultek Engineering) and stretched to 0.2-g tension. Tissues were allowed to equilibrate for 1 h with physiological saline with an ISO-Flex stimulator controlled by a Master-8 pulse generator (A.M.P.I.). Unless otherwise specified, a single-pulse, 0.4-msec-duration electrical stimulus (0–60 V) was used to activate nerve fibers, causing motor neurons to release neurotransmitters onto smooth muscle cells. Postjunctional responses were recorded. The resting membrane potential (RMP) of each cell was measured, and its identity was confirmed by viewing carboxyfluorescein labeling in situ. For each preparation, the electrophysiological properties of four to eight cells were averaged for each test condition and then compared with other preparations.

Drugs Used

ATP (10 μM to 10 mM), atropine (1 μM), carbachol (1 nM to 20 μM), 2-nitro-L-arginine (L-NNa, 1 mM), nicardipine (2 μM), and sodium nitroprusside (SNP; 10 nM to 1 mM) (all from Sigma-Aldrich) were used as neurotransmitters. In the absence of SNP or ATP, tissues were exposed to carbachol (1 nM to 20 μM), atropine (1 μM), and carbachol (1 nM to 20 μM) for 10 min before adding SNP or ATP with 5-min intervals between administrations of increasing concentrations of each drug. The response to each drug (carbachol, SNP, and ATP) was quantified by comparing the peak response to a baseline control period. The maximum contractile response to carbachol was expressed as a percentage of the contraction induced by 10 μM carbachol. The dilating effects of SNP and ATP were expressed as a percentage of the maximum contractile response to carbachol.

Immunohistochemistry

Immunohistochemistry was performed as described previously (71, 77, 78). Briefly, distal colon tissues were cut open along the mesenteric border and pinned flat with the mucosal side up in a Sylgard-lined petri dish containing PBS and fixed with Zamboni’s fixative (2% formaldehyde containing 0.2% picric acid) overnight at 4°C. Tissues were then washed (3 × 10 min) with dimethyl sulfoxide (Sigma-Aldrich) followed by PBS (3 × 10 min). For labeling of α-smooth muscle actin (α-SMA), tissues were stored in 50:50 optimum cutting temperature (OCT) compound (Tissue-Tek) and sucrose solution for 24 h at 4°C and subsequently frozen in liquid nitrogen-cooled isopentane and OCT compound. Samples were stored at −80°C until they were cryosectioned (12 μm) onto glass slides for immunohistochemistry. After incubation with 10% normal donkey serum (Merck Millipore) for 1 h at room temperature, sections were labeled with primary antibody to detect α-smooth muscle actin (rabbit, 1:1,000; Abcam). Sections were then washed and incubated with fluorophore-conjugated secondary antibody Alexa Fluor 594 (donkey anti-rabbit, 1:200; Abacus ALS) for 2 h. Tissues were given a further 3 × 10 min wash with PBS, followed by a 2-min incubation with the fluorescent nucleic acid stain 4′,6-diamidino-2-phenylindole (DAPI; 14 nM; Invitrogen). Tissues received 1 final 10-min PBS wash before being mounted onto glass slides with fluorescent mounting medium (DAKO).

Imaging

Images were captured using a Nikon Eclipse Ti multichannel confocal laser scanning system. Immunolabeled sections were visualized and imaged by using filter combinations appropriate for the specific fluorophores such as DAPI (excitation wavelength 405 nm) and Alexa 594 (excitation wavelength 559 nm). Z-series images were acquired at a nominal thickness of 0.5 μm (512 × 512 pixels). The total number of DAPI-immunoreactive (IR) nuclei of α-SMA-IR cells was counted throughout the muscle thickness including both longitudinal and circular muscle layers (from the serosa to the junction with the submucosa) in 8 randomly captured images per preparation at ×40 magnification. Quantitative analyses were performed blindly. Data are expressed as the number of α-SMA-IR cells per 1-mm² area of distal colon.

Statistical Analysis

Results were analyzed using Prism software (version 5.0, GraphPad) and were presented as means ± SE. One-way or two-way ANOVA was used for group comparison of data, followed by a Tukey-Kramer post hoc test. Unpaired t-tests were used to compare two data sets. For smooth muscle contractility experiments, responses to carbachol, SNP, and ATP were fitted to sigmoid curves. The variable n in RESULTS refers to the number of animals used for each set of experiments. P < 0.05 was considered significant. RESULTS

Assessment of Colonic Inflammation

Fecal Lcn-2 was quantified by ELISA for Winnie and C57BL/6 mice. Lcn-2 is a component of granules in neutrophils and is expressed in response to a wide variety of proinflammatory stimuli (44). Thus fecal Lcn-2 is a highly sensitive and broadly dynamic marker of intestinal inflammation (16). In this study, minimal Lcn-2 was detected in fecal pellets from C57BL/6 mice at any time point (Fig. 1, A–C), confirming a lack of colonic inflammation. Levels of fecal Lcn-2 in samples from all Winnie mice used in this study were consistently higher than C57BL/6 mice at each time point confirming presence of chronic inflammation in the Winnie mice (Fig. 1,
A–C, \( P < 0.001 \) for all). Some variation in Lcn-2 levels was observed in individual Winnie mice, but not in C57BL/6 mice (examples for \( n = 6 \)/group are presented in Fig. 1A). All Winnie mice demonstrated clinical symptoms of colitis, including chronic diarrhea, rectal bleeding, and weight loss. Gross morphological damage was assessed in cross sections to further substantiate the level of inflammation in the distal colon. Consistent with earlier observations (23, 36, 55, 71), morphological damage was assessed in cross sections to further substantiate the level of inflammation in the distal colon. Altered Gastrointestinal Transit in Winnie Mice

To determine differences in GI transit between Winnie mice (\( n = 8 \)) and C57BL/6 mice (\( n = 8 \)), radiographic images were used to track barium sulfate from the stomach to the cecum [orocecal intestine transit time (OCTT)], as well as from the cecum to the anus [colonic transit time (CTT)] (Fig. 2, A and B). Total transit time did not differ between C57BL/6 (221.3 ± 25.7 min) and Winnie (225.7 ± 19.1 min) mice. However, this masked substantial changes in transit within different gut regions. In C57BL/6 mice, OCTT was 164.4 ± 24.5 min, and CTT was 56.9 ± 4.5 min. In Winnie mice, although not statistically significant, OCTT was prolonged (190.0 ± 18.0 min) compared with C57BL/6 mice (Fig. 2C). On the other hand, CTT was significantly reduced in Winnie mice (40.0 ± 3.6 min, \( P < 0.05 \), Fig. 2C). Diarrhea in Winnie mice is evident by long size of fecal mass moving from the cecum to the anus compared with the short defined pellets observed in C57BL/6 mice (white arrows, Fig. 2, A and B).

Colonic Motility

Patterns of colon contraction. Three types of motor patterns [colonic migrating motor complexes (CMMCs), short contractions, and fragmented contractions] were identified and analyzed in the colon of Winnie and C57BL/6 mice. CMMCs were defined as propagating contractions appearing first at the proximal end and then sequentially over >50% of the distance to the distal end of the colon (74, 75; Fig. 3A). Contractions that propagated <50% of the length of the colon were defined as short contractions (Fig. 4A). Fragmented contractions were defined as phasic nonpropagating contractions occurring concurrently at various locations within the colon. They were easily distinguishable on spatiotemporal maps as distinct segmental contractions (solid arrows) and relaxations (dashed arrows; Fig. 5A).

The total number of contractions was similar in the colons of C57BL/6 and Winnie mice at all levels of intraluminal pressure (0 cmH\(_2\)O, C57BL/6 = 17.7 ± 1.4 contractions/10 min and Winnie = 23.0 ± 2.2 contractions/10 min; 1 cmH\(_2\)O, C57BL/6 = 16.5 ± 1.7 contractions/10 min and Winnie = 22.3 ± 2.4 AJP-Gastrointest Liver Physiol \( \cdot \) doi:10.1152/ajpgi.00210.2016 \( \cdot \) www.ajpgi.org
Downloaded from www.physiology.org/journal/ajpgi at Monash Univ (130.194.148.031) on September 19, 2019.
contractions/10 min; 2 cmH₂O, C57BL/6 = 18.1 ± 1.6 contractions/10 min and Winnie = 22.1 ± 1.3 contractions/10 min). However, further analysis of various types of motor patterns revealed significant differences between Winnie and C57BL/6 mice.

The number of CMMCs was lower in the colon from Winnie mice (n = 13) at all levels of intraluminal pressure than in C57BL/6 colon (n = 8, P < 0.05 for all, Table 1, Fig. 3, A and B). Similarly, the proportion of contractions identified as CMMCs was less in maps generated from the colons of Winnie than C57BL/6 colons (P < 0.001 for all, Table 1, Fig. 3C).

Both the frequency and proportion of short contractions (Fig. 4A) were higher in the colons from Winnie mice than those from C57BL/6 mice (P < 0.05 for all, Table 1, Fig. 4, B and C). With increasing intraluminal pressure, the number and proportion of short contractions did not change significantly in the colons of both groups (Table 1, Fig. 4, D–F). No significant changes in the frequency of short contractions were observed in either proximal or middle sections of the colon from both C57BL/6 and Winnie mice (Fig. 4.
D and E). However, increasing intraluminal pressure (>1 cmH2O) reduced the frequency of short contractions in the distal colon from C57BL/6 mice (P < 0.05, Fig. 4F), but not Winnie mice, so the frequency of short contractions was significantly higher in Winnie mice at increased levels of intraluminal pressure and was significantly different between the two genotypes (P < 0.05, Table 1, Fig. 4F).

The frequency and proportion of fragmented contractions were significantly higher in Winnie mice than in C57BL/6 mice, regardless of the intraluminal pressure (Table 1, Fig. 5, B and C). Increased intraluminal pressure initiated heightened numbers and proportion of fragmented contractions in both groups. Because of the low number of fragmented contractions in C57BL/6 colons at 0 cmH2O, we analyzed this in a slightly different way and compared the number of preparations exhibiting at least one fragmented contraction in the recording period between the two genotypes. At a baseline intraluminal pressure, 33 ± 18% of C57BL/6 colons exhibited fragmented contractions, increasing to 44 ± 17 and 56 ± 17% at 1 and 2 cmH2O, respectively. In contrast, more colons from Winnie mice exhibited fragmented contractions at baseline and 1-cmH2O intraluminal pressure (P < 0.05 for both, 77 ± 10%); this increased to 85 ± 10% at 2 cmH2O (Fig. 5D).

Speed and length of colonic contractions. CMMCs propagated more rapidly in the colons from Winnie mice when intraluminal pressure was increased from 0 to 2 cmH2O (P < 0.05, Table 1, Fig. 6A), but there were no changes in CMMC speed in colons from C57BL/6 mice. When intraluminal pressure was increased from baseline to 1 cmH2O, the propagation speed of short contractions remained constant in both Winnie and C57BL/6 colons (Table 1, Fig. 6B). At 2 cmH2O, short contractions propagated more rapidly in C57BL/6 than in Winnie colons (P < 0.01), but the propagation speed increased significantly in each genotype (Table 1, Fig. 6B). The speed of propagation of fragmented contractions did not differ between C57BL/6 and Winnie mice; faster contraction speeds were recorded as intraluminal pressure was increased to 2 cmH2O in both groups (Table 1, Fig. 6C).

CMMCs propagated over similar distances in the colon in both C57BL/6 and Winnie mice regardless of intraluminal pressure. Similarly, short and fragmented contraction lengths did not differ between Winnie and C57BL/6 mice (Table 1).

Phases of contraction in the colon. The individual properties of contractions were compared between C57BL/6 (n = 8) and Winnie (n = 13) mice at the proximal, middle, and distal regions of the colon by tracking the diameter independent of the type of contraction (32). Several parameters were analyzed from these traces including duration of contraction, quiescence (time at the baseline resting diameter between contractions), and contraction interval (time from the beginning to the end of the contraction-relaxation cycle; Fig. 7, A–D).

In the proximal colon, the duration of contraction was similar in Winnie and C57BL/6 mice, reducing in both groups as intraluminal pressure increased (1 cmH2O, P < 0.01; 2 cmH2O, P < 0.05; Table 2), whereas the time of quiescence decreased in Winnie mice only (P < 0.05, Table 2). Subsequently, at 2 cmH2O, quiescence was shorter in the colons from Winnie mice compared with the colons from C57BL/6 mice (P < 0.05, Table 2). The contraction interval in the proximal colon decreased as intraluminal pressure increased in
both Winnie and C57BL/6 mice (P < 0.05 for both, Table 2). In the middle colon, there were no changes in the duration of contraction or quiescence in both C57BL/6 and Winnie mice. As a result, there were no differences measured in the contraction intervals of the middle colon in either group (Table 2).

The total constriction and relaxation activity (duration of contraction) was greater in the distal colons from Winnie mice than from C57BL/6 mice across all levels of pressure (P < 0.05 for all, Table 2, Fig. 7E). Quiescence of the distal colon was decreased in Winnie mice at 2 cmH2O of intraluminal pressure (P < 0.05) compared with C57BL/6 mice (Table 2, Fig. 7F). Similarly, contraction intervals were shorter in Winnie mice than in C57BL/6 mice at 2 cmH2O (P < 0.05, Table 2).

To investigate possible mechanisms underlying these changes in the patterns and properties of colonic motility induced by intestinal inflammation, we examined three possible causes: changes in neuromuscular transmission, changes in intestinal smooth muscle function, and changes in intestinal smooth muscle structure. These were examined in segments of the distal colon only, since most changes in the phases of contraction were evident in this section of the colon.

Neuromuscular transmission. To evaluate the role of neuromuscular transmission in colonic dysmotility observed in Winnie mice, intracellular recordings from circular smooth muscle cells in the C57BL/6 and Winnie mouse colon were compared. Focal electrical stimulation of nerve fibers innervating the circular muscle layer evoked junction potentials in muscle cells.
Inhibitory and excitatory neuromuscular junction potentials were identified as purinergic fast inhibitory junction potentials (fIJPs), nitrergic slow inhibitory junction potentials (sIJPs), and cholinergic excitatory junction potentials (EJPs) using various antagonists.

Single-pulse electrical stimuli evoked fIJPs in control physiological solution (Fig. 8A). In both C57BL/6 and Winnie mouse colon, the amplitudes of these fIJPs increased with larger electrical stimuli (single-pulse, 0.4-ms duration, 0 – 60 V, data not shown). The amplitudes of fIJPs evoked by a 30-V electrical stimulus differed significantly between the two genotypes (C57BL/6, 24.4 ± 1.2 mV; Winnie, 17.9 ± 2.8 mV; P < 0.05, n = 12 and 14, Fig. 8B). Addition of the purinergic P2Y1 receptor antagonist, MRS2500 (1 μM), inhibited the fIJPs.

A short, high-frequency train of electrical stimuli (3 pulses at 20 Hz, 20 V, 0.5-ms duration) was used to generate sIJPs (Fig. 8C). The amplitudes of sIJPs did not differ between the C57BL/6 and Winnie colon (−6.8 ± 0.8 vs. −6.6 ± 0.8 mV, n = 12 and 14, Fig. 8D). Addition of a nitric oxide synthase inhibitor L-NNA (1 mM) in the presence of MRS2500 (1 μM) blocked the sIJPs.

High-frequency stimulus trains (3 pulses, 20 Hz, 20 V, 0.5-ms pulse duration) in the presence of MRS2500 and L-NNA, blocking the fast and slow IJPs, evoked EJPs (Fig. 8E). The amplitudes of EJPs in colonic smooth muscle cells of C57BL/6 mice (7.7 ± 0.7 mV, n = 11) were greater than those from the Winnie mice (5.9 ± 0.9 mV, P < 0.05, n = 14, Fig. 8F). In both genotypes, EJPs were inhibited by the muscarinic antagonist atropine (1 μM; C57BL/6, from 7.4 ± 0.7 to
To investigate the role of smooth muscle function in colonic dysmotility observed in Winnie mice, we examined circular smooth muscle contractile responses to common excitatory and inhibitory agents. Addition of carbachol (1 nM to 20 μM) to the organ bath containing excised colonic rings evoked a dose-dependent and significant increase in contractile force in both C57BL/6 (n = 6) and Winnie (n = 6) mice. However, the magnitude of contractions

1.4 ± 0.2 mV; Winnie, from 6.8 ± 0.8 to 1.3 ± 0.1 mV, P < 0.001 for both, n = 10 and 11).

In control Krebs solution, the resting membrane potentials (RMP) of colonic smooth muscle cells from C57BL/6 (−41.9 ± 0.7 mV, n = 12) and Winnie (−40.7 ± 1.5 mV, n = 14) mice were not significantly different. Addition of MRS2500, L-NNA, and atropine did not affect RMP.

Smooth muscle responses to carbachol, SNP, and ATP. To investigate the role of smooth muscle function in colonic dysmotility observed in Winnie mice, we examined circular smooth

Table 1. Parameters of different types of colonic contractions

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<th>Intraluminal Pressure</th>
<th>C57BL/6</th>
<th>Winnie</th>
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<tr>
<td>0 cmH2O</td>
<td>8.9 ± 1.0</td>
<td>4.9 ± 1.1</td>
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<td>1 cmH2O</td>
<td>9.5 ± 0.8</td>
<td>5.5 ± 1.1</td>
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<tr>
<td>2 cmH2O</td>
<td>10.1 ± 0.9</td>
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**CMMC**

Frequency, /10 min

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Proportion, %

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Length, mm

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**Short contractions**

Frequency, /10 min

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<td>2.7 ± 1.8</td>
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Proportion, %

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<td>1.7 ± 0.1</td>
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Length, mm

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<td>5.5 ± 0.6</td>
<td>5.5 ± 0.5</td>
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**Fragmented contractions**

Frequency, /10 min

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Proportion, %

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<th>C57BL/6</th>
<th>Winnie</th>
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<td>1.5 ± 0.7</td>
<td>2.6 ± 0.4</td>
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Length, mm

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<tr>
<td>23.9 ± 2.1</td>
<td>25.6 ± 1.3</td>
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Values are means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001 compared with C57BL/6 at the same level of intraluminal pressure. *P < 0.05, **P < 0.01 compared with Winnie at 2 cmH2O.

Fig. 6. CMMC and short and fragmented contraction speed in Winnie and C57BL/6 mice. The speed of CMMCs (A), short contractions (B), and fragmented contractions (C) was measured from spatiotemporal maps generated from the colons of C57BL/6 (n = 5) and Winnie (n = 13) mice at baseline, 1-cmH2O, and 2-cmH2O intraluminal pressure. *P < 0.05, **P < 0.01, ***P < 0.001.
evoked by carbachol was significantly lower in preparations from Winnie mice compared with those from C57BL/6 mice at 5 μM (P < 0.001), 10 μM (P < 0.001), and 20 μM (P < 0.001) doses (Fig. 9, A and B). Traces of contractility at a specific location along the colon plotted changes in the gut width against time (B and D). The duration of contractile activity (E) and quiescence (F) was measured from traces of colonic motor patterns at distal section (E and F) of the C57BL/6 (n = 8) and Winnie (n = 13) mouse colon. *P < 0.05.

**DISCUSSION**

This study provides the first analysis of in vivo GI transit and in-depth analysis of colonic function in an animal model of hyperplasia of smooth muscle cells. The changes in muscle function described above may be due to morphological changes in muscle structure. We have previously reported thickening of the distal colon muscle wall in Winnie mice (71). To determine whether chronic inflammation causes hyperplasia of smooth muscle cells in Winnie mice, cross sections of the distal colon were labeled with α-SMA antibody to label smooth muscle cells and DAPI to label nuclei (Fig. 10, A and B). The numbers of α-SMA-IR circular and longitudinal muscle cells counterstained with DAPI were significantly increased in the muscle layers of the colon in the cross-section preparations from Winnie (618 ± 16 α-SMA-IR cells/mm², n = 5) compared with C57BL/6 (329 ± 9 α-SMA-IR cells/mm², P < 0.001, n = 5) mice (Fig. 10C).
spontaneously occurring chronic intestinal inflammation. In this study, chronic inflammation in the distal colon of Winnie mice was confirmed by high fecal Lcn-2 protein levels measured over a 4-wk period and distinct gross morphological damage to the colon. Here, we report that Winnie mice exhibit alterations in small intestinal and colonic transit, in patterns of colonic motility, and in parameters of contractions. Furthermore, we provide novel data about the mechanisms underlying changes in Winnie colonic motility implicating impaired neuromuscular transmission and altered contractile activity and morphology of colonic smooth muscle cells.

In this study, Lcn-2 was increased in Winnie mice over 4 consecutive weeks together with persistent diarrhea. Acute diarrhea lasting for <14 days is generally associated with bacterial, viral, or parasitic infection. On the other hand, diarrhea that persists for at least 4 wk is most likely caused by alterations in GI transit and motility (18). Similar to IBD and irritable bowel syndrome-diarrhea predominant (IBS-D) patients, chronic diarrhea in Winnie mice was associated with increases in colonic transit time (12, 19, 37, 52, 79). Compared with healthy control mice, orocecal small intestine transit time was also altered in Winnie mice in this study. Previous studies examining murine gastrointestinal transit times via the movements of radiopaque markers, as well as other methods (i.e., phenol red meal test), have reported that the orocecal or small intestinal transit time is greater than colonic transit time in wild-type or control mice (59, 68). Furthermore, in experimentally induced models of intestinal inflammation such as TNBS-induced colitis (88), croton oil-induced diarrhea (40), and intraperitoneal lipopolysaccharide injection (22, 54), small intestinal transit is reported to be inhibited. It is considered that increased orocecal transit times may be due to electrical uncoupling and focal increase in slow-wave frequency, which lead to orally propagating contractions slowing the transit in the proximal small intestine (20). Consistent with our results, many clinical studies report delays in small intestine transit to be correlated with IBD (28, 61, 79). Moreover, it has been suggested that changes in intestinal transit resulting from inflammation, particularly faster colonic transit time, correlate with changes in colonic motility (13, 73).

Colonic dysmotility, hypersensitivity, and dysfunction result from intestinal inflammation and reflect changes in smooth muscle function and/or the ENS (1, 38, 64, 83, 94, 95). In human studies, the majority of data were obtained from muscle resected at the time of operation. Therefore investigation of colonic motility in IBD patients is usually limited to analyzing colonic segments rather than the whole colon (45, 94). Hence it is unsurprising that most of our knowledge regarding motility dysfunction in colonic inflammation has come from animal models. Changes in motor function have been described in animals with acute colitis induced by a variety of inflammatory stimuli, including infection, chemical irritation, and immune activation. These studies have mostly investigated smooth muscle contractility in sections of the colon rather than analysis of whole colon motility (1, 42, 43, 95). Smooth muscle contractility has been examined in colon sections from animal models of chronic colitis, such as IL10-/- mice, as well as from mice with chronic DSS-induced colitis (1, 65, 66). Although these experiments provide valuable information about the functional properties of isolated colonic smooth muscles, evaluation of the role of the ENS in inflammation-induced colonic dysmotility can only be achieved via studies of the whole organ. There are currently no in-depth analyses of isolated whole colon motility in a murine model of chronic colitis.

Our spatiotemporal maps of isolated colonic motility revealed distinct rhythmic recurring patterns of motor activity in control C57BL/6 mice, consistent with previous studies (9, 10, 74, 86). Alterations in both spatial and temporal characteristics of colonic motility were evident in Winnie mice. Notably, colonic motility in the C57BL/6 and Winnie mouse differed in the absence of external stimuli, as well as in response to rising intraluminal pressure. Winnie mice showed a high number of fragmented contractions in the colon. Fragmented contractions in the colon of C57BL/6 mice have previously been associated with variations in nitrergic innervation in different colonic regions (17, 96). Importantly, our study is the first to report fragmented contractions in an experimental model of colitis. Previous studies have shown that the membrane potential of the circular layer is maintained under tonic neurogenic inhibition in the interval between CMMCs (26, 85). Periodic withdrawal of tonic inhibition, or disinhibition, can lead to generation of contractions (85). Therefore, coinciding with a decrease in CMMCs, the amplified occurrence of fragmented contractions may indicate disruption to neural inhibition and circuitry regulating CMMC generation.

We observed an increased frequency of short or segmenting contractions between CMMCs in the Winnie mice colon. Spa-
Initially disorganized short contractions were also observed in C57BL/6 mice. This is consistent with previous studies describing this type of motor pattern in normal intestinal motility (75). The segmenting motor pattern varies from peristalsis in that it comprises only stationary or very short distance contractions. Hence it is considered to be a specialized motor pattern for mixing and absorption (29, 39). Although short contractions were evident in all regions of the Winnie and C57BL/6 mouse colon, they were most frequent at the distal end with no constant or predictable pattern of occurrence. Under normal conditions, short contractions have been reported to make up a high proportion of activity in the distal colon (4); however, we observed an exaggerated increase in spontaneous activity in the Winnie distal colon.

The chronic intestinal inflammation in the Winnie mouse colon is associated with fewer propulsive propagating contractions and promotion of short contractions. In Winnie mice, loss of Muc2 leads to a thinner mucus layer allowing increased intestinal permeability and thus enhanced susceptibility to luminal toxins normally within the gut. The extended mixing period observed in the colons from Winnie mice might suggest slower colonic transit, contrary to what was seen in vivo. However, the faster CMMC propagation seen in this study suggests a mechanism for the faster colonic transit in vivo and the nonsecretory diarrhea and changes to absorption described in ulcerative colitis (93). This would also contribute to soft stools and increased fecal water content observed in Winnie mice (71). An increased propagation of contraction would reduce the contact time of fecal material with the inflamed mucosa and diminish absorption of water and electrolytes (80). Thus clinical symptoms such as diarrhea may be associated with inhibited absorption in the colon of Winnie mice. This requires further investigation.

**Fig. 8.** Intracellular electrophysiological recordings of excitatory and inhibitory junction potentials from colonic smooth muscle cells. Representative traces of purinergic fast inhibitory junction potentials (fIJPs; *stimulus artifact; A). Quantitative analysis of fIJP amplitudes in the colonic smooth muscle cells from C57BL/6 (n = 12) and Winnie (n = 14) mice (B). Representative traces of nitrergic slow inhibitory junction potentials (sIJPs; C). Amplitudes of sIJP in the colonic smooth muscle cells from C57BL/6 (n = 12) and Winnie (n = 14) mice (D). Representative traces of excitatory junction potentials (EJPs; E). Amplitudes of EJPs in the colonic smooth muscle cells from C57BL/6 (n = 12) and Winnie (n = 14; F) mice. *P < 0.05.
Altered colonic motility in *Winnie* mice may result from challenges to ENS output and/or the regulatory mechanisms of smooth muscle cells. Chronic inflammation-induced changes to motility in *Winnie* mice are associated with changes to neuromuscular transmission, including decreased purinergic fIPs and cholinergic EJPs. The amplitude of fIPs in the *Winnie* colon was significantly different from those in C57BL/6 mice. The fIPs are mediated by ATP acting at P2Y1 receptors and represent the rapid purinergic component of the IJP (58). A reduction in purinergic inhibitory transmission in the inflamed colon is consistent with previous reports in guinea pig TNBS-colitis and mouse DSS-colitis (76, 89), where it has been concluded that an inflammation-induced decrease in the synthesis and release of purines contributes to a reduced purinergic neuromuscular transmission (76). Inhibition of P2Y1 receptors slows propulsive motility in TNBS-inflamed guinea pigs (89). Exogenously applied ATP to precontracted distal colon rings produced comparable responses in *Winnie* and C57BL/6 mice (58). A reduction in purinergic inhibitory transmission in the inflamed colon is consistent with previous reports in guinea pig TNBS-colitis and mouse DSS-colitis (76, 89), where it has been concluded that an inflammation-induced decrease in the synthesis and release of purines contributes to a reduced purinergic neuromuscular transmission (76). Inhibition of P2Y1 receptors slows propulsive motility in TNBS-inflamed guinea pigs (89). Exogenously applied ATP to precontracted distal colon rings produced comparable responses in *Winnie* and C57BL/6 mice (58). A reduction in purinergic inhibitory transmission in the inflamed colon is consistent with previous reports in guinea pig TNBS-colitis and mouse DSS-colitis (76, 89), where it has been concluded that an inflammation-induced decrease in the synthesis and release of purines contributes to a reduced purinergic neuromuscular transmission (76). Inhibition of P2Y1 receptors slows propulsive motility in TNBS-inflamed guinea pigs (89). Exogenously applied ATP to precontracted distal colon rings produced comparable responses in *Winnie* and C57BL/6 mice (58). A reduction in purinergic inhibitory transmission in the inflamed colon is consistent with previous reports in guinea pig TNBS-colitis and mouse DSS-colitis (76, 89), where it has been concluded that an inflammation-induced decrease in the synthesis and release of purines contributes to a reduced purinergic neuromuscular transmission (76). Inhibition of P2Y1 receptors slows propulsive motility in TNBS-inflamed guinea pigs (89). Exogenously applied ATP to precontracted distal colon rings produced comparable responses in *Winnie* and C57BL/6 mice (58).
EJPs are predominantly mediated by acetylcholine (ACh) released from cholinergic neurons acting at smooth muscle receptors to cause contraction (87). The amplitudes of EJPs were reduced in the Winnie mouse colon, in contrast to previous studies of the effects of acute TNBS- and DSS-induced colitis on neuromuscular transmission which reported no change in EJP amplitudes (76, 89). Our data are consistent with our previous finding of reductions in density of cholinergic nerve fibers innervating the distal colon of Winnie mice (71), something that has also been described in ulcerative colitis patients (41).

Smooth muscle contractility in response to cholinergic stimulation is also impaired in the Winnie distal colon. In contrast, no change in ACh-mediated contractile force was reported in the colon of IL-10−/− mice with chronic inflammation (66). IL10−/− mice provide an IBD model that closely resembles human Crohn’s disease (35), whereas Winnie mice appear to model ulcerative colitis (23). In addition, these authors used colonic sections, rather than colonic rings as employed in our study. Intestinal muscle hypocontractility is seen in isolated colonic muscle strips from both patients with ulcerative colitis and animal models of acute colonic inflammation (2, 31, 45, 53, 69, 73, 81, 82).

Our study identified changes in both excitatory neuromuscular transmission and the smooth muscle response to carbachol suggesting that colonic dysmotility in Winnie mouse may result from both alterations to ENS output and smooth muscle cell function. Cholinergic innervation mediates the rapid component of the CMMC (11). Thus reduced CMMC frequency in Winnie mouse colon may be due to impaired cholinergic output from the ENS.

The slow nitrergic component of the IJP (sIJP) involves activation of NO and cyclic GMP (89). Winnie and C57BL/6 mice had similar sIJP in the distal colon of Winnie mice to be unchanged when compared with C57BL/6 mice (71). Normal nitrergic sIJP have been reported in acute TNBS-colitis (89). The reasons for this apparent protection of nitrergic neuromuscular transmission are uncertain.

Exogenously applied NO donor SNP evoked a reduced level of smooth muscle relaxation in the distal colon from Winnie mice. NO-mediated relaxation of distal colon smooth muscle is also impaired in chronically inflamed IL10−/− mice (66). Similar findings have been observed in acute intestinal inflammation where smooth muscle relaxation in response to NO...
donor was decreased (72, 92). Release of inhibitory neurotransmitters suppresses contractile activity of the circular muscle (24, 75, 85). Subsequent to downregulatory mechanisms, smooth muscle response to NO is inhibited by high endogenous NO production as demonstrated by increased concentrations of NO in intestinal mucosa of IBD patients (56, 70, 90).

An impaired smooth muscle cell response may be associated with changes to muscle structure (6, 60). We found that the number of smooth muscle cells in the Winnie distal colon was significantly greater than in the C57BL/6 colon and have previously reported an increase in the muscle thickness of the distal colon in Winnie mice (71). A thickened intestinal wall occurs because of both hypertrophy and hyperplasia of the smooth muscle cells and is characteristic of inflammation, in human disease and in animal models of intestinal inflammation (5, 6, 27). It has been suggested that an increased number of cells with an altered contractile nature may challenge normal intestinal motility and is a cumulative risk for future obstruction, stricture formation, and fibrosis (60). Chronic inflammation in the Winnie distal colon would accentuate hyperplasia of smooth muscle cells in the colon wall, which may be associated with changes in smooth muscle function. Cooperation between neurally induced pacemaker activity by interstitial cells of Cajal and enteric neural programs in the Winnie mouse colon needs to be further investigated.

In conclusion, this is the first study to provide analyses of intestinal transit and whole colon motility in an animal model of spontaneous chronic colitis. We found that cholinergic and purinergic neuromuscular transmission, as well as the smooth muscle cell responses to cholinergic and nitricergic stimulation, is altered in the chronically inflamed Winnie mouse colon. The reduced inhibitory purinergic responses are probably a prejunctional event, whereas diminished inhibitory nitricergic responses in the Winnie mouse colon may be due to a postjunctional mechanism. Diminished excitatory responses occurred both prejunctionally and postjunctionally. The changes to intestinal transit and colonic function in the Winnie mouse we identified are similar to those seen in IBD patients; thus this model is highly representative of human IBD and should be useful for studying chronic intestinal inflammation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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