Invited Review

Activation of 5-HT$_4$ receptors facilitates neurogenesis of injured enteric neurons at an anastomosis in the lower gut

Miyako Takaki[1,2,3], Kei Goto[2], Isao Kawahara[2] and Junich Nabekura[4]

[1]Department of Physiology II, Nara Medical University, School of Medicine, Kashihara, Nara, Japan
[2]Department of Molecular Pathology, Nara Medical University, School of Medicine, Kashihara, Nara, Japan
[3]Department of Orthopedic Surgery, Nara Medical University, School of Medicine, Kashihara, Nara, Japan
[4]Division of Homeostatic Development, Department of Developmental Physiology, National Institute for Physiological Sciences, Okazaki, Aichi, Japan

Abstract

Two-photon microscopy (2PM) can enable high-resolution deep imaging of thick tissue by exciting a fluorescent dye and protein at anastomotic sites in the mouse small intestine *in vivo*. We performed gut surgery and transplanted neural stem cells (NSC) from the embryonic central nervous system after marking them with the fluorescent cell linker, PKH26. We found that neurons differentiated from transplanted NSC (PKH [+]) and newborn enteric neurons differentiated from mobilized (host) NSC (YFP [+]) could be localized within the granulation tissue of anastomoses. A 5-HT$_4$-receptor agonist, mosapride citrate (MOS), significantly increased the number of PKH (+) and YFP (+) neurons by 2.5-fold (*P*<0.005). The distribution patterns of PKH (+) neurons were similar to those of YFP (+) neurons. On the other hand, the 5-HT$_4$-receptor antagonist, SB-207266 abolished these effects of MOS. These results indicate that neurogenesis from transplanted NSC is facilitated by activation of 5-HT$_4$-receptors. Thus, a combination of drug administration and cell transplantation could be more beneficial than exclusive cell transplantation in treating Hirschsprung's disease and related disorders including post rectal cancer surgery. The underlying mechanisms for its action were explored using immunohistochemistry of the longitudinal mouse ileum and rat rectal preparations including an anastomosis. MOS significantly increased the number of new neurons, but not when co-administered with either of a protein tyrosine kinase receptor, c-RET two inhibitors. The c-RET signaling pathway contributes to enteric neurogenesis facilitated by MOS. In the future, we would perform functional studies of new neurons over the thick granulation tissue at anastomoses, using *in vivo* imaging with 2PM and double transgenic mice expressing a calcium indicator such as GCaMP6 and channelrhodopsin.

Key words: 5-HT$_4$ receptors, enteric neurons, granulation tissue, neural stem cells, neurogenesis

Corresponding author: Prof. Miyako Takaki Ph.D., AGAF Department of Orthopedic Surgery, Nara Medical University, School of Medicine, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan
Phone: +81-744-29-8873 Fax: +81-744-25-6449 e-mail: mtakaki@naramed-u.ac.jp
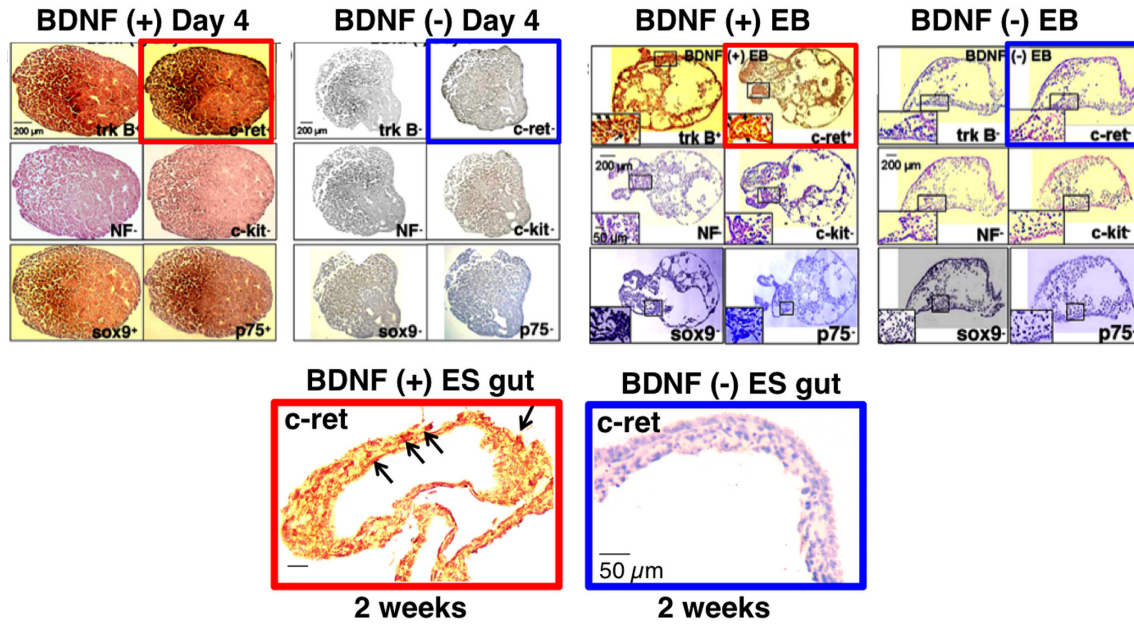
Fig. 1. Brain-derived neurotrophic factor (BDNF) facilitates c-ret expression in day 4, and an embryoid body (EB) in hanging drop culture and ES gut. Immunoreactivity associated with trkB, c-ret, sox9 and p75, but no NF or c-kit immunoreactivity, was identified after 4 days, treated with BDNF (BDNF+Day 4). No trkB, c-ret, NF, c-kit, sox9 or p75 immunoreactivity was seen after 4 days if not treated with BDNF (BDNF–Day 4). Immunoreactivity associated with trkB and c-ret, but no NF, c-kit, sox9 or p75 immunoreactivity, was identified in an EB treated with BDNF (BDNF+EB). No trkB, c-ret, NF c-kit, sox9 or p75 immunoreactivity was seen in another EB not treated with BDNF (BDNF–EB). C-ret immunoreactivity was apparent in an ES gut differentiated from EB (BDNF+) after 2 weeks in outgrowth culture (BDNF+ES gut). No c-ret immunoreactivity was detected in ES guts differentiated from EB (BDNF–) after 2 weeks in outgrowth culture (BDNF–ES gut). This figure was reproduced and modified from ref. (1) with permission.

Introduction

Before considering the thrust of this review, we have better to consider the role of brain-derived neurotrophic factor (BDNF) in the regeneration of impaired enteric neurons and the associated problems both in culture and *in vivo*.

Brain-derived neurotrophic factor (BDNF) as a regenerative agent for the impaired enteric nervous system (ENS)

Using an embryoid body (EB) culture system, we developed a functional organ-like cluster, a ''gut'', from mouse embryonic stem (ES) cells (ES gut). By adding BDNF only during EB formation, a gene encoding the Ret receptor tyrosine kinase, c-ret expression was facilitated by BDNF in day 4 in both the EB and ES gut. This resulted, for the first time, in the *in vitro* formation of enteric neural ganglia with connecting nerve fiber tracts (the enteric nervous system [ENS]) in the ES gut (Fig. 1) (1). This ENS is differentiated from enteric neural crest-derived cells (ENCDC). The ES gut with ENS exhibited strong peristalsis-like movements. Moreover, focal electrical stimulation of ES guts with ENS elicited propagated increases in intracellular Ca^{2+} concentration ([Ca^{2+}]$_i$) at single or multiple sites that were attenuated by atropine or abolished by tetrodotoxin (1). These results suggest *in vitro* formation of physiologically functioning enteric cholinergic excitatory neurons. Thus BDNF facilitates physiologically functioning ENS differentiation.
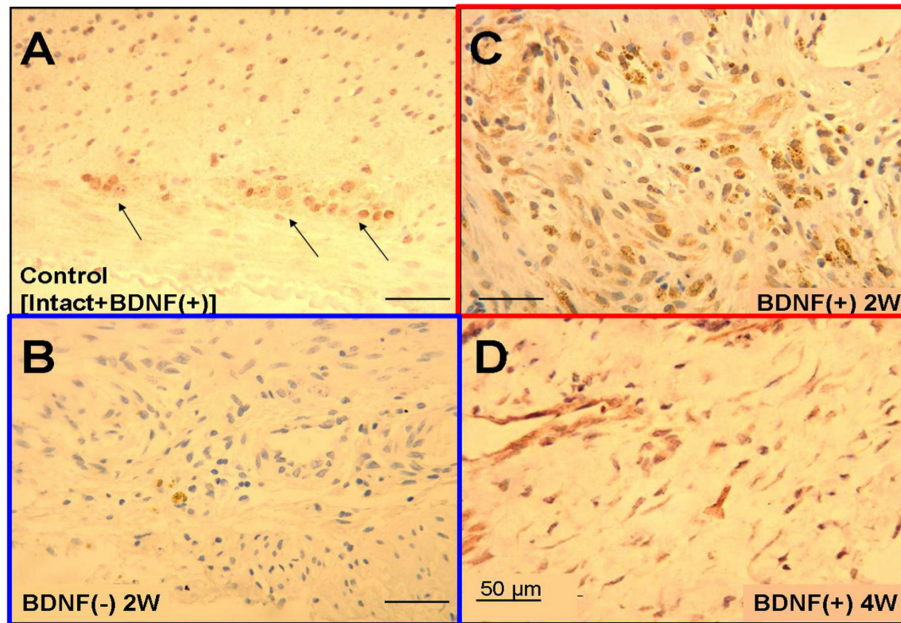
Fig. 2. Representative images of immunostaining for tyrosine receptor kinase B (TrkB) in the intact rectum treated with BDNF for 2 weeks (wk) [Intact+BDNF(+)](A) and in the newly formed granulation tissue treated with saline for 2 wk [BDNF(-)2W](B) and treated with BDNF for 2 wk [BDNF(+)2W](C) and treated with BDNF for 4 wk [BDNF(+)4W](D). A: TrkB-positive cells indicated by arrows were observed in myenteric ganglia of the intact rectum. B: TrkB-positive cells were rarely observed in the granulation tissue within the 2-mm rectal anastomotic site. C: TrkB-positive cells were more frequently observed than NF-positive cells in this region. D: Number of TrkB-positive cells decreased. This figure was reproduced and modified from ref. (3).

BDNF facilitates ENS regeneration in vivo

The defecation reflex, composed of a recto-rectal reflex contraction (R-R reflex) and the recto-internal anal sphincter reflex relaxation (R-IAS reflex) (2), is impaired after rectal transection and anastomosis (3). This surgery simulates a lower anterior resection for rectal cancer. Rectal transection and anastomosis cause sectioning of intrinsic reflex nerve pathways in the rectum. Eight weeks after this rectal surgery, the defecation reflex recovered to the control level, accompanied with regeneration of reflex pathways. Two weeks after local treatment with BDNF (10^{-6} g/ml) at the rectal anastomotic site, however, the R-IAS reflex 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 ENS with local application of BDNF (3). BDNF facilitates physiologically functioning ENS regeneration *in vivo*.

Problems of BDNF as a regenerative drug for ENS in vivo

However, BDNF up-regulated its receptor, TrkB at the rectal anastomosis (Fig. 2) (3). Up-regulation of TrkB is thought to relate to an increased risk of metastasizing rectal cancer. Furthermore, BDNF can exacerbate the inflammation generated at the rectal anastomosis after surgery. Therefore, we should give up using BDNF as a therapeutic drug for ENS regeneration after rectal cancer surgery.
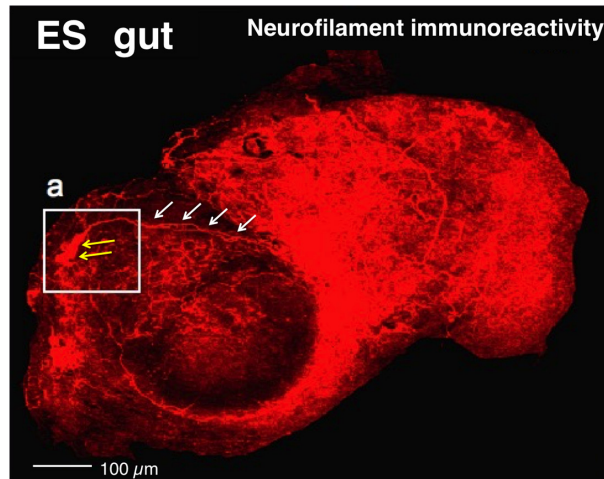
Fig. 3. Immunohistochemical detection of neurofilaments (NF) in a dome-like structure in the embryonic stem (ES) gut after 14–21 day outgrowth culture differentiated from embryoid bodies (EBs) treated with 1 µmol l^{-1} MOS. Yellow arrows indicate newly differentiated neurons. White arrows indicate nerve fibers. Not all structures labeled by red color are neurons, but is the result of non-specific binding. This figure was reproduced and modified from ref. (4) with permission.

A 5-HT$_4$-receptor (SR4)-agonist as a new candidate for ENS regeneration

In ES gut: In spontaneously contracting ES guts, dense distributions of interstitial cells of Cajal (ICC) (c-kit positive cells; gut pacemaker cells) and smooth muscle cells were discernibly identified with no treatment, but discernible enteric neural networks were not observed. In these ES guts, we also succeeded in forming dense enteric neural networks by using an SR4-agonist, mosapride citrate (MOS; 1–10 µmol l^{-1}) which was added during EB formation (Fig. 3) (4). Addition of an SR4-antagonist, GR113808 (GR; 10 µmol l^{-1}) abolished the SR4-agonist-induced formation of enteric neural networks. MOS (1 µmol l^{-1}) up-regulated the expression of the mRNA of SR4 and GR (10 µmol l^{-1}) abolished this up-regulation (4). 5-HT per se exerted similar effects to those of the SR4-agonist, but was less potent. These results suggest that the SR4-agonist differentiated enteric neural networks, which was mediated via activation of SR4 in the ES gut. Some SR4-agonists could therefore be promising drugs for ENS regeneration *in vivo*.

In vivo rectum: In the rectum of guinea pigs, MOS also promoted the regeneration of the neural circuits of the impaired myenteric plexus and the recovery of the defecation reflex (5). Furthermore, MOS generated neurofilament (NF)-, SR4- and 5-bromo-2'-deoxyuridine (BrdU)-positive cells, and as predicted, MOS formed neural networks in the newly formed granulation tissue at the anastomotic site 2 weeks after the enteric nerve circuit insult. Using possible neural stem cell markers, it was found that both anti-distal less homeobox 2 (DLX2)- and p75-positive cells as well as NF-positive cells increased during the same time period. MOS did not exert any effects at all on the intact rectum. All actions by MOS were inhibited by the specific SR4-antagonist, GR (10 µmol l^{-1}) (5). These results indicate that activation of enteric neural SR4 promotes reconstruction of enteric neural circuits leading to the recovery of the defecation reflex in the rectum, and that this reconstruction possibly involves neural stem cells. These findings suggest that treatment with SR4-agonists could be a novel therapy for generating new enteric neurons to rescue aganglionic gut disorders including post rectal cancer surgery, instead of BDNF.
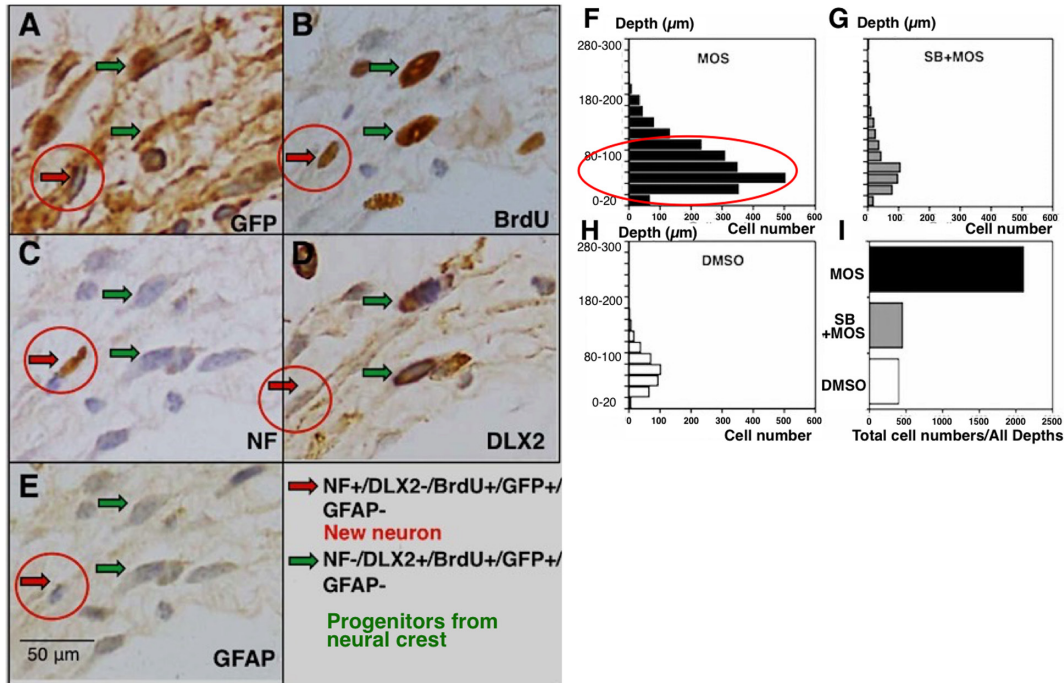
Fig. 4. Immunoreactivity in the granulation tissue at the anastomosis (A-E) and distribution of total number of new neurons at the anastomosis (F-I). This figure was reproduced and modified from ref. (6). Abbreviations: see ref. (6).

In vivo imaging newborn neurons within the thick granulation tissues

Newborn neurons are typically distributed within the thick granulation tissues. The granulation tissues are newly formed connective tissues composed of fibroblasts and blood capillaries after the transection and anastomosis of the rectum (3). Traditional fluorescence microscopy including confocal microscopy is unsuitable for high-resolution deep imaging of the 300–400 µm thick granulation tissue, as previously reported (6). Two-photon-excited fluorescence microscopy (2PM), overcomes this limitation by providing enhanced optical penetration. We previously confirmed the expression of green fluorescent protein (GFP) in the cytoplasm of enteric neurons of the ileum of Thy1-GFP mice (7). Using 2PM and Thy1-GFP mice, we three-dimensionally obtained *in vivo* images of reconstructed enteric neural circuits within the thick tissue in the ileum in their native environment (8).

Neurogenesis from progenitors of the neural crest was promoted by oral application of the SR4- agonist, MOS (Fig. 4). The number of newly generated neurons observed in mice treated with MOS for one week was 421,689 per 864,900 mm^2, which was significantly greater than that observed in preparations treated with MOS plus an antagonist or in 4 week vehicle controls (6). Most neurons were located within 100 µm of the surface (Fig. 4F) (6). These results suggest that activation of enteric neural SR4 by MOS also promotes formation of new enteric neurons in the anastomotic thick granulation tissue in the living mouse terminal ileum. Functional studies of these new enteric neurons remain to be investigated.

A combination of SR4-agonist administration and cell transplantation as a more beneficial treatment

As demonstrated in the guinea pig rectum (5), the study in the ileum also revealed that MOS-activated neural SR4 facilitates neurogenesis from mobilized neural stem cells (NSC) (6). In the next-step study, we hypothesized that NSC from the hippocampus and subventricular zone (SVZ) of mouse embryos could be transplanted into the gut to achieve neurogenesis (9). We aimed to determine whether activation of SR4 by MOS promotes neurogenesis from transplanted NSC in their native environment after ileal surgery in Thy1 promoter yellow fluorescent protein (YFP) mice using 2PM, since we have already confirmed the expression of cytoplasmic YFP in enteric neurons (10).

Validation of the quality of NSC for transplantation

Before cell transplantation, we examined effects of MOS on NSC in 4 day cultures to verify the quality of the NSC. Neurospheres were formed after 4 days of culture in NSC growth medium in control. We then examined the effects of BDNF on NSC in culture, because BDNF facilitated formation of enteric neural networks in ES guts, but glia-derived neurotrophic factor (GDNF) did not (1). BDNF (10 ng ml^{-1}) weakly facilitated outgrowth of projections from neurospheres, but MOS (1 μmol l^{-1}) more potently facilitated outgrowth of projections from neurospheres. GR (10 μmol l^{-1}), the selective SR4-antagonist, abolished these MOS-induced effects on neurospheres (10). We judged that these NSC could be used for cell transplantation.

In vivo images of the anastomotic region in MOS-treated YFP mice

Immediately after surgery, 2×10^5 NSC labeled with PKH26 was transplanted through the tail vein (10). Following that, the mice were given either i) 0.1% DMSO solution (vehicle), ii) MOS (100 μmol l^{-1}) in vehicle or iii) a selective SR4- antagonist for oral administration (SB-207266 [SB: 10–50 μmol l^{-1}]) plus MOS (100 μmol l^{-1}) in vehicle, to drink each day for 2 weeks (6, 10).

A Ti-sapphire laser (MaiTai Hp, Spectral Physics, Mountain View, CA, USA) was tuned to the excitation wavelength (950 nm) for YFP (yellowish green fluorescence) and for PKH26 (yellow-orange fluorescence)(6, 10). Neurons were counted in each optical section of each of 9 fields, where we could differentiate two types of neurons as either orange (PKH26 +) or yellow (YFP+) (10).

In Thy1-YFP mice treated with MOS (100 μmol l^{-1}), 2 weeks after gut surgery and NSC transplant, YFP+ neurons that possibly differentiated from the mobilized (host) NSC were observed around the knot in the thick granulation tissue, at 1–201 μm deep from the serosal surface. PKH+ neurons that possibly differentiated from the transplant NSC, were also observed in the same site in almost all mice tested (10).

Confirmation of neurogenesis from the transplant NSC

After *in vivo* imaging with 2PM, longitudinal ileum sections including the anastomosis were observed under a confocal microscope in MOS-treated mice. These indicated that PKH26 (red fluorescence) (+) aggregates were found at the border of the granulation tissue but not outside the granulation tissue (10). These aggregates of the transplant NSC seem to have been mobilized to the granulation tissue. It seems likely that some factors such as chemokines from the granulation tissue had provoked the mobilization of the transplant NSC.

PGP9.5-immunopositive (+) cells, some of which formed neural ganglia, were observed in the granulation tissue. The same section showed both PGP9.5 immuno-positivity and PKH fluorescence (10). This indicated that both PKH26 (+) and PGP9.5 (+) cells corresponded to the neurons differentiated from the transplanted NSC.
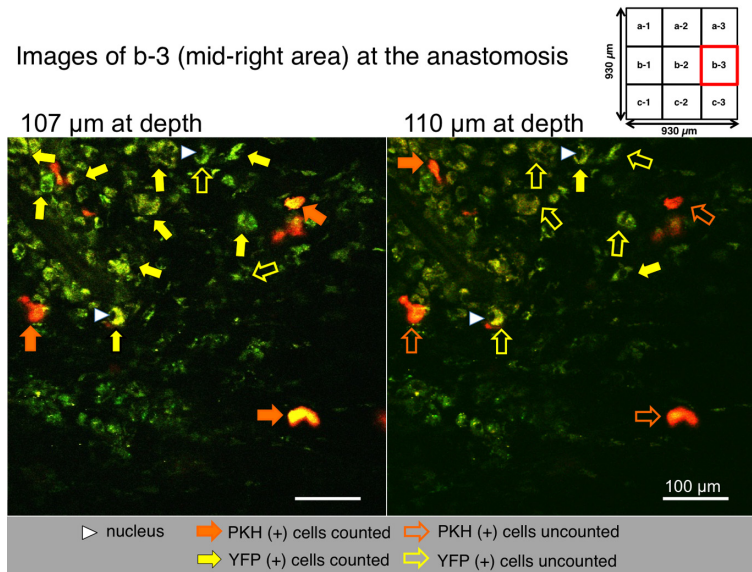
Fig. 5. Two-PM images of anastomotic region in a neural stem cell (NSC)-transplanted and MOS-treated YFP mouse for 2 weeks. PKH26 fluorescence (+) /YFP fluorescence (+) [PKH26 (+)/YFP (+)] neurons were distributed in each 9 field (a-1~c-3; field size: 310 µm × 310 µm) around the knot at the anastomosis. Mid-right areas (b-3) were demonstrated at depths of 107 and 110 µm. Each white arrowhead indicates a nucleus. Orange solid arrows indicated PKH26 (+) neurons and yellow solid arrows indicated YFP (+) neurons (not all). Overlapped neurons between both depths were not counted as indicated by each open arrow. This figure was reproduced and modified from ref. (10).

Quantitative analysis of new neurons differentiated from the transplant and host NSC

PKH26 (+) neurons (shown by solid orange arrows) or YFP (+) neurons (shown by solid yellow arrows) could be accurately counted in samples of the mid-right area (b-3) at depths of 107 µm and 110 µm from the serosal surface (Fig. 5). Around the knot of thread at the anastomosis, 59 PKH (+) neurons and 693 YFP (+) neurons were counted in 9 fields (from a-1 to c-3) × 140 optical sections (= with a total z-axis depth of 140 µm) (10).

The average cell numbers of PKH26 (+) and YFP (+) neurons were not significantly different between each of the nine fields. The significantly facilitating effects of neurogenesis by MOS were observed in 3 fields ($P<0.05$ or $P<0.005$) on PKH26 (+) neurons and in 6 fields ($P<0.05$ or $P<0.005$) on YFP (+) neurons. The significantly antagonizing effects of SB on PKH26 (+) and YFP (+) neurons were observed in 5 fields ($P<0.05$ or $P<0.005$) (10).

PKH26 (+) or YFP (+) neurons were distributed at a depth of 0–160 µm. The significantly facilitating effect of MOS on neurogenesis was observed at a depth of 60–80 µm ($P<0.05$) for PKH26 (+) neurons and at a depth of 80–100 µm ($P<0.005$) for YFP (+) neurons. The significantly antagonizing effects of SB were observed at a depth of 80–100 µm ($P<0.005$) for PKH26 (+) neurons and at a depth of 40–80 µm ($P<0.005$) for YFP (+) neurons. No differences were observed between the distributions of PKH26 (+) neurons or YFP (+) neurons (10).

The total number of PKH26 (+) neurons was one-tenth of that of YFP (+) neurons; MOS significantly increased the total number of PKH26 (+) neurons to 2.5-fold ($P<0.005$), and that of YFP (+) neurons to a similar 2.5-fold ($P<0.005$), SB completely antagonized these effects of MOS on both PKH26 (+) ($P<0.005$) and YFP
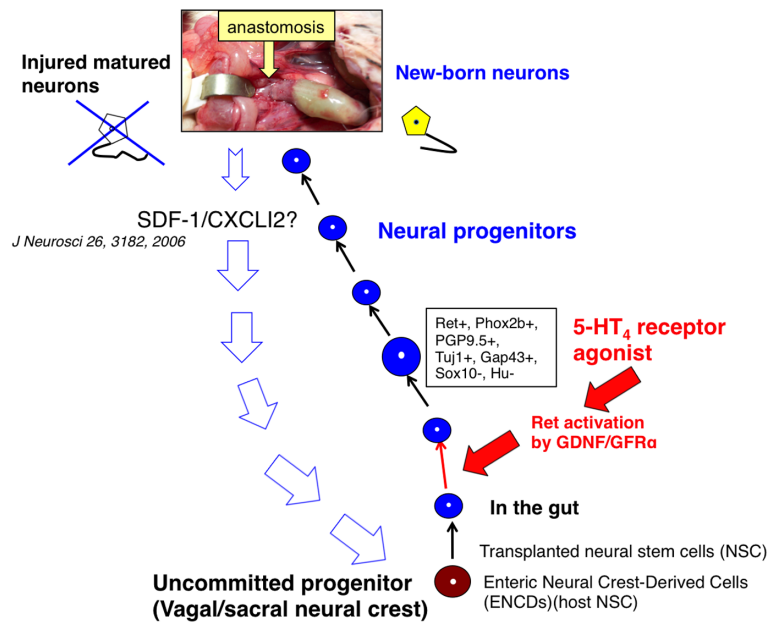
Fig. 6. Supposed underlying mechanism for neurogenesis from transplant neural stem cells (NSC) and host NSC at the anastomosis. SDF-1/CXCL12: chemokine stromal cell-derived factor-1; GDNF: glia-derived neurotrophic factor; GFRα: GDNF family receptor α.

(+) neurons ($P<0.005$) (10).

The transplant NSC (PKH+ cells) and host NSC (YFP+ cells) are mobilized to the anastomosed area and either/or survived, proliferated, and finally differentiated into neurons (9, 11, 12). MOS clearly accelerated these processes after surgery (Fig. 6). We speculated that these processes might be initially activated probably by release of chemokines from the anastomosis as shown in hippocampal slices (13). This reference has reported that chemokine stromal cell-derived factor-1 (SDF-1)/CXCL12 regulates the migration of neural progenitors to neuro-inflammatory site (Fig. 6) (13). We have not examined the contribution of chemokines to the initiation of the above process at this stage, although we plan to do so in future studies. The effects of MOS on neurogenesis from the transplant and host NSC were completely antagonized by the SR4-antagonist, which indicates that MOS facilitates the formation of new neurons via SR4 activation.

The distribution pattern of PKH (+) neurons was similar to that of YFP (+) newborn enteric neurons. This result suggests the possibility that both host NSC from neural crest (6) and transplant NSC migrate from the outside into the deep granulation tissue via the blood stream, as in the colonic ENS, which is developed by a population of trans-mesenteric ENCDCs (14).

Thus, a combination of drug administration and cell transplantation could be more beneficial than cell transplantation alone in treating Hirschsprung's disease (HSCR) and related disorders.

⸺ Underlying mechanism for an SR4-agonist facilitated neurogenesis ⸺

HSCR and related disorders occur due to the failure in development of ENS. HSCR pathogenesis is caused by mutations in genes encoding the Ret receptor tyrosine kinase (RET) and endothelin receptor type B (EDNRB) (11, 15). We have previously reported that MOS increases the mRNA level of RET in the cells mobilized into an implanted gel sponge in a rat subcutaneous model (not a gut model); this increase in RET mRNA was

completely blocked by treatment with an SR4-antagonist (12). RET seems to be the target molecule of MOS and RET inhibitors could suppress MOS-induced neurogenesis (16).

We investigated whether SR4 activation induced c-RET activation and/or protein kinase A (PKA) activation by elevating cAMP levels (16). MOS was orally administered to rodents for 2 weeks after enteric nerve circuit insult via lower gut transection and anastomosis, together with the RET inhibitors withaferin A (WA) and RPI-1 or the PKA inhibitor H89. Then PGP9.5-positive cells were examined in the newly formed granulation tissue at the anastomotic site. MOS significantly increased the number of new neurons, but not when co-administered with WA or RPI-1. Co-administration of H89 failed to alter MOS-induced increases in neurogenesis (16). These results suggest that the c-RET signaling pathway contributes to the enteric neurogenesis which is facilitated by MOS. But the contribution of PKA activation seems unlikely.

Genes and/or other markers enable many of the putative progenitor stages of enteric neuronal development to be recognized. *Ret* encodes a transmembrane receptor kinase, RET, that dimerizes when activated by a complex that includes a member of the GDNF family of ligands and a preferred glycosylphosphatidyl-inositol-anchored co-receptor, GDNF family receptor α (GFRα) (17). A common RET/GDNF/GFRα1-dependent progenitor gives rise to committed lineages both, enteric neurons (17–19) and glia from ENCDCs (11).

SR4 is a GPCR coupled to G protein G_s-cAMP cascades (20). MOS increased the number of c-RET-positive cells and c-RET mRNA in the implanted GS of rats mentioned above (16). c-RET is a GDNF receptor tyrosine kinase (RTK). GPCR-mediated signaling pathways have been reported to include transactivation of RTKs; the differential involvement of RTKs and downstream signaling pathways activated in response to GPCR-mediated stimulation elicits a variety of cellular effects during development, proliferation, differentiation, survival, repair and synaptic transmission in the CNS (21). Therefore, the GPCR SR4 would be expected to cross-communicate with the RTKs c-RET in the ENS (see Fig. 7) (22).

From the above results, we obtained direct evidence that c-RET activation contributes to the MOS-induced facilitation of enteric neurogenesis, although the detailed downstream pathway from c-RET was not identified. WA (23) and RPI-1 (24) inhibit total and phospho-RET levels and activation of ERKs and AKT, whereas RPI-1 also inhibits activation of phospholipase Cγ (PLCγ) (24). It seems likely that there are no differences in the inhibitory effects of WA and RPI-1 on enteric neurogenesis. Therefore, the downstream pathways via AKT and ERK1/2 may be more important than others (see Fig. 7) (22).

In the literature (22, 25, 26), the detailed downstream has been reported as follows: RET activation results in phosphorylation of several residues, including Y1015 and Y1062. GRB2 (Growth factor receptor-bound protein 2) and PLCγ are required for proliferation and/or differentiation of ENS precursors. RAC, RHO and CDC42 regulate ENCDC migration and proliferation. Kinesin-like protein KIF26A, Sprouty2 (SPRY2), and phosphatase and tensin homolog deleted from chromosome 10 (PTEN) are negative regulators of RET signaling (see Fig. 7).

PKA is thought to integrate RET and EDNRB signaling pathways by RET phosphorylation at S696 (26) or S697 (22) and subsequent activation of RAC (26, 27). Furthermore, it has been previously reported that the activation of PKA and cAMP responsive element binding protein (CREB) mediates SR4-promoted enteric neuronal survival after administration of the SR4-agonists RS67506 and tegaserod (28). According to microarray analysis for DNA of 1 μmol l^{-1} MOS-treated neurospheres, levels of CREB and GDNF/GFRα1 were increased to higher than twofold (our unpublished data). Taken together, the possibility for PKA activation by cAMP elevation induced by SR4 activation could not be completely excluded, though the above result showed no effect of the PKA inhibitor H89 on enteric neurogenesis enhanced by MOS (16).
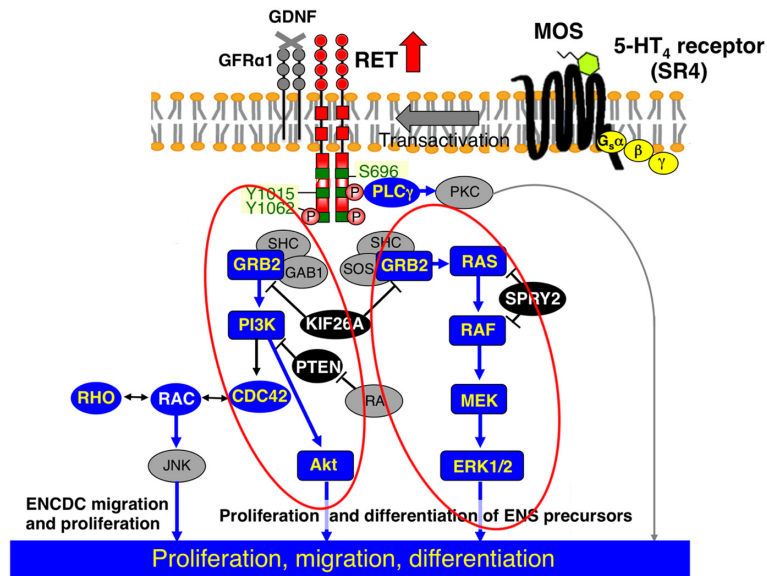
Fig. 7. Molecular mechanism for neurogenesis activated by 5-HT₄ receptors by MOS. Main intracellular pathways activated by proto-oncogene tyrosine-protein kinase receptor Ret (RET) are shown. MOS activates a 5-HT4 receptor (SR4), G protein coupled receptor (GPCR). This GPCR possibly does transactivation of RET. RET activation results in phosphorylation of several residues, including Y1015 and Y1062. Growth factor receptor-bound protein 2 (GRB2) and phospholipase C gamma (PLCγ) are required for proliferation and/or differentiation of enteric nervous system (ENS) precursors. RAC, RHO and CDC42 regulate enteric neural crest-derived cells (ENCDCs) migration and proliferation. Kinesin-like protein KIF26A (KIF26A), Sprouty2 (SPRY2) and phosphatase and tensin homolog (PTEN) are negative regulators of RET signalling: KIF26A binds to GRB2 and prevents RAS-ERK and phosphoinositide 3-kinase (PI3K) signalling, whereas SPRY2 binds to RAS and RAF and blocks activation of the RAS-ERK pathway. PTEN regulates proliferation via PI3K and Akt. Retinoic acid (RA) reduces PTEN levels in migrating ENCDCs and hence blocks the inhibitory effect of PTEN on RET signalling. Two circled pathways supposed to be more important than others. This figure was reproduced and modified from ref. (22) and ref. (26) with permission. GAB1: GRB2-associated binding protein 1; GDNF: glial cell line-derived neurotrophic factor; GFRα1: GDNF family receptor α1; GRB2: growth factor receptor-bound protein 2; JNK: c-Jun-NH2-terminal kinase; PKC: protein kinase C; S: serine; Y: tyrosine.

Perspectives: preliminary functional studies of new neurons from transplant neural stem cells

Recently, Ca²⁺ imaging using GFP-based genetically encoded Ca²⁺ indicators (GECIs) has been introduced as an alternative to using chemically synthesized fluorescent Ca²⁺ indicators (29). High-sensitivity and fast-responsiveness GECIs, termed G-CaMP6 and G-CaMP8 were developed by mutagenizing existing G-CaMPs (29). Thy1-promoter G6-2A-mCherry transgenic mice expressing GCaMP6 as a GECI and mCherry were used in this study. Nonlinear optical microscopy, 2PM can provide deeper optical penetration (several hundred μm) in *ex vivo* and *in vivo* preparations. We have used this approach and obtained clear three-dimensional imaging of newborn enteric neurons that were endogenously generated after lower gut transection and anastomosis in Thy1-promoter GFP/YFP mouse. Neurogenesis has been promoted by oral application of the SR4-agonist, MOS. Most neurons were located within 100 μm of the surface. However, whether these neurons are physiologically functioning has not been investigated.
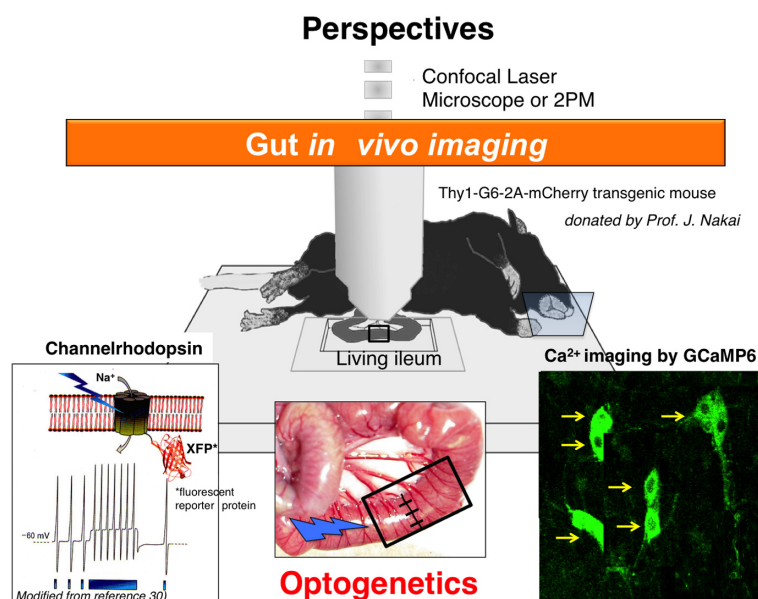
Perspectives

Fig. 8. Perspectives: Functional studies of new born neurons at the anastomosis.

Recently, optical tools to investigate cellular activity in the intestinal wall have been proposed (30). Upon blue illumination, channelrhodopsins will conduct cations, which in neurons, will result in Na$^+$ influx and a depolarization of the cell. Using either brief or longer light pulses a single action potential or sustained depolarization can be elicited (30). We are planning to investigate cellular activity of new neurons regenerated after impairment of ENS using Thy1-promoter G6-2A-mCherry transgenic mice (double transgenic mouse) specifically expressing channelrhodopsins in enteric neurons (Fig. 8). Although this plan is difficult to accomplish, we have already succeeded in performing Ca^{2+} imaging in Thy1-promoter G6-2A-mCherry transgenic mice under isoflurane anesthesia.

Acknowledgments

Conflict of interest

The authors declare that they have no conflicts of interest.

References

1) Takaki M, Nakayama S, Misawa H, Nakagawa T, Kuniyasu H. *In vitro* formation of enteric neural network structure in a gut-like organ differentiated from mouse embryonic stem cells. Stem Cells. 2006; 24: 1414–22.

2) Yamanouchi M, Shimatani H, Kadowaki M, Yoneda S, Nakagawa T, Fujii H, Takaki M. Integrative control of rectoanal reflex in guinea pigs through lumbar colonic nerves. Am J Physiol Gastrointest Liver Physiol. 2002; 283: G148–56.

3) 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. 2008; 294: G1084–93.

4) Takaki M, Misawa H, Matsuyoshi H, Kawahara I, Goto K, Zhang G-X, Obata K, Kuniyasu H. *In vitro* enhanced differentiation of neural networks in ES gut-like organ from mouse ES cells by a 5-HT$_4$-receptor activation. Biochem Biophys Res Commun. 2011; 406: 529–33.

5) Matsuyoshi H, Kuniyasu H, Okumura M, Misawa H, Katsui R, Zhang G-X, Obata K, Takaki M. A 5-HT$_4$-receptor activation-induced neural plasticity enhances *in vivo* reconstructs of enteric nerve circuit insult. Neurogastroenterol Motil. 2010; 22: 806–13.

6) Goto K, Kato G, Kawahara I, Luo Y, Obata K, Misawa H, Ishikawa T, Kuniyasu H, Nabekura J, Takaki M. *In vivo* imaging of enteric neurogenesis in the deep tissue of mouse small intestine. PLoS ONE. 2013; 8: e54814.

7) Feng G, Mellor RH, Bernstein M, Keller-Peck C, Nguyen QT, Wallace M, Nerbonne JM, Lichtman JW, Sanes JR. Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. Neuron. 2000; 28: 41–51.

8) Wake H, Moorhouse AJ, Jinno S, Kohsaka S, Nabekura J. Resting microglia directly monitor the functional state of synapses *in vivo* and determine the fate of ischemic terminals. J Neurosci. 2009; 29: 3974–80.

9) Micci M-A, Kahrig KM, Simmons RS, Sama SK, Espejo-Navarro MR, Pasricha PJ. Neural stem cell transplantation in the stomach rescues gastric function in neuronal nitric oxide synthase–deficient mice. Gastroenterology. 2005; 129: 1817–24.

10) Goto K, Kawahara I, Inada H, Misawa H, Kuniyasu H, Nabekura J, Takaki M. Activation of 5-HT$_4$ receptors facilitates neurogenesis from transplanted neural stem cells in the anastomotic ileum. J Physiol Sci. 2015; 65: in press. doi :10.1007/s12576-015-0396-1.

11) Gershon MD. Developmental determinants of the independence and complexity of the enteric nervous system. Trends in Neuroscience. 2010; 33: 446–56.

12) Kawahara I, Kuniyasu H, Matsuyoshi H, Goto K, Obata K, Misawa H, Fujii H, Takaki M. The 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. 2012; 302: G588–97.

13) Belmadani A, Tran PB, Ren D, Miller RJ. Chemokines regulate the migration of neural progenitors to sites of neuroinflammation. J Neuroscience. 2006; 26: 3182–91.

14) Nishiyama C, Uesaka T, Manabe T, Yonekura Y, Nagasawa T, Newgreen DF, Young HM, Enomoto H. Trans-mesenteric neural crest cells are the principal source of the colonic enteric nervous system. Nature Neuroscience. 2012; 15: 1211–8.

15) Hofstra RMW, Osinga J, Buys CHCM. Mutations in Hirschsprung disease: When does a mutation contribute to the phenotype. Eur J Hum Genet. 1997; 5: 180–5.

16) Goto K, Kawahara I, Kuniyasu H, Takaki M. A protein tyrosine kinase receptor, c-RET signaling pathway contributes to the enteric neurogenesis induced by a 5-HT$_4$ receptor agonist at an anastomosis after transection of the gut in rodents. J Physiol Sci. 2015; 65: 377–83.

17) Enomoto H, Hughes I, Golden J, Baloh RH, Yonemura S, Heuckeroth RO, Johnson EM Jr, Milbrandt J. GFRalpha1 expression in cells lacking RET is dispensable for organogenesis and nerve regeneration. Neuron. 2004; 44: 623–36.

18) Uesaka T, Jain S, Yonemura S, Uchiyama Y, Milbrandt J, Enomoto H. Conditional ablation of GFRalpha1 in postmigratory enteric neurons triggers unconventional neuronal death in the colon and causes a

Hirschsprung's disease phenotype. Development. 2007; 134: 2171–81.

19) Uesaka T, Nagashimada M, Yonemura S, Enomoto H. Diminished Ret expression compromises neuronal survival in the colon and causes intestinal aganglionosis in mice. J Clin Invest. 2008; 118: 1890–8.

20) Kobayashi K, Ikeda Y, Haneda E, Suzuki H. Chronic fluoxetine bidirectionally modulates potentiating effects of serotonin on the hippocampal mossy fiber synaptic transmission. J Neurosci. 2008; 28: 6272–80.

21) Shah BH, Catt KJ. GPCR-mediated transactivation of RTKs in the CNS: mechanisms and consequences. Trends Neurosci. 2004; 27: 48–53.

22) Takaki M, Goto K, Kawahara I. The 5-hydroxytryptamine 4 receptor agonist-induced actions and enteric neurogenesis in the gut. J Neurogastroenterol Motil. 2014; 20: 17–30.

23) Samadi AK, Mukerji R, Shah A, Timmermann BN, Cohen MS. A novel RET inhibitor with potent efficacy against medullary thyroid cancer *in vivo*. Surgery. 2010; 148: 1228–36.

24) Cuccuru G, Lanzi C, Cassinelli G, Pratesi G, Tortoreto M, Petrangolini G, Seregni E, Martinetti A, Laccabue D, Zanchi C, Zunino F. Cellular effects and antitumor activity of RET inhibitor RPI-1 on MEN2A-associated medullary tyroid carcinoima. J Natl Cancer Inst. 2004; 96: 1006–14.

25) Lake JI, Heuckeroth RO. Enteric nervous system development: migration, differentiation, and disease. Am J Physiol Gastrointest Liver Physiol. 2013; 305: G1–24.

26) Obermayr F, Hotta R, Enomoto H, Young HM. Development and developmental disorders of the enteric nervous system. Nat Rev Gastroenterol Hepatol. 2013; 10: 43–57.

27) Asai N, Fukuda T, Wu Z, Enomoto A, Pachnis V, Takahashi M, Costantini F. Targeted mutation of serine 697 in the Ret tyrosine kinase causes migration defect of enteric neural crest cells. Development. 2006; 133: 4507–16.

28) Liu M-T, Kuan Y-H, Wang J, Hen R, Gershon MD. 5-HT$_4$ receptor-mediated neuroprotection and neurogenesis in the enteric nervous system of adult mice. J Neurosci. 2009; 29: 9683–99.

29) Ohkura M, Sasaki T, Sadakari J, Gengyo-Ando K, Kagawa-Nagamura Y, Kobayashi C, Ikegaya Y, Nakai J. Genetically encoded green fluorescent Ca^{2+} indicators with improved detectability for neuronal Ca^{2+} signals. PLoS ONE. 2012; 7: e51286–315.

30) Boesmans W, Hao MM, Vanden Berghe P. Optical tools to investigate cellular activity in the intestinal wall. J Neurogastroenterol Motil. 2015; 21 (3): 337–51.