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The gastrointestinal tract is innervated by extrinsic sympathetic, parasympathetic and sensory nerve fibers as well as by intrinsic fibers from the neurons in myenteric and submucosal ganglia embedded into the gastrointestinal wall. Morphological and functional studies of intestinal innervation in animal models are important to understand the pathophysiology of inflammatory bowel disease (IBD). The recently established *Winnie* mouse model of spontaneous chronic colitis caused by a point mutation in the *Muc2* mucin gene develops inflammation due to a primary epithelial defect. *Winnie* mice display symptoms of diarrhea, ulcerations and rectal bleeding similar to those in IBD. In this study, we investigated myenteric neurons, noradrenergic, cholinergic and sensory nerve fibers in the distal colon of *Winnie* (*Win/Win*) mice compared to *C57/BL6* and heterozygote littermates (*Win/Wt*) using histological and immunohistochemical methods. All *Win/Win* mice used in this study had inflammation with signs of mucosal damage, goblet cell loss, thickening of muscle and mucosal layers, and increased
CD45-immunoreactivity in the distal colon. The density of sensory, cholinergic and noradrenergic fibers innervating the myenteric plexus, muscle and mucosa significantly decreased in the distal colon of Win/Win mice compared to C57/BL6 and Win/Wt mice, while the total number of myenteric neurons as well as subpopulations of cholinergic and nitrergic neurons remained unchanged. In conclusion, changes in the colon morphology and innervation found in Winnie mice have multiple similarities with changes observed in patients with ulcerative colitis.
Alterations in the distal colon innervation in Winnie mouse model of spontaneous chronic colitis

Ahmed A. Rahman¹ · Ainsley M. Robinson¹ · Valentina Jovanovska¹ · Rajaraman Eri² · Kulmira Nurgali¹,³

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Abstract The gastrointestinal tract is innervated by extrinsic sympathetic, parasympathetic and sensory nerve fibers as well as by intrinsic fibers from the neurons in myenteric and submucosal ganglia embedded into the gastrointestinal wall. Morphological and functional studies of intestinal innervation in animal models are important to understand the pathophysiology of inflammatory bowel disease (IBD). The recently established Winnie mouse model of spontaneous chronic colitis caused by a point mutation in the Muc2 mucin gene develops inflammation due to a primary epithelial defect. Winnie mice display symptoms of diarrhea, ulcerations and rectal bleeding similar to those in IBD. In this study, we investigated myenteric neurons, noradrenergic, cholinergic and sensory nerve fibers in the distal colon of Winnie (Win/Win) mice compared to C57/BL6 and heterozygote littermates (Win/Wt) using histological and immunohistochemical methods. All Win/Win mice used in this study had inflammation with signs of mucosal damage, goblet cell loss, thickening of muscle and mucosal layers, and increased CD45-immunoreactivity in the distal colon. The density of sensory, cholinergic and noradrenergic fibers innervating the myenteric plexus, muscle and mucosa significantly decreased in the distal colon of Win/Win mice compared to C57/BL6 and Win/Wt mice, while the total number of myenteric neurons as well as subpopulations of cholinergic and nitric neurons remained unchanged. In conclusion, changes in the colon morphology and innervation found in Winnie mice have multiple similarities with changes observed in patients with ulcerative colitis.

Keywords Myenteric neurons · Spontaneous chronic colitis · Winnie mice · Distal colon · Innervation

Introduction Inflammatory bowel disease (IBD) is a chronic inflammatory condition characterized by frequent relapses of disease activity and periods of remission that affect individuals throughout life (Podolsky 2002). The etiology of IBD is not completely understood. However, it is generally agreed that altered immunological function, resulting from a complex interplay between genetic susceptibility and certain environmental factors, contributes significantly to the initiation and progression of gastrointestinal (GI) inflammation (Kaser et al. 2010). IBD is comprised of two main immunologically and histopathologically different diseases, ulcerative colitis and Crohn’s disease. A non-conventional TH2 immune response associated with epithelial barrier dysfunction has been implicated in the pathogenesis of ulcerative colitis, while elevated level of cytokines such as interferon-γ, interleukin-2, and tumor necrosis factor-α in the gut consistent with a TH1 type response plays an important role in the pathogenesis of Crohn’s disease (Strober et al. 2007). Ulcerative colitis causes inflammation and ulcers in the mucosal lining of the large intestine whereas all layers of the gut wall may be affected in Crohn’s disease. Inflammation in the gut causes breakdown of intestinal barrier function and abnormal secretion, changes in muscle contractility, neurotransmission, visceral sensation and motility patterns leading to symptoms of diarrhea, cramping and pain (Belai et al. 1997; Jönsson 2000).
Several lines of evidence indicating abnormal structural and functional alterations in the intrinsic and extrinsic innervation of the GI tract associate with symptoms of IBD (Bernardini et al. 2012; Geboes and Collins 1998; Lindgren et al. 1991, 1993; Moynes et al. 2014). The enteric nervous system (ENS), residing within the gut wall and innervating mucosa, muscles and glands, controls GI functions such as nutrient absorption, secretion, GI sensation and propulsion of the contents along the gut (intestinal motility) (Furness 2012). Intestinal inflammation induces morphological and functional changes in the ENS (Mawe et al. 2009). Functional changes involve shifts in the amount and proportion of neurotransmitters and neuropeptides in the ENS of IBD patients and animal models of intestinal inflammation (Boyer et al. 2005; Neunlist et al. 2003; Winston et al. 2013). Alterations in electrophysiological properties of enteric neurons and in neurotransmission between enteric neurons and to the intestinal muscles have been found in animal models of intestinal inflammation (Linden et al. 2003; Lomax et al. 2005; Nurgali et al. 2007, 2009, 2011). These changes in the ENS correlate with intestinal dysmotility (Krauter et al. 2007; Lomax et al. 2007a; Winston et al. 2013). Alterations in the structure and function of cholinergic and noradrenergic nerve fibers innervating the gut wall and synapsing on the enteric neurons have been observed in patients with IBD (Straub et al. 2008), as well as in animal models of intestinal inflammation (Lomax et al. 2010; Swain et al. 1991). Studies of inflammation-induced damage to the ENS and extrinsic nerve fibers in the inflamed intestine are of great significance for understanding mechanisms underlying intestinal dysfunctions and identification of new targets for effective therapies.

More than 60 experimental animal models have been established to study IBD, including genetically engineered mice, chemically-induced models, congenic mouse strains, and immune cell transfer models (Mizoguchi 2012). Although these models do not reproduce the complexity of human disease, they are valuable tools for studying many important aspects of the pathophysiological mechanisms and the effects of emerging therapeutic strategies (Wirtz and Neurath 2007). Most of the experimental models induce acute colitis, while there are only a few animal models of chronic intestinal inflammation. Recently, the Winnie mouse model of colitis has been developed. In this model, chronic intestinal inflammation results from a primary intestinal epithelial defect conferred by a point mutation rather than a deletion in the Muc2 mucin gene (Eri et al. 2011; Heazlewood et al. 2008). In humans, expression of Muc2 is reduced or depleted in Crohn’s disease (Buisine et al. 2001), while in active ulcerative colitis, Muc2 production and secretion are reduced (Van Klinken et al. 1999). Winnie mice (C57/BL6 background) show abnormal Muc2 biosynthesis causing changes in a mucus layer, increased intestinal permeability and greatly enhanced susceptibility to luminal inflammation-inducing toxins. They develop mild spontaneous inflammation in the distal colon with symptoms comparable to human IBD by 6 weeks of age; inflammation progresses over time and results in severe colitis by the age of 16 weeks (Eri et al. 2011; McGuckin et al. 2011). Colitis in Winnie mice is chronic with periods of remissions and relapses similar to human IBD. Previous studies consist mainly of histopathological and immunological changes in the GI tract of Winnie mice, but a study of the intestinal innervations in this model has not been performed. Since Winnie mice closely mimic human chronic colitis, the aim of the present investigation was to evaluate the intrinsic and extrinsic intestinal innervation, especially in the distal colon.

Materials and methods

Animals

Winnie (Win/Win) (19–26 g, 12–16 w.o., n = 16), heterozygote littermates (Win/Wt) (20–25 g, n = 16) and age-matched C57/BL6 (26–30 g, n = 16) mice were obtained from Monash Animal Services (Melbourne, Australia). All mice were housed in a temperature-controlled environment with 12 h day/night cycles at the animal holding room at the Western Center for Health, Research and Education (Melbourne, Australia). All animal experiments in this study complied with the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved by the Victoria University Animal Experimentation Ethics Committee. Mice were killed by cervical dislocation. Subsequent procedures were carried out in vitro.

Immunohistochemistry and histology

Immunohistochemistry was performed as described previously (Robinson et al. 2014; Wafai, et al. 2013). Briefly, segments of the distal colon were processed in two different ways: (1) wholemount longitudinal muscle-myenteric plexus (LMM) preparations, and (2) cross-sections of the distal colon. In general, the colon was exposed through a midline laparotomy and a 5-cm section 2 cm from the anus was collected from each animal. Immediately following dissection, colon tissues were placed in oxygenated phosphate-buffered saline (PBS, pH 7.2) containing L-type Ca2+ channel blocker, nicardipine (3 μM). Segments of the distal colon were then cut open along the mesenteric border and pinned flat with the mucosal side up in a Sylgard-lined Petri dish (maximally stretched for LMM preparations while unstretched for cross-sections). Tissues for LMM preparations and cryostat cross-sections were fixed with Zamboni’s fixative (2 % formaldehyde containing 0.2 % picric acid) overnight at 4 °C and subsequently washed.
with dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Australia) (3×10 min) followed by PBS (3×10 min). Tissues for histology were fixed in 10 % buffered formalin overnight at 4 °C and stored in 70 % ethanol until embedding.

For LMMP preparations, tissues were dissected to expose the myenteric plexus by removing the mucosa, submucosa and circular muscle layers. Following a 1 h incubation in 10 % normal donkey serum (Merck Millipore, MA, USA) at room temperature, tissues were incubated with primary antibodies against protein gene product (PGP) 9.5 (chicken, 1:500; Abcam, Cambridge, UK), CD45 (rat, 1:500; Biolegend, USA), neuronal nitric oxide synthase (nNOS) (goat, 1:500; Novus Biologicals, USA), choline acetyl transferase (ChAT, Goat, 1:500; Merck Millipore, Australia), vesicular acetylcholine transporter (VACHT) (goat, 1:500; Merck Millipore, Australia) and tyrosine hydroxylase (TH) (sheep, 1:1000; Merck Millipore, Australia). Anti-PGP9.5 antibody is a widely used marker for labeling all neuronal cell bodies (Kajimoto et al. 1992); anti-CD45 antibody is a pan leukocyte marker; anti-nNOS antibody identifies predominantly inhibitory muscle motor neurons and some interneurons (Qu et al. 2008); anti-ChAT antibody labels excitatory muscle motor neurons and interneurons; anti-VACHT antibody identifies cholinergic fibers (Qu et al. 2008; Weihe et al. 1996); and anti-TH antibody identifies noradrenergic fibers (Nagatsu 1989).

The tissues were then washed briefly in PBS-Triton (0.1 %) and incubated with secondary antibodies: Alexa Fluor 594 (donkey anti-chicken, 1:200), Alexa Fluor 488 (donkey anti-goat, 1:200, donkey anti-sheep, 1:200) (Jackson ImmunoResearch, PA, USA) and mounted onto glass slides with fluorescent mounting medium (DAKO, Australia).

For cryostat cross-section preparations, tissues were cryoprotected (30 % sucrose/phosphate buffer; Sigma-Aldrich, Australia) overnight at 4 °C then transferred to 50 % Optimal Cutting Temperature compound (OCT; Tissue-Tek; Sakura Finetek, Torrance, CA, USA) in 30 % sucrose/phosphate buffer solution for 12 hr prior to freezing in 100 % OCT. Tissues were sectioned at a 20-μm thickness (at least 15 sections from each animal) using a cryostat microtome (Leica CM1850, St. Gallen, Switzerland) and mounted onto glass slides. Cross-sections were incubated with species-specific primary antibodies to detect CD45 (rat, 1:500; Bioworld), calcitonin gene-related peptide (CGRP) (rabbit, 1:3000; Sigma-Aldrich, MO, USA), TH (sheep, 1:1000; Merck Millipore, Australia) and VACHT (goat, 1:500; Merck Millipore, Australia). Intrinsic primary afferent neurons contain and release CGRP (Grider 2003). Additionally, approximately 85 % of spinal afferents and less than 5 % of vagal afferents contain CGRP, thus anti-CGRP antibody is recognized as a marker for sensory afferent fibers (Kressel et al. 1994). Sections were then washed in PBS and incubated with fluorophore-conjugated secondary antibodies Alexa Fluor 594 (donkey anti-rabbit, 1:200; Abacus ALS, Australia), Alexa Fluor 488 (donkey anti-rat, donkey anti-goat, 1:200; Jackson ImmunoResearch, PA, USA) and FITC (donkey anti-sheep, 1:200; Abacus ALS).

For histology, tissues were embedded in paraffin, sectioned at 5 μm, deparaffinized, cleared, and rehydrated in graded ethanol concentrations. For hematoxylin and eosin (H&E) staining, sections were immersed in Xylene (3×4 mins, 100 % ethanol (3 min), 90 % ethanol, (2 min), 70 % ethanol (2 min), rinsed in tap water, hematoxylin (4 min), rinsed in tap water, Scott’s tap water (1 min), eosin (6 min), rinsed in tap water, 100 % ethanol (2×1 min), xylene (2×3 mins) and mounted on glass slides with DPX mountant.

**Imaging**

Images were captured using a Nikon Eclipse Ti multichannel confocal laser scanning system (Nikon, Japan). Immunolabeled sections were visualized and imaged by using filter combinations appropriate for the specific fluorophores such as FITC, Alexa 488 or 594 (488 or 559 nm excitation). Images (512×512 pixels) were obtained with 2×0 (dry, 0.75) or 40× (oil immersion, 1.3) lenses. In order to obtain Z-series, neuronal structures were imaged by collecting ten consecutive optical sections at 1-μm intervals. H&E stained colon sections were visualized using an Olympus microscope (Olympus BX53) and images were captured with CellSense™ software.

**Quantitative analysis of immunohistochemical and histological data**

Images were analyzed using Image J software (National Institute of Health, Bethesda, MD, USA). Muscle and mucosal thicknesses were quantified as described previously (Miampamba and Sharkey 1998). Muscle thickness was measured from the serosa to the submucosa including both longitudinal and circular muscle layers, while mucosal thickness was measured from the submucosa to the luminal surface of mucosa. Infiltration of leukocytes within the colon mucosa was assessed by measuring the density of CD45-immunoreactive (IR) cells per area (average of 8 areas of 500 μm² per animal at ×20 magnification). Image J software was employed to adjust color images from RGB to 8 bit, after which thresholding to a consistent value was applied to obtain the percentage area of CD45-immunoreactivity. Histological scores were developed from the following parameters: 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), epithelial damage and ulceration (0–3) (average of 8 areas of 500 μm² per animal). Nerve fibers in cross-sections of the distal colon were measured from...
8 images per preparation at ×20 magnification (total area 2 mm²). Images were changed from RGB to 8 bit and made binary prior to particle analysis with Image J software. Myenteric neurons and CD45-IR leukocytes were counted in wholemount preparations within a 2-mm² area by randomly capturing 8 images per preparation at ×20 magnification. The number of neurons per ganglion was quantified in images at ×40 magnification; the number of neurons was averaged per 10 ganglia in each preparation.

Analysis of fecal water content and colon length

Fecal water content was calculated following stool collection from animals in all groups. After collection, stools were immediately weighed (wet weight) and left to air dry. Three days later, stools were re-weighed (dry weight) and the difference between wet and dry weight was calculated. Mouse colon length (from cecum to anus) was measured immediately after dissection from the animal by placing the colon parallel to a ruler and recording its size.

Statistical analysis

Statistical analysis was conducted with Prism (v.5.0; GraphPad Software, La Jolla, CA, USA). All values are expressed as mean ± standard error of the mean. Paired t tests were used to analyze the difference in weight, muscle and mucosal thickness. One-way ANOVA for multiple group comparison followed by the Bonferroni’s or Tukey-Kramer post hoc tests was used to analyze the differences between all groups. P<0.05 was considered significant.
## Results

### Winnie mouse model of colitis

All Win/Win mice used in this study displayed symptoms of intestinal inflammation: perianal inflammation and bleeding, soiled fur and soft fecal consistency, not forming pellets compared to control mice (Fig. 1a–d). The fecal water content (wet weight minus dry weight) of stools from Win/Win mice was greater than in stools from C57/BL6 and Win/Wt mice (P<0.001 for both, n=6/group; Fig. 1e; Table 1). The body weight of all mice was monitored daily for a period of 7 days prior to culling. Average body weight of Win/Win mice (23±1.0 g) was less compared to C57/BL6 (27±0.2 g, P<0.01) and Win/Wt (28±0.3 g, P<0.05) mice (n=12/group; Fig. 1f). Immediately following dissection, the length of the colon was measured and recorded showing Win/Win mice to have longer colons compared to C57/BL6 (data not shown). In cross-sections, gross morphological differences were observed in Win/Win mice (Fig. 2a–c). No abnormalities were observed in the histology of the colon from C57/BL6 mice while only very mild inflammatory changes were observed in Win/Wt mice (Fig. 2a–c).

Thickening of the muscle layer is also considered as a histological index of inflammation (Miampamba and Sharkey 1998). The thickness of the colonic muscle layer (total thickness of longitudinal and circular muscles) was significantly higher in Win/Win mice (50±1.4 μm, n=4) compared to both C57/BL6 (44±0.6 μm, P<0.05, n=4) and Win/Wt mice (45±1.2 μm, P<0.01, n=4; Fig. 2d). Win/Win mice had a significantly thicker mucosal layer (271±5 μm, n=4) compared to C57/BL6 (206±4 μm, P<0.001, n=4) and Win/Wt mice (197±6 μm, P<0.01, n=4; Fig. 2e).

The severity of inflammation was evaluated by immunolabelling with anti-CD45 antibody specific to the leukocyte common antigen in cross-sections of the colon (Fig. 2f-h). Quantification of leukocyte density in the distal colon revealed a higher level of leukocyte infiltration within the mucosa of Win/Win mice compared to C57/BL6 and Win/Wt mice (P<0.01 for both, n=6/group; Fig. 2i; Table 1). Infiltration of CD45-IR cells to the level of myenteric ganglia was studied in wholemount LMMP preparations. Quantitative analysis did not reveal significant difference between the groups (data not shown). In cross-sections, gross morphological damage was assessed to further substantiate the level of inflammation in the distal colon of mice from all groups. Histological scoring of H&E-stained sections incorporated individual scores for various parameters: aberrant crypt architecture, increased crypt length, goblet cell depletion, general leukocyte infiltration, crypt abscesses and epithelial damage and ulceration.

Assessment of all parameters revealed histological scores to be significantly higher in sections from Win/Win mice compared to sections from C57/BL6 and Win/Wt mice (P<0.001 for all, n=6/group; Fig. 2j; Table 1).

### Table 1 Evaluation of intestinal inflammation

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<th>C57/BL6</th>
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<td>Diarrhea</td>
<td>Absent (hard pellets)</td>
<td>Absent (hard pellets)</td>
<td>Prominent (loose stool)</td>
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<td>Fecal water content (wet weight minus dry weight, g)</td>
<td>0.14±0.005</td>
<td>0.15±0.004</td>
<td>0.24±0.005***</td>
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<td>Colon length (from caecum to anus, cm)</td>
<td>6.2±0.2</td>
<td>6.4±0.2</td>
<td>8.3±0.5***††</td>
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<tr>
<td>Density of CD45+ leukocytes in colon cross-sections (average within 2 mm² per animal, %)</td>
<td>6.6±0.5</td>
<td>8.0±0.9</td>
<td>12.9±1.5***</td>
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<td>Parameters for histological scoring (n=4/group)</td>
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<td>Aberrant crypt architecture (0–3)</td>
<td>0.33±0.05</td>
<td>0.42±0.21</td>
<td>2.33±0.06***</td>
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<td>Increased crypt length (0–3)</td>
<td>0.25±0.04</td>
<td>0.33±0.02</td>
<td>2.33±0.05***</td>
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<td>Goblet cell depletion (0–3)</td>
<td>0.25±0.03</td>
<td>0.33±0.08</td>
<td>2.25±0.03***</td>
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<td>General leukocyte infiltration (0–3)</td>
<td>0.17±0.1</td>
<td>0.33±0.1</td>
<td>2.25±0.12***</td>
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<tr>
<td>Crypt abscesses (0–3)</td>
<td>0.25±0.15</td>
<td>0.25±0.15</td>
<td>2.50±0.15***</td>
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<tr>
<td>Epithelial damage and ulceration (0–3)</td>
<td>0.33±0.34</td>
<td>0.42±0.14</td>
<td>2.55±0.35***</td>
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<tr>
<td>Overall histological score (out of 18)</td>
<td>3.1±0.6</td>
<td>4.4±0.4</td>
<td>14.4±0.6***</td>
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***P<0.001 compared to both C57/BL6 and Win/Wt groups

†† P<0.001 compared to C57/BL6 group

† P<0.01 compared to Win/Wt group
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Cell Tissue Res

(Abtract)
Changes in the density of cholinergic nerve fibers in the distal colon of Winnie mice

Anti-VACHT antibody was used as a marker for cholinergic fibers accumulating acetylcholine in synaptic vesicles (Qu et al. 2008; Weihe et al. 1996) in both cross-sections and wholemount LMMP preparations of the distal colon. The density of cholinergic fibers was decreased within both mucosal and muscle layers observed in the cross-section preparations from Win/Win (4.7±0.2 %, n=4) compared to C57/BL6 (6.4±0.3 %, P<0.05, n=4) and Win/Wt (6.1±0.4 %, P<0.05, n=4) mice (Fig. 3a–c, g). Similarly, a significant decrease in VACHT-IR fibers within the myenteric ganglia was observed in wholemount LMMP preparations of the distal colon from Win/Win (5.2±0.4 %, n=4) compared to C57/BL6 (8.6±0.6 %, P<0.01, n=4) and Win/Wt (7.7±0.7 %, P<0.05, n=4) mice (Fig. 3d–f, h).

Changes in the density of noradrenergic and sensory nerve fibers in the distal colon of Winnie mice

TH immunoreactivity was used to label sympathetic fibers in the gastrointestinal tract (Lourenssen et al. 2005; Straub et al. 2005).
noradrenergic and dopaminergic neurons (Li et al. 2004; Qu et al. 2008). In our study, TH-IR neurons were not found in the distal colon of C57/BL6, Win/Win or Win/Wt mice. To assess changes in the expression of noradrenergic fibers, we evaluated the density of TH-IR fibers in both cross-sections and wholemount LMMP preparations of the distal colon in Win/Win mice compared to C57/BL6 and Win/Wt mice (Fig. 4a–i). We were not able to quantify the fiber density in cross-sections due to very sparse amount of TH-IR fibers (Fig. 4a–f). However, quantitative analysis of TH-IR fibers was performed in wholemount preparations. A significant decrease in the density of TH-IR fibers within myenteric ganglia was observed in wholemount preparations of the distal colon from Win/Win (3.2±0.2 %, n=4) compared to C57/BL6 (6.6±0.7 %, P<0.01, n=4) and Win/Wt (7.3±0.5 %, P<0.001, n=4) mice (Fig. 4g–i, m).

Immunolabeling using anti-CGRP antibody was carried out to reveal sensory nerve fibers in the colon cross-sections. CGRP-IR nerve fibers were widely distributed in the mucosa, submucosal and myenteric plexuses of the colon in C57/BL6 and Win/Wt mice (Fig. 4j–l). A significant decrease in the density of CGRP-IR nerve fibers was observed in the colon cross-section preparations from Win/Win (3.3±0.1 %, n=4) compared to C57/BL6 and Win/Wt mice (3.4±0.2 %, n=4).

Myenteric neurons were labeled using antibody against protein gene product (PGP) 9.5. Images of PGP9.5-IR neurons within 0.25 mm² area (a–c) and within myenteric ganglia (d–f). Scale bars (a–c) 100 μm, (d–f) 50 μm. No significant differences in the average number of myenteric neurons counted per 2 mm² area (g) or in the average number of myenteric neurons per ganglion (average of 10 ganglia per animal) (h) have been observed between all groups. Data are expressed as mean ± SEM. Numbers of animals per group are shown in parentheses.
compared to C57/BL6 (9.4±0.4 %, P<0.001, n=4) as well as Win/Wt (8.7±0.1 %, P<0.01, n=4) mice (Fig. 4j–l, n).

No changes in the total number of myenteric neurons and subpopulations of nNOS-IR and cholinergic neurons in the distal colon of Winnie mouse

The total number of neurons in wholemount LMMP preparations of the distal colon was counted using the pan-neuronal marker PGP9.5. The mean number of neurons per surface area (2 mm²) of the colon was similar in Win/Win (548±7, n=4), C57/BL6 (559±16, n=4) and Win/Wt (568±18, n=4) mice (Fig. 5a–c, g). No significant differences were observed in the average number of myenteric neurons per ganglion between Win/Win (22±2.4, n=4), C57/BL6 (23±2.3, n=4) and Win/Wt (24±3.2, n=4) mice (Fig. 5d–f, h).

The number of nNOS-IR neurons in wholemount LMMP preparations of the distal colon was quantified. Immunofluorescence staining showed no significant difference in the average number of nNOS-IR neurons per surface area (2 mm²) between C57/BL6, Win/Wt and Win/Win mice (C57/BL6: 390±14; Win/Wt: 398±14; Win/Win: 389±8; n = 4/group) (Fig. 6a–c, g) or in the mean number of neurons per ganglion (C57/BL6: 12±1.5; Win/Wt: 13±2.2; Win/Win: 14±1.3; n = 4/group) (Fig. 6d–f, h).

The number of cholinergic neurons in wholemount LMMP preparations of the distal colon was counted using anti-ChAT antibody. No significant difference was observed in the mean number of ChAT-IR neurons per surface area (2 mm²) of the colon between C57/BL6 (535±19, n=4), Win/Wt (522±21, n=4) and Win/Win mice (504±19, n=4) (Fig. 7a–c, g). Similarly, the average number of ChAT-IR neurons per ganglion was comparable in C57/BL6 (34±2.6, n=4), Win/Wt (37±3.8, n=4) and Win/Win mice (34±2.9, n=4) (Fig. 7d–f, h).

Fig. 6 Number of myenteric nitrergic neurons in the distal colon. Nitric oxide synthase-immunoreactive (NOS-IR) neurons were counted within 2 mm² area and within each ganglion in wholemount preparations of the distal colon. Examples of myenteric NOS-IR neurons within 0.25 mm² area (a–c) and within myenteric ganglia (d–f). Scale bars (a–c) 100 μm, (d–f) 50 μm. No significant differences in the number of NOS-IR neurons per area (g) and per ganglion (h) have been found between all groups. Data are expressed as mean ± SEM. Numbers of animals per group are shown in parentheses.
Discussion

Animal models of IBD have provided significant contributions to understanding pathophysiological mechanisms as well as development of novel therapeutic strategies for IBD (Mizoguchi and Mizoguchi 2010; Xavier and Podolsky 2007). Although animal models have their limitations and do not reproduce all the pathogenetic and clinical features of human IBD, each animal model provided an invaluable tool to study complex physiological and biochemical disease aspects that are difficult to address in humans (Elson et al. 1995; Dothel et al. 2013; Grisham 1993). The Winnie mice used in our study develop inflammation in the colon with multiple similarities to human ulcerative colitis, including goblet cell pathology, depleted mucus layer, and distal gradient of colitis as well as a characteristic immune profile (Eri et al. 2011; Heazlewood et al. 2008; Lourensens et al. 2005; McGuckin et al. 2011). The manifestation of clinical symptoms in Winnie mice starts at the age of 6 weeks when animals become young adults. Colitis in Winnie mice has a chronic and relapsing nature, a major feature of human IBD. Winnie mice carry only a point mutation in Muc2 gene leading to spontaneous colitis unlike chemically-induced models and some other chronic models which require pathogens to develop colitis (e.g., IL-10−/− mice) (Uhlig and Powrie 2009; Wirtz and Neurath 2007). In this study, we used 12- to 16-week-old Winnie mice all of which had active colitis with symptoms of perianal bleeding and diarrhea confirmed by increased fecal water content and lack of weight gain. By this age, chronic inflammation induced morphological changes in the colon including increase in its length and marked thickening of the intestinal wall which may contribute to colonic dysmotility present in these mice (unpublished data). Together with the mucosal damage and leukocyte infiltration observed in all Winnie mice in this study, these are the hallmark features of chronic intestinal inflammation. Muscular hypertrophy, changes in colon length and similar...
gloss morphological changes have been described previously in
other models of chronic intestinal inflammation (Grisham
1993; Elson et al. 1995; Rivera-Nieves et al. 2003).

Inflammation in Winnie mice was associated with sig-
nificant structural damage to the colonic innervation
which was investigated for the first time in this study.
The results of this study have demonstrated decrease in
the density of cholinergic, noradrenergic and sensory
nerve fibers projecting to the myenteric plexus, and un-
changed total number of neurons and numbers of
nitricergic and cholinergic neurons in the myenteric plex-
us of the distal colon from Winnie mice.

Significant reduction of the density of VACH-IR cho-
linergic nerve fibers observed in the colon tissues from
Winnie mice in our study is consistent with previous work
conducted in the colon tissues from ulcerative colitis pa-

tients (Jönsson et al. 2007). It has been established that
acetylcholine attenuates the release of pro-inflammatory
cytokines (Borovikova et al. 2000; Ulloa 2005) and there-
by could control systemic inflammation and modulate im-
mune response. Other studies further demonstrated that
cholinergic pathways also modulated experimental colitis
in rats by using acetylcholinesterase inhibitors (Miceli and
Jacobson 2003) or vagotomy (Ghia et al. 2006). Previous
studies showed impaired release of acetylcholine from the
inflamed rat intestine (Collins et al. 1989). Alterations in
functions of cholinergic fibers in Winnie mice should be
further investigated.

Noradrenergic neurons of the sympathetic celiac and
the superior mesenteric ganglia innervate the smooth
muscles and enteric ganglia in the colon and modulate
motility, secretion, blood flow, and immune system activ-
ation (Cervi et al. 2014; Lomax et al. 2010; Miolan and
Niel 1996; Straub et al. 2008; Vasin’ et al. 2008). Results
of our study demonstrated that the density of noradrener-
gic nerve fibers identified by TH immunoreactivity was
significantly reduced in the colon tissues from Winnie
mice. This is consistent with the results of previous stud-
ies in patients with Crohn’s disease (Belai et al. 1997;
Straub et al. 2008), and in mouse models of DSS and
TNBS-induced colitis (Lomax et al. 2007b; Straub et al.
2005). A large body of evidence obtained from animal
models of gastrointestinal inflammation indicated marked
changes in sympathetic neuronal excitability (Dong et al.
2008), neurotransmitter release (Blandizzi et al. 2003;
Swain et al. 1991) and structure of noradrenergic nerve
fibers (Dvorak et al. 1980; Dvorak and Silen 1985;
Magro et al. 2002; Straub et al. 2008). Decreased colonic
mucosal norepinephrine concentration was observed in
Crohn’s disease patients (Magro et al. 2002). In addition,
colitis impairs noradrenergic regulation of submucosal
arterioles and mesenteric arteries (Birch et al. 2008; Lo-
max et al. 2007b).

Similar to previous studies (Lourensse et al. 2005;
Straub et al. 2005), no TH-IR cell bodies were found in
the myenteric plexus of Winnie or C57/BL6 mice. How-
ever, TH-IR neurons were previously reported to be pre-

sent in the mouse and human gut. TH immunoreactivity
was observed in a very small proportion (less than 0.5 %)
of noradrenergic neurons in the ileum of adult Balb/c
mice (Qu et al. 2008). TH-IR neurons constituted about
9 % of myenteric and 13 % of submucosal neurons in the
ileum of adult CD-1 mice (Li et al. 2004). TH-IR
myenteric and submucosal neurons were found in humans
throughout the gastrointestinal tract, but most frequently
in the esophagus (Wakabayashi et al. 1989). In our study,
cell bodies of TH-IR neurons were not found in the distal
colon of C57/BL6 or Winnie mice. Whether this discrep-
ancy is due to the differences between species and mouse
strains or regional differences needs to be further investi-
gated. However, the presence of TH-IR fibers from intrin-
sic TH-positive neurons should not be excluded as we
have not investigated the entire length of the intestine.

Anti-CGRP antibody was used to label sensory fibers
including extrinsic spinal and vagal primary sensory af-

dent as well as intrinsic sensory fibers containing and
releasing CGRP (Grider 2003; Kressel et al. 1994; Qu
et al. 2008). CGRP was found to be reduced in the
inflamed bowel in animal models of chemically-
induced colitis (Eysselein et al. 1991; Miamampa
et al. 1992; Miampamba and Sharkey 1998). Moreover,
tissues from patients with Crohn’s disease and ulcerative
colitis also showed decrease in the number and density
of CGRP-positive nerve fibers in the colonic mucosa
(Koch et al. 1987; Eysselein et al. 1992).

Our data demonstrated that, although significant re-
duction in all types of fibers analyzed in this study was
observed, CGRP-IR fibers were the most affected com-
pared to other types of fibers in Winnie mice with the
loss of about 65 % of CGRP-IR fibers in cross-sections.
CGRP-IR sensory fibers extensively supply the mucosa
and submucosa where chronic inflammation is the most
prominent in Winnie mice. CGRP released from sensory
nerve fibers plays important anti-inflammatory and pro-
tective roles: it facilitates mucus production and controls
blood flow in the gastrointestinal mucosa (Holzer 2007).
It has been suggested that during inflammation there is
a sustained increased release of CGRP, leading to de-
pletion of CGRP fibers (Eysselein et al. 1992). Loss of
CGRP-IR sensory fibers and sensory neuron dysfunction
impair mucosal protection (Holzer 2007). The loss of
about 52 % of TH-IR noradrenergic fibers within
myenteric ganglia observed in our study might contrib-
ute to impairment of motility, secretion, blood flow and
gastrointestinal immunity in Winnie mice which needs to
be further investigated. It was suggested that the loss of
noradrenergic fibers is a pro-inflammatory signal in the chronic phase of the intestinal inflammation (Straub et al. 2008). On the other hand, the density of VACHT-IR cholinergic fibers was less reduced in cross-sections (by 27%) of the colon from Winnie mice. This might be due to the extensive projection of cholinergic fibers to the muscle layer where immune infiltration is less prominent. Nevertheless, significant reduction of VACHT fibers within the myenteric ganglia (by 40%) suggests that excitatory cholinergic neurotransmission is reduced, leading to impaired motility and symptoms of diarrhea observed in Winnie mice. Given the potent anti-inflammatory effect of the cholinergic innervation (Matteoli and Boeckxstaens 2013), the loss of VACHT fibers may have an important impact on immune homeostasis in Winnie mice.

Whether the loss of nerve fibers in Winnie mice is a result of chronic inflammation or if Winnie mice are born with altered intestinal innervation which contributes to initial pathological changes in the mucosa leading to inflammation needs to be further elucidated.

Our findings showed that the number of myenteric neurons remained unchanged in the Winnie mouse distal colon. There is a great degree of controversy in the literature regarding the number of myenteric neurons in the inflamed intestine. Some studies reported increase, while others reported decrease or no change in the number of myenteric neurons in tissues from animal models of intestinal inflammation and from IBD patients (Table 2). Decreased number of neurons in the myenteric plexus in both wholemount preparations and cross-sections was reported in most animal models of chemically-induced intestinal inflammation, except a study reporting no changes in wholemount preparations and cross-sections of the colon from rats with DSS-induced colitis (Winston et al. 2013). In human tissues, most of the studies were performed in transverse or cross-sections showing either increase, decrease or no change in the number of myenteric neurons (Table 2). A study performed in wholemount preparations of the colon from patients with ulcerative colitis demonstrated no changes in the number of myenteric neurons (Neunlist et al. 2003). This is consistent with the results of our study in wholemount preparations of the distal colon from Winnie mice with chronic inflammation. Our results demonstrated that the number of cholinergic (ChAT-IR excitatory muscle motor and interneurons) and nitrergic (NOS-IR inhibitory muscle motor and interneurons) neurons in the myenteric plexus were unaltered in the distal colon of Winnie mice. These results are consistent with findings in rats with DSS-induced colitis (Winston et al. 2013) and in patients with ulcerative colitis (Neunlist et al. 2003). Further studies need to investigate the gene expression of proteins regulating the synthesis of ACh and NO which can be altered, even though the number of neurons is not changed, leading to intestinal dysmotility (Winston et al. 2013). However, reductions in the density of nerve fibers observed in Winnie mice might lead to decreased neuropeptide release and changes in the neurotransmission affecting gastrointestinal functions. Functional studies investigating these changes in Winnie mice are warranted in the future.

In conclusion, the present study demonstrates that colitis in Winnie mice is associated with significant impairment of distal colon innervation. Changes in the cholinergic, noradrenergic and sensory innervation observed in Winnie mice were similar to those observed in ulcerative colitis patients.

### Table 2

<table>
<thead>
<tr>
<th>Species</th>
<th>Pathology/model</th>
<th>Tissues studied</th>
<th>Preparations</th>
<th>Number of neurons</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Ulcerative colitis</td>
<td>Colon</td>
<td>Transverse sections</td>
<td>Threefold increase</td>
<td>(Storsteen et al. 1953)</td>
</tr>
<tr>
<td>Human</td>
<td>Crohn’s disease</td>
<td>Ileum</td>
<td>Transverse sections</td>
<td>Threefold increase</td>
<td>(Davis et al. 1955)</td>
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<tr>
<td>Human</td>
<td>Ulcerative colitis</td>
<td>Colon</td>
<td>Cross-sections</td>
<td>61% decrease</td>
<td>(Bernardini et al. 2012)</td>
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<tr>
<td>Human</td>
<td>Ulcerative colitis</td>
<td>Colon</td>
<td>Cross-sections</td>
<td>No change</td>
<td>(Neunlist et al. 2003)</td>
</tr>
<tr>
<td>Human</td>
<td>Ulcerative colitis</td>
<td>Colon</td>
<td>Transverse sections</td>
<td>No change</td>
<td>(Villanacci et al. 2008)</td>
</tr>
<tr>
<td>Guinea-pig</td>
<td>TNBS</td>
<td>Colon</td>
<td>Wholmounts</td>
<td>15% decrease</td>
<td>(Linden et al. 2005)</td>
</tr>
<tr>
<td>Guinea-pig</td>
<td>TNBS</td>
<td>Ileum</td>
<td>Wholmounts</td>
<td>17% decrease</td>
<td>(Nurgali et al. 2011)</td>
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<tr>
<td>Mouse</td>
<td>DNBs</td>
<td>Colon</td>
<td>Wholmounts</td>
<td>50% decrease</td>
<td>(Boyer et al. 2005)</td>
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<tr>
<td>Rat</td>
<td>DNBS</td>
<td>Colon</td>
<td>Cross-sections</td>
<td>50% decrease</td>
<td>(Sanovic et al. 1999)</td>
</tr>
<tr>
<td>Rat</td>
<td>TNBS</td>
<td>Colon</td>
<td>Cross-sections</td>
<td>Decrease or absent (qualitative analysis)</td>
<td>(Poli et al. 2001)</td>
</tr>
<tr>
<td>Rat</td>
<td>TNBS</td>
<td>Colon</td>
<td>Wholmounts</td>
<td>33% decrease</td>
<td>(Lin et al. 2005)</td>
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<td>Rat</td>
<td>TNBS</td>
<td>Colon</td>
<td>Wholmounts</td>
<td>20% decrease</td>
<td>(Samelli et al. 2009)</td>
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<tr>
<td>Rat</td>
<td>DSS</td>
<td>Colon</td>
<td>Wholmounts</td>
<td>No change</td>
<td>(Winston et al. 2013)</td>
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</table>
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Conflict of interest The authors of this manuscript do not have any potential conflicts to disclose.

Role of authors AAR performed experiments, analyzed data and wrote the manuscript. AMR performed experiments, analyzed data and contributed to manuscript writing. VJ contributed to processing tissues for immunohistochemical and histological studies. RE and KN developed the concept and edited the manuscript. KN obtained funding and supervised the study.

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AUTHOR'S PROOF!

AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES.

Q1. Please check if the affiliations are presented correctly.
Q2. Miampamba et al. 1998 has been changed to Miampamba and Sharkey 1998 as per the reference list. Please check if okay.
Q3. Note that all seven missing reference details queried in this table have been added to the Reference List by the Copyeditor. Author to check and confirm.