Anatomical and Functional Changes to the Colonic Neuromuscular Compartment after Experimental Spinal Cord Injury

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Abstract

A profound reduction in colorectal transit time accompanies spinal cord injury (SCI), yet the colonic alterations after SCI have yet to be understood fully. The loss of descending supraspinal input to lumbosacral neural circuits innervating the colon is recognized as one causal mechanism. Remodeling of the colonic enteric nervous system/smooth muscle junction in response to inflammation, however, is recognized as one factor leading to colonic dysmotility in other pathophysiological models. We investigated the alterations to the neuromuscular junction in rats with experimental high-thoracic (T3) SCI. One day to three weeks post-injury, both injured and age-matched controls underwent *in vivo* experimentation followed by tissue harvest for histological evaluation. Spontaneous colonic contractions were reduced significantly in the proximal and distal colon of T3-SCI rats. Histological evaluation of proximal and distal colon demonstrated significant reductions of colonic mucosal crypt depth and width. Markers of intestinal inflammation were assayed by qRT-PCR. Specifically, *Icam1*, *Ccl2* (MCP-1), and *Ccl3* (MIP-1 α) mRNA was acutely elevated after T3-SCI. Smooth muscle thickness and collagen content of the colon were increased significantly in T3-SCI rats. Colonic cross sections immunohistochemically processed for the pan-neuronal marker HuC/D displayed a significant decrease in colonic enteric neuron density that became more pronounced at three weeks after injury. Our data suggest that post-SCI inflammation and remodeling of the enteric neuromuscular compartment accompanies SCI. These morphological changes may provoke the diminished colonic motility that occurs during this same period, possibly through the disruption of intrinsic neuromuscular control of the colon.

Keywords: colonic motility; myenteric plexus; neurogenic bowel; smooth muscle; tunica muscularis

Introduction

SPINAL CORD INJURY (SCI) triggers profound physiological alterations in multiple organ systems of the individual. In addition to the motor and sensory losses, autonomic nervous system regulation of the cardiovascular system and visceral organs is compromised severely. Bowel dysfunction, often referred to as neurogenic bowel, is one of the most prevalent and clinically recognized comorbidities associated with SCI¹ and is manifested as diminished colonic transit, constipation, evacuation dyssynergy, and overflow incontinence. Subsequently, bowel dysfunction is a major physical and psychological challenge that reduces quality of life.^{2,3} Surveys among the SCI population often rank colorectal, bladder, and sexual dysfunction as significant obstacles and prioritize recovery of bowel function above the ability to walk.^{4–6} Despite this priority, pre-clinical investigation of bowel dysfunction is profoundly lacking.

Bowel dysfunction is a multi-factorial consequence of SCI. Focus is often directed at the result of damage to the supraspinal regulation of somatic^{7,8} and autonomic circuitry of the spinal

cord^{9–11} and is frequently compartmentalized as post-SCI deficits in storage and evacuation. The gastrointestinal tract, including the colon, however, is unique in that it has its own extensive intrinsic nervous system known as the enteric nervous system (ENS). The ENS has the ability to function quasi-autonomously and provides the local reflex motor programming necessary for secretion, propulsive movement of colonic contents, and colonic segmentation.¹² Colonic compliance and the propulsive capacity of this neuromuscular interface are dependent ultimately on the muscular properties of the intestinal wall.

Experimental models of inflammatory bowel disease (IBD) have demonstrated pathophysiology of the neuromuscular compartment. It has been proposed that recurring inflammatory insults to the colon provoke thickening of the muscle wall and progressive fibrosis, both of which impact compliance and propulsive capacity. Significant morphological changes after experimentally induced colitis, assessed six days or 21 days after induction, include thickening of the muscle layer as well as increased collagen deposition throughout the tunica muscularis.¹³ Myenteric alterations were also noted and may explain the disrupted motor activity that

persists long after resolution of the inflammatory bout (reviewed in¹⁴). Because of the profound and persistent physiological changes after SCI, inferring parallel mechanistic changes with IBD must be made with caution. Integrated data on the colonic pathophysiology of inflammatory and fibrotic processes associated with SCI in humans recently have received systematic, although semiquantitative, investigation and identified an increase in collagen within the longitudinal muscle layer as well as a general reduction in myenteric neuronal density.¹⁵

Determining the suitability of a validated animal model of SCI to investigate the acute and long-term physiological changes in the neuromuscular compartment is necessary for identifying appropriate therapeutic strategies for the SCI population. In the present study, we used our established rodent model of T3 spinal level SCI to test the hypothesis that T3-SCI would provoke pathophysiological tissue remodeling and a reduction in colonic contractility. We investigated whether: (1) experimental T3-SCI leads to diminished local colonic smooth muscle contractility; (2) T3-SCI provokes alterations in the muscular compartment similar to that observed after colitis; (3) T3-SCI provokes pathophysiological changes to the colonic lumen; (4) T3-SCI results in an increase of colonic collagen infiltration; and (5) T3-SCI results in the loss of enteric neurons.

Methods

All procedures followed National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee at the Penn State Hershey College of Medicine. Male Wistar rats ≥ 8 weeks old (total $n = 104$, Hsd:WI, Stock 001, Harlan, Indianapolis, IN) on entrance into the experiment, and initially weighing 175–200 g were used and double housed in a room maintained at 21–24°C and a 12:12-h light-dark cycle with food and water provided *ad libitum*.

Surgical procedures and animal care

Rats were assigned randomly for SCI or surgical control surgery, and each group was divided further into three-day ($n = 20$ total) and three-week ($n = 20$ total) post-surgical study groups. For studies of colonic inflammation, two additional T3-SCI or surgical control cohorts of one-day ($n = 16$ total) and seven-day ($n = 16$ total) post-surgical duration were generated as well as a separate set of three-day ($n = 16$ total) and three-week ($n = 16$ total) T3-SCI or surgical control cohorts. Before surgical procedures, rats were anesthetized deeply with isoflurane (2–3%, 1 L/min O₂) as necessary to achieve areflexia (absence of palpebral reflex) and were maintained at 35.5–37.5°C on a feedback-controlled heating block; rectal temperature was monitored continuously (TCAT 2LV, Physitemp Instruments, Clifton, NJ).

As described previously,¹⁶ all animals were administered ophthalmic ointment to both eyes, buprenorphine SR (1 mg/kg, subcutaneously [SC], Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA) to alleviate post-operative pain, and antibiotic agents (Enroflox, 10 mg/mL concentration at 1 mL/kg SC, Bayer, Shawnee Mission, KS) to reduce post-surgical infection before any surgical manipulation. Once a deep plane of anesthesia was achieved, the skin overlying vertebral thoracic levels 1–3 (T1–T3) was shaved and cleaned with three alternating applications of Nolvasan (chlorhexidine acetate, Fort Dodge Animal Health, Fort Dodge, IA) and ethanol. The surgical site was incised 3–4 mm along the midline, and the underlying spinous processes were cleared of all musculature. Using fine tipped rongeurs, spinal T3 was exposed via laminectomy of the T2 spinous process, which extended laterally to the T2 transverse processes.

The rats were placed in the Infinite Horizon controlled impact device (Precision Systems and Instrumentation, LLC, Lexington, KY) and clamped via the T1 and T3 spinous processes. Once se-

cure, a rapid 300 kDyn force (15 sec dwell time) was applied to the cord and overlying dura. Procedures for the control animals were the same as for spinal injury except that the spinal cord and surrounding dura mater were not disturbed after laminectomy. On completing the surgical procedure, the muscle tissue overlying the lesion site was closed in anatomical layers with polyglactin suture and the skin closed with 9 mm wound clips. Wound clips were removed five to seven days after operation as necessary. Animals were administered warmed supplemental fluids (5 mL lactated Ringer solution, SC) and placed in an incubation chamber maintained at 37°C until the effects of anesthesia had subsided.

After operation, animals were single housed in corn cob filled animal housing tubs on a warming unit (Gaymar T-pump, Stryker, Kalamazoo, MI) to maintain a warmed environment ($\sim 25^\circ\text{C}$). Because of the necessity for single housing, each cage contained segments of 8 cm diameter plastic tubes as environmental enrichment. Rats that received T3-SCI received subcutaneous supplemental fluids (5–10 mL lactated Ringer solution) twice daily, and antibiotic agents (Enroflox, 10 mg/kg) once daily for five days after operation. Manual compression of the bladder was performed at least twice daily in animals with T3-SCI until the return of spontaneous voiding. The ventrum of control animals was inspected daily without need for manual compression of the bladder.

Because of the reduction in locomotor capacity after T3-SCI, a reservoir of chow was placed at head level to facilitate ease of access for feeding. All T3-SCI rats ingested a measureable amount each day, thereby confirming that access to chow was available. Body weight and food intake were measured daily for all animals as an index of well-being. Animals were fasted overnight before experimentation and euthanasia.

In vivo physiological surgical instrumentation

After three days or three weeks after the initial surgical procedure, animals were fasted overnight, water provided *ad libitum*, before being deeply re-anesthetized for *in vivo* physiological instrumentation with thiobutabarbital (Inactin; Sigma, St. Louis, MO; 75–150 mg/kg intravenously [IV]), which does not affect long-term cardiovascular¹⁷ or gastrointestinal¹⁸ autonomic function. Animals were placed on a feedback-controlled warming pad (TCAT 2LV, Physitemp Instruments) and maintained at $37 \pm 1^\circ\text{C}$ for the duration of the experiment.

Once fully anesthetized for physiological instrumentation, the animal was intubated tracheally by way of a 1–2-cm midline incision on the ventral side of the neck caudal to the mandible toward the sternal notch. Tracheal intubation maintains an open airway and facilitates clearing of respiratory secretions if necessary. The underlying strap muscles were separated using blunt dissection at the midline to expose the trachea. The exposed trachea was isolated from the underlying esophagus to place a loop of 3-0 Ethilon^{TM} suture between the trachea and esophagus to form a ligature. The trachea was opened ventrally by making a small incision in the membrane between two of the cartilaginous rings of the trachea just caudal to the thyroid gland. A small piece of polyethylene tubing (PE-270, 5 mm in length and beveled at one end) was inserted into the trachea and secured in place with the ligature. The strap muscles were returned to their proper anatomical location, and the overlying skin was secured around the tracheal tube with 3-0 Ethilon.

Pressure transducer implantation consisted of two 3.5F Mikro-Tip® catheter pressure transducers (Millar SPR-524, Millar Instruments; Houston, TX) that were inserted into the proximal and distal colon. For the proximal colon, a midline laparotomy was made to locate the cecum. Once isolated, a small incision was made immediately distal to the cecum, and the transducer was inserted approximately 2 cm. Surgical glue was used to secure the catheter and seal the incision. For the distal colon, the probe was lubricated and introduced into the rectum and distal colon such that the tip of the transducer was positioned approximately 4 cm from the rectum.

Because excessively manipulating the colon can provoke changes in colon physiology (see¹⁹), the transducer was placed in an area of colon that was sufficiently absent of fecal pellets (≥ 1 cm) to minimize handling of the tissue by the experimenter. Fecal pellets from the distal colon were displaced only if absolutely necessary. Signals from Mikro-Tip catheters were amplified and recorded continuously on a computer using SciWorks DataWave software (DataWave Technologies; Loveland, CO), and after 1 h of stabilization, 10 min of baseline colonic responses were recorded from both the proximal and distal colon.

Tissue harvest

At the conclusion of *in vivo* physiological experiments, deeply anesthetized rats received a pneumothorax and were exsanguinated via cutting the left ventricle of the heart. For each animal, colonic tissue (proximal and distal) was isolated and collected. Tissue samples were preserved in one of two manners: (1) Fresh tissue samples were placed in aluminum foil and then immediately flash frozen in liquid nitrogen, or (2) for hematoxylin and eosin (H&E), Masson trichrome staining, or immunohistochemistry, tissue samples were post-fixed in 4% paraformaldehyde for 24 h and then transferred to 70% ethanol for an additional 24 h before paraffin embedding.

At the completion of intestinal tissue collection, the vertebrae encompassing the spinal cord lesion level were removed, and the entire vertebral tissue sample was post-fixed overnight in refrigerated phosphate buffered saline (PBS) containing 4% paraformaldehyde. After three to seven days, the dorsal surface of the individual vertebrae was removed and the spinal cord extracted for re-emersion in fresh refrigerated PBS containing 4% paraformaldehyde and 30% sucrose before embedding for cryosectioning.

All rats designated for quantitative reverse transcription polymerase chain reaction (qRT-PCR) quantification ($n=8$ per each surgical condition $\times$ four post-operative time points) were euthanized without previous physiological experimentation. To gain a greater temporal picture of the inflammatory process, animals were grouped into one-, three-, and seven-day and three-week post-operative cohorts. Deeply anesthetized rats were exsanguinated via decapitation at the same time (09:00–10:00). For each animal, colonic tissue (proximal only) was isolated and placed in aluminum foil and then immediately flash frozen in liquid nitrogen.

Histological processing

For histological staining of T3-SCI lesion extent, tissue was cryosectioned (40 μ m thick), and alternating sections were mounted on gelatin coated slides. To compare lesion severity with the spinal cords of control animals, spinal cord sections were stained with Luxol fast blue (LFB) to visualize myelinated fibers. The LFB-stained slides were imaged digitally on a Zeiss Axioscope light microscope and Axiocam CCD camera, imported into Adobe Photoshop and contrast digitally adjusted to allow consistent identification of LFB-stained (i.e., spared) white matter.

For individual images, the boundaries of the tissue slice were outlined to determine cross-sectional area. A separate threshold histogram was generated, and the pixels corresponding to LFB staining above background were selected. These pixels were quantified and expressed per unit cross-sectional area.²⁰ The lesion epicenter was defined as the section with the least proportion of LFB-stained tissue. The proximity of the T3 lesion center to the cervical enlargement precluded an appropriate determination of spinal cord cross-sectional area in undamaged tissue rostral to the injury (i.e., damaged tissue extended into the cervical enlargement as described in.²¹ Therefore, it was necessary that the cross-sectional area of the intact spinal cords at T3 of comparably sized animals be determined for normalization purposes. The LFB-stained myelin in injured tissue was then expressed as a percent of the total spinal cord cross-sectional area as would be predicted by the intact tissue.

Whole colon histology and histochemistry were performed using paraformaldehyde-fixed tissue samples from the colon. Two to three discontinuous portions of the sample tissue were placed in the same embedding cassette and processed in an automated Tissue-Tek VIP processor and paraffin-embedded with a Tissue-Tek TEC embedding station (Sakura Finetek USA, Torrance, CA). The *en bloc* samples were cross-sectioned serially at 6 μ m, and multiple sets of nonadjacent serial sections were mounted on Plus-slides (Thermo Fisher Scientific, Pittsburgh, PA) and processed for H&E staining or histochemical staining for collagen (Masson trichrome; Abcam; Cambridge, MA). All slides were marked with a non-identifying numeric code to prevent observer bias during analysis.

Image analysis of the tunica muscularis from both the proximal and distal colon cross sections were captured in three different, randomly selected, regions at 20 $\times$ magnification. Using the Zeiss image acquisition software ZEN 2, the entire tissue area and the area of only the tunica muscularis was recorded as described previously.²² Collagen content of the tunica muscularis was quantitatively estimated by Masson trichrome histochemical stain to differentiate collagen fibers (blue) and muscle fibers (red) within the proximal and distal colonic tunica muscularis.

Colonic H&E sections were analyzed for quantification of colonic crypt depth and width using the same Zeiss Zen software. A minimum of three different, randomly selected, regions imaged at 20 $\times$ magnification were used. To maintain consistency of analysis, only crypts on naturally occurring submucosal folds were selected. In addition, a subset of colonic H&E sections was examined by an American College of Veterinary Pathologists diplomate also blinded to treatment and the consistency of results verified across observers. Tissues sections were analyzed by measuring the crypt depth and width and calculating crypt depth:width ratio.

Enteric neuronal immunohistochemistry was performed using serially adjacent 6 μ m sections of the paraffin embedded colon tissue; samples were taken and subjected to heat-mediated antigen retrieval in 10 mM sodium citrate buffer for 20 min. Nonspecific antibody binding was reduced by incubation in UltraVision Protein Block (TA060PBQ; Thermo Scientific) for 5 min at room temperature. The sections were incubated for 24 h at 4°C in a primary antibody cocktail that comprised antihuman neuronal protein C and D antibody (mouse monoclonal anti-HuC/D; dilution 1:200; Life Technologies, A21271) and 2% bovine serum albumin (BSA). HuC/D is used commonly as an enteric pan-neuronal marker (see²³), and was employed as such in our studies. After the incubation, the tissue underwent three 2 min washes in wash buffer and then was incubated in a secondary antibody cocktail for 2 h at room temperature (anti-mouse immunoglobulin G [IgG]-peroxidase antibody; dilution 1:100; Sigma-Aldrich, A0168 and 2% BSA). Labeling was localized using diaminobenzadine (DAB) and sections were counterstained with hematoxylin.

Using a previously described protocol to determine the number of neurons per enteric ganglion area,²⁴ images of HuC/D positive ganglia from each tissue section were analyzed using ImageJ software (NIH, Bethesda, MD). Individual ganglia demonstrate variable cross-sectional size within the intact myenteric plexus, and this might be reflected in what region of the ganglion appears in the plane of the section. While reduction of the ganglion area could be expected as a result of neuronal loss, differences in cross-sectional area are deemed to be a greater source of variability. To normalize quantification of the number of neurons, the total ganglion area was measured (as μ m²), the number of HuC/D positive neurons within that ganglion were counted, and calculated as the number of neurons per unit area.

Ribonucleic acid (RNA) isolation, RT reaction, and qRT-PCR

The qRT-PCR was used to quantify the level of inflammatory mediators present at the assigned time points. Tissue sections from the proximal colon were analyzed for intercellular adhesion

molecule-1 (*Icam1*), monocyte chemotactic protein (*Ccl2*), and macrophage inflammatory protein-1 α (*Ccl3*), after T3-SCI and control surgical procedures. Nomenclature is presented according to Rat Genome Nomenclature Committee guidelines (www.informatics.jax.org/mgihome/nomen/gene.shtml) along with more common, informal usage. These molecules are reported commonly in the scientific literature and were selected as reliable biomarkers of gastrointestinal pathophysiology.²⁵

Using a previously reported protocol,¹⁶ whole colon tissue sections were used for RNA isolation. Briefly, frozen tissue was homogenized in TRIzol (Invitrogen, Carlsbad, CA) followed by RNeasy Microkit procedures (Qiagen, Valencia, CA). The RT was conducted using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). For RT, $\sim 1 \mu\text{g}$ of RNA from each sample was added to random primers ($10\times$), dNTP ($25\times$), MultiScribe reverse transcriptase ($50 \text{ U}/\mu\text{L}$), RT buffer ($10\times$), and RNase Inhibitor ($20 \text{ U}/\mu\text{L}$) and incubated in a thermal cycler (Techne TC-412, Barloworld Scientific, Burlington, NJ) for 10 min at 25°C , then for 120 min at 37°C . Primers for *Actb* (β -actin) were a QuantiTect Primer Assay (Rn_Actb_1_SG QuantiTect Primer Assay QT00193473, Qiagen, Frederick, MD). Primers for *Icam1*, *Ccl2* (MCP-1), and *Ccl3* (MIP-1 α) were designed using Primer Express (Applied Biosystems, Foster City, CA). The forward and reverse primer pairs were reported previously.¹⁶

Real-time PCR used SYBR Green $2\times$ Master Mix (Qiagen) according to previous description.¹⁶ The quantity of *Icam1*, *Ccl2* (MCP-1), and *Ccl3* (MIP-1 α) mRNA was based on a standard curve and normalized to *Actb* (β -actin) mRNA (ABI QuantStudio 12KFlex with available OpenArray block, Applied Biosystems). The suitability of *Actb* as an internal control was assessed through analysis of the raw data between groups, and no variability of *Actb* was detected.

Statistical analysis

The occurrence of outliers was tested using a Grubbs test macro written in GraphPad (GraphPad Software, La Jolla, CA). No significant outliers were identified. All subsequent statistical calculations were performed using SigmaPlot software (version 12.5; Systat Software, Inc., San Jose, CA). Between group results from anatomical or *in vivo* studies were compared by analysis of variance (ANOVA) and Tukey *post hoc* analysis. Results are expressed as means $\pm$ standard error of the mean (SEM) with significance defined as $p < 0.05$.

Results

At the termination of the experiment, the severity of experimental T3-SCI was quantified before further data analysis. The extent of the

FIG. 1. Spinal cord injury (SCI) at T3 results in a significant decrease of the frequency and amplitude of basal giant migrating contractions within the proximal colon. Representative traces from (A) three-day and (B) three-week surgical control and T3-SCI rats. After three weeks, the frequency (C) and the amplitude (D) of giant migrating contractions were significantly reduced in the injured animals ($*p < 0.05$; C & D). Values expressed as mean $\pm$ standard error of the mean.

lesion epicenter was determined based on the reduction of LFB-stained white matter at the T3 spinal cord segment. The percent area of white matter at the lesion epicenter of three-day T3-SCI rats was $7 \pm 1\%$ and was significantly reduced in comparison with T3-control animals ($76 \pm 1\%$; $p < 0.05$). At three weeks, when the post-injury progression of the lesion epicenter has relatively stabilized and the lesion boundaries are more clearly defined,²⁶ the percent area of white matter at the lesion epicenter of three-week T3-SCI rats was $7 \pm 1\%$ and was significantly reduced in comparison with T3-control animals ($77 \pm 1\%$; $p < 0.05$). These data are comparable to the injury extent reported previously and indicate the severity of our injury model.^{21,27–29} One rat had 25% remaining white matter and was dropped from the experiment while all remaining T3-SCI rats were included for analysis.

Frequency and amplitude of baseline colonic contractions are reduced in T3-SCI rats

Contractions within the proximal colon appeared reduced in three-day T3-SCI rats ($n = 10$) compared with three-day controls but not significantly ($n = 10$, Fig. 1A, $p = 0.07$). This overall reduction was observed in the rats tested three weeks after the sur-

gical procedure ($n = 10$ T3-SCI, $n = 10$ control, Fig. 1B). After three weeks, frequency of contractions was significantly lower after T3-SCI (Fig. 1C, $p < 0.05$), and the mean amplitude of contractions was also significantly lower (Fig. 1D, $p < 0.05$).

Simultaneous testing of distal colon contractions revealed a more profound reduction in T3-SCI rats (Fig. 2A). The frequency of distal colon contractions after three days recovery was significantly lower after T3-SCI (Fig. 2C, $p < 0.05$) as well as the mean amplitude of contractions in three-day T3-SCI rats (Fig. 2D, $p < 0.05$). After three weeks of recovery, the T3-SCI rats still displayed deficits in distal colon contractions (Fig. 2B). While the frequency of distal colon contractions in three-week T3-SCI rats remained significantly lower than surgical controls (Fig. 2C, $p < 0.05$), the amplitude of the distal colon contractions was not different between injured and control rats ($p > 0.05$), however.

These results indicate that T3-SCI in the rat diminishes the spontaneous contractility of the colon, especially in the distal colon. Fewer contractions and diminished force could result in longer transit time of fecal material, possibly leading to constipation and impaction.

FIG. 2. Spinal cord injury (SCI) at T3 results in a significant decrease of the frequency and amplitude of basal giant migrating contractions within the distal colon. Representative traces from (A) three-day and (B) three-week surgical control and T3-SCI rats. At both time points, the frequency (C) of giant migrating contractions was significantly reduced in the injured animals ($*p < 0.05$). The amplitude of giant migrating contractions in the distal colon (D) was only significantly reduced in three-day T3-SCI rats ($*p < 0.05$). Values expressed as mean $\pm$ standard error of the mean.

T3-SCI changes the depth of mucosal crypts within the colon

After T3-SCI, we observed morphological changes of the mucosal crypts within the proximal and distal colon harvested from the animals used for physiological recordings. Compared with the proximal colons of controls, three-day T3-SCI rats had a nonsignificant reduction in crypt depth (Table 1, $p=0.07$) while after three weeks, T3-SCI rats had significantly shorter mucosal crypts (Table 1, $p<0.05$). In the distal colon, there was no significant difference in mucosal crypt depth between three-day T3-SCI rats and three-day controls. Mucosal crypt depth, however, was significantly shallower in three-week T3-SCI rats in comparison with three-week controls (Table 1, $p<0.05$). Further, the distal colon crypt depth was significantly lower between three-day and three-week rats with T3-SCI (Table 1, $p<0.05$). The crypt width within the proximal and distal colon did not significantly differ in both the three-day or three-week T3-SCI rats compared with controls (Table 1, $p<0.05$). It should be emphasized that the shorter distal colon crypt depth of three-week controls versus three-day controls was not statistically different ($p=0.1$).

T3-SCI increases colonic expression of inflammatory markers

To quantify colonic inflammation, total RNA was isolated to analyze expression of inflammatory markers commonly linked with gastrointestinal inflammatory processes.²⁵

In our experimental T3-SCI conditions, colonic *Ccl2* (MCP-1) expression was significantly different between T3-SCI and control only at three days (Fig. 3; $p<0.05$). Expression of the chemokine *Ccl3* (MIP-1a) demonstrated a significant increase (Fig. 3; $p<0.05$) at three days and seven days after T3-SCI. After 3-weeks, however, *Ccl3* expression was not significantly different between T3-SCI and control ($p>0.05$). The post-SCI expression of *Icam1* demonstrated a significant increase at three days and seven days after T3-SCI that also returned to stable levels within three weeks (Fig. 3; $p<0.05$).

TABLE 1. T3-SPINAL CORD INJURY PROVOKES A REDUCTION OF MUCOSAL CRYPT DEPTH IN THE DISTAL COLON AT THREE WEEKS AFTER INJURY

	Experimental groups	
	Control	T3-SCI
3-day		
Proximal Colon		
Crypt depth (μm)	172.9 $\pm$ 2.6	159.5 $\pm$ 3.5
Crypt width (μm)	39.7 $\pm$ 1.9	39.5 $\pm$ 0.9
Distal Colon		
Crypt depth (μm)	274.8 $\pm$ 9.3	252.8 $\pm$ 11.0
Crypt width (μm)	41.1 $\pm$ 3.1	42.2 $\pm$ 2.3
3-week		
Proximal Colon		
Crypt depth (μm)	181.4 $\pm$ 5.0	154.9 $\pm$ 6.1*
Crypt width (μm)	38.0 $\pm$ 1.2	35.1 $\pm$ 1.7
Distal Colon		
Crypt depth (μm)	241.6 $\pm$ 10.4	202.5 $\pm$ 6.4*#
Crypt width (μm)	33.9 $\pm$ 2.8	37.5 $\pm$ 1.7

SCI, spinal cord injury.

* $p<0.05$ vs. control, # $p<0.05$ vs. 3-day T3-SCI.

FIG. 3. Expression levels of inflammatory markers in the colon after T3-spinal cord injury (SCI). (A) Colonic *Ccl2* (MCP-1) mRNA expression was significantly altered only at three days after injury. (B) Expression of *Ccl3* (MIP-1a) mRNA expression demonstrated a significant (between-groups) elevation in T3-SCI rats at three and seven days compared with animals matched at the same post-operative time point. (C) *Icam-1* mRNA expression was also significantly elevated in T3-SCI rats at three and seven days compared with animals matched at the same post-operative time point. (* $p<0.05$ A, B, & C). Values expressed as mean $\pm$ standard error of the mean.

These results indicate that animals with T3-SCI demonstrate an inflammatory cascade within the colon that overlaps the onset of diminished smooth muscle contractions. The inflammatory cascade is not persistent and, unlike the reduction of smooth muscle contractions, resolves within three weeks.

Tunica muscularis of the colon undergoes morphological changes after T3-SCI

After T3-SCI, the thickness and collagen content of the tunica muscularis is significantly affected in both the proximal and distal colon harvested from the animals used for physiological recordings (Fig. 4). In the proximal colon, the tunica muscularis was significantly thicker in the three-week T3-SCI rats ($n = 10$) in comparison with three-week control rats as well as three-day rats (Fig. 5A, $p < 0.05$). There was no significant difference in thickness between the three-day T3-SCI rats and controls (Fig. 5A, $p > 0.05$). In the distal colon, the tunica muscularis was significantly thicker in the three-week T3-SCI rats versus controls (Fig. 5B, $p < 0.05$). Compared with controls, collagen infiltration of the smooth muscle was significantly greater in three-week T3-SCI rats (Fig. 5C, $p < 0.05$). In the distal colon of the same rats, collagen content was increased significantly in three-week T3-SCI rats (Fig. 5D, $p < 0.05$).

These results indicate that T3-SCI initiates progressive morphological changes to the muscular compartment of the proximal and distal colon. Increased muscle thickness and collagen reduce the compliance of the colon wall and may contribute to impaired motility.

T3-SCI results in loss of myenteric neurons within the colon

Experimental T3-SCI induced a progressive loss of myenteric neurons in the colon (Fig. 6). Quantitative analysis within the proximal colon showed that the number of HuC/D immunoreactive myenteric neurons normalized with regard to ganglionic area was significantly decreased three days after T3-SCI by 20% (Fig. 7A, $p < 0.05$). The distal colon of three-day T3-SCI rats revealed a similar, significant decrease of HuC/D immunoreactive myenteric neurons by 15% (Fig. 7B, $p < 0.05$).

As with other aspects of the neuromuscular compartment, this reduction was progressive such that the number of HuC/D immunoreactive neurons were lower three weeks after T3-SCI. Compared with surgical controls, enteric neurons in the proximal colon were significantly decreased by 35% in injured rats (Fig. 7A, $p < 0.05$). Within the myenteric ganglia of the distal

FIG. 4. Representative images of Masson trichrome-stained proximal and distal colon sections after control (top), three-day T3-spinal cord injury (SCI) (middle), or three-week T3-SCI (bottom). T3-SCI provokes an increase in muscle thickness (red) and collagen (blue, denoted by arrowheads within smooth muscle) infiltration after three weeks post-injury throughout the proximal and distal colon. ($\times 400$, scale bar, 500 μm).

FIG. 5. Graphic summary of the effects of T3-SCI on muscle thickness of the (A) proximal and (B) distal colon. Proximal and distal colon thickness demonstrated a significant increase only at three weeks after injury. Collagen infiltration of smooth muscle was significantly increased in the (C) proximal and (D) distal colon at three weeks after injury. Tissue analysis was normalized to equal area across all specimens, and collagen was expressed as the optical density of blue-labeled tissue within the normalized region of interest. (* $p < 0.05$ A, B, C, & D). Values expressed as mean $\pm$ standard error of the mean.

colon, a similar significant neuronal decrease of 36% was observed (Fig. 7B, $p < 0.05$).

These results indicate that T3-SCI initiates progressive morphological changes to the enteric motoneurons of the proximal and distal colon. Decreased neural input to the smooth muscle of the colon reduces the excitatory and inhibitory regulation of the colon wall and may contribute to impaired motility.

Discussion

In the present study, we have shown that experimental SCI provokes functional colonic impairment accompanied by remodeling of the colonic neuromuscular compartment. Our experimental data indicate that: (1) High thoracic (spinal level T3) SCI disrupts normal contractility of both the proximal and distal colon until at least three weeks post-injury; (2) colonic mucosal crypt depth was diminished for the proximal and distal colon at three weeks post injury; (3) T3-SCI induced a significant elevation in the colonic expression of inflammatory cytokine transcripts for *Icam1*, *Ccl2*, and *Ccl3*; (4) thickness of the smooth muscle layer was significantly increased in the proximal and distal colon by three weeks post-injury; (5) collagen deposition within the tunica muscularis increased significantly along the entire length of the colon beginning by three weeks post-injury, and (6) myenteric neuron density

was progressively reduced over three weeks after T3-SCI in both the proximal and distal colon.

Across species, the colon is a functionally heterogeneous organ serving several distinct and complex processes that vary depending on species digestive requirements. The intrinsic anatomical and physiological characteristics between humans and animals must be appreciated when extrapolating the results of these studies. Anatomically, the nomenclature identifying the ascending, transverse, descending, and sigmoid regions of the colon that are associated with the bipedal anatomy of humans is inappropriate for most quadrupedal animals. In particular, ascending and descending regions are not present, and this report follows the accepted terminology that is suitable for the laboratory rat, which identifies a prominent cecum and divides the colon as a proximal and a distal segment.

Functionally, the proximal-most region of the colon consists of the cecum, which serves as an important site of microbial digestion in hindgut fermenters such as rats.³⁰ The proximal colon serves to mix and store liquid feces, absorb excess fluid and electrolytes whereas the distal colon serves mainly to propel and expel dehydrated feces (reviewed in³¹). While these processes require slow propulsion and mixing, luminal contents must also be moved along relatively longer distances to limit the excessive reabsorption of water. The neuromuscular apparatus of the colon is designed to generate three unique types of contractile response to achieve these requirements.

FIG. 6. Representative images of HuC/D-immunoreactive tissue sections from the proximal and distal colon control (top), three-day T3-SCI (middle), or three-week T3-SCI (bottom). T3-SCI provokes a progressive reduction in HuC/D immunoreactivity in the myenteric ganglia (denoted by arrowheads). ($\times 400$, scale bar, 500 μm).

Rhythmic phasic contractions predominate in fasting and fed states and are characterized by slow waves and contractile electrical complexes to promote mixing and slow net distal propulsion of luminal contents. Giant migrating contractions are large amplitude contractions that occlude the lumen and propagate without inter-

ruption over relatively long distances to produce mass movements. Commonly, these contractions precede defecation. Tonic contractions increase the tone of the colonic wall, and it is believed that the reduction in lumen size enhances the propulsive and mixing movements of the phasic contractions and giant migrating contractions.

FIG. 7. Graphic summary of the effects of T3-spinal cord injury (SCI) on enteric neuronal HuC/D-immunoreactive neuronal numbers expressed as per unit area (10,000 mm^2) of myenteric ganglion. The number of HuC/D-positive neurons was progressively reduced after injury in the (A) proximal and (B) distal colon. (* $p < 0.05$ A, B). Values expressed as mean $\pm$ standard error of the mean.

Each of these fundamental contractile reflexes differs among species, both in duration and frequency as well as the neuromuscular interface that generates them. Specifically, unlike the finding for dogs and humans,^{32,33} phasic contractions are regarded as relatively inconsequential in the rat model where the predominant motor activity of the colon is composed of giant migrating contractions.³⁴ While this difference imposes certain limits on the cross-species comparability of our data, the mechanisms of reduced propulsive movement of colonic contents by giant migrating contractions are very clinically relevant to the SCI population. The giant migrating contractions comprising our luminal pressure data from neurally intact surgical controls are consistent with these previous reports in rodents.^{34–37}

The advantages in spatial resolution by quantifying giant migrating contractions with subminiature vascular pressure transducers over more traditional balloon pressure transducers has been discussed previously.^{35,38} Specifically, the present approach permitted simultaneous measurements within a 5–7-mm region of both the proximal and distal colon in contrast to the 2–3-cm of colon recorded frequently with a balloon manometer. Our observation of significantly fewer giant migrating contractions in both the proximal and distal colon of T3-SCI rats suggests that the profound fecal impaction reported previously³⁹ and that we also observe in our T3-SCI rats is not solely because of outlet obstruction produced by dyssynergy or hyperreflexia within the internal and external anal sphincters.⁷ Whereas anal sphincter reflexes are often presumed to be segmental in nature,^{7,40,41} diminished colonic contractions are associated with derangements in ENS function,^{42–45} and we propose that derangements of the enteric neuromuscular interface are likely the mechanism responsible for the reduced contractions after SCI.

In experimental preparations of normal or inflamed colon, giant migrating colonic contractions continue to occur in *ex-vivo* bath preparations,⁴⁶ thereby reinforcing the quasi-autonomous role of the ENS and revealing dysfunction of this enteric neuromuscular apparatus in disease.⁴⁷ The inconsistent differences in the amplitude of contractions in the proximal and distal colon may be the result of multiple neuronal and muscular changes within the tissue, and it would be overly speculative to try to ascertain the relative contributions of these compartments at this time.

Our previous observations have identified an upregulation of upper gastrointestinal expression of inflammatory cytokine transcripts for *Icam1* and *Ccl3* after T3-SCI.¹⁶ Our present data confirm that the upregulation of *Icam1*, *Ccl2*, and *Ccl3* after T3-SCI extends to the colon and that this colonic inflammatory response may be of longer duration compared with the upper gastrointestinal tract. Our previous report demonstrated further that reduced basal vascular perfusion of the upper gastrointestinal tract through the superior mesenteric artery accompanied this inflammatory response.¹⁶ The characteristic reduction in arterial blood pressure after SCI^{48,49} leads us to propose that a similar state of hypoperfusion likely exists for the inferior mesenteric supply to the distal colon. Reports suggest that the microvasculature of the ileum and colon are more susceptible to pathophysiological alterations, leading to or accompanying myenteric neuronal loss, in other disease models^{50,51} and are potential contributors to enteric pathophysiology in our model of SCI.

Further, the rapidly emerging understanding of the gastrointestinal microbiome suggests that changes in bacterial distribution and genetic composition may lead to derangements in the health and normal function of the gastrointestinal tract.⁵² Analyzing the regional microbial distribution within the gastrointestinal tract of Wistar rats, the species and strain used in this study, revealed a shift in phylum colonization of the colon after the experimental induc-

tion of diabetes.⁵³ At the present, post-SCI changes in microbial colonization have only been demonstrated for the mouse,⁵⁴ and further investigation is warranted.

Colonic mucosal layer pathologies, as evidenced by blunting of crypts, often accompany gastrointestinal disease or injury and may be the result of reactive oxygen species or dysregulation of gastrointestinal peptide synthesis and release (see⁵⁵). Our findings demonstrate that the temporal changes of the mucosal layer, as evidenced by proximal and distal colon crypt depth, occur up to three weeks later than the initial inflammatory cascade. It remains unclear when apoptosis or diminished proliferation of crypt cells occurs after T3-SCI and whether these pathophysiological changes resolve in longer-term animals.

In contrast to colonic mucosal and neuromuscular remodeling, post-SCI alterations in bladder wall thickness and collagen have received greater attention,^{56–58} and similar alterations in vascular smooth muscle are emerging.⁵⁹ In the present study, we observed an early onset of muscle wall thickening after T3-SCI that persisted in animals tested at three weeks after injury. Abnormal smooth muscle structure in response to inflammatory conditions is a hallmark of numerous pathophysiological states. The mechanisms leading to thickening of intestinal smooth muscle involve a complex interplay of epithelial barrier dysfunction, inflammatory cytokines, extracellular proteases, and oxidative imbalance.^{60–62} Each of the above mechanisms initiates smooth muscle hyperplasia and hypertrophy as well as collagen deposition. In animal models of inflammatory colitis, it has been shown that 2,4-dinitrobenzenesulfonic acid administration provokes muscle wall thickening and increased collagen deposition as early as six days.¹³ Our data recapitulate these findings in the context of a unique pathophysiological, rather than chemical, challenge. While our experiments revealed a significant increase in colonic wall thickness occurring by three weeks after T3-SCI, the duration of smooth muscle thickening, and the point at which such thickening becomes mechanically pathophysiological to impair colonic compliance, remains uncertain. The clinical literature, however, frequently reports that colonic compliance is diminished in individuals with chronic SCI.^{63–65}

Recently, an analysis of archival tissue from the spina bifida and SCI populations revealed neuromuscular remodeling of the colon.¹⁵ That study reported increased fibrosis of the smooth muscle, in the absence of changes to smooth muscle proteins, but no direct quantification of tissue thickness. While these changes in patient tissue are supportive of our observations, they are limited by an absence of patient demographics relating to injury severity, level, and duration, each of which are consistently controlled in our experimental animal model. Nonetheless, we propose that remodeling of smooth muscle remains an active process occurring frequently after SCI.

As discussed previously, proper colonic motility is dependent on the ENS for propulsive contractility. The diminished colonic contractions, combined with the morphological changes shown in this study, are consistent with other functional disorders such as colitis,^{66,67} slow-transit constipation,⁶⁸ ischemia and reperfusion,⁶⁹ and general aging.^{70,71} Some studies indicate a generalized pathology of the ENS or of the extrinsic innervation of the gastrointestinal tract, but numerous studies have shown that neurons that contain neuronal nitric oxide synthase (nNOS neurons) are more susceptible to damage than other enteric neurons.^{72–74}

The majority of nNOS-positive neurons provide local inhibitory innervation of the longitudinal muscle, circular muscle, and muscularis mucosa.⁷⁵ Specifically, colonic motility is the result of the opposing contributions of a cholinergic excitatory and a non-adrenergic, noncholinergic inhibitory circuit that includes these

nNOS-positive neurons.¹² The HuC/D protein is a neuron-specific protein that is used routinely to identify myenteric motor neurons; however, it labels all neurons but not specific phenotypes. This study demonstrates a significant decrease in enteric neuron numbers within the colon of T3-SCI rats, but further investigation is needed to determine whether there is a loss of a particular subpopulation of enteric neurons, specifically nNOS neurons.

It is clear that neurogenic bowel after SCI is a multi-factorial process. Our data complement recently reported human data by experimentally controlling injury location, severity, and duration to demonstrate a functional reduction in colonic giant migrating contractions in rats. These data further demonstrate a progressive impairment of the colonic neuromuscular interface immediately after T3-SCI that is consistent with observations based on archival tissue from humans with SCI. Because this interface is the final common pathway for functional contractions of the colon, changes to the neuromuscular interface must be considered to maximize the efficacy of therapeutic interventions designed to alleviate the colonic dysfunction observed in the SCI population.

Acknowledgments

The authors wish to acknowledge their gratitude to Joel L. Coble, Gina M. Deiter, and Emily N. Blanke for their assistance in multiple capacities. Dr. Timothy K. Cooper, DVM, PhD, provided expert analysis of histological sections as well as insightful direction during the early conceptualization of these studies.

This work was supported by National Institutes of Health Grant NINDS 49177 and Craig H. Neilsen Foundation Senior Research award (295319).

Author Disclosure Statement

No competing financial interests exist.

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