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SHORT COMMUNICATION

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

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Abstract We previously reported that a serotonin 4 (5-HT_4) receptor agonist, mosapride citrate (MOS), increased the number of c-RET-positive cells and levels of c-RET mRNA in gel sponge implanted in the necks of rats. The 5-HT_4 receptor is a G protein coupled receptor (GPCR) coupled to G protein G_s-cAMP cascades. We investigated the possibility that 5-HT_4 receptor activation induced c-RET activation and/or PKA activation by elevating cAMP levels. Rodents were orally administered MOS by adding it to drinking water for 2 weeks after enteric nerve circuit insult via gut transection and anastomosis, together with the RET inhibitors withaferin A (WA) and RPI-1 or the PKA inhibitor H89. We then examined PGP9.5-positive cells 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. In conclusion, the c-RET signaling pathway contributes to enteric neurogenesis facilitated by MOS, though the contribution of PKA activation seems unlikely.

Keywords 5-HT_4-receptor · Granulation tissue · Enteric neuron · RET · PKA

Abbreviations

EDN3	Endothelin 3
ENCDCs	Enteric neural crest-derived cells
ENS	Enteric nervous system
EDNRB	Endothelin B receptor
GDNF	Glial cell line-derived neurotrophic factor
GFRα1	GDNF family receptor α1
GAB1	GRB2-associated binding protein 1
GRB2	Growth factor receptor-bound protein 2
JNK	C-Jun-NH_2-terminal kinase
KIF26A	Kinesin-like protein
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C gamma
PTEN	Phosphatase and tensin homolog deleted from chromosome 10
RA	Retinoic acid
RET	Proto-oncogene tyrosine-protein kinase receptor Ret
S	Serine
Y	Tyrosine
SPRY2	Sprouty2

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Introduction

The enteric nervous system (ENS) provides the intrinsic innervation of the bowel, consisting of 2 layers of ganglia and fibers encircling the gastrointestinal tract. The ENS is vital for life and is capable of autonomous regulation of motility and secretion. The ENS originates in the neural crest, cells of which invade, proliferate, and migrate within the intestinal wall until the entire bowel is colonized with enteric neural crest derived cells (ENCDCs) [1]. Molecules controlling this process, including glial cell-derived neurotrophic factor (GDNF) and its receptor RET, endothelin-3 (EDN3) and its receptor endothelin receptor type B

(EDNRB), and transcription factors such as sex determining region Y-box10 (SOX10) and paired-like homeobox 2b (PHOX2B), are required for ENS development in humans [1–3].

Although several therapeutic challenges exist for aganglionic gut disorders, we previously explored a small candidate molecule promoting enteric neurogenesis following insult to a neural circuit in the ENS [4]. If such a candidate is effective by oral application, then administration of such a drug could easily repair any deficiency in the ENS. Therefore, we examined a serotonin 4 (5-HT_4)-receptor (R) agonist, mosapride citrate (MOS) as a candidate. We administered MOS to an anastomosis locally [5, 6] or orally by addition to drinking water [6, 7] in rodents for 1–2 weeks after enteric nerve circuit insult [5–7]. We observed a MOS-dependent increase in the number of neurofilament-, 5-HT_4-R- and 5-bromo-2′-deoxyuridine (BrdU)-positive new neurons in the newly formed granulation tissue at the anastomosis [5–7]. Possible neural stem cell markers, anti-distal less homeobox 2 (DLX2)-, and p75 neurotrophin receptor-positive cells were also found at the anastomosis after treatment with MOS [5–7]. In addition, in a gut-like organ induced by mouse embryonic stem cells [8], MOS succeeded in forming dense enteric neural networks [9]. These results indicated that activation of enteric neural 5-HT_4-R by MOS promotes in vivo and in vitro formation of new enteric neurons, and that treatment with MOS could be a novel therapy for generating new enteric neurons to rescue aganglionic gut disorders.

The molecular mechanism for enteric neurogenesis facilitated by MOS, however, has remained unclear. MOS significantly increased the number of DLX2-, 5-HT_4-R-, and c-RET-positive cells after local administration by a gel sponge (GS) implanted in the neck [6]. DLX2- and 5-HT_4-R-positive cells are neural stem cells [6], and c-RET-positive cells are ENCDCs [8]. Therefore, it seems likely that MOS differentiates ENCDCs [10] into neural stem cells. In addition, MOS increased DLX2, 5-HT_4-R, and c-RET mRNA levels in a manner blocked by a selective 5-HT_4-R antagonist, GR113808 [5, 6, 9]. In the present study, we aimed to obtain direct evidence for a contribution of the c-RET signaling pathway to enteric neurogenesis facilitated by the 5-HT_4-R agonist, MOS. Data have been partly reported in the abstract form [11].

Materials and methods

All surgical and experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and reviewed and approved by the animal care and use committee of Nara Medical University (permission number: 10781, 10950). Balb/C female mice ($n = 9$; body weights: 17–25 g), were anesthetized with an intraperitoneal injection of Nembutal (50 mg kg^{-1}) and the abdomen was opened by a lower midline laparotomy. The ileum was transected 5–6 cm from ileo-cecal sphincter and an end-to-end 1-layer anastomosis was performed [7]. Body temperature was maintained at 36–37 °C using a heating pad. After recovery from the surgical procedure, mice drank daily either 0.1 % dimethyl sulfoxide (DMSO) vehicle ($n = 3$, DMSO group), MOS (100 μM) in vehicle alone ($n = 3$, MOS group) or MOS (100 μM) in vehicle. The last group also received an intraperitoneal (IP) injection of a selective RET inhibitor, withaferin A (WA; ChromaDex Inc., Irvine, CA, USA) (8 mg $kg^{-1}day^{-1}$) [12] ($n = 3$, WA + MOS group) for 2 consecutive weeks [7]. WA was dissolved in DMSO at a stock concentration of 20 mM and stored at −80 °C. Fresh WA solution was prepared daily for each injection. In mice ileum, severe adhesion would occur with local treatment by GS, and GS could not be inserted tightly between any organs like in the rat rectum [6]. For this reason we avoided local treatment by GS.

Male Wistar rats ($n = 24$; body weights: 300–500 g) were anesthetized with an intra-peritoneal injection of Nembutal (40 mg kg^{-1}) and the abdomen was opened by a lower midline laparotomy. The surgical approach was performed to spare extrinsic inputs from the lumbar colonic nerves. The rectal transection was performed 4 cm from anal verge and an end-to-end 1-layer rectal anastomosis was performed [6]. The 24 rats were divided into 2 groups ($n = 12$ rats each) treated with a 2-indolinone Ret tyrosine kinase inhibitor RPI-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) [13] or with H89 (Tocris Bioscience, Mineapolis, MN, USA). RPI-1 was dissolved in 0.1 % DMSO. H89 (*N*-[2-[[3-(4-bromophenyl)-2-propenyl] amino] ethyl]-5-isoquinoline-sulfonamide dihydrochloride) is a cAMP-dependent protein kinase A (PKA) inhibitor [14] and it was dissolved in 0.1 % DMSO. Local treatment (LT) was performed by implantation of a GS [W × L: 0.6 × 1.6 cm; each upper half (0.8 cm) and lower half (0.8 cm); soaked solution volume = 0.1 ml] right after surgery [6]. The GS was inserted tightly between the rectum and seminal vesicle and wrapped around the anastomotic site. For this reason the rectum is appropriate for local treatment by GS. This GS was not completely absorbed until 4 weeks after implantation.

Experiments using RPI-1 included a DMSO ($n = 3$ rats, LT with 0.1 % DMSO), MOS ($n = 3$ rats, drinking water containing 100 μM MOS + LT with 0.1 % DMSO), RPI-1 + MOS ($n = 3$ rats, drinking water containing 100 μM MOS + LT with 150 μM RPI-1) and RPI-1 ($n = 3$ rats, LT with 150 μM RPI-1 alone) groups. Experiments using H89 included a DMSO ($n = 3$ rats, LT with 0.1 % DMSO), MOS ($n = 3$ rats, drinking water containing

100 μM MOS + LT with 0.1 % DMSO), H89 + MOS ($n = 3$ rats, drinking water containing 100 μM MOS + LT with 30 μM H89) groups and a group which received H89 alone ($n = 3$ rats, LT with 30 μM H89).

To ascertain the effects of RET inhibitors, we chose WA for an IP injection in mouse ileum and RPI-1 for local treatment in rat rectum to compare the present results with our previous data in mice [7] and rats [6] and to explore whether enteric neurogenesis both in mouse ileum and rat rectum occurs in a similar manner.

Fixed frozen blocks and sections of mouse and rat tissue for immunohistochemistry (IHC) were obtained from Genostaff Co., Ltd (Tokyo, Japan). The ileum or rectum, including an anastomotic site, was fixed with 4 % paraformaldehyde at 4 °C for 16 h, and embedded in Cryo Mount 1 (MUTO PURE CHEMICALS CO. LTD, Tokyo, Japan) by their proprietary procedures. Two blocks were obtained from each tissue. Consecutive 6-μm longitudinal sections, including an anastomotic site, were cut from each block. Tissue sections were washed with PBS to remove embedding compound during IHC. Antigen retrieval was performed by heat treatment at 80 °C for 40 min with citrate buffer, pH 6.0. Endogenous peroxidase blockade was accomplished using 0.3 % H_2O_2-methanol for 30 min, followed by incubation with Protein Block (DAKO Corp., Carpinteria, CA, USA) and an avidin/biotin blocking kit (VECTOR LABORATORIES, INC., CA, USA). The sections were incubated with mouse monoclonal antibody for PGP9.5 (anti-PGP9.5) [clone 13C4/13C4] (cat. Ab8189, 0.4 μg ml^{-1}, Abcam plc., Cambridge, UK) at 4 °C overnight. They were incubated with biotin-conjugated goat anti-rabbit Ig (DAKO Corp.) diluted 1:600, for 30 min at room

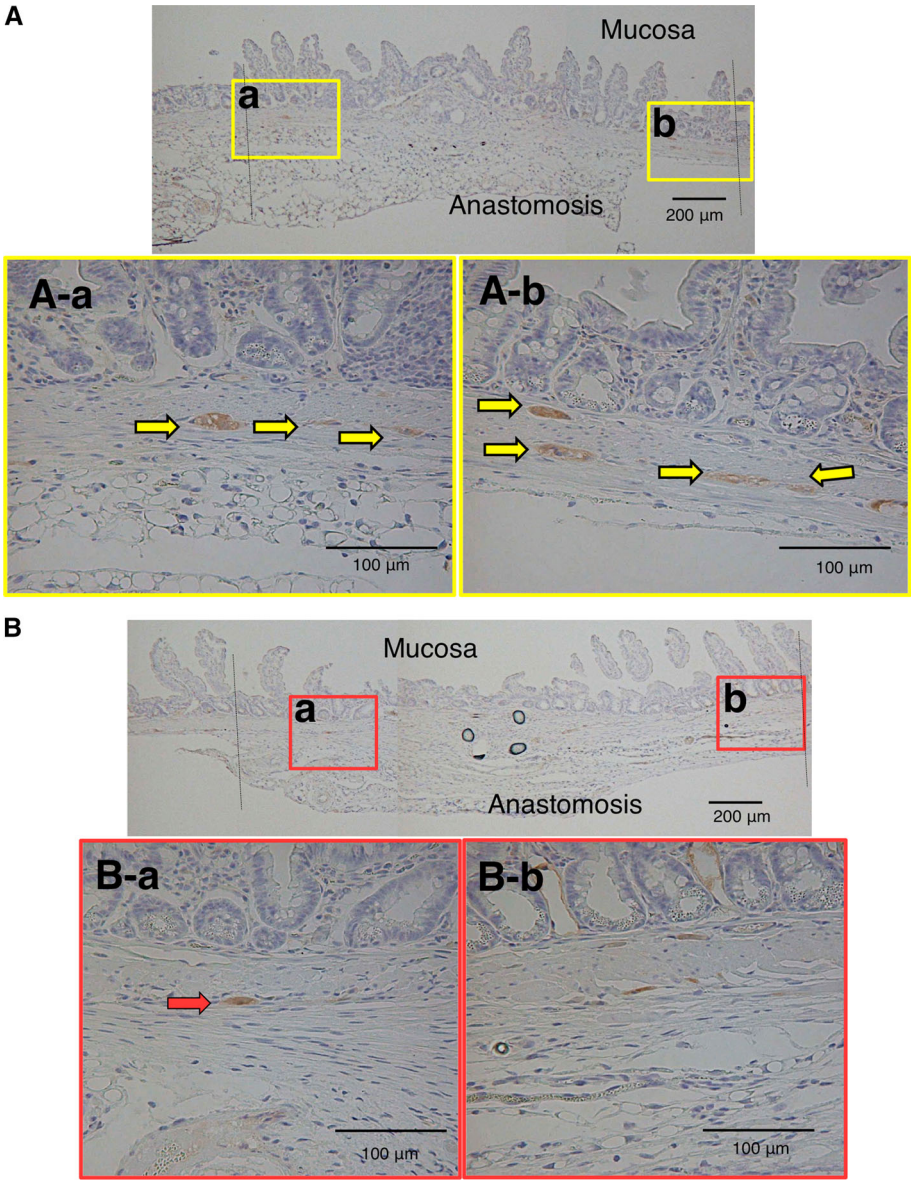


Fig. 1 Immunohistochemical detection of PGP 9.5-positive cells at the anastomosis in the ileum of a mouse that drank mosapride citrate (MOS) (100 μM) (**A**) and a mouse treated with a selective RET inhibitor, withaferin A (WA) (8 mg $kg^{-1}day^{-1}$) intraperitoneal injection + MOS (100 μM)-drinking water (**B**) for 2 weeks after surgery

temperature, followed by the addition of peroxidase-conjugated streptavidin (NICHIREI BIOSCIENCES INC., Tokyo, Japan) for 5 min. Peroxidase activity was visualized by diaminobenzidine solution (DAKO Corp.). The sections were counterstained with Mayer's hematoxylin (MUTO PURE CHEMICALS CO. LTD), dehydrated, and then mounted with Malinol (MUTO PURE CHEMICALS CO. LTD). The number of solitary cells or ganglia at the anastomosis was counted in each section of the 2 blocks.

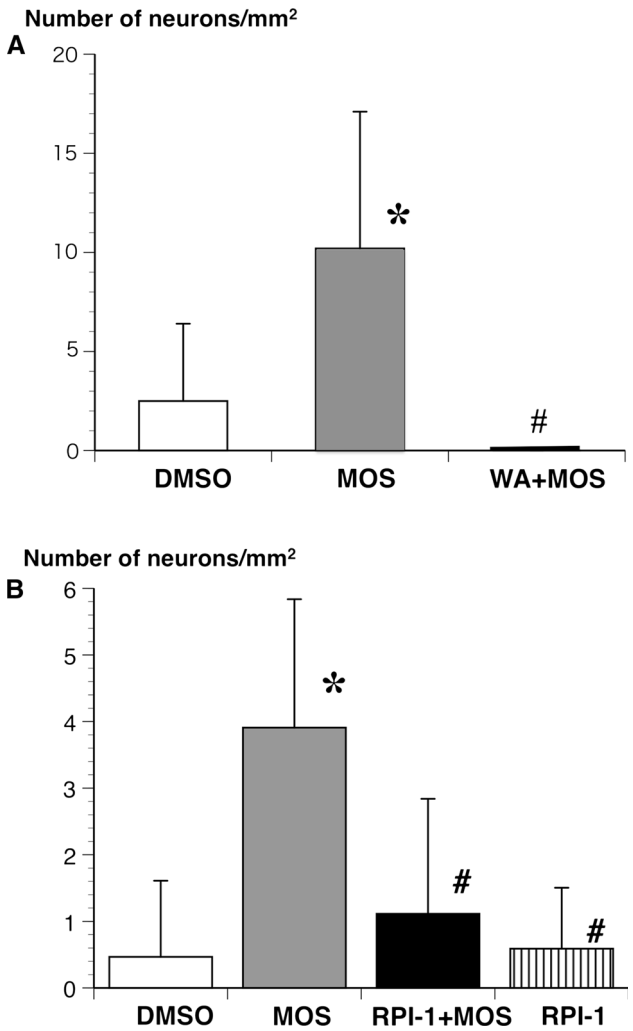


Fig. 2 The average number of neurons observed at the anastomosis in the ileum at each of 2 levels of 3 mice in MOS group ($n = 6$) was significantly larger than that in both the DMSO group ($n = 6$) ($^{*}P < 0.05$) and the WA + MOS group ($n = 6$) ($^{\#}P < 0.005$) for 2 weeks after surgery (**A**). There was no significant difference in the average number of neurons between the DMSO group and the WA + MOS group. The average number of neurons observed at the anastomosis in the rectum at each of 2 levels of 3 rats in MOS group ($n = 6$) was significantly larger than that in the DMSO group ($n = 6$) ($^{*}P < 0.01$) and in both a 2-indolinone Ret tyrosine kinase inhibitor, RPI-1 + MOS group [local treatment (LT) with RPI-1 (150 μM) + MOS (100 μM)-drinking water rats] ($n = 6$) and RPI-1 group [local treatment (LT) with RPI-1 (150 μM) rats] ($n = 6$) ($^{\#}P < 0.01$) for 2 weeks after surgery (**B**)

Multiple comparisons were performed by 1-way analysis of variance (ANOVA) with post hoc Bonferroni's and/or Dunnett's test. A value of $P < 0.05$ was considered statistically significant. All data are expressed as the mean ± SD.

Results

Many PGP9.5-positive neurons were observed at the anastomosis in the ileum of a mouse in MOS group as shown in Fig. 1A. However, only 1 PGP9.5-positive neuron was observed at the anastomosis in the ileum of a mouse in the WA + MOS group as shown in Fig. 1B. The average number

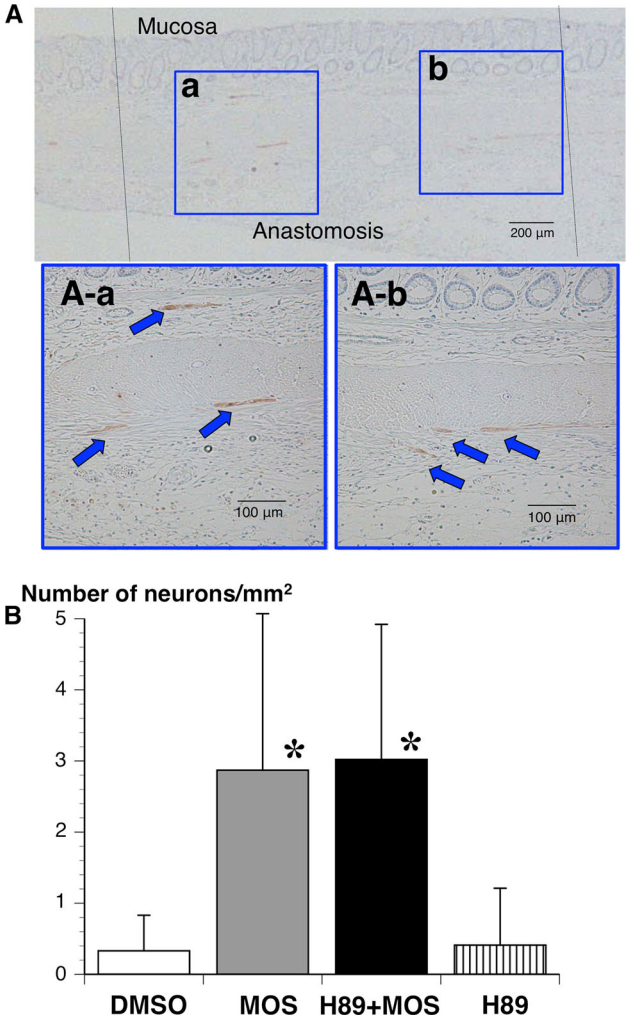


Fig. 3 Immunohistochemical detection of PGP 9.5-positive cells at the anastomosis in the rectum of LT with a cAMP-dependent protein kinase A (PKA) inhibitor, H89 (30 μM) + MOS (100 μM)-drinking water rat for 2 weeks after surgery (**A**). The average number of neurons observed at the anastomosis in the rectum at each of 2 levels of 3 rats in the MOS group ($n = 6$) and H89 + MOS group ($n = 6$) was each significantly larger than that in the DMSO group ($n = 6$) ($^{*}P < 0.05$) for 2 weeks after surgery (**B**). There was no significant difference in the average number of neurons between the anastomosis in the MOS and H89 + MOS groups (**B**)

of neurons (ganglia or solitary cell) observed at the anastomosis in the ileum at 2 levels of 3 mice in the MOS group ($n = 6$) was significantly larger than that in both the DMSO group ($n = 6$) ($P < 0.05$) and the WA + MOS group ($n = 6$) ($P < 0.005$) (Fig. 2A). There was no significant difference in the average number of neurons between the DMSO group and the WA + MOS group (Fig. 2A). The average number of neurons observed at the anastomosis in the rectum at 2 levels of 3 rats in the MOS group ($n = 6$) was significantly larger than that in both the DMSO ($n = 6$) ($P < 0.01$) and RPI-1 + MOS groups ($n = 6$) ($P < 0.01$) (Fig. 2B).

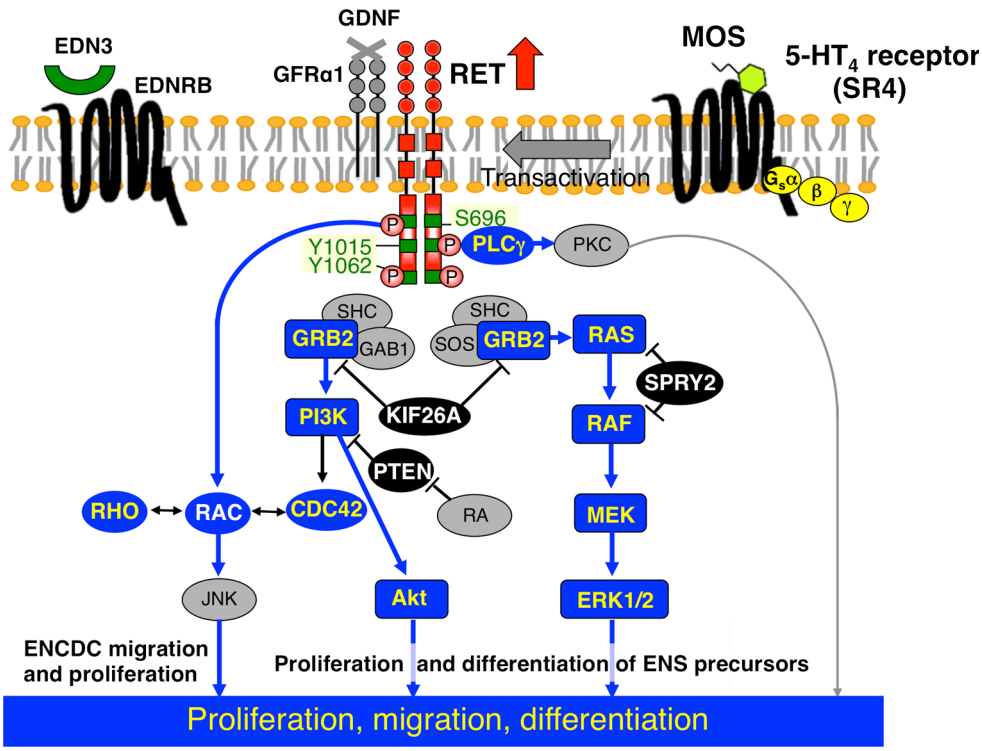
Many PGP9.5-positive neurons were observed at the anastomosis in the rectum of a rat in the H89 + MOS group as shown in Fig. 3A. The average number of neurons observed at the anastomosis in the rectum at 2 levels of 3 rats in the MOS ($n = 6$) and H89 + MOS groups ($n = 6$) was each significantly larger than that in the DMSO group ($n = 6$) ($P < 0.05$) (Fig. 3B). There was no significant difference in the average number of neurons between the anastomosis in the MOS and H89 + MOS groups (Fig. 3B).

Discussion

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) [15]. A common RET/GDNF/GFRα1-dependent progenitor gives rise to committed lineages of both enteric neurons [15–17] and glia from ENCDCs [1].

5-HT_4-R is a GPCR coupled to G protein G_s-cAMP cascades [18]. MOS increased the number of c-RET-positive cells and c-RET mRNA in the implanted GSs of rats mentioned above [6]. 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 [19]. Therefore, the GRCR 5-HT_4-R would be expected to cross-communicate with the RTKs c-RET in the ENS (see Fig. 4) [20].

From the present results, we obtained direct evidence that c-RET activation contributes to the MOS-induced enhancement of enteric neurogenesis, although the detailed downstream pathway from c-RET was not identified. WA [12] and RPI-1 [13] inhibit total and phospho-RET levels and activation of extracellular signal-regulated kinases (ERKs) and serine/threonine-specific protein kinase (AKT), whereas RPI-1 also inhibits activation of phospholipase Cγ (PLCγ) [13]. It seems likely that there are no



Modified from Nat Rev Gastroenterol Hepatol 10(1), 43-57, 2013[39]

Fig. 4 Possible mechanism for MOS-induced facilitation of enteric neurogenesis modified from a previously proposed mechanism [2, 3, 19] by the present results

differences in the inhibitory effects of WA and RPI-1 on enteric neurogenesis, although application method and species used are different. The downstream pathway via AKT and ERK1/2 may be important.

In the literature [2, 3, 20], the detailed downstream pathway has been reported as follows: RET activation results in phosphorylation of several residues, including Y1015 and Y1062. 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, and phosphatase and tensin homologs deleted from chromosome 10 (PTEN) are negative regulators of RET signaling (see Fig. 4).

PKA is thought to integrate RET and EDNRB signaling pathways by RET phosphorylation at S696 [3] or S697 [20] and subsequent activation of RAC [3, 21]. Furthermore, it has been previously reported that the activation of PKA and cAMP responsive element binding protein (CREB) mediates 5-HT_4-R-promoted enteric neuronal survival after administration of the 5-HT_4-R agonists RS67506 and tegaserod [14]. We also performed microarray analysis of DNA extracted from neurospheres of embryonic neural stem cells from the mouse hippocampus and subventricular zone after treatment with 1 μM MOS for 4 days. According to this analysis for DNA of 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 5-HT_4-R activation could not be completely excluded, though the present result showed no effect of the PKA inhibitor H89 on enteric neurogenesis enhanced by MOS.

In conclusion, mosapride citrate (MOS) facilitated enteric neurogenesis at the anastomosis after gut surgery (transection and anastomosis), mediated via activation of the 5-HT_4 receptor and c-RET signaling.

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Conflict of interest The authors declare no potential conflicts of interest.

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