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Gastrointestinal dysfunction and enteric neurotoxicity following treatment with anticancer chemotherapeutic agent 5-fluorouracil

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Key Points

- 5-FU is the first-line chemotherapy for colorectal cancer; its use is associated with severe long-term gastrointestinal side-effects. Mechanisms underlying 5-FU-induced gastrointestinal dysfunction have not been investigated in depth.
- This is the first study in a mouse model demonstrating that short-term 5-FU treatment induces increased gastrointestinal transit associated with acute intestinal inflammation, which may lead to persistent changes in the ENS contributing to delayed gastrointestinal transit and colonic dysmotility.
- These findings provide insight into the mechanisms underlying chemotherapy-induced gastrointestinal dysfunction.

Abstract

Background *The use of the anticancer chemotherapeutic agent 5-fluorouracil (5-FU) is often limited by nausea, vomiting, constipation, and diarrhea; these side-effects persist long after treatment. The effects of 5-FU on enteric neurons have not been studied and may provide insight into the mechanisms underlying 5-FU-induced gastrointestinal dysfunction.* ***Methods*** *Balb/c mice received intraperitoneal injections of 5-FU (23 mg/kg) 3 times/week for 14 days. Gastrointestinal transit was analysed in vivo prior to and following 3, 7, and 14 days of 5-FU treatment via serial x-ray imaging. Following 14 days of 5-FU administration, colons were collected for assessment of ex vivo colonic motility, gross morphological structure, and immunohistochemical analysis of myenteric neurons. Fecal lipocalin-2 and CD45$^+$ leukocytes in the colon were analysed as markers of intestinal inflammation.* ***Key Results*** *Short-term administration of 5-FU (3 days) increased gastrointestinal transit, induced acute intestinal inflammation and reduced the proportion of neuronal nitric oxide synthase-immunoreactive neurons. Long-term treatment (7, 14 days) resulted in delayed gastrointestinal transit, inhibition of colonic migrating motor complexes, increased short and fragmented contractions, myenteric neuronal loss and a reduction in the number of ChAT-immunoreactive neurons after the inflammation was resolved. Gross morphological damage to the colon was observed following both short- and long-term 5-FU treatment.* ***Conclusions & Inferences*** *Our*

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results indicate that 5-FU induces accelerated gastrointestinal transit associated with acute intestinal inflammation at day 3 after the start of treatment, which may have led to persistent changes in the ENS observed after days 7 and 14 of treatment contributing to delayed gastrointestinal transit and colonic dysmotility.

Keywords *5-fluorouracil, 5-FU, colonic motility, enteric neuropathy, gastrointestinal transit, myenteric neurons.*

Abbreviations: 5-FU, 5-fluorouracil; CMMC, colonic migrating motor complex; CRC, colorectal cancer; ENS, enteric nervous system; FC, fragmented contraction; IR, immunoreactive; SC, short contraction.

INTRODUCTION

Colorectal cancer (CRC) is a leading cause of cancer related mortality,[1–3] leading to over 1 million deaths annually[4]; it is one of the most frequently diagnosed cancers worldwide. Due to late stage diagnosis approximately 50% of patients have metastasis to surrounding tissues, most frequently to the liver.[5] Therefore, dual therapy consisting of chemotherapy and surgical resection is common.[6]

Since the discovery of fluoropyrimidines in the 1950's, 5-fluorouracil (5-FU) has been the backbone of therapy for many solid tumors and is considered as the standard first line therapy for metastatic CRC.[7] 5-fluorouracil is an analogue of uracil with a fluorine atom at the C-5 position in place of hydrogen.[8] Upon entering a cell, 5-FU is converted intracellularly to several active metabolites: fluorodeoxyuridine monophosphate, fluorodeoxyuridine triphosphate, and fluorouridine triphosphate. These active metabolites exert their cytotoxic effects via misincorporation of fluoronucleotides into RNA and inhibition of nucleotide synthetic enzyme thymidylate synthase.[9]

Combination therapies including 5-FU are reported to improve response rates for advanced CRC by 40–50%,[10,11] however, severe gastrointestinal side-effects such as nausea, vomiting, constipation, and diarrhea remain significant hurdles in the clinical application of 5-FU. It has been suggested that 5-FU-induced gastrointestinal dysfunction results from inflammation, epithelial degradation, and intestinal ulceration triggering intestinal mucositis.[12] However, recent evidence suggests that 5-FU induced gastrointestinal dysmotility outlasts intestinal mucositis.[13] One system that has been overlooked in 5-FU-induced gastrointestinal dysfunction is the enteric nervous system (ENS), embedded within the wall of the gastrointestinal tract and controlling its functions.[14] Neuronal loss and phenotypic changes within the ENS following chemotherapeutic administration of other agents, cisplatin and oxaliplatin, have been found to result in downstream effects on gastrointestinal muscle tone and transit.[15,16]

The aims of this study were to investigate the effects of short- and long-term 5-FU treatment on: (i) gastrointestinal transit time and gastric emptying; (ii) *ex vivo* colonic motility functions; (iii) histological architecture of the colonic mucosa; (iv) level of inflammation in the colon; and (v) the total number of myenteric neurons and subpopulations of inhibitory and excitatory neurons controlling colonic motility.

MATERIALS AND METHODS

All procedures were approved by the Victoria University Animal Experimentation Ethics Committee and performed in accordance with the guidelines of the National Health and Medical Research Council *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*.

Animals

Male Balb/c mice aged 6–8 weeks (18–25 g) supplied from the Animal Resources Centre (Perth, Australia) were used for the experiments. Mice had free access to food and water and were kept under a 12 h light/dark cycle in a well-ventilated room at an approximate temperature of 22 °C. Mice acclimatized for up to 7 days prior to the commencement of *in vivo* intraperitoneal injections. A total of 42 mice were used for this study.

In vivo 5-FU injections

Mice received intraperitoneal injections of 5-FU (23 mg/kg; Sigma-Aldrich, Castle Hill, NSW, Australia), 3 times a week via a 26 gauge needle. 5-FU was dissolved in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich) to make 1 M/L stock solution refrigerated at −20 °C. The stock was then defrosted and diluted with sterile water to make 0.1 M/L (10% DMSO) solutions for intraperitoneal injections. The dose of 5-FU was calculated to be equivalent to standard human dose per body surface area.[17] The low doses of 5-FU (10–40 mg/kg) have been shown to have antitumor efficacy in mouse models of cancer.[18] Sham-treated mice received 10% DMSO in sterile water via intraperitoneal injection three times a week via a 26 gauge needle. The injected volumes were calculated to the body weight; the maximum volume did not exceed 200 μL per injection. Mice were euthanized via cervical dislocation at 3 (2 treatments), 7 (3 treatments), and 14 (6 treatments) days after the first injection and colon was collected for *in vitro* experiments.

Gastrointestinal transit

Prior to performing x-ray imaging, animals were trained/conditioned for oral gavage using a non-irritating substance such as 0.9% w/v saline (volume 0.1–0.4 mL); this was repeated at least three times with each animal with at least 24 h between each training.

The training/conditioning with restraint were done by placing the restrainer into the mouse cages at least 24 h prior to the x-ray procedure. Gastrointestinal transit was studied by x-ray prior to first treatment (day 0) and following 3, 7, and 14 days of 5-FU treatment. The contrast agent, 0.4 mL of suspended barium sulfate (X-OPAQUE-HD, 2.5 g/mL), was administered via oral gavage. Radiographs of the gastrointestinal tract were performed using a HiRay Plus Porta610HF x-ray apparatus (JOC Corp, Kanagawa, Japan; 50 kV, 0.3 mAs, exposure time 60 ms). Mice were immobilized in the prone position by placing them inside a transparent plastic restraint tube with partly open front side for breathing which comfortably restrains animal movement essential for maximum of 1–2 min for successful x-ray imaging. X-rays were captured using Fujifilm cassettes (24 × 30 cm) immediately after administration of barium sulfate (T0) every 5 min for the first hour, every 10 min for the second hour, then every 20 min through to 480 min (T480). Animals were closely monitored during and after all procedures. Images were developed via a Fujifilm FCR Capsula XLII and analysed using eFilm 4.0.2 software. Speed of gastrointestinal transit was calculated as time in minutes taken to reach each region of the gastrointestinal tract (stomach, small intestines, cecum, and large intestines). Organ emptying was calculated as the time taken for complete barium emptying from specific gastrointestinal regions (stomach, small intestines).[19–21]

Colonic motility experiments

The entire colon was removed from day 14 sham and 5-FU-treated mice and set up in organ-bath chambers to record motor patterns *in vitro*.[15] Briefly, the colon was placed into warmed (35 °C), oxygenated physiological saline until the fecal pellets were expelled. The empty colon was cannulated at both ends and arranged horizontally in an organ-bath chamber. The proximal end of the colon was connected to a reservoir containing oxygenated physiological saline to maintain intraluminal pressure. The distal end was attached to an outflow tube that provided a maximum of 2 cm H_2O back-pressure. Organ baths were continuously superfused with oxygenated physiological saline solution and preparations were left to equilibrate for 30 min. Contractile activity of each segment was recorded with a Logitech Quickcam Pro camera positioned 7–8 cm above the preparation. Videos (2 × 20 min) of each test condition were captured and saved in *avi* format using VirtualDub software (Avery Lee, Cambridge, MA, USA) (version 1.9.11).

Colonic migrating motor complexes (CMMCs) were defined as propagating contractions directed from the proximal to the distal end of the colon which travelled more than 50% of the colon length.[22–24] Contractions that propagated less than 50% colon length were considered to be short contractions (SCs). Incomplete non-propagating phasic contractions occurring concurrently at different parts of the colon rather than propagating over the length of the colon were defined as fragmented contractions (FCs). Recordings were used to construct spatiotemporal maps using in-house edge detection software.[25] Spatiotemporal maps plot the diameter of the colon at all points during the recording allowing contractile motor patterns to be analysed with Matlab software (Mathworks, Natick, MA, USA) (version 12).

Drugs used

Hexamethonium Bromide (HEX; Sigma-Aldrich) and tetrodotoxin (TTX) (Abcam, Cambridge, MA, USA) were prepared as stock solutions and diluted in physiological saline daily before addition to preparations.

Histology

The colon was harvested and placed in a 10% formalin solution overnight and then transferred into 70% ethanol the following day. Paraffin embedded colon sections were cut 5 μm thick and de-waxed in a 60 °C oven for 30 min. To examine the morphological changes to the colon, a standard Hematoxylin and Eosin staining protocol was followed.[26,27]

Immunohistochemistry in wholemount preparations

Colon sections (2–3 cm) were placed in oxygenated phosphate-buffered saline (PBS; pH 7.2) containing nicardipine (3 μM; Sigma-Aldrich) for 20 min to inhibit smooth muscle contractions. Samples were cut open along the mesenteric border, cleared of their contents, maximally stretched and dissected mucosa up to expose the myenteric plexus attached to the longitudinal muscle layer. Tissues were fixed with Zamboni's fixative (2% formaldehyde, 0.2% picric acid) overnight at 4 °C. Preparations were cleared of fixative by washing 3 × 10 min with DMSO followed by 3 × 10 min washes with PBS. Fixed tissues were stored at 4 °C in PBS for a maximum of 5 days.

Wholemount preparations were incubated with 10% normal donkey serum (Chemicon, EMD Millipore Corporation, Billerica, MA, USA) for 1 h at room temperature. Tissues were then washed (2 × 5 min) with PBS and incubated with primary antibodies against Protein Gene Product 9.5 (PGP9.5) (chicken, 1 : 500; Abcam), neuronal nitric oxide synthase (nNOS; goat, 1 : 500; Abcam), and choline acetyl transferase (ChAT; goat, 1 : 200; Abcam) overnight at 4 °C. Tissues were then washed in PBS (3 × 10 min) before incubation with species-specific secondary antibodies labeled with different fluorophores: donkey anti-chicken Alexa 594 (1 : 200; Jackson Immuno Research Laboratories, West Grove, PA, USA) and donkey anti-goat Alexa 488 (1 : 200; Jackson Immuno Research Laboratories) for 2 h at room temperature. Wholemount preparations were given 3 × 10 min final washes in PBS and then mounted on glass slides using fluorescent mounting medium (DAKO, Glostrup Municipality, Hovedstaden, Denmark). Wholemount preparations were observed under a Nikon Eclipse Ti laser scanning microscope (Nikon, Tokyo, Japan), eight randomly chosen images from each preparation were captured with a 20× objective and processed using NIS Elements software (Nikon). The number of PGP9.5, nNOS, and ChAT immunoreactive neurons was quantified in the myenteric ganglia within a 2 mm^2 area of each preparation.

Immunohistochemistry in cross-sections

Colon sections (1–2 cm) were placed in oxygenated PBS containing nicardipine (3 μM; Sigma-Aldrich) for 20 min to inhibit smooth muscle contractions. Samples were cut open along the mesenteric border, cleared of their contents, and pinned mucosa up without stretching. Tissues were fixed with Zamboni's fixative overnight at 4 °C. Preparations were cleared of fixative by washing 3 × 10 min with DMSO (Sigma-Aldrich) followed by 3 × 10 min washes with PBS. After washing, tissues were embedded in 100% OCT and frozen using liquid nitrogen (LN_2) and isopentane (2-methyl butane) and stored in −80 °C freezer. Tissues were cut at 20 μm section thickness using Leica CM1950 cryostat (Leica Biosystems, Wetzlar, Hesse, Germany), adhered to slides and allowed to rest for 30 min at room temperature before processing.

Cross-section preparations were incubated with 10% normal donkey serum (Chemicon) for 1 h at room temperature. Tissues

were then washed (2 × 5 min) with PBS and incubated with primary antibodies against CD45 (Rat, 1 : 500; BioLegend, San Diego, CA, USA), overnight at 4 °C. Sections were then washed in PBS (3 × 10 min) before incubation with secondary antibodies labeled with fluorophore donkey anti-rat Alexa 488 (1 : 200; Jackson Immuno Research Laboratories) for 2 h at room temperature. The sections were given 3 × 10 min final washes in PBS and then cover slipped using fluorescence mounting medium (DAKO). Sections were viewed under a Nikon Eclipse Ti laser scanning microscope (Nikon), eight randomly chosen images from each preparation were captured with a 20× objective and processed using NIS Elements software (Nikon). The number of $CD45^+$ immunoreactive cells was quantified within a 2 mm^2 area in every colonic section.

Imaging

Three dimensional (z-series) images of wholemount preparations were taken using a Nikon Eclipse Ti laser scanning microscope (Nikon). Fluorophores were visualized using excitation filters for Alexa 594 Red (excitation wavelength 559 nm), Alexa 488 (excitation wavelength 473 nm), and Alexa 405 (excitation wavelength 405 nm). Z-series images were taken at step size of 1.75 μm (1600 × 1200 pixels).

Quantification of fecal lipocalin-2

Fresh fecal pellets were collected from mice after 3, 7, and 14 days of 5-FU administration and stored immediately at −80 °C. Fecal samples were thawed and suspended in PBS with 0.1% Tween (100 mg/mL) overnight prior to processing. The next day samples were centrifuged at 13.8 *g* at 4 °C for 10 min. Samples were then agitated and centrifuged for another 5 min at 12 000 rpm at 4 °C. Supernatant was collected and lipocalin-2 levels were quantified using DuoSET ELISA Mouse Lipocalin-2 kit (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

Data were assessed using two-way ANOVA, Welch's two-tailed *t*-test, and Student's two-tailed *t*-test. Analyses were performed using Graph Pad Prism (Graph Pad Software Inc., San Diego, CA, USA). Data are presented as mean ± SEM. Value differences were considered statistically significant at $p < 0.05$.

RESULTS

Altered gastrointestinal transit following 5-FU administration

To determine the effects of 5-FU administration on gastrointestinal transit, series of radiographic images were used to track barium sulfate throughout the gastrointestinal tract before the first injection (day 0), after two injections (day 3), three injections (day 7), and six injections (day 14; Figs 1 and 2, Table 1). The speed of barium movement was calculated by tracing barium entry from one part of the gastrointestinal tract to the next. After 3 days of 5-FU administration, movement of barium in both the cecum and colon was faster than before treatment (Fig. 2A), however, after 7 and 14 days of 5-FU administration barium movement was significantly delayed compared to day 0 in the cecum and colon (Fig. 2A, Table 1).

Although tracing barium movement allowed analysis of real time transit speed, gastrointestinal organ filling and emptying does not happen simultaneously, thus we further analysed the time taken for complete barium emptying from specific regions.

No changes in gastric emptying time were observed at day 3 of 5-FU administration, but significant delays in gastric emptying were seen after 7 and 14 days of 5-FU administration (Fig. 2B, Table 1). Intestinal emptying was faster after 3 days of 5-FU administration, but significantly delayed after both 7 and 14 days of 5-FU administration (Fig. 2C, Table 1).

Pellet formation time was decreased after 3 days of 5-FU administration (Fig. 2D), but significantly increased after 7 and 14 days of 5-FU administration (Fig. 2D). Thus, increased intestinal transit was observed after short-term 5-FU treatment, while prolonged treatment induced delays in gastrointestinal transit.

Changes in colonic motility following 5-FU treatment

To investigate effects of 5-FU treatment on colonic motility, excised colons were studied in organ bath experiments at day 14 of 5-FU treatment. The total number of contractions (including all types of motor patterns in the colon: CMMCs, short and FCs, Fig. 3A) was increased in colons from 5-FU-treated animals compared to sham-treated mice (sham: 27.7 ± 1.4, 5-FU: 36.1 ± 2.1, $p < 0.01$, Fig. 3B). To determine if this increase was due to changes in a specific type of motor activity, the frequency, proportion, and propagation speed were analysed for each type of motor pattern.

Colonic migrating motor complexes were defined as sustained anally directed contractions propagating more than 50% of the colon length. Colonic migrating motor complexes are mediated mainly by myenteric neurons, although inputs from the mucosa may modulate their activity.[28] *In vivo* treatment with 5-FU was associated with a decrease in the frequency of CMMCs in comparison with sham-treated mice (sham: 11.2 ± 0.6, 5-FU: 3.5 ± 1.0, $p < 0.0001$, Fig. 3B). Similarly, the proportion of CMMCs following 5-FU treatment was significantly decreased compared to sham-treated mice (sham: 41.1 ± 3.0%, 5-FU: 12.4 ± 3.8%, $p < 0.0001$, Fig. 3C). No significant difference in the speed of CMMCs was found between sham-treated and 5-FU-treated mice (sham: 2.9 ± 0.2 mm/s, 5-FU: 2.3 ± 0.3 mm/s, Fig. 3D).

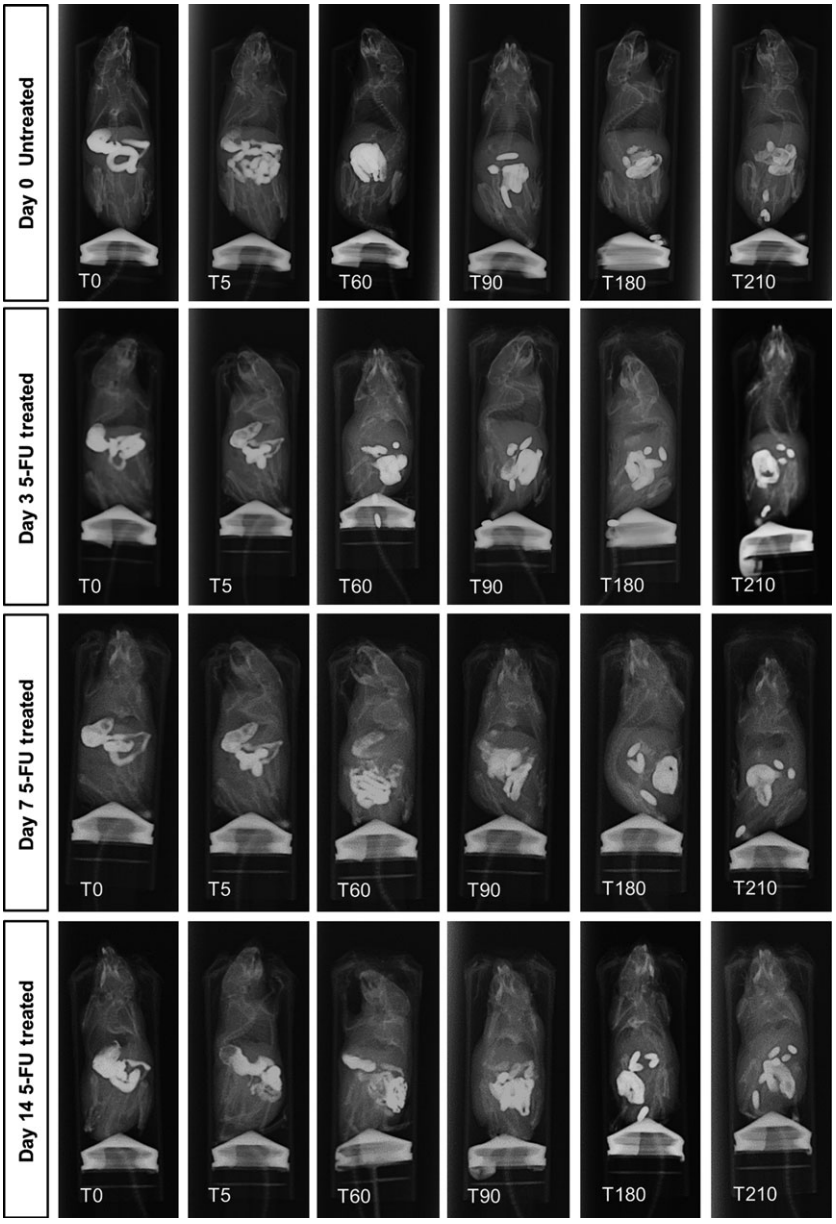

Figure 1 X-ray images following repeated *in vivo* 5-FU (23 mg/kg, 3 times/week) administration. Representative x-ray images obtained from mice 0–210 min after intragastric barium sulfate (0.4 mL, 2.5 mg/mL) administration at day 0 (prior to 1st injection) and following 3, 7, and 14 days of 5-FU administration.

Contractions that propagated less than 50% of the colon length were termed SCs (Fig. 3A). Treatment with 5-FU resulted in both increased frequency (sham: 10.1 ± 1.1, 5-FU: 20.5 ± 3.0, $p < 0.01$, Fig. 3B) and increased proportion (sham: 36.0 ± 2.2%, 5-FU: 54.2 ± 4.1%, $p < 0.01$, Fig. 3C) of SCs in the colon. A significant increase in the speed of SCs was found following 5-FU administration when compared to sham-treated mice (sham: 0.7 ± 0.0 mm/s, 5-FU: 1.9 ± 0.3 mm/s, $p < 0.01$, Fig. 3D). Incomplete contractions that did not propagate, but rather occurred concurrently over the length of the colon were defined as FCs. Both the frequency (sham: 6.4 ± 0.8, 5-FU: 11.8 ± 1.2, $p < 0.01$, Fig. 3B) and the proportion (sham: 22.9 ± 2.3%, 5-FU: 32.5 ± 1.8%, $p < 0.01$, Fig. 3C) of FCs were significantly higher in 5-FU-treated compared to sham-treated mice. No significant difference in the speed of FCs was found between sham-treated and 5-FU-treated mice (sham: 1.5 ± 0.1 mm/s, 5-FU: 2.1 ± 0.3 mm/s, Fig. 3D).

To investigate whether neurogenic components are involved in the recorded colonic motor patterns, a voltage-gated sodium channel blocker TTX and a nicotinic cholinergic antagonist HEX were added into the organ baths. TTX (1 μM, 20 min) abolished CMMCs, SCs, and FCs in colons from both sham and 5-FU-treated mice (Fig. 3E), indicating that these motility patterns are neurogenic. However, a persistent TTX-resistant

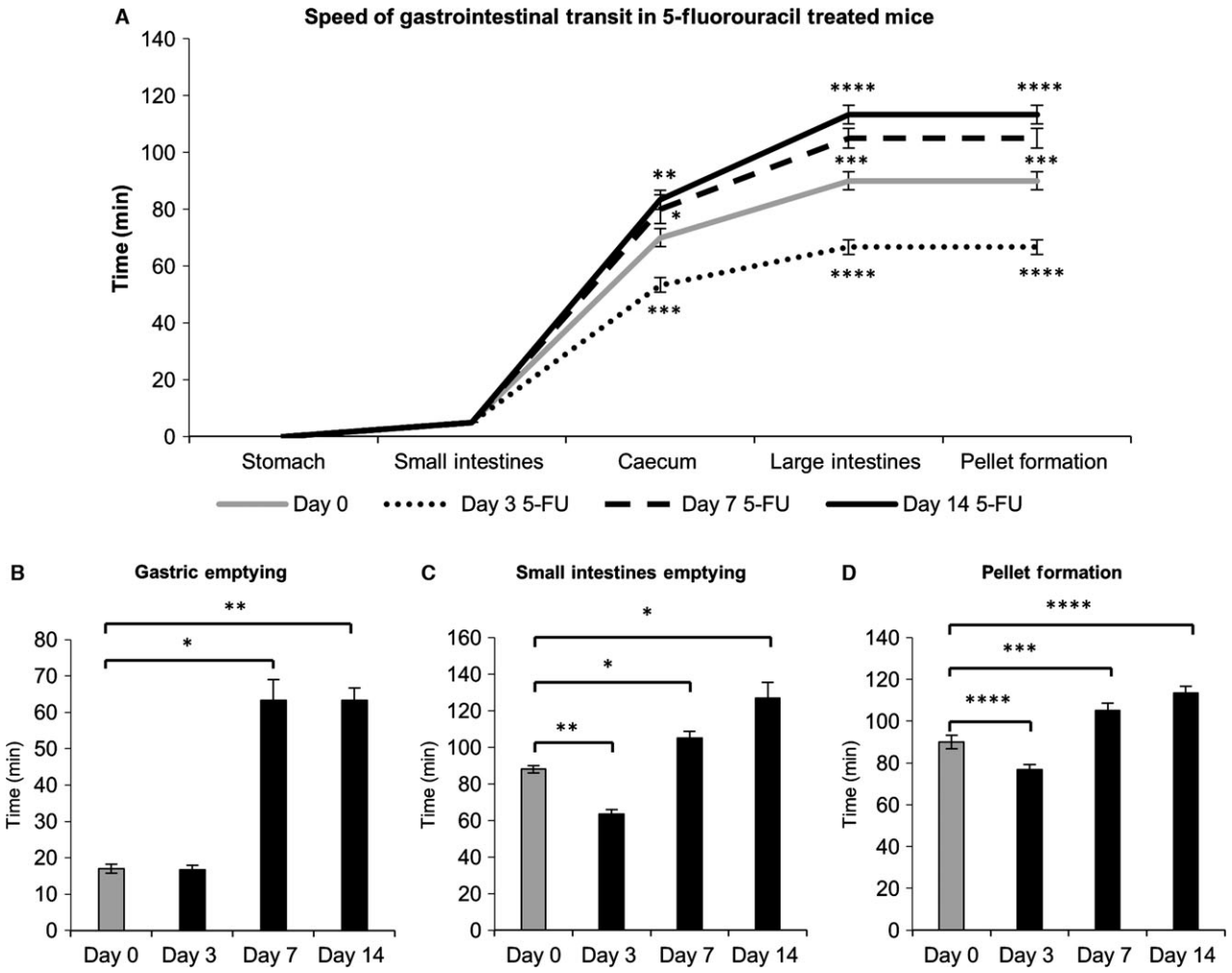

Figure 2 Gastrointestinal transit time, gastric and intestinal emptying following repeated *in vivo* 5-FU administration. (A) Time (min) taken for barium sulfate to reach the stomach, small intestines, cecum, and large intestines before (day 0) and at 3, 7, and 14 days following 5-FU administration. (B) Time (min) taken for complete emptying of barium from the stomach. (C) Time (min) taken for complete emptying of barium from the small intestines. (D) Time (min) taken to form first pellet before (day 0) and at 3, 7, and 14 days following 5-FU administration. Data represented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, $n = 6$ per group/time point.

tonic constriction in the distal part of the colon blocked by HEX (100 μM, 20 min) was observed in sham-treated mice. In 5-FU-treated mice, TTX-resistant tonic constrictions were prominent in both proximal and distal parts of the colon; the proximal tonic constriction was not blocked by application of HEX (Fig. 3E).

Morphological damage to the colon following 5-FU administration

Colonic architecture from sham-treated animals at days 3, 7, and 14 appeared healthy with a visible brush border and uniform crypts (Fig. 4A–C). Histological examination of the colon at day 3 from the 5-FU-treated group demonstrated no obvious changes to the epithelial brush border; however, there was a severe loss of colonic crypts and goblet cells and cellular infiltration within the lamina propria when compared to the sham-treated group (Fig. 4A and A′). By days 7 and 14, there was thickening of the epithelial brush border indicative of regeneration of the colonic crypts in the 5-FU-treated groups, but they still appeared short, distended, and disorganized with an increased number of goblet cells (Fig. 4B′ and C′) when compared to colon from the sham-treated group (Fig. 4B and C).

Intestinal inflammation following 5-FU treatment

To investigate if 5-FU treatment causes inflammation, immune cell infiltration in the colon and the concentration of a neutrophil gelatinase-associated protein,

Table 1 Speed of transit and emptying following repeated *in vivo* 5-fluorouracil administration

Parameters measured	Day 0	Day 3	Day 7	Day 14
Speed of transit (time to reach each region, min)				
Stomach	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Small intestines	5 ± 0	5 ± 0	5 ± 0	5 ± 0
Cecum	70 ± 3	53 ± 3***	80 ± 5*	83 ± 4**
Large intestines	90 ± 3	66 ± 3****	105 ± 4***	113 ± 4****
Time for complete barium emptying (min)				
Gastric emptying	17 ± 1	16 ± 1	63 ± 6*	63 ± 3**
Intestinal emptying	88 ± 2	63 ± 3**	105 ± 4*	126 ± 8*
Pellet formation	90 ± 3	66 ± 3****	105 ± 4***	113 ± 4****

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, significantly different to Day 0 ($n = 5$ per/group).

lipocalin-2, a highly sensitive biomarker for intestinal inflammation[29,30] in fecal samples were analysed.

Immune cells in colonic-cross sections were labeled with a pan leukocyte marker anti-CD45 antibody following 3 (Fig. 5A and A′), 7 (Fig. 5B and B′), and 14 (Fig. 5C and C′) days of sham and 5-FU treatment. Total numbers of CD45 positive cells were counted within a 2 mm^2 area. A significant increase in the number of CD45 positive cells was found in the colon following 3 days of 5-FU administration (99 ± 2, $p < 0.0001$) when compared to sham (48 ± 1, Fig. 5D). No significant changes in the number of CD45 positive leukocytes were found after 7 (sham: 49 ± 2, 5-FU: 53 ± 3) and 14 (sham: 51 ± 4, 5-FU: 54 ± 0.1) days of treatment (Fig. 5D). A significant increase in the concentration of lipocalin-2 was observed at day 3 after 5-FU administration (1592 ± 160 ng, $p < 0.05$) compared to sham-treated mice (883 ± 100 ng), but not at days 7 (sham: 946 ± 230 ng, 5-FU: 867 ± 210 ng) and 14 (sham: 1122 ± 160 ng, 5-FU: 881 ± 120 ng) (Fig. 5E).

Reduction in the total number of myenteric neurons and changes in neuronal subpopulations following administration of 5-FU

To investigate any changes to the total number of myenteric neurons, wholemount preparations of the colon were labeled with anti-PGP9.5 antibody to count neurons within a 2 mm^2 area. Repeated *in vivo* administration of 5-FU induced myenteric neuronal loss at days 7 (sham: 1225 ± 6; 5-FU: 1150 ± 5, $p < 0.001$) and 14 (sham: 1229 ± 3; 5-FU: 1091 ± 5, $p < 0.001$), but not at day 3 (sham: 1234 ± 4; 5-FU: 1231 ± 10, Fig. 6).

To determine if 5-FU administration was associated with changes in subpopulations of myenteric neurons controlling intestinal muscle activity, inhibitory muscle motor, and interneurons IR for nNOS (Fig. 7) and neurons IR for ChAT (Fig. 8) were analysed.

Fewer nNOS-IR neurons were observed at days 3 (sham: 357 ± 7; 5-FU: 286 ± 3, $p < 0.0001$) and 14 (sham: 380 ± 11; 5-FU: 310 ± 3, $p < 0.0001$) following 5-FU administration (Fig. 7A′ and C) when compared to sham (Fig. 7A and C). However, no reduction in the number of nNOS-IR neurons from the 5-FU-treated group was observed at day 7 (sham: 363 ± 5; 5-FU: 341 ± 4; Fig. 7B, B′ and D). The proportion of nNOS-IR neurons was reduced on day 3 (sham: 29.5 ± 0.3%; 5-FU: 23.8 ± 0.3%) when compared to sham ($p < 0.01$, Fig. 7E), but not on days 7 (sham: 29.3 ± 0.6; 5-FU: 29.7 ± 0.3) and 14 (sham: 30.9 ± 0.9; 5-FU: 28.5 ± 0.4, Fig. 7E).

The total number of ChAT-IR neurons increased on day 3 (sham: 294 ± 4; 5-FU: 319 ± 4), but decreased on days 7 (sham: 322 ± 2; 5-FU: 300 ± 4) and 14 (sham: 320 ± 3; 5-FU: 282 ± 5) following 5-FU administration (Fig. 8A′, B′, C′, and D) when compared to the sham-treated group (Fig. 8A–D). However, no significant difference in the proportion of ChAT-IR neurons was observed at any time point after 5-FU administration began (Fig. 8E).

DISCUSSION

This study is the first to examine 5-FU-induced enteric neuropathy and its effect on gastrointestinal function. Our results show that 5-FU administration causes a significant increase in gastrointestinal transit time at day 3, but delays in intestinal and gastric emptying at days 7 and 14. Significant reductions in the frequency and proportion of CMMCs, but increased frequency and proportions of short and FCs were prominent after 14 days of 5-FU treatment. All types of contractions were abolished by TTX; a residual tonic constriction in the distal colon was blocked by HEX in both sham and 5-FU-treated mice. A TTX-resistant tonic constriction of the proximal colon seen in 5-FU–treated mice was not blocked by HEX. Severe crypt ablation and mucosal destruction occurred in the colon at day 3. Consistent crypt disorganization and hypoplasia persisted throughout the experimental period; however, mucosal barrier regeneration was evident after both 7 and 14 days. Acute inflammation, confirmed by increased levels of fecal lipocalin-2 and CD45 positive leukocytes in the colon, was seen on day 3 of 5-FU treatment but subsided by days 7 and 14. Loss of myenteric neurons was seen on days 7 and 14, accompanied by

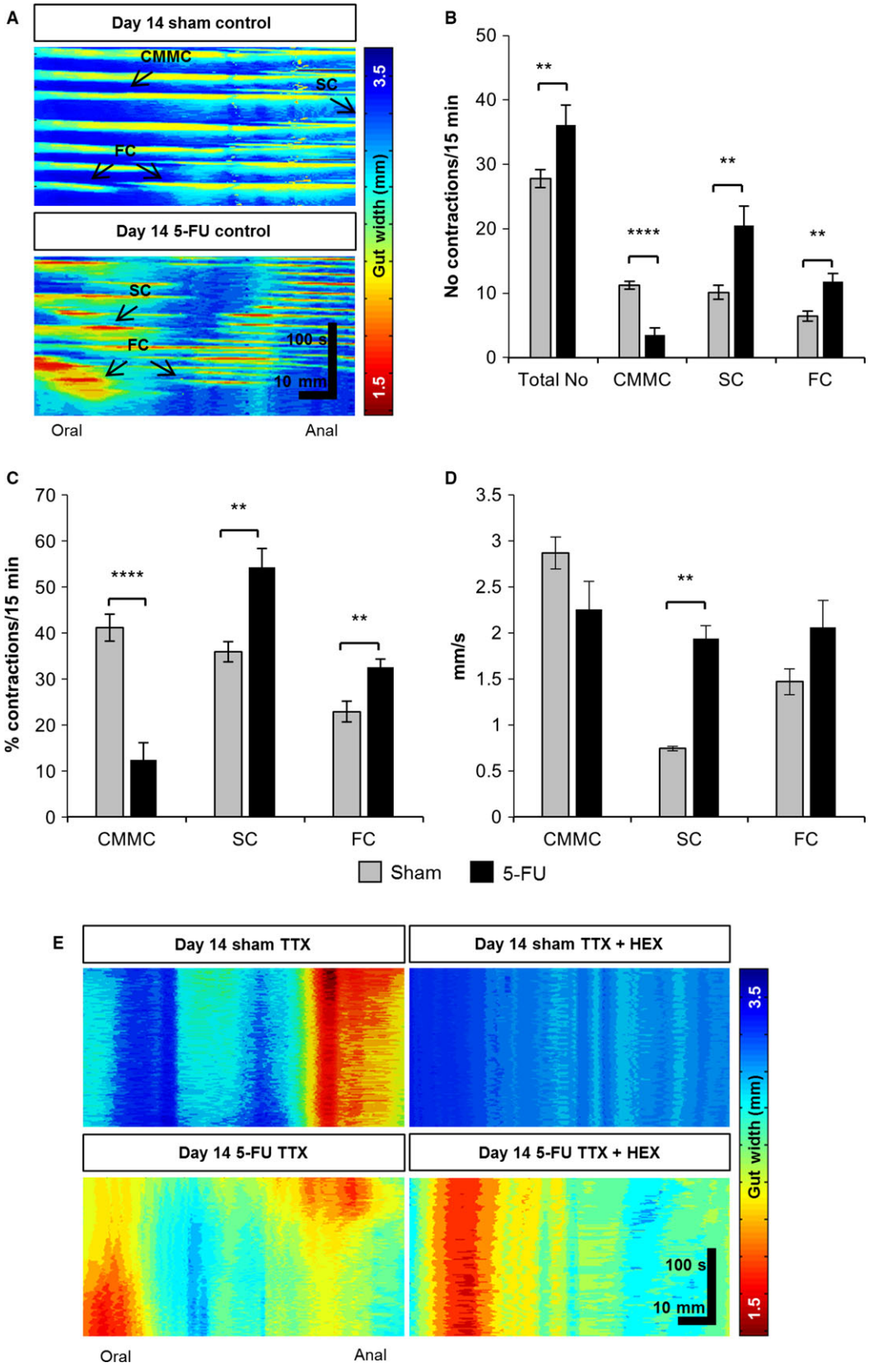

Figure 3 Total number, proportion, and speed of different contractions following repeated *in vivo* 5-FU administration. (A) Representative spatiotemporal maps generated from digital video recordings of colonic motility from sham and 5-FU-treated mice. Each contraction can be seen as a reduction in the gut width (red), while relaxation as an increase in the gut width (blue). Colonic migrating motor complexes (CMMCs) propagate >50% of the colon length, short contractions (SCs) propagate <50% of the colon length and fragmented contractions (FCs) are interrupted by period(s) of relaxation during contraction. (B) Total number of contractions including all types of contractile activity in the colons from sham and 5-FU-treated mice. (C) The proportion of CMMCs, SCs, and FCs to the total number of contractions. (D) The speed of CMMCs, SCs, and FCs. Data represented as mean ± SEM. $^{**}p < 0.01$, $^{****}p < 0.0001$, $n = 6$ per group/time point. (E) Spatiotemporal maps from day 14 sham and 5-FU-treated mice after addition of tetrodotoxin (TTX, 1 μM, 20 min) and hexamethonium (HEX, 100 μM, 20 min).

reduced numbers of ChAT-IR neurons at these time points.

Although 5-F U is routinely used in the treatment of CRC and is known to cause gastrointestinal side-effects, the neurological mechanisms underlying these side-effects have not been studied.[31,32] Our study demonstrates that, while short-term 5-FU treatment accelerates overall gastrointestinal transit without a change in gastric emptying, more prolonged treatment results in significant delays to overall gastrointestinal transit, gastric emptying and intestinal emptying. Our findings are consistent with previous studies showing that short- and long-term administration of the chemotherapeutic agent cisplatin differentially alters gastrointestinal transit.[19,20] Our findings suggest that mechanisms underlying these differential changes in transit are different. Accelerated gastrointestinal transit observed after 3 days of treatment was associated with acute intestinal inflammation and reduced proportions of nNOS-IR neurons. Intestinal inflammation was resolved after days 7 and 14 when delays to overall gastrointestinal transit, gastric, and intestinal emptying, and colonic dysmotility were observed. The loss of myenteric neurons observed at these time points might contribute to the functional changes in the gastrointestinal tract. Gastric and intestinal transit has previously been investigated using radioactivity retention technique in rats where it was found that delayed gastric empting and increased fundus and duodenum muscle contractions occurred 3 and 15 days after a single high dose of 5-FU (150 mg/kg) injection.[13] However, our study is the first to use radiographic analysis to evaluate overall gastrointestinal transit alongside ENS damage following 5-FU administration in mice. Postinflammatory delays to gastric and intestinal emptying have been reported in a plethora of gastrointestinal disorders.[33–35] Gastric emptying of solids is significantly delayed in patients with postinfectious functional dyspepsia, irritable bowel syndrome, non-obstructive Crohn's disease and colitis.[34–38]

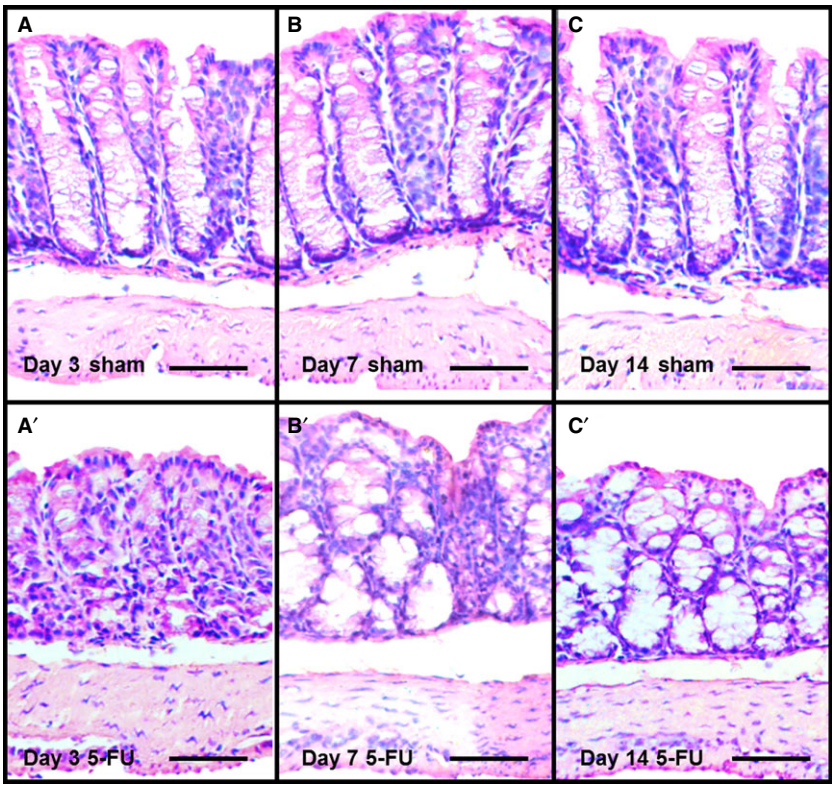

Figure 4 Gross morphological changes in the colon following repeated *in vivo* 5-FU administration. H&E staining in the colon from sham and 5-FU -treated mice at 3 (A, A′), 7 (B, B′), and 14 (C, C′) days. Scale bar = 100 μm, $n = 4$ per group/time point.

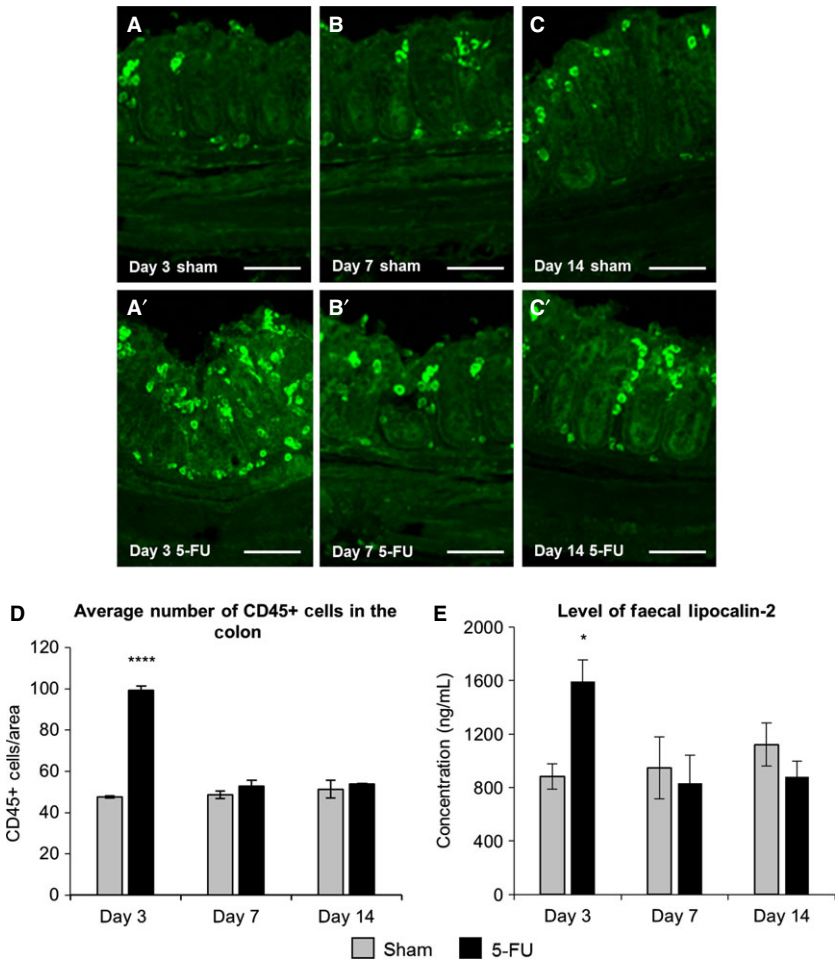

Figure 5 Inflammatory markers in the colon and fecal pellets. Cross-sections of the colon labeled with antibody against $CD45^+$ leukocytes (green) following 3 (A, A′), 7 (B, B′), and 14 (C, C′) days of sham or 5-FU treatment, scale bar = 50 μm. Average number of $CD45^+$ cells in the colon was counted per 2 mm^2 at 3, 7 and 14 days in both sham and 5-FU treated mice. (D) Concentration of lipocalin-2 (ng/mL) in fecal pellets collected following 3, 7, and 14 days of 5-FU administration (E). Data represented as mean ± SEM. *$p < 0.05$, ****$p < 0.0001$, $n = 4$ per group/time point.

Mucosal inflammation, epithelial degradation and intestinal ulceration, manifesting as mucositis, following 5-FU administration have been suggested as causes of gastrointestinal dysfunction.[12] Our results show that, while short-term 5-FU treatment was associated with destruction of the epithelial brush border and severe loss of colonic crypts and goblet cells, chronic treatment did not worsen epithelial damage. In fact regeneration of mucosa was evident during chronic 5-FU treatment, although crypts still appeared shorter, distended, and disorganized. These results are consistent with previous findings in the rat duodenum following 3 days of 5-FU treatment, where loss of crypt architecture, shortening of villi and inflammatory infiltration in the lamina propria was reported.[13] This was followed by the restoration of intestinal villi and crypts following 15 days of 5-FU treatment, but with sparse neutrophil infiltration in the muscular layer.[13] Such evidence indicates that duodenal mucosal inflammation resolves during 15 days of 5-FU treatment.

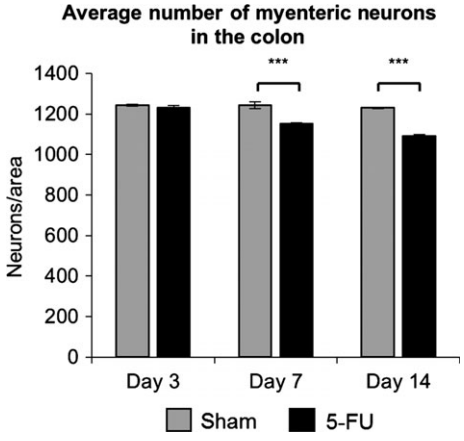

Figure 6 Effect of repeated *in vivo* 5-FU administration on the total number of myenteric neurons. Average number of PGP9.5-IR neurons in the colon was counted per 2 mm^2 at 3, 7, and 14 days in both sham and 5-FU-treated mice. Data represented as mean ± SEM. ***$p < 0.001$, $n = 6$ per group/time point.

Both *in vitro* and *in vivo* treatment with 5-FU promote the attraction of inflammatory cells.[39] Intestinal inflammation is associated with gastrointestinal dysmotility both at the site of inflammation and at distant non-inflamed sites.[40–42] Further to this a

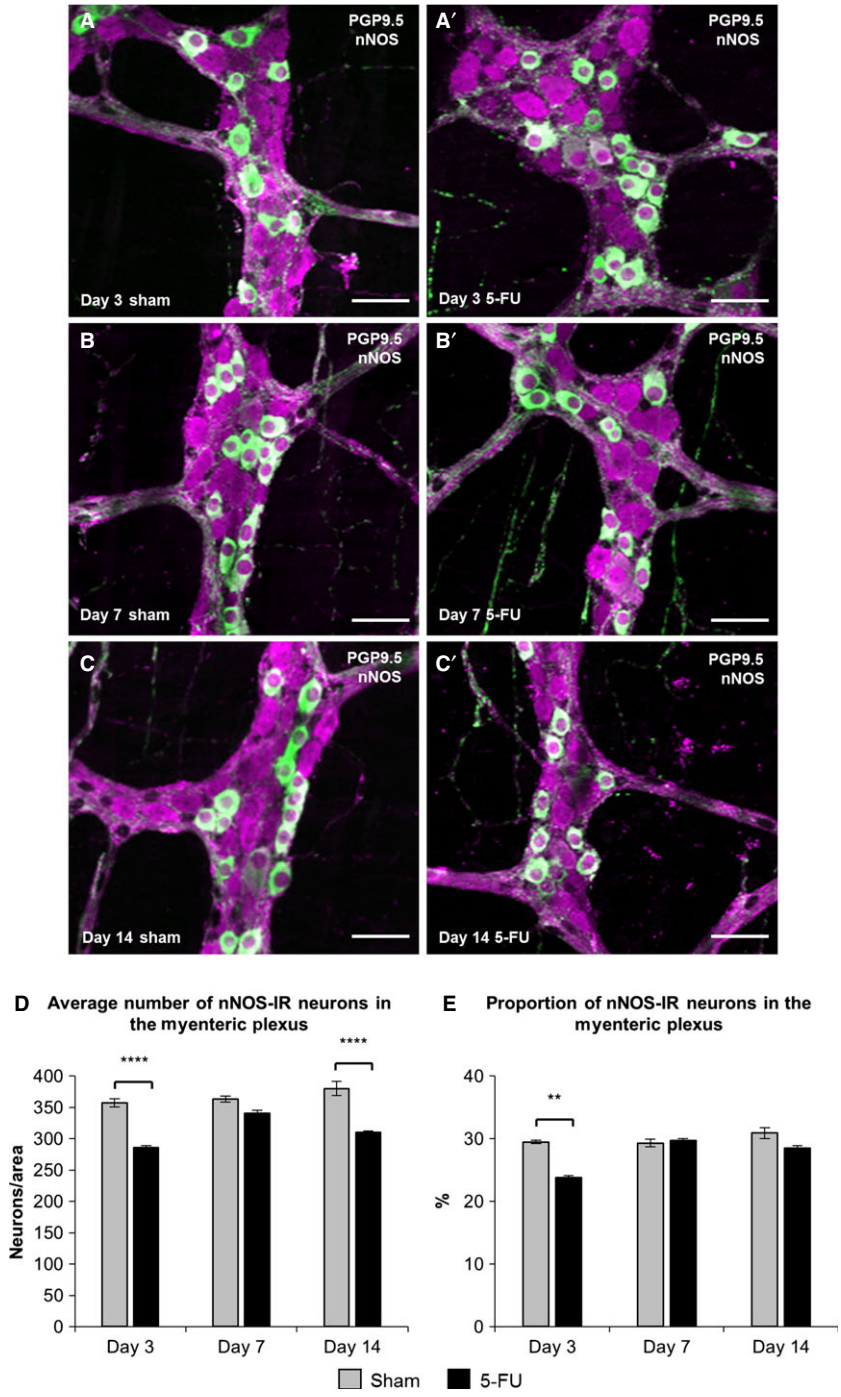

Figure 7 Effect of repeated *in vivo* 5-FU administration on average number and proportion of nNOS-IR myenteric neurons. Wholemount preparations of myenteric neurons in colon following 3 (A, A′), 7 (B, B′), and 14 (C, C′) days of sham or 5-FU treatment, scale bar = 50 μm. (D) Average number of nNOS-IR neurons (green in A–C) in the colon was counted per 2 mm^2 at 3, 7 and 14 days in both sham and 5-FU treated mice. (E) Proportion of nNOS-IR neurons (green in A–C) to the total number of PGP9.5-IR myenteric neurons (magenta in A–C) in the colon was counted at 3, 7, and 14 days in both sham and 5-FU-treated mice. Gray column: sham-treated, black column: 5-FU-treated. Data represented as mean ± SEM. $^{**}p < 0.01$, $^{****}p < 0.0001$, $n = 6$ per group/time point.

number of inflammatory mediators have been implicated in both short- and long-term smooth muscle contractility changes.[43] Accordingly, many gastrointestinal side-effects experienced during and after 5-FU administration have been attributed to inflammation-induced mucosal damage which we observed in the rat model of acute toxicity induced by a high dose (150 or 300 mg/kg) of 5-FU (R. Abalo, unpublished data) and in this study after a short-term treatment of mice with a low dose (23 mg/kg) of 5-FU. However, 5-FU-induced gastrointestinal dysmotility appears to outlast intestinal mucositis,[13] indicating that a more complex and multifaceted pathophysiology is at play.

Our results show that chronic 5-FU administration results in significant neuronal loss in the myenteric plexus of the colon at days 7 and 14 when intestinal

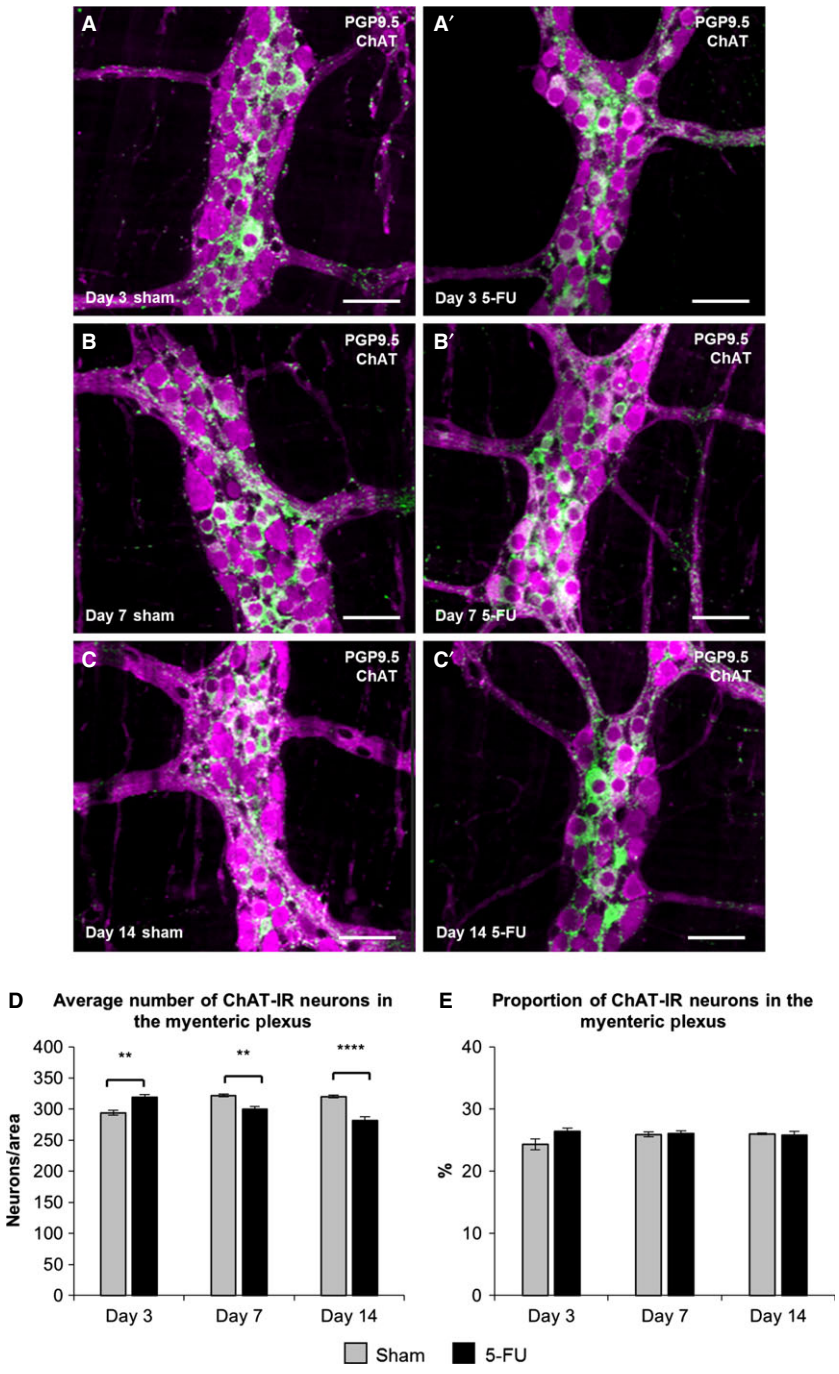

Figure 8 Effect of repeated *in vivo* 5-FU administration on the average number and proportion of ChAT-IR myenteric neurons. Wholemount preparations of myenteric neurons in the colon following 3 (A, A′), 7 (B, B′), and 14 (C, C′) days of sham or 5-FU, scale bar = 50 μm. (D) Average number of ChAT-IR neurons (green in A–C) in the colon was counted per 2 mm^2 at 3, 7, and 14 days in both sham and 5-FU-treated mice. (E) Proportion of ChAT-IR neurons (green in A–C) was counted to the total number of PGP9.5-IR myenteric neurons (magenta in A–C) in the colon was counted per 2 mm^2 at 3, 7, and 14 days in both sham and 5-FU treated mice. Gray column: sham-treated, black column: 5-FU-treated. Data represented as mean ± SEM. $^{**}p < 0.01$, $^{****}p < 0.0001$, $n = 6$ per group/time point.

inflammation subsided. Our findings are consistent with previous studies investigating effects of platinum-based anticancer chemotherapeutics, which also demonstrated that long-term treatments induce loss of myenteric neurons.[15,16] However, 5-FU treatment induced less severe damage to myenteric neurons with 12% loss at day 14 compared to 25% of myenteric neuronal loss at day 14 of oxaliplatin treatment.[15] Although previous studies demonstrated that the loss of enteric neurons does not necessarily lead to changes in CMMCs[44,45] our results demonstrated that the loss of myenteric neurons correlated with colonic dysmotility in 5-FU-treated mice. The loss of myenteric neurons leads to long-term alterations in gastrointestinal functions as was shown in rats after cisplatin treatment[16] and mice after oxaliplatin treatment.[15] 5-FU treatment inhibited CMMCs, but the total number of contractions of the isolated colon was significantly increased due to an increase in the number and

proportion of short and FCs. Short and FCs play a vital role in the construction of productive motor patterns in the healthy intestine.[25] Short distance contractions result in a segmenting motor pattern essential for mixing and absorption of colonic contents.[46] Alteration in short segmenting contractions induces intestinal dysmotility.[47]

Our results demonstrated that all types of contractions were neurogenic; residual tonic constriction in the distal colon was blocked by HEX in both sham and 5-FU-treated mice. Tetrodotoxin-resistant tonic constriction in the proximal colon observed in 5-FU–treated mice was not blocked by HEX. These results suggest that there is TTX-insensitive tonic excitatory drive to the smooth muscle that presumably involves at least one nicotinic synapse and hence must involve neurons with TTX-resistant action potentials like those described by Foong *et al.* in the mouse submucosal plexus.[48] This activity seems to be differentially sensitive to 5-FU. Thus, initial acute inflammation caused by 5-FU treatment may have led to persistent changes in the ENS and gut dysfunction. Similarly, in animal models of intestinal inflammation, long-term gut dysfunction persists long after resolution of acute inflammation due to neuronal loss, axonal damage, and neuronal dysfunction in the myenteric plexus leading to changes in synaptic transmission between neurons and to the smooth muscles.[26,49–54]

We found that long-term treatment with 5-FU induced loss of both excitatory (ChAT-IR) and inhibitory (nNOS-IR) neurons in the myenteric plexus. However, only nNOS-IR neuron proportions were reduced at day 3 post 5-FU treatment, which is consistent with increased intestinal transit at this time point. Loss of nNOS-IR neurons was also observed in cisplatin and oxaliplatin-treated animals[15,16] however, ChAT-IR neurons were not investigated in these studies. Thus, our results suggest that the loss of neurons after 5-FU treatment is not restricted to a specific neurochemically defined subpopulation. Differential effects of various chemotherapeutic compounds on enteric neurons might be attributed to the differences in their mechanisms of action with platinum-based compounds having direct neurotoxicity due to platinum adducts to DNA[55] compared to inflammation-induced neuropathy associated with 5-FU treatment.[13]

Damage to the intestinal smooth muscles, alterations in neuromuscular transmission, smooth muscle sensitivity to neurotransmitters and morphological and functional changes of interstitial cells of Cajal might be also involved in 5-FU-induced dysmotility and these require further investigation.

In conclusion, this study is the first to demonstrate that 5-FU administration induces accelerated gastrointestinal transit associated with acute intestinal inflammation at day 3 post-treatment, which may have led to persistent changes in the ENS and gastrointestinal dysfunctions: delayed gastrointestinal transit, gastric and intestinal emptying, and colonic dysmotility. The loss of myenteric neurons observed at days 7 and 14 of treatment may contribute to the functional changes in the gastrointestinal tract. Further research investigating the effects of 5-FU on the electrophysiological properties of myenteric neurons is warranted.

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTION

RMM conception and design, collection, analysis and interpretation of data, manuscript writing; VS collection, analysis and interpretation of data, manuscript revision; ED interpretation of data, manuscript revision; RA interpretation of data, manuscript revision; JCB interpretation of data, manuscript revision; KN conception and design, interpretation of data, manuscript revision. All authors approved final version of the manuscript.

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