A new possibility for repairing the anal dysfunction by promoting regeneration of the reflex pathways in the enteric nervous system

Katsui R, Kojima Y, Kuniyasu H, Shimizu J, Koyama F, Fujii H, Nakajima Y, Takaki M. A new possibility for repairing the anal dysfunction by promoting regeneration of the reflex pathways in the enteric nervous system. Am J Physiol Gastrointest Liver Physiol 294: G1084–G1093, 2008. First published February 28, 2008; doi:10.1152/ajpgi.00345.2007.—Moderate rectal distension elicits recto-rectal reflex contractions and simultaneous recto-intestinal internal anal sphincter reflex relaxations that together comprise the defecation reflex. Both reflexes are controlled by 1) pelvic nerves, 2) lumbar colonic nerves, and 3) enteric nervous system. The aim of the present study was to explore a novel approach to repairing the defecation reflex dysfunction by using the plasticity of the enteric nervous pathways. Experiments were performed in anesthetized guinea pigs with ethyl carbamate. The rectum 30 mm oral from the anal verge was transected without damage to extrinsic nerves, and subsequent end-to-end one-layer anastomosis was performed. Recovery of the defecation reflex and associated reflex pathways were evaluated. Eight weeks after sectioning of intrinsic reflux nerve pathways in the rectum, the defecation reflex recovered to the control level, accompanied with regeneration of reflex pathways. The 5-HT₄-receptor agonist mosapride (0.5 and 1.0 mg/kg) significantly (P < 0.01) enhanced the recovered defecation reflex 8 wk after surgery. Two weeks after local treatment with brain-derived neurotrophic factor (BDNF: 10⁻⁶ g/ml) at the rectal anastomotic site, the recto-intestinal internal anal sphincter reflex relaxations recovered and some bundles of fine nerve fibers were shown to interconnect the oral and anal ends of the myenteric plexus. These results suggested a possibility for repairing the anal dysfunction by promoting regeneration of the reflex pathways in the enteric nervous system with local application of BDNF. 

Brain-derived neurotrophic factor; internal anal sphincter

Methods and Materials

Animal preparation. Experimental procedures followed the regulations of and were approved by the animal care and use committee of Nara Medical University. Under anesthesia with Nembutal 40 mg/kg ip in 44 male guinea pigs, the abdomen was opened by a lower midline laparotomy. The operation was performed to spare extrinsic input from the lumbar colonic nerves. The rectum was transected (RT) 3 cm from anal verge and an end-to-end one-layer rectal anastomosis (RA) was performed in 28 guinea pigs. Furthermore, we performed the local treatment with saline or BDNF at the anastomotic site by application of gelatin sponge (GS; 6–7 mm × 10 mm) absorbed saline or BDNF (10⁻⁶ g/ml) in another 16 guinea pigs. This concentration of BDNF potentiates formation of enteric neural networks from murine embryonic stem cells (18). GS was inserted tightly between the rectum and seminal vesicle. This GS was completely absorbed 4 wk after application.

Measurement of the defecation reflex. Experiments were performed on 52 male guinea pigs (8 intact and 44 operated guinea pigs; body wt 482 ± 39 g, range 356–508 g) anesthetized with ethyl carbamate.
Fig. 1. Each representative set of tracings of the recto-rectal (R-R) reflex response and the recto-internal anal sphincter (R-IAS) reflex response in an intact guinea pig (A) and in each guinea pig at 1 (B), 2 (C), 3 (D), 4 (E), and 8 wk (F) after rectal transection (RT) and subsequent anastomosis (RA). Rectal distension was performed for 5 min at 20-min intervals. Each rectal reflex response is indicated by a solid circle (●).

Fig. 2. A: summarized data of R-R and R-IAS reflex indexes in intact guinea pigs (n = 8) and each guinea pig at 1–8 wk (n = 4–8) after RT + RA. After 8 wk, mean R-IAS reflex index was not significantly different from that in intact guinea pigs. *P < 0.01 vs. control. #P < 0.01 vs. 1 wk after RT + RA. B and C: summarized data of frequency (B) and amplitude (C) of R-R and R-IAS reflex responses in intact guinea pigs and each guinea pig at 1–8 wk after RT and RA. *P < 0.01 vs. intact. #P < 0.05 vs. after 8 wk. ###P < 0.01 vs. after 8 wk. D: lower abdominal view of the rectal anastomosis.
(0.7–1.0 g/kg ip), artificially ventilated, and immobilized with gallamine (0.1 mg/kg iv). Rectal motility was recorded with a warm water-filled balloon that was attached to flexible polyethylene tubing connected to a pressure transducer. The 1.5-cm-long balloon was introduced into the rectum 5 cm oral to the anus. During experiments the tubing was loosely fixed to a metal rod to prevent evacuation of the balloon through the anus.

Gradual and sustained rectal distension for 5 min at intervals of 20 min was performed as previously reported (9, 16, 26). This rectal distension method simulated the physiological distension of the rectum comparable to two pieces of fresh feces (9, 17, 26). During infusion of water into the balloon for up to 0.6 ml for 24 s no reflex response was evoked, but subsequent sustained rectal distension for 4 min 36 s evoked rectal reflex responses.

The motility of the IAS was recorded with a custom-made strain-gauge force transducer, similar to that used by Mizutani and Nakayama (13). We have previously validated that this transducer records motility of the IAS independently of rectal motility (9, 16, 26). The trial for typical reflex response was repeated three times in each experiment. A reproducible reflex response was obtained in intact and operated animals 1–4 and 8 wk after surgery throughout the experiments by the present protocol. The reflex response was selected from one to three trials and evaluated by mean amplitude, frequency, and “reflex index.” Mean systemic arterial blood pressure was maintained between 100 and 150 mmHg throughout the experiment, and PO2, PCO2, and pH were maintained within the physiological range by changing the tidal volume and rate of artificial ventilation. The body temperature was maintained normal at 36–37°C with a heating pad.

Analysis of reflex index. All data were acquired using a personal computer (Fujitsu, Tokyo, Japan) via an analog-to-digital converter (Digidata 1322A, Axon Instruments, Foster City, CA) at 166.7 Hz, filtered at 10 Hz with Axoscope 7 (Axon Instruments, Foster City, CA). The R-R reflex and the R-IAS reflex areas were calculated with Origin 6.1J (OriginLab, Northampton, MA) (Fig. 1A) (9, 16). “Reflex area” is expressed as positive values for rectal contractions and IAS relaxations. The measurement of the reflex area could detect any changes either in amplitude, duration, or frequency of the reflex response. The reflex index is expressed as a relative ratio to mean reflex area in intact guinea pigs or operated guinea pigs treated with saline or the control reflex area before mosapride (=1.0) (9, 16, 26).

Drugs. The following drugs were used: mosapride citrate (kindly donated by Dainippon Sumitomo Pharmaceutical, Osaka, Japan), gallamine triethiodide (Sigma, St. Louis, MO), ethyl carbamate (Wako Pure Chemical Industries, Osaka, Japan), and BDNF (Upstate, Charlottesville, VA). Mosapride dissolved in a solution containing 50% dimethylsulfoxide (DMSO) was injected intravenously, and thus final DMSO concentration was less than 1%.

Immunohistochemistry. After functional studies, whole mounts of the rectum including an anastomotic site were prepared by removing the mucosa and submucosa, and partly removing the circular muscle layer. They were then fixed in acetone (4°C, 1 h) for c-Kit and...
neurofilament (NF) immunohistochemistry. After fixation, preparations were washed for 30 min in PBS (0.1 M, pH 7.4). Nonspecific antibody binding was reduced by incubation for 12 h in 10% normal goat serum in PBS containing 0.3% (vol/vol) Triton-X 100 at room temperature. Tissues were incubated 48 h at 4°C with a rat monoclonal antibody raised against c-Kit protein (ACK45, 5 μg/ml in PBS, BD Biosciences, San Jose, CA) and with a rabbit polyclonal antiserum to label NF (5 μg/ml in PBS, BIOMOL International LP, Philadelphia). Immunoreactivity for Kit was detected by use of Alexa Fluor 488-conjugated secondary antibody (Alexa Fluor 488 goat anti-rat; Molecular Probes, Eugene, OR; 1:200 in PBS for 48 h in the dark at room temperature) and that for NF was detected by use of Texas Fig. 4. Summarized data of effects of mosapride on R-R and R-IAS reflexes evaluated by the reflex index in the same guinea pigs (n = 4) at 2 (A) and 8 wk after RT+RA (B). After the regeneration of nerve fibers interconnecting with oral and anal sites over the anastomosis (=8 wk), mosapride enhanced the R-IAS reflex response. *P < 0.05 and **P < 0.01 vs. control by ANOVA and Dunnett’s and Bonferroni’s tests.

Fig. 5. A: representative sets of tracings of the R-R and R-IAS reflex response. At 2 wk after RT+RA without gelatin sponge (GS) and brain-derived neurotrophic factor (BDNF) [GS(−)BDNF(−)] (left), 2 wk after treatment with GS absorbed saline at the anastomotic site [GS(+)]BDNF(−)] (middle), and 2 wk after treatment with GS absorbed BDNF (10⁻⁶g/ml) at the anastomotic site [GS(+)BDNF(+)] (right). B: effects of treatment with BDNF on mean R-R and R-IAS reflex indexes in the same guinea pigs 2 wk after RT+RA (n = 6 each). **P < 0.01 vs. GS(+)BDNF(−). C: representative images of immunostaining for neurofilament in the anastomotic site treated without BDNF (top) and with BDNF (middle) for 2 wk after RT+RA are shown. Some bundles of fine nerve fibers interconnected the oral cut end with the anal end of the myenteric plexus (see arrowheads). Bottom left: cut end of the myenteric plexus in the posterior wall untreated with BDNF of the same rectum. Bottom right: cut end of the myenteric plexus in the anterior wall treated with BDNF of the same rectum. More numerous regenerative nerve fibers were observed. Arrowheads indicate cut ends of nerve fibers in the myenteric plexus. Calibration bar: 200 μm.
Red-conjugated secondary antibody (Texas Red-goat anti-rabbit: MP Biomedicals, Aurora, OH; 1:100 in PBS for 48 h in the dark at room temperature) (18). Tissues were examined with a Bio-Rad MRC 600 (Hercules, CA) confocal microscope. Confocal micrographs are digital composites of Z-series scans of 10–15 optical sections through a depth of 100–150 μm. Final images were constructed with Comos software (Bio-Rad).

For immunohistochemistry of tissue sections, the rectum including an anastomotic site was fixed with 4% paraformaldehyde at 4°C and was embedded in paraffin. Consecutive 4-μm sections were cut from each block, and immunostaining was performed by the immunoperoxidase technique following antigen retrieval with pepsin (DAKO, Carpinteria, CA) treatment for 20 min at room temperature. After endogenous peroxidase block by 3% H2O2-methanol for 15 min, specimens were rinsed with PBS and incubated with a primary antibody diluted with Washing Solution (BioGenex, San Ramon, CA) at room temperature for 2 h. The specimens were rinsed with PBS and incubated at room temperature for 1 h with secondary antibody conjugated to peroxidase diluted at 0.5 μg/ml (Medical & Biotechnological Laboratories, Nagoya, Japan). The sections were then rinsed with PBS and color developed by diaminobenzidine solution (DAKO).

Sections were counterstained with Meyer’s hematoxylin (Sigma Chemical, St. Louis, MO). Antibodies used in primary reaction and the working concentrations were as followed: anti-tyrosine receptor kinase B (TrkB) (clone H-181, 1 μg/ml, Santa Cruz Biotechnology) as a BDNF receptor (6, 18); anti-NF (clone 2F11, reacting with 70-, 100-, and 200-kDa proteins, 0.5 μg/ml, DAKO) (18), anti-distal-less homeobox 2 (DLX2) (Abcam, cat. ab18188, 0.5 μg/ml, Tokyo, Japan) (24), and anti-p75 (intracellular domain, 0.5 μg/ml, Upstate, Lake Placid, NY) as enteric neural stem cell markers (18); and anti-PCNA (clone PC10, DAKO) as a cell proliferating marker that is specifically expressed in cell nuclei during the S phase (1).

Detection of regenerated enteric neurons. To identify neuronal cell proliferation, 5-bromo-2’-deoxyuridine (BrdU, 1 mg/ml solution; Sigma) was added to the drinking water for 2 wk for four animals, i.e., three BDNF-treated and one saline-treated operated animals, until the day before they were euthanized. For detection of regenerated enteric neurons, the immunohistochemistry with anti-NF antibody was performed as described above. After being rinsed in PBS, the specimens were pretreated with sodium chloride sodium citrate solution for 2 h at 65°C, followed by partial denaturation of double-stranded DNA with 2 M HCl for 30 min at 37°C. To reveal BrdU, the sections were incubated with a rat monoclonal antibody raised against BrdU (Abcam) overnight at 4°C. The specimens were rinsed in 0.1 M TE (pH 7.8) followed by routine immunohistochemistry.

Statistical analysis. Statistical significance of differences between means was estimated by nonpaired or paired t-test, Mann-Whitney test, or one-way ANOVA and followed by multiple comparisons by Bonferroni or Dunnett’s post hoc test. A P value of <0.05 was considered statistically significant.

RESULTS

Changes in R-R and R-IAS reflexes 1–8 wk after RT+RA. Figure 1 shows representative examples of R-R and R-IAS reflexes 1–8 wk after RT+RA (Fig. 2D) compared with reflex responses in an intact guinea pig. The initial transient increase in rectal intraluminal pressure by gradual distension (for 24 s) was TTX insensitive, and this phase is excluded from reflex area. The subsequent sustained rectal distension (for 4 min, 36 s) evoked reflex responses superimposed on a sustained, passively generated pressure of 80–100 mmHg. One week after the surgery, the R-IAS reflex response was markedly attenuated whereas the R-R reflex response was unchanged (Fig. 1B), although a residual R-IAS reflex mediated via extrinsic nerves was observed. During 2–8 wk after surgery, the R-IAS reflex response gradually returned to the intact level with unchanged R-R reflex (Fig. 1, C–F).

The mean R-R reflex index was unchanged during 1–8 wk after the surgery whereas the mean R-IAS reflex index was significantly decreased during the first 4 wk after the surgery (n = 4–8; P < 0.01) and returned to the intact level after 8 wk (Fig. 2A). The mean frequencies of R-R and simultaneous R-IAS reflex responses were unchanged throughout 1–8 wk after RT+RA (Fig. 2B). The mean amplitude of the R-IAS reflex response significantly decreased after 1–4 wk relative to that in intact guinea pigs but returned to the intact level after 8 wk (n = 4 each, P < 0.01). The mean amplitude in R-R reflex response was unchanged throughout 1–8 wk (Fig. 2C).

Immunoreactivity for NF and c-Kit. Representative images of immunostaining for NF in the rectum 2, 4, and 8 wk after RT+RA are shown in Fig. 3. After 2 wk, enteric neurons in the rectal anastomotic site were severely damaged and the neural network was disrupted. Fine nerve fibers were observed emerging from damaged nerve stumps (Fig. 3A). At 8 wk, numerous bundles of fine nerve fibers were observed crossing anastomotic site within the rectal myenteric plexus (Fig. 3C).

After 2 wk, the dense myenteric ICC (ICC-MY) network was observed at the anastomotic site, but myenteric plexus was disrupted (Fig. 3D). At oral side, intramuscular ICC (ICC-IM) was also observed because the circular muscle remained intact (Fig. 3Du). It appeared that both ICC networks regenerated...
across the anastomotic site after 2 wk (Fig. 3D, b and c). Thus reorganization of the ICC network across the site of anastomosis preceded that of the myenteric plexus.

Effects of mosapride on R-R and R-IAS reflex responses after RT+RA. We evaluated effects of mosapride at early (2 wk) and late (8 wk) time points following RT+RA (Fig. 4). Mean R-R reflex indexes increased by mosapride (0.1–1.0 mg/kg) in a dose-dependent manner after 2 wk. The mean R-R reflex index significantly increased by mosapride (1.0 mg/kg) to 2.2 ± 0.9 (P < 0.05). However, the mean R-IAS reflex index was not affected by mosapride (0.1–1.0 mg/kg) (Fig. 4A). After 8 wk, R-R reflex indexes were significantly enhanced by mosapride (0.5 and 1.0 mg/kg) to 1.72 ± 0.24 (P < 0.01) and 1.52 ± 0.07 (P < 0.01), respectively, and mean R-IAS reflex indexes were also significantly increased (0.5 mg/kg, 1.83 ± 0.31, P < 0.01; 1.0 mg/kg, 1.76 ± 0.45, P < 0.05; Fig. 4B).

Effects of BDNF on R-IAS reflex responses associated with the regeneration of NF-positive fibers after RT+RA. We have recently reported that BDNF induced differentiation of enteric neuronal network structure from neural crest-derived cells from embryonic stem cells (18). Therefore, we tested whether the application of BDNF (10^−6 g/ml) at the anastomotic site accelerated the regeneration of damaged enteric nerves after RT+RA. Although the R-R reflex response was unchanged, the R-IAS reflex response was markedly enhanced in the BDNF group 2 wk after RT+RA (Fig. 5A).

**Fig. 7.** Representative images of immunostaining for NF (A) and tyrosine receptor kinase B (TrkB; B) in the intact rectum treated with BDNF for 2 wk [Intact+BDNF(+)] and in the newly formed granulation tissue within the 2-mm rectal anastomotic site treated with saline for 2 wk [BDNF(−)2W] and treated with BDNF for 2 wk [BDNF(+2W)] and treated with BDNF for 4 wk [BDNF(+4W)]. A: NF-positive cells indicated by arrows were observed in myenteric ganglia of the intact rectum. NF-positive cells were not found in the granulation tissue within the 2-mm rectal anastomotic site [BDNF(−)2W]. Many NF-positive cells indicated by arrows were found in this region [BDNF(+2W)]. NF-positive cells exhibited elongated cell processes and connected them to the neighboring nerve cells without increase in number of cells [BDNF(+4W)]. B: TrkB-positive cells indicated by arrows were observed in myenteric ganglia of the intact rectum [Intact+BDNF(+)]. TrkB-positive cells were rarely observed in the granulation tissue within the 2-mm rectal anastomotic site [BDNF(−)2W]. TrkB-positive cells were more frequently observed than NF-positive cells in this region [BDNF(+2W)]. Number of TrkB-positive cells decreased [BDNF(+4W)]. C: representative images of immunostaining for a neural stem cell marker in the central nervous system, distal-less homeobox (DLX2). No DLX2-positive cells were found in the granulation tissue within the 2-mm rectal anastomotic site [BDNF(−)2W]. Many DLX2-positive cells were observed in this region [BDNF(+2W)]. Number of DLX2-positive cells decreased [BDNF(+4W)]. D: summarized effects of BDNF on numbers of NF-, TrkB- and DLX2-positive cells in the granulation tissue within 2 mm anastomotic site. Mean values were obtained from 5 high-powered fields (HPF: 0.2 mm^2). *P < 0.05 vs. BDNF(−) by Mann-Whitney test. #P < 0.05 vs. BDNF(+2W) by Mann-Whitney test.
reflex index significantly increased by BDNF ($P < 0.01$), whereas the mean R-R reflex index was unaffected by BDNF treatment (Fig. 5B).

Some bundles of fine nerve fibers traversed the oral and anal ends of the myenteric plexus in the rectal anastomotic site treated with BDNF for 2 wk after RT+RA (middle panel in Fig. 5C). No changes were observed in the already regenerated ICC networks (data not shown). Furthermore, we compared the BDNF-treated anterior wall with the BDNF-untreated posterior wall in the same rectal anastomotic site. In the BDNF-treated anterior wall, more myenteric nerve fiber regeneration was observed (bottom panels in Fig. 5C).

Additional effects of BDNF on NF-, TrkB-, DLX2-, p75-, PCNA-, BrdU-positive cells in the anastomosis 2–4 wk after RT+RA. In sections of intact rectum that had been treated with BDNF, a normal distribution of myenteric ganglia was observed, indicating that BDNF did not penetrate from the intact serosa into the rectum to exert its action (Fig. 6A). In the rectal anastomotic site treated with saline (Fig. 6B) or with BDNF (Fig. 6C) for 2 wk after RT+RA, myenteric ganglia disappeared within about 3 mm of the anastomosis, consistent with observations in the whole mount preparations. Granulation tissue (new connected tissue formed by growth of fibroblasts and blood capillaries into injured tissue) was formed at the rectal anastomotic site treated with either saline or BDNF for 2–4 wk after RT+RA (Fig. 6, B and C).

A normal-appearing density and distribution of NF-positive cells was found in myenteric ganglia in the intact rectum, indicating that BDNF did not affect undisturbed myenteric ganglia. In the untreated granulation tissue at the rectal anastomotic site, NF-positive cells were not observed (0.6 ± 0.9). In contrast, in the tissue treated with BDNF, many NF-positive cells were observed 2 wk after RT+RA (13.6 ± 8.2), although no clusters of ganglia were seen. The NF-positive cells exhibited elongated cell processes that appeared to project to neighboring NF-positive cells. At 4 wk, there was no further increase in the number of NF-immunoreactive cells (Fig. 7A). These results indicate that BDNF penetrated into the rectum from the anastomosis to form or cause migration of neurons into the granulation tissue and then initiate the formation of neural networks.

Cells immunoreactive for the BDNF receptor, TrkB, were observed in myenteric ganglia in the intact rectum as previously reported in human adult enteric ganglia (6). TrkB-positive cells were rarely found in the granulation tissue at the anastomotic site in saline-treated animals, whereas TrkB-positive cells were more numerous than NF-positive cells in this site of BDNF-treated animals. The number of TrkB-positive cells decreased after 4 wk (Fig. 7B).

After 2 wk, cells immunoreactive for DLX2, a marker of neural stem cells in central nervous system (24), were frequently found in the granulation tissue at the anastomotic site of BDNF-treated animals. However, number of DLX2-positive cells greatly diminished after 4 wk (Fig. 7C).

After 2 wk, the mean numbers of NF-, TrkB-, and DLX2-positive cells significantly increased in the granulation tissue within 2 mm of the anastomotic site treated with BDNF compared with untreated preparations (Fig. 7D). However, the increase in TrkB- and DLX2-positive cells was transient as the number of these cells decreased after 4 wk, whereas number of NF-positive cells was unchanged (Fig. 7D).

DLX2-positive cells (A) shows concurrent expression of p75- (B) and PCNA-positive cells (C) in the granulation tissue within 2 mm anastomotic site treated with BDNF for 2 wk (Fig. 8), although PCNA-positive cells may partly include fibroblasts with proliferating activity.
BrdU-positive cells were identified in the granulation tissue within the 2-mm rectal anastomotic site treated with BDNF for 2 wk (Fig. 9). Some of the BrdU-positive cells (A) are also NF-immunoreactive (B), although BrdU-positive cells also appeared to include fibroblasts, endothelial cells, and macrophages (B). Cells immunoreactive for BrdU and NF were not detected in the granulation tissue within 2 mm anastomotic site treated with saline for 2 wk (C, D).

DISCUSSION

The most important findings of the present study are as follows: 1) At 8 wk after sectioning of intrinsic reflex nerve pathways, the defecation reflex was completely recovered, and this was accompanied with regeneration of reflex pathways. 2) Administration of BDNF at the anastomotic site accelerated neural regeneration and reflex recovery. 3) BDNF might have formed or caused migration of neurons into the newly formed granulation tissue at the anastomotic site. These results suggest the possibility for developing a novel therapy for restoring the postsurgical anal dysfunction (simulated lower anterior resection for rectal cancer) by promoting the regeneration of the reflex pathways.

It appears that some species differences exist in process of enteric nerve regeneration. In canines that had colorectal anastomoses after lower anterior resection, the defecation reflex recovered after 6 mo associated with regeneration of nerve trunks and bundles at the anastomotic site (7). In the small intestine of the guinea pig, however, migration of the myoelectric complex was observed associated with regeneration of enteric nerve fibers across the anastomotic site 60 days (~8 wk) after interruption of the myenteric plexus by intestinal transection. The reflex recovery was proceeded by progressive growth of nerve fibers in the anastomotic region that included early extension of nerve fibers 7–15 days after surgery, some nerve fibers crossing the anastomotic site 23–28 days after surgery, and many nerve fibers crossing the anastomotic site 57–60 days after surgery (3). This process is quite similar to sequence of events reported here, demonstrating that the recovery of R-R and R-IAS reflexes was associated with regeneration of enteric nerve pathways crossing the anastomotic site 56 days after sectioning intrinsic reflex nerve pathways.

We have previously reported that rectal distension evokes a cholinergic reflex contraction in the rectum (R-R) and nitric reflex relaxation in the internal anal sphincter (R-IAS) in a reproducible manner in the guinea pig (26). The present results indicate that nerve fibers crossing the anastomotic region provide a link between the oral cholinergic excitatory pathway and the anal nitric inhibitory pathway.

Progressive reorganization of the myenteric plexus for one year has been reported following RT+RA in the ileum of the guinea pig (23). Outgrowth of nerve fibers from the severed stumps was detected 2 wk after transection. Six weeks later, numerous bundles of fine nerve fibers were observed interconnecting the oral and anal cut ends of the myenteric plexus. Myenteric ganglion cell bodies require 12 mo for complete regeneration in the guinea pig. These findings suggest that myenteric ganglion cell bodies, unlike myenteric nerve fibers, require a longer term of reconstruction than previously believed after transection and anastomosis of the ileum of the guinea pig (23), although progressive recovery of reflex responses was not investigated in any of these models.

Recovery of electrical rhythmicity, neural responses mediated by ICC, and ICC networks in the mouse ileum have been evaluated 5 and 24 h after surgery (resection and anastomosis) (27). Five hours after surgery, electrical rhythmicity, neural responses, and ICC networks are disrupted. After 24 h, elec-

![Fig. 9. Representative images of immuno-staining for 5-bromo-2’-deoxyuridine (BrdU) and NF. BrdU-positive cells were found in the granulation tissue within the 2-mm rectal anastomotic site treated with BDNF for 2 wk (BDNF(+)2W). A contingent of BrdU-positive cells (A) are also immunoreactive for NF (B). These BrdU/NF-positive cells were not found in the granulation tissue within the 2-mm anastomotic site treated with saline for 2 wk (BDNF(−)2W) (C and D). Arrows: cells double labeled for BrdU and NF.](http://ajpgi.physiology.org/)
trical rhythmicity largely recovered and ICC networks appeared normal at all regions examined, although the recovery of myenteric neurons was not evaluated (27). In the small intestine of adult guinea pigs, ICCs regenerated and restored the normal distribution 5 mo after semitranssection and anastomosis (12). The present results showing that the dense ICC networks distributed across the anastomotic site 2 wk after RT+RA, suggesting that regeneration of ICC networks preceded that of myenteric neurons. In contrast, recent studies in the mouse colon have shown that even 70 days after the ablation of myenteric plexus and ICC-MY with the detergent benzalkonium chloride (BAC), no ICCs are present in the BAC-treated area (2). The difference between this and our result is partially related to the different ablation process of myenteric plexus and ICCs.

The role of ICCs in the defecation reflex is still controversial (14, 22). Correlations between the number of ICCs and defecation disturbance were assessed for the neorectum after anterior resection of the rectum (14). Expression of ICCs in the neorectum did not recover to preoperative levels over time. There was no correlation between the number of ICCs and the time interval from the initial anterior resection of the neorectum, nor was there any relationship between the number of ICCs and defecation disturbance. However, there is a report showing that, in ICC-deficient mutant mice, the rectoanal relaxation reflex was largely attenuated. This result indicates that ICC has a distinct role in the rectoanal relaxation reflex (22). In the present study, the distribution of ICC-IM and ICC-MY had recovered 2 wk after RT+RA, but nerve components of the R-IAS reflex pathway had not. Accordingly, we could not determine whether ICCs have a distinct role on the rectoanal relaxation reflex.

In the gastrointestinal tract, stimulation of 5-HT4 receptors has pronounced effects on various functions including peristaltic reflex (9, 16). We also have previously shown that mosapride, a 5-HT4 receptor agonist, moderately enhances the incidence of both R-R and R-IAS reflex responses after chronic extrinsic denervation. This strongly suggests that 5-HT4 receptors are located on the nerve terminals in myenteric ganglia impinging on myenteric motor neurons (9).

In the present study, after the regeneration of reflex pathways (=8 wk after RT+RA), mosapride (0.1–1.0 mg/kg) enhanced the R-IAS reflex response in a dose-dependent manner, but not before the regeneration of reflex pathways (at the 2-wk time point). This indicated that mosapride facilitates the R-IAS reflex response mediated via the regenerated nerve fibers crossing the anastomotic site to interconnect the oral excitatory pathway with anal inhibitory pathway (26).

Several recent reports demonstrate that BDNF enhances colonic motility and peristalsis in rats (4). The transient nature of this response indicates that it is unlikely that this effect contributes to the regeneration of enteric nerve pathways. BDNF, however, promoted enteric neural network differentiation in the gutlike organ from murine embryonic stem cells (18), suggesting the possibility that application of BDNF at the anastomotic site accelerates the regeneration of the enteric nerve pathways via the neurotrophic action on the cut end of the nerves and/or soma. This concept is supported by the recovery of the reflex responses and interconnection of NF immunoreactivity across the anastomotic site.

Furthermore, we found neurons in the granulation tissue at the anastomotic site treated with BDNF. These neurons expressed neural stem cell markers such as p75 in the enteric nervous system and DLX2 in the central nervous system (24). These neurons also expressed the cell proliferating marker, PCNA. BrdU/UNF concurrent labeling cells were found in the granulation tissue at the anastomotic site treated with BDNF. Taken together, BDNF might have formed neurons from neural stem cells in the newly formed granulation tissue at the anastomotic site and/or promoted migration of neurons into the site.

In most patients, the rectoanal inhibitory reflex recovers 1–2 years after the lower anterior resection of the rectum for carcinoma (15, 25). The recovery of the reflex is based on the regeneration of the enteric nerves crossing the anastomotic site (25). The possibility that an intrinsic defecation reflex mediated via enteric nervous system recovers after the lower anterior resection of the rectum is presented here. The local treatment with BDNF at the anastomotic site shortens the regeneration period of the enteric nerve pathway. In clinical settings, mosapride actually augmented lower gastrointestinal tract motility and thereby ameliorated constipation probably via a neural mechanism (11). Results reported here further confirmed that mosapride enhanced the defecation reflex response in the guinea pig mediated via enteric neural 5-HT4 receptors (9).

In conclusion, the results presented here suggest the possibilities for repairing the anal dysfunction by the recovery of damaged reflex pathways and for developing a new therapy by promoting the recovery of damaged reflex pathways with local application of BDNF.

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