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Comparison of effects of a selective 5-HT reuptake inhibitor versus a 5-HT$_4$ receptor agonist on in vivo neurogenesis at the rectal anastomosis in rats

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Kawahara I, Kuniyasu H, Matsuyoshi H, Goto K, Obata K, Misawa H, Fujii H, Takaki M. Comparison of effects of a selective 5-HT reuptake inhibitor versus a 5-HT$_4$ receptor agonist on in vivo neurogenesis at the rectal anastomosis in rats. Am J Physiol Gastrointest Liver Physiol 302: G588–G597, 2012. First published December 22, 2011; doi:10.1152/ajpgi.00284.2011.—It was recently reported that activation of enteric neural 5-HT$_4$ receptors (SR4) promotes reconstruction of enteric neural circuit injury in distal gut of guinea pigs and that this reconstruction involves neural stem cells. We aimed to explore a novel approach using a selective serotonin reuptake inhibitor (SSRI), which increases endogenous 5-HT; to repair enteric nerve fiber injury in the rat distal gut. Enteric nerve fiber injury was performed by rectal transection and subsequent end-to-end one-layer anastomosis. The SSRI fluvoxamine maleate (100 µmol/l) was applied locally at the anastomotic site to compare with the 5-HT$_4$ agonist mosapride citrate (100 µmol/l) (applied for patent) applied locally and orally. Unlike mosapride, fluvoxamine failed to promote the regeneration of the nerve fiber tract across the anastomosis. Furthermore, fluvoxamine did not generate anti-distal-less homeobox 2 (DLX2)- and anti-SR4-positive cells (neural stem cells) or anti-neurofilament (NF)-positive cells (neural cells) in newly formed granulation tissue at the anastomosis, whereas these cell types were observed in mosapride-treated preparations. In contrast to its effects in guinea pigs, mosapride generated 5-bromo-2’-deoxyuridine (BrdU)-positive neural cells in ganglia sites 3 mm oral and anal from the anastomosis 2 wk after nerve fiber injury. All actions of mosapride were observed after local and or oral applications. These findings indicate that local SSRI treatment does not induce in vivo nerve fiber tract growth across the anastomosis in the rat distal gut. Mosapride induces nerve fiber tract growth across the anastomosis, mediated through enteric neural stem cells possibly from neural crest-derived stem cells or mesenchymal stem cells in the bone marrow.

selective serotonin reuptake inhibitor; neural stem cell; serotonin-4 receptor; enteric nervous system

The enteric nervous system (ENS), which is composed of the neurons and nerve fibers in the gastrointestinal tract, has independent intrinsic reflex circuits regulating motility and secretion, although it exhibits many features resembling the central nervous system (CNS) (5). Enteric neurons are as phenotypically diverse as those of the CNS and include a variety of neurotransmitters found in the CNS, and the number of neurons in the ENS is equivalent to that in the spinal cord (5). Recently, we reported that brain-derived neurotrophic factor (BDNF) induces the formation of enteric neural networks from murine embryonic stem (ES) cells (21). Furthermore, we found that after rectal transection local in vivo application of BDNF improved distension-evoked reflexes of the internal anal sphincter (IAS) (6). We also demonstrated that BDNF facilitates the regeneration of the reflex nerve pathway and enteric neurogenesis from neural stem cells in the granulation tissue at the anastomotic site (6). As a repairing mechanism for the neural circuit insult, we suggested that the neural stem cells have a pivotal role in enteric neurogenesis.

We have also found that a 5-HT$_4$ receptor agonist, mosapride (MOS), enhances in vivo the defecation reflex composed of the intrinsic rectal cholinergic contraction reflex and IAS nitrergic relaxation reflexes mediated through physiologically active enteric neural 5-HT$_4$ receptors in guinea pigs (9, 10, 19). It has also been reported that 5-HT$_4$ receptor stimulation increases neuronal numbers and the length of neurites in enteric neurons developing in vitro from immunoselected neural crest-derived precursors (12). As further support for a role of 5-HT$_4$ receptors we have demonstrated that enteric neural 5-HT$_4$ receptor stimulation promotes in vivo reconstruction of an enteric neural circuit, leading to the recovery of the impaired defecation reflex in the distal gut in guinea pigs, and that this reconstruction possibly involves neural stem cells (15, 16, 28). Consistent with this finding, 5-HT$_4$ receptor-mediated neuroprotection and neurogenesis in the ENS have been shown in adult mice (13).

5-HT can stimulate 5-HT$_4$ receptors (20) as well as other subtypes of 5-HT receptors. Selective serotonin reuptake inhibitors (SSRIs) are widely used as antidepressants. SSRIs can increase endogenous 5-HT in the gastrointestinal tract (1). The endogenous 5-HT increased by SSRI can act on enteric neural 5-HT$_4$ receptors, as well as other subtypes of 5-HT receptors (14). We therefore compared the abilities of the 5-HT$_4$ receptor agonist MOS and the SSRI fluvoxamine maleate to promote neurogenesis from nerve fiber tract injury in rats. We also evaluated effects of local application (MOS-LA) versus oral

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application (MOS-PO) of MOS in rats to explore the possibility of clinical application.

**METHODS**

**Animal preparation.** All surgical and experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Pub. No. 85-23, revised 1996) and were reviewed and approved by the animal care and use committee of Nara Medical University. The abdomens of 45 male rats (body wt: 300–500 g) were opened by lower midline laparotomy under anesthesia with Nembutal (40 mg/kg ip). This surgical approach was performed to spare extrinsic inputs from the lumbar colonic nerves. Rectal transection (RT) 4 cm from the anal verge and an end-to-end spare extrinsic inputs from the lumbar colonic nerves. Rectal Nembutal (40 mg/kg ip). This surgical approach was performed to the anastomotic site was performed by application of gelatin sponge (GS) [W x L: 0.6 cm x 1.6 cm; each upper half (0.8 cm) and lower half (0.8 cm) was wrapped around the anastomotic site; soaked solution volume = 0.1 ml] (n = total of 20 rats) right after surgery. GS was inserted tightly between the rectum and the seminal vesicle. This GS was not completely absorbed until 4 wk after application.

Additional experiments involved subcutaneous implantation of GS (W x L: 0.5 cm x 0.5 cm; thickness: 0.25 mm) at the neck for 3 days. GS was treated with 0.1 ml of 0.1% DMSO (n = 5 rats), 100 μmol/l MOS (n = 5 rats), 100 μmol/l SSRi (n = 5 rats), or 50 μmol/l GR113808 (GR) + 100 μmol/l MOS (n = 3 rats). GS was removed after a solution containing 100% DMSO were diluted to 1,000-fold with a final DMSO concentration of 0.1%. This concentration of DMSO did not exert any effects on experimental results.

**Measurement of defecation reflex.** Experiments were performed on nine male guinea pigs (5 for MOS and 4 for SSRi; body wt: 330–430 g) anesthetized with ethyl carbamate (0.7–1.0 g/kg ip), artificially ventilated, and immobilized with gallamine (0.1 mg/kg iv). Two weeks after RT and RA, 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. Gradual and sustained rectal distension for 5 min at intervals of 20 min was performed as previously reported (6, 9, 15, 29).

The motility of the IAS was recorded with a custom-made strain gauge force transducer similar to that described previously (6, 9, 15, 29). The reflex response composed of recto-rectal (R-R) and recto-anal sphincter (R-IAS) reflexes was evaluated by “reflex index” as previously reported (15).

**Drugs.** The following drugs were used: fluvoxamine maleate (SSRi; Wako Pure Chemical Industries, Osaka, Japan), mosapride citrate (MOS; kindly donated by Dainippon-Sumitomo Pharmaceutical, Osaka, Japan), GR113808 (GR; Wako Pure Chemical Industries), gallamine triethiodide (Sigma, St. Louis, MO), and ethyl carbamate (Wako Pure Chemical Industries). mosapride (SSRI; Wako Pure Chemical Industries, Osaka, Japan), mosapride citrate (MOS; kindly donated by Dainippon-Sumitomo Pharmaceutical, Osaka, Japan), GR113808 (GR; Wako Pure Chemical Industries), gallamine triethiodide (Sigma, St. Louis, MO), and ethyl carbamate (Wako Pure Chemical Industries). MO, SSRi, and GR dissolved in a solution containing 100% DMSO were diluted to 1,000-fold with a final DMSO concentration of 0.1%. This concentration of DMSO did not exert any effects on experimental results.

**Immunohistochemistry.** The rectal whole mount preparations were fixed in 4% paraformaldehyde (4°C, overnight) or 99.5% acetone (4°C, 1 h) to detect neurofilament (NF) or neuronal nitric oxide synthase (nNOS). Thereafter, the mucosa and submucosa were removed from respective preparations, which were washed for 30 min in PBS (0.01–0.1 mol/l, pH 7.4) and then incubated for 3–12 h at 4°C in 10% normal donkey serum in PBS containing 0.3% (vol/vol) Triton X-100 (PBS-TX) to reduce nonspecific antibody binding. After incubation for 2 days at 4°C with a rabbit polyclonal antiserum to label NF (clone 2F11, reacting with 70-, 160-, and 200-kDa proteins, 0.5 μg/ml; DAKO) (21) or with a goat polyclonal antibody to label NOS (ProSci, Poway, CA) (5), NF and nNOS immunoreactivities were detected with Alexa Fluor 594- and Alexa Fluor 488-conjugated secondary antibody, respectively. Tissues were examined with an Olympus FV1000 (Tokyo, Japan) confocal microscope. Confocal micrographs are digital composites of Z-series scans of 10–15 optical sections through a depth of 10–20 μm or 100–150 μm. Final images were constructed with the FV10-ASW software application (version 1.7, Olympus).

For immunohistochemistry of sectioned preparations, the rectum including an anastomotic site and GS was fixed with 4% paraformaldehyde at 4°C and embedded in paraffin. Consecutive 4-μm sections were cut from each block. Immunostaining was performed by the immunoperoxidase technique after antigen retrieval with microwave treatment (15 min twice in pH 6.0 citrate buffer) for proliferating cell nuclear antigen (PCNA) and treatment with pepsin (DAKO, Carpenteria, CA) for 20 min at room temperature for distal-less homeobox 2 (DLX2), serotonin receptor 4 (SR4), and NF or c-RET. After endogenous peroxidase blockade with 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, as previously reported (6, 16). 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, color-developed with diaminobenzidine (DAB) solution (DAKO), and counterstained with Mayer’s hematoxylin (Sigma). Antibodies used in primary reaction and working concentrations were as follows: anti-NF (clone 2F11, reacting with 70-, 160-, and 200-kDa proteins, 0.5 μg/ml; DAKO) (21), anti-SR4 (clone N-16; the epitope is located at the N-terminus of human SR4, 0.5 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA) (16), and anti-DLX2 (catalog no. ab18188, 0.5 μg/ml; Abcam, Tokyo, Japan) (6, 16, 27) as enteric neural stem cell markers, anti-PCNA (clone PC10; DAKO) as a cell proliferating marker that is specifically expressed in cell nuclei during the S phase (3), and anti-c-RET (rabbit polyclonal, 2 μg/ml; Assay Biotechnology, Sunnyvale, CA) as a neural crest-derived stem cell marker.

**Quantitative reverse transcription-polymerase chain reaction.** Extraction of total RNA was carried out with the RNeasy Mini Kit (Qiagen Genomics, Bothell, WA), and total RNA (1 μg) was synthesized with the ReverTra Ace-a-RT Kit (Toyobo, Osaka, Japan). qRT-PCR was performed by StepOne Real-Time PCR Systems (Applied Biosystems, Foster City, CA) using Fast SYBR Green Master Mix (Applied Biosystems) and analyzed by the relative standard curve quantification method. PCR condition was set according to the provider’s instructions. ACTB mRNA was amplified for internal control (GenBank accession no. NM001101). Each amplification reaction was evaluated by a melting curve analysis. For visualizing PCR products, agarose gel electrophoresis was performed with ethidium bromide staining.

Primer sets were upper 5′-ACT CAT GCC CAT TTC CT-3′ and lower 5′-CGG ACA TGG CGC TCA GT-3′ for mouse SR4 (NM_012853.1), upper 5′-TCC TAC TCC GGC AAA AG-3′ and lower 5′-GCC CGC CAT CAC CTC TT-3′ for mouse DLX2 (NM_00119746.1), upper 5′-CGG ACAG CGT GGG CAG CC-3′ and lower 5′-GGC TGG CCA GTG CGC CG-3′ for mouse NF (NM_017029.1), upper 5′-CCA AAT CAA GAG AAA GT-3′ and lower 5′-TCA GAG CAA AAG TTA GC-3′ for mouse PCNA (NM_022381.3), and upper 5′-GGG CCC CGA GTG TGA GG-3′ and lower 5′-CCT CAC ACT CGG GCG GC-3′ for mouse c-RET (AF042830.2).
Detection of regenerated enteric neurons. To identify neuronal cell proliferation, 5-bromo-2-deoxyuridine (BrdU, 1 mg/ml solution; Sigma or Nacalai Tesque, Kyoto, Japan) was added to the drinking water for 1–2 wk for 12 animals: 2 were fluvoxamine treated by LA, 2 and 3 were MOS treated by LA and PO, and 2 and 3 were 0.1% DMSO treated by LA and PO. After rinsing 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 mol/l 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 mol/l Tris-EDTA (pH 7.8), followed by routine immunohistochemistry.

Statistical analysis. Statistical significance of differences between means was determined by Mann-Whitney U-test or one way or two-way ANOVA, followed by multiple comparisons by Bonferroni or Fisher’s protected least significant difference (PLSD) post hoc test. A P value of <0.05 was considered statistically significant.

RESULTS

Effects of fluvoxamine and mosapride by local application on regeneration of NF- and nNOS-positive fibers after nerve fiber tract injury. Myenteric neurons in the rectal anastomotic site were severely damaged and the neural network was disrupted after 2 wk, although myenteric neurons at oral and anal sites of the anastomotic site appeared to be intact after treatment with vehicle and the SSRI fluvoxamine (Fig. 1, A and B). However, after treatment with MOS for 2 wk, bundles of fine nerve fibers were observed traversing the oral and anal ends of the myenteric plexus, as previously described in guinea pigs (15, 16) (Fig. 1, C and D).

In contrast, in the rectal anastomotic site treated with GR and MOS for 2 wk, these patterns of new axon growth were not observed (Fig. 2, Ab and Bb).

Additional effects of SSRI and MOS-LA on anti-PCNA-, DLX2-, SR4-, and NF-positive cells in granulation tissue sections at anastomotic site 1–2 wk after nerve fiber tract injury. The granulation tissue defined as new tissue formed by growth of fibroblasts and blood capillaries into injured tissue was newly formed at the rectal anastomotic site 1–2 wk after RT + RA. In the granulation tissue at the anastomotic site treated with SSRI or MOS for 1–2 wk, myenteric networks observed in the intact rectal region were absent in the region within 1–3 mm of the anastomosis, consistent with observations in the whole mount preparations.

In the granulation tissue at the rectal anastomotic site treated with SSRI, after 1–2 wk PCNA-positive cells were observed as in the tissue treated with MOS but DLX2-, SR4-, and NF-positive cells were only observed sparsely in the tissue treated with SSRI (Fig. 3, A and B).

After 1–2 wk, PCNA-positive cells including fibroblasts with proliferating activity were frequently found in the granulation tissue within the anastomotic site treated with SSRI (83 ± 18 and 72 ± 16 cells/mm²) and MOS (72 ± 11 and 47 ± 6 cells/mm²) (Fig. 3C). However, after 1 wk, cells immunoreactive for DLX2 and SR4 were significantly diminished in the granulation tissue treated with SSRI compared with MOS [16 ± 4 vs. 42 ± 5 cells/mm² (P < 0.05) and 6 ± 2 vs. 12 ±
Fig. 3. Representative set of images of immunostaining for proliferating cell nuclear antigen (PCNA), distal-less homeobox 2 (DLX2), serotonin receptor 4 (SR4), and NF. Representative images of immunostaining for PCNA, DLX2, NF, and SR4 at the anastomotic site treated with MOS-LA (A) and SSRI-LA (B) for 1 wk and 2 wk after RT + RA are shown. PCNA-positive cells were detected similarly in A and B, but DLX2-, SR4-, and NF- positive cells were less detected in the granulation tissue within the anastomotic site after SSRI-LA (B). In particular, SR4- and NF-positive cells were not detected 2 wk after SSRI-LA (right) but were detected as indicated by arrows after MOS-LA (A, right). Calibration bars, 100 μm. C: summarized effects of MOS-LA and SSRI-LA on numbers of PCNA-, DLX2-, SR4-, and NF-positive cells in the granulation tissue within 2 mm of anastomotic site. Mean values were obtained from 5 high-powered fields (HPFs; 0.2 mm²). *P < 0.05 vs. MOS-LA by Mann-Whitney U-test.

Comparison of effects of MOS and SSRI on mobilization of anti-PCNA-, DLX2-, SR4-, and c-RET-positive cells into subcutaneously implanted GS at the neck. MOS significantly increased the number of DLX2-, SR4-, and c-RET-positive cells in the implanted GS, but SSRI markedly decreased the number of DLX2- and SR4-positive cells compared with control and MOS (Fig. 4), like SSRI applied at the anastomotic site (Fig. 3). No NF-positive cells were observed. DLX2- and SR4-positive cells are neural stem cells, and c-RET-positive cells are neural crest-derived stem cells (21). Therefore, it seems likely that MOS differentiates neural crest-derived stem cells (17) or mesenchymal stem cells mobilized from the bone marrow into neural stem cells and that SSRI suppresses mobilization of neural crest-derived stem cells or mesenchymal stem cells and/or differentiation into neural stem cells. These results were confirmed by similar results by qRT-PCR (Fig. 5). In addition, GR antagonized MOS-induced increases of mRNA of DLX2, SR4, and c-RET to 0.92 ± 0.43-fold, 1.14 ± 0.34-fold, and 1.18 ± 0.21-fold of control, respectively.

Comparison of effects of LA vs. PO delivery of MOS on regeneration of NF- and nNOS-positive fibers after nerve fiber tract injury. After treatment with 0.1% DMSO by oral application for 2 wk, no bundles of fine nerve fibers were observed within the anastomotic site (Fig. 6A). However, after treatment with MOS-PO for 2 wk, fine nerve fibers were observed traversing the oral and anal ends of the myenteric plexus (Fig. 6Ba) as after MOS-LA for 2 wk (Fig. 1, C and D). nNOS-positive fine nerve fibers were also observed (Fig. 6Bb).

BrdU-positive cells were identified in sites 3 mm oral and anal from the anastomotic sites treated for 2 wk with MOS-LA (Fig. 7A) and MOS-PO (Fig. 7B), unlike in guinea pigs (16). Summarized data of BrdU positivity indicated that MOS-PO significantly increased BrdU-positive cells from MOS-LA in 5 mm oral and 5 mm anal sites but not in 3 mm oral and 3 mm anal sites (Fig. 7C). In contrast, SSRI-LA showed significantly less BrdU immunoreactivity in all sites (Fig. 7C). Although BrdU-positive cells also appeared to include fibroblasts, endothelial cells, and macrophages, BrdU-positive cells forming ganglia include newly formed neurons.

Comparison of additional effects of MOS on anti-PCNA-, DLX2-, SR4-, and NF-positive cells in granulation tissue sections at anastomotic site 1–2 wk after nerve fiber tract injury between PO and LA. After 1–2 wk, PCNA-positive cells were frequently found in the granulation tissue within the anastomotic site in preparations from animals treated with MOS-PO and MOS-LA, although PCNA-positive cells decreased after 2 wk. Cells immunoreactive for DLX2, SR4, and NF were also frequently found in the granulation tissue treated with MOS-PO and MOS-LA, but DLX2-, SR4-, and NF-positive cells after 1 wk were more frequently found in the tissue treated with MOS-PO than in that treated with MOS-LA. After 2 wk, the appearance of DLX2-, SR4-, and NF-positive cells was comparable in the tissues treated with MOS-PO and MOS-LA (Fig. 8, A and B). SR4-positive cells in the tissues treated with MOS-PO were found in a ganglion (Fig. 8B).

After 1–2 wk, the number of PCNA-positive cells was similar in granulation tissue within the anastomotic site in preparations from animals treated with MOS-PO (72 ± 10 and 38 ± 5) and MOS-LA (72 ± 11 and 47 ± 6). The number of cells immunoreactive for DLX2, SR4, and NF was signifi-
cantly larger in granulation tissue treated with MOS-PO than in that treated with MOS-LA after 1 wk \([54 \pm 5 \text{ vs. } 42 \pm 5 \text{ (}\text{P}<0.05\text{)}\text{, }18 \pm 2 \text{ vs. } 12 \pm 2 \text{ (}\text{P}<0.05\text{)}\text{, and } 9 \pm 2 \text{ vs. } 2 \pm 0 \text{ (}\text{P}<0.05\text{)}\text{]} \) (Fig. 8C). However, there were no significant differences in the number of DLX2-, SR4-, and NE-positive cells between MOS-PO and MOS-LA after 2 wk except for the number of SR4-positive cells (Fig. 8C).

**Measurement of defecation reflex.** Figure 9A shows representative examples of R-R and R-IAS reflex recoveries at 2 wk after nerve fiber tract injury in a guinea pig treated with MOS-LA. In contrast, the R-IAS reflex response remained abolished and the R-R reflex was unchanged in a guinea pig treated with SSRI-LA (Fig. 9B). Mean reflex index of R-IAS reflex was 0 for SSRI-LA \((n = 4)\), whereas it was \(-0.8\) for MOS-LA \(n = 5\) (15).

**DISCUSSION**

The results reported here indicate that SSRI does not induce in vivo nerve fiber tract growth across a surgical anastomosis, but MOS-LA and MOS-PO promoted a 5-HT4 receptor-mediated neural regeneration, since effects of MOS-LA and MOS-PO were antagonized by 5-HT4 receptor antagonists GR (see Fig. 2) and SB-207266 (our unpublished data in guinea pigs and mice), respectively. There were no significant differences in nerve fiber tract growth action between MOS-LA and MOS-PO after 2 wk, suggesting that the clinical application of MOS is promising.

SSRI can increase endogenous 5-HT in the gastrointestinal tract (1). Increased endogenous 5-HT can act on enteric neural 5-HT4 receptors as well as other subtypes of 5-HT receptors such as 5-HT3 receptors (14). In fact, there is evidence supporting the idea that a variety of subtypes of 5-HT receptors play roles in the inflammatory response (2, 7, 26). 5-HT3 receptors in in vitro nodose ganglion play proinflammatory roles (7), whereas enteric neuronal 5-HT4 receptors induce anti-inflammatory actions via \(\alpha7\)nACh receptors on muscularis macrophages associated with postop-

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Fig. 4. Immunostaining for PCNA, DLX2, SR4, and c-RET after 3 days following the subcutaneous implantation of gel sponge at the neck. A: representative images of immunostaining for PCNA, DLX2, SR4, and c-RET after 3 days following the subcutaneous implantation of gel sponge \((W \times L: 0.5 \text{ cm} \times 0.5 \text{ cm}; \text{thickness: } 0.25 \text{ cm})\) at the neck. Gel sponge absorbed 0.1% DMSO (Control), 100 \(\mu\text{mol/l MOS}\), and 100 \(\mu\text{mol/l SSRI}\). Calibration bar, 100 \(\mu\text{m}\). B: summarized effects of Control, MOS, and SSRI on numbers of PCNA-, DLX2-, SR4-, and c-RET-positive cells in the gel sponge. Mean values were obtained from 10 HPFs \((0.2 \text{ mm}^2)\). *\(P<0.05\) vs. Control, †\(P<0.05\) vs. MOS by Mann-Whitney \(U\)-test.
Fig. 5. qRT-PCR analysis for mRNA of PCNA, DLX2, SR4, and c-RET after 3 days following the subcutaneous implantation of gel sponge at the neck. Gel sponge absorbed 0.1% DMSO (Control), 100 μmol/l MOS, 100 μmol/l SSRI, and 50 μmol/l GR + 100 μmol/l MOS. A: PCNA mRNA. B: DLX2 mRNA. C: SR4 mRNA. D: c-RET mRNA. GR antagonized effects of MOS. NF mRNA was not detected. *P < 0.05 vs. Control by Fisher’s protected least significant difference (PLSD) post hoc test in D and by Bonferroni post hoc test in C. #P < 0.05 vs. SSRI by Fisher’s PLSD post hoc test in B and D and by Bonferroni post hoc test in C.

Fig. 6. Immunostaining for NF (red) and neuronal nitric oxide synthase (nNOS, green) in the rectal myenteric plexus 2 wk after RT+RA. A: 2 wk after vehicle in drinking water (Cont-PO), no bundles of fine nerve fibers can be seen between the oral and anal cut ends of the myenteric plexus. B: after MOS in drinking water (100 μmol/l; MOS-PO), numerous NF-positive bundles of fine nerve fibers can be seen between the oral and anal cut ends of the myenteric plexus as shown by arrows (a). Numerous nNOS-positive nerve fibers as shown by arrows are present at the anastomosis (b).
erative ileus (26). Regardless of the roles of increased endogenous 5-HT in inflammation, there is a possibility for diverse actions of increased endogenous 5-HT on in vivo induction of nerve fiber tract growth across the anastomosis mediated through different subtypes of 5-HT receptors.

5-HT per se enhanced in vitro differentiation of neural networks in ES cell-induced gutlike organ in mouse (20). Moreover, it has been described that 5-HT2B receptors play a role in promotion of neuronal precursor differentiation of ENS during development in mice (4). However, in the present study, SSRI did not induce in vivo nerve fiber tract growth. It is speculated that promoting effects of increased endogenous 5-HT on the nerve fiber tract growth across the anastomosis may be masked by activation of different subtypes of 5-HT receptors with suppressive effects. In addition, the increase of endogenous 5-HT may not be attainable in this region.

On the other hand, the finding that SSRI treatment induced significant decreases in number of PCNA-, DLX2-, and SR4-positive cells in the implanted GS at the neck may suggest the possibility that SSRI suppresses the mobi-

Fig. 7. Representative images of immunostaining for 5-bromo-2-deoxyuridine (BrdU) in MOS-LA (A) and MOS-PO (B). Myenteric ganglionic cells labeled for BrdU (indicated by 6 thick arrows) were found in the rectal tissue within sites 3 mm oral and 3 mm anal from rectal anastomotic site in rats treated with 100 μmol/l MOS-LA and MOS-PO for 2 wk. Calibration bar, 100 μm. C. summarized data of BrdU positivity in the rectal tissue within sites 3–5 mm oral and anal from rectal anastomotic site. Open bars, MOS-LA; gray bars, MOS-PO; filled bars, SSRI-LA. *P < 0.005 vs. MOS-LA; #P < 0.005 vs. SSRI-LA; §P < 0.005 vs. MOS-PO.

Fig. 8. Representative images of immunostaining for PCNA, DLX2, SR4, and NF in newly formed granulation tissue within the anastomotic site treated with MOS-LA (A) and MOS-PO (B) for 1 wk and 2 wk. PCNA-positive cells were detected similarly in A and B, but DLX2-, SR4- and NF-positive cells were more frequently detected at 1 wk in B. DLX2-, SR4-, and NF-positive cells were detected similarly at 2 wk in A and B except for SR4-positive cells. Calibration bar, 100 μm. C. mean values were obtained from 5 HPFs (0.2 mm²). PCNA-positive cells were detected similarly in MOS-LA and MOS-PO, but DLX2-, SR4-, and NF-positive cells were significantly increased at 1 wk and SR4-positive cells were significantly increased at 2 wk in the granulation tissue in MOS-PO. *P < 0.05 vs. MOS-LA by Mann-Whitney U-test.
lization of neural crest-derived stem cells or mesenchymal stem cells and/or differentiation into neural stem cells (see Figs. 4 and 5). In the present RT + RA model, a similar mechanism may participate in the lack of induction of nerve fiber tract growth across the anastomosis by SSRI-LA.

Furthermore, recent studies suggested that a serotonergic depressant, fluoxetine, can reverse the established state of neuronal maturation in the adult hippocampus (8) and promote gliogenesis during neural differentiation in mouse ES cells (11). Therefore, these actions of SSRI may suppress enteric neural differentiation/development as repairing mechanism even in the present adult rat.

The MOS-induced significant increase in number of DLX2-, SR4-, and c-RET-positive cells and mRNA of DLX2, SR4, and c-RET in the implanted GS at the neck could support the possibility that MOS differentiated neural crest stem-derived stem cells (17) or bone marrow mesenchymal stem cells into neural stem cells as repairing mechanisms (see Figs. 4 and 5). In the present RT + RA model, a similar mechanism may participate in induction of nerve fiber tract growth across the anastomosis by MOS-LA and by MOS-PO.

Furthermore, surprisingly, unlike in guinea pigs, MOS-LA and MOS-PO generated BrdU-positive neural cells in ganglia 3–5 mm distant from the anastomotic site 2 wk after RT + RA. The cell proliferative ability of rat neural stem cells seems to be higher than that in guinea pig.

To detect whether any drug treatments promote reconstruction of enteric neural circuit injury in the distal gut, it should be determined whether the IAS relaxation reflex (22–25, 29) can completely recover, as seen in guinea pigs (6, 15, 16). However, we did not detect a marked R-IAS relaxation reflex even in intact rats. The cell proliferative ability of rat neural stem cells seems to be higher than that in guinea pig.

As SERT knockout rats (Slc6a41Hubr) (18) are available and promising for further study.

Therefore, we used guinea pigs instead of rats to evaluate the reflex index for R-IAS reflex as previously reported (15). The R-IAS reflex response remained abolished with unchanged R-R reflex in guinea pigs treated for 2 wk with SSRI-LA (Fig. 9B), whereas R-IAS reflex recovered to ~0.8 of intact control (≈1.0) in guinea pigs treated with MOS-LA, consistent with the previous report (15).

In conclusion, SSRI does not induce in vivo nerve fiber tract growth across the anastomosis in the rat distal gut, whereas MOS does induce nerve fiber tract growth across the anastomosis, mediated through enteric neural 5-HT4 receptors.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: I.K., H.K., H. Matsuyoshi, K.G., K.O., H. Misawa, and M.T. performed experiments; I.K., H.K., and M.T. analyzed data; I.K., H.K., and M.T. prepared figures; I.K., H.K., H. Matsuyoshi, K.G., K.O., H. Misawa, H.F., and M.T. approved final version of manuscript; H.K. and M.T. interpreted results of experiments; H.F. and M.T. conception and design of research; M.T. drafted manuscript; M.T. edited and revised manuscript.
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