

## Impairment and restoration of spontaneous contractile activity of longitudinal smooth muscles in the TNBS-inflamed hamster distal colon

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### ABSTRACT

In the present study, we aimed to determine how inflammation affects spontaneous motility in the longitudinal direction of a hamster colon preparation. Trinitrobenzene sulfonic acid (TNBS) injected into the distal colon caused diarrhea 4–7 days after the treatment, but diarrhea was not observed in hamsters kept for 4 weeks. At 1 week after induction of colitis, spontaneous motility in the longitudinal direction was strongly suppressed. Contraction of longitudinal smooth muscles induced by electrical field stimulation was impaired, but not that induced by exogenously applied acetylcholine, indicating that acute inflammation preferentially impairs neurotransmissions with a minor effect on contractility of the longitudinal smooth muscle itself. The spontaneous motility reappeared in the colonic preparation isolated from the hamster maintained for 4 weeks after induction of colitis. The reappearance of the motility accompanied cholinergic and nitrenergic regulations of contractile activity. These results demonstrated that impairment and following restoration of spontaneous contractile activity of longitudinal smooth muscles in the TNBS-inflamed distal colon of the hamster may depend on the damage and recovery of neural factors, rather than alteration of muscle contractility.

The enteric nervous system (ENS) plays an essential role in the regulation of gastrointestinal function, with the ability to function independently from the central nervous system (7). During inflammatory processes of the intestine, the regulation by ENS is disrupted, leading to functional disorders such as diarrhea (22, 23, 26). Influence of inflammation of the intestine is not restricted to the mucosa but extends to the submucosa and muscularis externa, thus influencing neurotransmission and muscle contractility

(27). In fact, indiscriminate loss of adrenergic, cholinergic, purinergic and nitrenergic neurotransmission has been reported in the inflamed intestine (3, 8, 10, 18). Furthermore, myogenic contractions induced by high  $K^+$  and carbachol were decreased in trinitrobenzene sulfonic acid (TNBS)-induced colitis (11), indicating substantial damage of smooth muscle during the inflammatory process. In addition, the network between interstitial cells of Cajal (ICC) and the myenteric plexus is also disrupted by inflammation (4, 17, 29). Collectively, impairments of the ENS, ICC-myenteric network and smooth muscle contractility may cause disorder of intestinal motility.

In addition to the acute structural and functional changes, persistent alterations in gastrointestinal function are commonly observed after the resolution of intestinal inflammation (1). These include altered

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motility patterns, abnormal secretion and changes in visceral sensation (15, 22, 23). Recently it has been shown in the guinea pig colon that sustained alterations in enteric neural signaling, such as increased amplitude of fast excitatory postsynaptic potential in submucosal S neurons and shortened action potential durations and decreased afterhyperpolarization in AH neurons, were promoted following transient intestinal inflammation (16). Thus, the apparent recovery from inflammation is not necessarily accompanied by complete restoration of neural components.

Peristaltic reflex in the intestine can be classified into two phases; preparatory phase and emptying phase (13, 14). The preparatory phase is mainly achieved by contraction of longitudinal smooth muscle, whereas the emptying phase depends on the coordinated contraction of circular smooth muscle. Previous studies have shown that inflammation alters circular smooth muscle contractile properties and responses to neurotransmitters or nerve stimulation in the colon (5, 18, 25). In the present study, we aimed to determine how inflammation affects spontaneous motility in the longitudinal direction of a hamster colon preparation, with the expectation of better understanding of the effects of inflammation on colonic motility. Our results showed that spontaneous longitudinal contraction is strongly disrupted at 1 week after TNBS treatment but can be restored within 4 weeks. The disruption of spontaneous contraction seemed to originate in the impairment of the ENS rather than muscle contractility itself, and the recovery would be related to a restoration of neural components in the ENS.

## MATERIALS AND METHODS

*Experimental animals.* Syrian golden hamsters, 8–12 weeks of age and weighing 90–130 g, purchased from Seven-Sangyo (Gifu, Japan) were used. Upon receiving every group of experimental animals, they were housed for acclimatization in our colony at  $22 \pm 2^\circ\text{C}$ , with a 12-h light cycle, and given free access to laboratory chow (LABO MR Stock, Nihon-Nosan, Yokohama, Japan) and water. Care and experimental procedures were approved by the Animal Care and Use Committee of Gifu University, and all efforts were made to minimize animal suffering and to reduce the number of animals used.

*Induction of colitis.* Colitis was induced according to the method described previously (19) with slight modifications. After an overnight fasting, animals

were anaesthetized with pentobarbitone (50 mg/kg, ip). Then they were given 10 mg of TNBS dissolved in 0.25 mL of 40% ethanol (v/v) by means of a silicon catheter inserted 5 cm through the anus. The animals were maintained in a head-down position for 2 min to prevent leakage of the intracolonic instillate. The hamsters were maintained in their home cages and used at 1 week or 4 weeks after TNBS administration.

*Tissue preparation.* Colonic segments for mechanical recordings were prepared as previously described (6). Animals were anesthetized and exsanguinated via the carotid artery. The abdominal cavity was opened immediately and a 3–4-cm-long segment of the distal colon (2 cm advance to anus) was dissected out and immersed in physiological salt solution (PSS; see below) at room temperature. The intraluminal contents were flushed using a small cannula filled with PSS.

*Mechanical recordings.* Each segment of distal colon (2–3 cm in length) was mounted in longitudinal orientation in an organ bath (10 mL in capacity) filled with PSS (pH 7.4). The solution was continuously bubbled with 95% O<sub>2</sub> + 5% CO<sub>2</sub> gas mixture and maintained at 37°C. The distal end of each segment was tied to organ holders and the proximal end was secured with a silk thread to an isometric force transducer. The preparation was stimulated electrically by means of two platinum electrodes, one of which was placed in the lumen of the preparation and the other in the bathing solution as described previously (20). Supramaximal rectangular pulses of 0.3 ms in duration were delivered by using an electric stimulator (model SEN-3301; Nihon Kohden, Tokyo, Japan) with a frequency spectrum of 20 Hz for 1 s. Contractile activity was recorded isometrically with a force transducer (T7-30-240; Orientec, Tokyo, Japan). An initial tension of 1.5 g was applied to the colonic preparations, which were subsequently allowed to equilibrate for 45–60 min. At the end of this period, the tension created by the segment was considered as the resting tension and no further mechanical adjustment was made during experimentation. Isometric responses were filtered and amplified by an amplifier (AS1202; NEC, Tokyo, Japan) and recorded using a PowerLab system (model 2/25; AD Instruments, Bella Vista NSW, Australia).

*Morphological study.* Segments of distal colon were obtained at 1 week or 4 weeks after the induction of

inflammation by TNBS treatment. Tissue was fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline for 7 h at 4°C. The fixed tissue was embedded in paraffin, sectioned transversely at 5  $\mu$ m in thickness, and mounted on a glass slide. The sections were stained with hematoxylin and eosin and examined with light microscopy. Images were captured with a digital camera (Pro 600ES; Pixera Corporation, Los Gatos, CA, USA) mounted on the microscope and adjusted for brightness and contrast in Adobe Photoshop Elements 3.0 (Adobe Systems, San Jose, USA).

**Solutions and drugs.** During experiments, tissues were maintained in PSS (modified Krebs solution) consisting of (in mM): NaCl 118.4, KCl 4.7, CaCl<sub>2</sub> 2.5, MgSO<sub>4</sub> 1.2, KH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25 and glucose 11.7. N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME), acetylcholine (ACh) chloride and atropine sulfate salt monohydrate were obtained from Sigma (St. Louis, MO, USA). TNBS and tetrodotoxin were obtained from Wako (Osaka, Japan). Drugs were dissolved in distilled water. The drug concentrations given in the text are final concentrations in the bath solution.

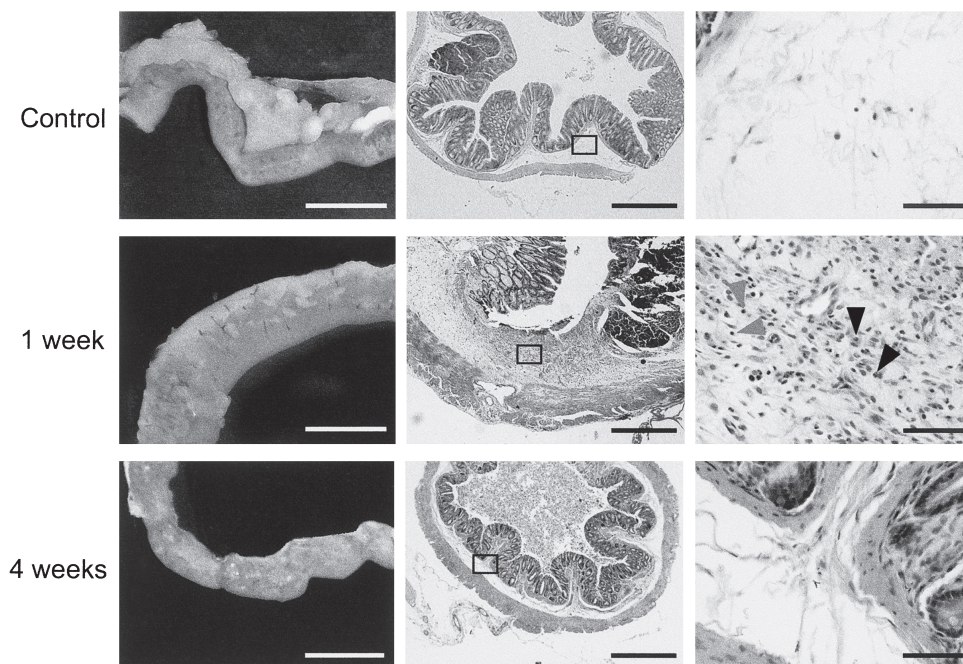
**Data analysis.** Data are presented as means  $\pm$  SD. Sample sizes (n) represent the number of experiments performed using different tissue preparations from different hamsters. The significance of differences between mean values was determined by one-way analysis of variance, and Scheffe's F test was used for *post-hoc* analysis. A *P* value less than 0.05 indicated statistical significance.

## RESULTS

### *General observations*

TNBS was injected into the distal colon of each hamster to induce colitis. One week after treatment with TNBS, body weight of hamsters decreased to  $80 \pm 6$  g from the initial weight of  $97 \pm 8$  g ( $n = 10$ ), whereas control animals gained approximately 10 g over the one-week period. Four weeks later, body weight of TNBS-treated animals was  $133 \pm 6$  g ( $n = 8$ ), which was comparable to that of the control animals.

Diarrhea occurred 4–7 days after TNBS treatment. The diarrhea induced by TNBS was not observed in hamsters kept for 4 weeks after TNBS treatment. Macroscopic observation showed that the



**Fig. 1** Representative macroscopic and microscopic photographs of the hamster distal colon before (control) and at 1 week or 4 weeks after treatment with trinitrobenzene sulfonic acid (TNBS). Micrographs shown in the center (low magnification) and right panels (high magnification) are haematoxylin/eosin-stained cross sections, the images of which are printed at the same final magnification. Rectangles in center panel are magnified in right panel, black arrowheads indicate neutrophils and grey arrowheads show lymphocytes. Scale bars, 10 mm (left panels) and 500  $\mu$ m (center panels) and 50  $\mu$ m (right panels).

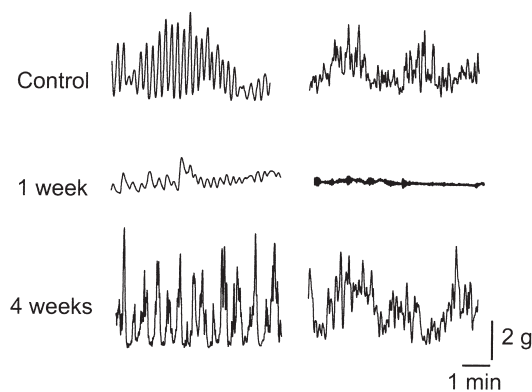
size of the distal colon at 1 week was larger than that of the control hamsters (Fig. 1). The colon of hamsters at 1 week after TNBS injection contained diarrheal feces. In contrast, the hamsters at 4 weeks after TNBS treatment had recovered in terms of colonic size and their colon contained well-formed solid feces as in control hamsters (Fig. 1).

#### *Histological findings*

Control hamsters displayed little evidence of inflammation in the distal colon (Fig. 1). At 1 week after TNBS treatment, erosion of the epithelium and hypertrophy of the smooth muscle layers were obvious. In addition, infiltration of inflammatory cells comprised mainly of neutrophils and lymphocytes was observed in the mucosal, submucosal and muscle layers (Fig. 1). At 4 weeks after TNBS injection, the erosion of the epithelium as well as the hypertrophy of the muscle layers were restored (Fig. 1). Infiltration of inflammatory cells was rarely found at this time point.

#### *Effect of TNBS treatment on spontaneous motility in the isolated distal colon*

The segment of the distal colon isolated from the control hamster exhibited spontaneous contractile motility (Fig. 2). There was a variation in the motility pattern between preparations. To show these variations, two typical examples were presented in Fig. 2. Some preparations (10 of 41 preparations) showed a relatively well-synchronized rhythmic motility (left panel in Fig. 2), whereas the others (31 of

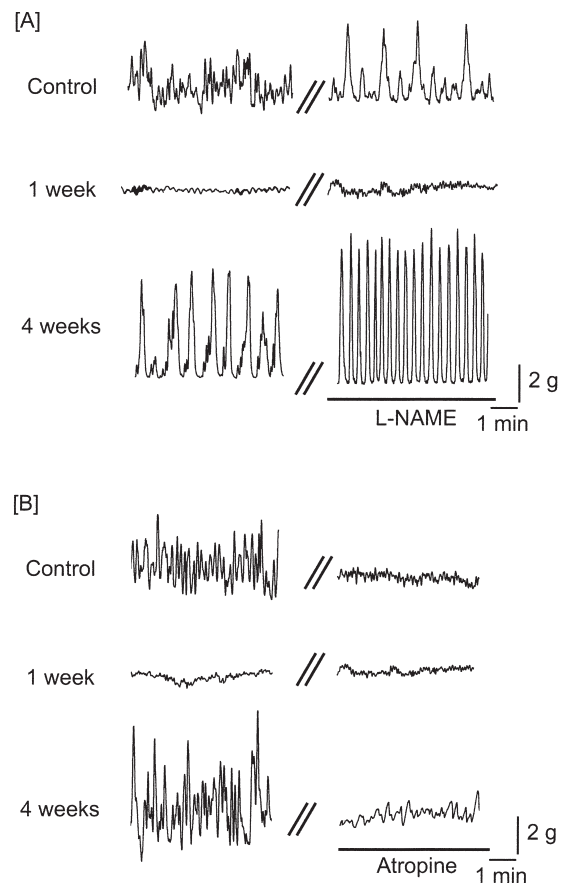


**Fig. 2** Representative traces of spontaneous contractions in the control and TNBS-treated hamster distal colons at 1 week or 4 weeks. The spontaneous contractions in the longitudinal direction were recorded isometrically. Two independent typical examples derived from different animals are shown for each condition. Left panel shows the synchronized rhythmic contraction whereas right panel shows irregular pattern of contraction.

41 preparations) showed an irregular pattern (right panel in Fig. 2). At 1 week after TNBS treatment, the spontaneous activity was strongly suppressed (Fig. 2). The suppressed spontaneous motility reappeared at 4 weeks after the initiation of colitis, with similar probability of variations to controls (Fig. 2).

#### *Effects of nitrenergic and cholinergic blockades on spontaneous motility in isolated segments of normal and TNBS-treated colon*

A nitric oxide (NO) synthase (NOS) inhibitor, L-NAME, was applied to see the contribution of nitrenergic component on spontaneous motility in isolated segments of normal and TNBS-treated colon. The concentration of L-NAME used (200  $\mu$ M) was



**Fig. 3** The effects of a nitric oxide synthase inhibitor ( $N^G$ -nitro-L-arginine methyl ester, L-NAME) or a muscarinic receptor antagonist (atropine) on rhythmic spontaneous contractions of the control and TNBS-treated hamster distal colons at 1 week or 4 weeks. Typical traces of the colonic spontaneous contractions in the longitudinal direction before and after application of L-NAME [A] or atropine [B] are shown. L-NAME and atropine were added to the organ baths at final concentration of 200  $\mu$ M and 1  $\mu$ M, respectively.



shown to be effective in similar experiments (6, 28). As shown in Fig. 3A, application of L-NAME increased the amplitude of spontaneous contractions. The blocker had little effect on the suppressed motility in the colon at 1 week after TNBS treatment (Fig. 3A). L-NAME application enhanced amplitude of spontaneous contractions in the colonic segment at 4 weeks after TNBS treatment (Fig. 3A).

Application of a muscarinic blocker, atropine (1  $\mu$ M), significantly reduced the amplitude of spontaneous contraction (Fig. 3B). The effect of atropine was not obvious in the colon at 1 week after TNBS treatment, but the drug suppressed spontaneous activity of the colon at 4 weeks after the treatment in a manner similar to that in the control (Fig. 3B).

#### Effect of TNBS treatment on EFS-induced mechanical responses in the isolated segment of the distal colon

EFS (20 V, 0.3 ms in duration, 20 Hz, for 1 s) induced a mechanical contractile response in the distal colon of control hamsters with an absolute force of  $3.4 \pm 0.4$  g ( $n = 10$ ) (Fig. 4). The EFS-induced contractile responses were severely impaired in the colon at 1 week after TNBS treatment with a force of  $1.4 \pm 0.5$  g ( $n = 7$ ) (Fig. 4). However, at 4 weeks after TNBS treatment, the mechanical responses to EFS were recovered to  $3.4 \pm 0.4$  g ( $n = 8$ ) (Fig. 4). Pretreatment with tetrodotoxin prevented all of the

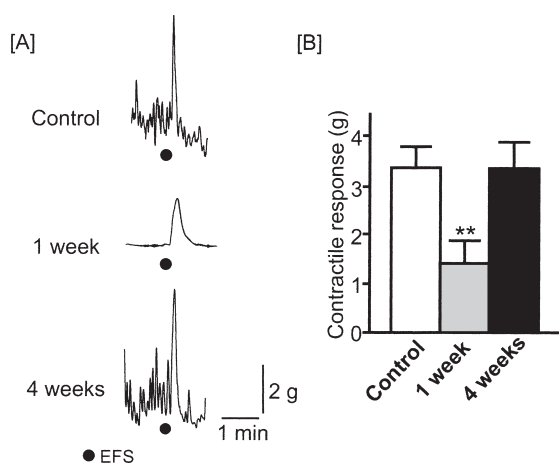
EFS-induced responses (data not shown), indicating that these responses were neurally mediated.

#### Effects of exogenously applied ACh on contractility of the isolated segments of normal and TNBS-treated colons

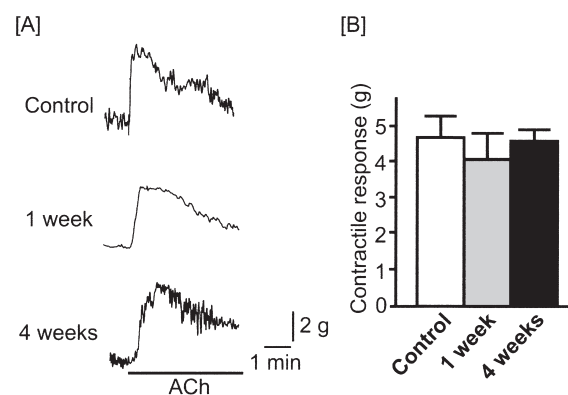
The relationship between time after TNBS enema and contractile response to ACh is presented in Fig. 5. Application of ACh to the organ bath at a final concentration of 10  $\mu$ M evoked contractions in the control distal colon. The longitudinal smooth muscle contractility after application of ACh remained constant in the colon at 1 week or 4 weeks after TNBS treatment. The tensions generated by the tissues from normal, colitic and recovered animals were  $4.5 \pm 0.3$ ,  $4.1 \pm 0.8$  and  $4.5 \pm 0.2$  g, respectively ( $n = 4-6$ ).

## DISCUSSION

In this study, we examined the effects of inflammation on spontaneous motility in the longitudinal direction of the colon. When a colon segment to be examined for its motility *in vitro* is isolated from the acute inflammatory phase, we can easily judge the severity and extend of colonic damage. To examine the restoration of motility disorder TNBS-injected animals would have to be kept until the expected colitis had recovered. However, there is no reliable marker to judge the severity and extent of prior colonic damage in the healed colon. We therefore considered it important to choose an experi-



**Fig. 4** Electrical field stimulation (EFS)-induced mechanical responses in the control and TNBS-treated hamster distal colons at 1 week or 4 weeks. [A] Typical responses to application of EFS. EFS was applied at 20 V, 0.3 ms pulse duration, 20 Hz, for 1 s at close circles indicated in the traces. The longitudinal mechanical responses were recorded isometrically. [B] Summary graphs of the EFS-evoked contractions ( $n = 7-10$ ). Each bar represents the mean  $\pm$  SD. \*\* $P < 0.01$ , compared to the control.



**Fig. 5** Acetylcholine (ACh)-evoked contractions in the control and TNBS-treated hamster distal colons at 1 week or 4 weeks. [A] Typical responses to exogenously applied ACh. ACh was applied to the organ bath at a final concentration of 10  $\mu$ M. The longitudinal mechanical responses were recorded isometrically. [B] Summary graphs of the ACh-evoked contractions ( $n = 4-6$ ). Each bar represents the mean  $\pm$  SD.

mental animal that responds to TNBS with a high reproducibility. In our preliminary experiments, TNBS-induced colitis was highly reproducible with relatively small individual difference in the hamster. Accordingly, we selected hamsters to induce experimental colitis. It has been reported that TNBS-induced inflammation in the colons of rats and guinea-pigs reached maximal severity at about 1 week and returned to normal in terms of the presence of hyperaemia, ulceration or bowel wall thickening within 4 weeks after TNBS injection (16, 11, 21). Consistent with the time course observed in rats and guinea-pigs, enlarged size of the colon at the acute inflammatory phase (1 week after TNBS treatment) in the hamster model was restored at 4 weeks after TNBS treatment as judged by macroscopic observation (Fig. 1). Since the size or weight of the colon segment is recognized to be a reliable and sensitive indicator of the severity of colonic damage (21), our macroscopic observation showed that colonic inflammation is apparently resolved within 4 weeks after TNBS treatment in hamsters. This view is supported by microscopic observation showing little evidence of inflammation at this time point (Fig. 1). Taken together, it may be appropriate to isolate colon specimens from the animals 4 weeks after TNBS treatment to examine the post-inflammatory changes in spontaneous motility. Thus, in the present study, we isolated colonic segments at 1 week and 4 weeks after TNBS treatment as representative samples for acute inflammatory phase and post-inflammatory phase, respectively.

At 1 week after induction of colitis, spontaneous motility in the longitudinal direction was strongly suppressed (Fig. 2). This result is consistent with results of previous studies showing that circular smooth muscle contractility had decreased at 3–7 days after induction of colitis in human and rats (9, 11, 12). It has been demonstrated that inflammation of the intestine causes impairment of adrenergic, cholinergic, purinergic and nitrgenic neurotransmission in the ENS (3, 8, 10, 18). Furthermore, TNBS-induced colitis is accompanied by substantial impairment of circular smooth muscle contractility (11). However, in the inflamed colon of the hamster, contraction of longitudinal smooth muscles in response to exogenously applied ACh was not impaired (Fig. 5). This demonstrates that contractility of longitudinal smooth muscle itself remains intact in spite of the presence of acute inflammatory events. Alternatively, neural components that control the longitudinal smooth muscle contractions would be impaired by the acute inflammation. This is prov-

en by the fact that the magnitude of EFS-induced mechanical response, which can be recognized as nerve-mediated contraction (3), was less than that in healthy controls at 1 week after TNBS treatment (Fig. 4). It is therefore reasonable to conclude that the suppression of the longitudinal spontaneous motility of the inflamed hamster colon is due to impairment of neurotransmissions rather than smooth muscle disorders.

Spontaneous motility reappeared in the colonic preparation isolated from the hamster maintained for 4 weeks after induction of colitis (Fig. 2). The reappearance of motility would depend on the recovery of neural components, because inhibition of cholinergic neurotransmission strongly inhibited spontaneous motility as observed in healthy controls (Fig. 3B). In addition, the results showing that EFS applied to the healed colon (4 weeks after TNBS treatment) elicited a mechanical response, which was similar to that observed in control preparations (Fig. 4), suggest that regulatory neural components of ENS can recover rapidly. In line with this, it has been demonstrated that intestinal inflammation induced a rapid axonal proliferation within longitudinal and circular muscle layers (24). However, it has recently been shown in the guinea pig colon that sustained alterations in enteric neural signaling were promoted following transient intestinal inflammation (16). Thus, the apparent recovery from inflammation is not necessarily accompanied by complete restoration of neural components. Pharmacological analysis to reveal the neural components responsible for the EFS-induced response in the healed colon is now in progress.

It has been reported that the presence of larger number of NOS-containing neurons in the proximal colon than that in the distal colon is attributable to the ability of the proximal colon to produce propulsive contractions of relatively large amplitude (28). This evidence shows that NO plays an important role in the regulation of contractile activity in the colon. A loss of nitrgenic neurons after induction of colitis has been reported (18). In addition, it has been demonstrated that the dominance of a source of NO is shifted from neural constitutive NOS in the control to inducible NOS in post-inflammation (2). Therefore, the behavior of nitrgenic components related to longitudinal contraction during the course of colitis is interesting. Involvement of nitrgenic neurons in colonic motility can usually be proven by using a NOS inhibitor (28). In fact, the amplitude of spontaneous contractions was elevated after application of the NOS inhibitor L-NAME in the

normal colon (Fig. 3A). L-NAME substantially elevated amplitude and increased frequency of the spontaneous contractions in the healed colon (Fig. 3A). These results suggest that the nitrenergic component that had been damaged during the acute inflammatory phase is recovered within 4 weeks after induction of colitis. It should be noted, however, that the present study does not provide a direct suggestion in terms of source of NO following the resolution of colitis. Further studies are needed to determine the possible long-term alterations in the regulatory mechanisms of longitudinal muscle motility in a previously inflamed colon. In addition, involvement of the ICC-myenteric network in the impairment and/or restoration of spontaneous longitudinal contractile activity in TNBS-treated animals remains to be determined.

In summary, we have demonstrated that spontaneous motility of longitudinal smooth muscles in the hamster colon is strongly suppressed during the acute inflammation caused by TNBS, although muscle contractility in response to ACh remains intact. The suppressed motility can recover within 4 weeks after the initiation of colitis. The suppression and the reappearance of spontaneous motility seem to depend on the damage and restoration of neural factors, respectively.

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