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Delivery of enteric neural progenitors with 5-HT₄ agonist-loaded nanoparticles and thermosensitive hydrogel enhances cell proliferation and differentiation following transplantation *in vivo*

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Abstract

Cell therapy offers an innovative approach for treating enteric neuropathies. Postnatal gut-derived enteric neural stem/progenitor cells (ENSCs) represent a potential autologous source, but have a limited capacity for proliferation and neuronal differentiation. Since serotonin (5-HT) promotes enteric neuronal growth during embryonic development, we hypothesized that serotonin receptor agonism would augment growth of neurons from transplanted ENSCs. Postnatal ENSCs were isolated from 2–4 week-old mouse colon and cultured with 5-HT₄ receptor agonist (RS67506)-loaded liposomal nanoparticles. ENSCs were co-cultured with mouse colon explants in the presence of RS67506-loaded (n=3) or empty nanoparticles (n=3). ENSCs were also transplanted into mouse rectum *in vivo* with RS67506-loaded (n=8) or blank nanoparticles (n=4) confined in a thermosensitive hydrogel, Pluronic F-127. Neuronal density and proliferation were analyzed immunohistochemically. Cultured ENSCs gave rise to significantly more neurons in the presence of RS67506-loaded nanoparticles. Similarly, colon explants had significantly increased neuronal density when RS67506-loaded nanoparticles were present. Finally, following *in vivo* cell delivery, co-transplantation of ENSCs with 5-HT₄ receptor agonist-loaded nanoparticles led to significantly

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increased neuronal density and proliferation. We conclude that optimization of postnatal ENSCs can support their use in cell-based therapies for neurointestinal diseases.

Keywords

Enteric neuropathies; cell therapy; serotonin; 5-HT₄ receptor agonist; liposomal nanoparticles; thermosensitive hydrogel

Introduction

The enteric nervous system (ENS) is an extensive network of neurons and glial cells within the wall of gastrointestinal (GI) tract and plays critical roles in regulating a variety of gut functions, including motility, secretion, and absorption [1]. The ENS is derived from neural crest cells that migrate along the GI tract, proliferate extensively, and differentiate into specific neuronal and glial subtypes [2]. Developmental disorders, injury or degenerative changes in the ENS lead to enteric neuropathies, including congenital aganglionosis (Hirschsprung disease; HSCR), intestinal pseudo-obstruction, esophageal achalasia, gastroparesis, neurogenic diarrhea and constipation, visceral hypersensitivity, inflammatory neuropathies, and neurotransmitter deficiencies [3]. Despite the prevalence and severity of these diseases, existing treatments are not curative, but rather only palliative, and often yield less than satisfactory results.

Cell-based therapies have the potential to treat enteric neuropathies by replacing damaged or diseased neurons or by delivering enteric neurons to a region that lacks them, as in HSCR. A number of recent studies have shown that ENS stem/progenitor cells (ENSCs) can be isolated, expanded in culture, and transplanted into intestinal tissue *in vitro* [4-6] and *in vivo* [7-9], including into animal models of enteric neuropathies [10-12]. However, the capacity of these cells to proliferate and migrate has been limited [12, 13], and may therefore be insufficient to restore gut function, emphasizing the need to engineer the cells and/or their microenvironment, and to optimize cell delivery methods, in order to maximize the success of cell replacement therapy.

Recent studies have highlighted the important role of serotonin (5-hydroxytryptamine; 5-HT) in promoting the survival and development of enteric neurons. Stimulation of 5-HT₄ receptors protects cultured enteric neurons from apoptosis and promotes their development *in vitro* [14]. Serotonin also promotes enteric neurogenesis during the development of the ENS [15, 16] and in the adult ENS *in vivo* [14]. We have previously shown that 5-HT₄ receptor agonism increases neuronal density and proliferation of cultured postnatal ENSCs [17], leading us to hypothesize that serotonin agonism could enhance the success of ENSC transplantation.

Optimization of cell delivery is also critical for successful cell-based therapy. Most studies to date have introduced neuronal precursor cells by laparotomy and direct injection into the gut wall [10, 11, 18-20]. Although some have demonstrated improved gut function following cell injection [10-12], there are significant drawbacks to this delivery method, such as substantial leakage from the puncture hole and poorly controllable cell spreading.

These factors lead to inefficient cell engraftment, random cell distribution, and poor reproducibility. Moreover, suspension of single cells into a delivery vehicle can lead to significant cell death, primarily due to anoikis [21].

Recent advances in bioengineering have produced numerous biomaterials that can overcome these issues related to ENS cell therapy for neurointestinal diseases. Micro/nanoparticle formulations have been successfully developed for delivering therapeutic molecules, such as drugs, antibiotics, growth factors, and cytokines [22]. Both natural and synthetic polymers can be used for nanoparticle fabrication and encapsulating bioactive molecules. Liposomes have been widely used for drug delivery systems (DDS), recently gaining popularity due to the clinical success of poly(ethylene glycol)-modified liposomal doxorubicin (PLD), known as Doxil®, the first FDA-approved nano-drug for treating breast and ovarian cancer and Kaposi's sarcoma [23, 24]. Therapeutic studies have shown that PLD is at least four times more effective than free drug and possesses greater anti-tumor effect [25]. Injectable thermosensitive hydrogel has also gained increasing attention in tissue engineering, drug delivery, and cell encapsulation. Pluronics F-127 (PF-127) is a commercially available thermosensitive hydrogel approved by the FDA and broadly studied as a DDS due to its unique features, including its temperature dependent solid-gel-solid transition behavior and non-toxic biodegradable formulation [26]. Formulation of PF-127-containing pharmaceutical agents prepared at lower temperature (<25°C) can be applied topically or injected subcutaneously, working as a sustained delivery depot of antibiotics [27], insulin [28], anti-inflammatory [29] or analgesic drugs [30]. PF-127 hydrogel has recently been tested for *in situ* delivery of neural precursor cells and has demonstrated improved cell survival and reduced host immune response [31-33]. Furthermore, hydrogel containing drug-loaded nanoparticles has been shown to provide sustained release of insulin [34, 35] and anticancer drugs [36, 37] *in vitro*, and cyclosporine A (CsA) *in vivo* [38].

In this study, we aimed to establish a feasible approach to deliver ENSCs to the gut wall in order to maximize cell engraftment and to enhance cell differentiation and proliferation. We achieved this goal using a combination of liposome-encapsulated 5-HT₄ receptor agonist and confinement of ENSCs and nanoparticles within a thermosensitive hydrogel. Successful expansion of ENSCs and optimization of cell delivery are major steps forward in bringing the field of enteric neuronal cell-based therapy closer to clinical application.

Materials and Methods

Animals

Experiments were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee. Actb-DsRed mice (strain Tg(CAG-DsRed*MST)1Nagy/J; Jackson Labs Stock #005441), in which all cells express red fluorescent protein [39], were purchased from Jackson Laboratory. All wild-type mice used in this study were C57BL/6 mice purchased from Jackson Laboratory.

Enteric neuronal stem/progenitor cell (ENSC) cultures

Enteric neuronal stem/progenitor cells (ENSC) were isolated and cultured as previously described [17, 20]. Briefly, gastrointestinal tracts were isolated from postnatal day 14-21 (P14-21) C57BL/6 or DsRed mice. The longitudinal muscle-myenteric plexus (LMMP) was isolated using microdissection. Note that the circular muscle is often included with the LMMP as it adheres to the myenteric plexus. Cells were dissociated with dispase (250 $\mu\text{g ml}^{-1}$; StemCell Technologies, Vancouver, Canada) and collagenase XI (1 mg ml^{-1} ; Sigma Aldrich, St. Louis, MO) at 37°C for 1 hour with gentle pipetting. The cell suspension was passed through a 40 μm cell strainer and cultured at a density of 50,000 cells ml^{-1} in proliferation medium, consisting of NeuroCult NSC Basal Medium (StemCell Technologies) supplemented with 20 ng ml^{-1} epidermal growth factor (EGF; StemCell Technologies) and 10 ng ml^{-1} basic fibroblast growth factor (bFGF; StemCell Technologies) for 7-10 days to form enteric neurospheres. Neurospheres were passaged at day 7 with gentle Accutase (StemCell Technologies) dissociation at 37°C for 20-30 minutes. Cells were re-plated into a 6-well Ultra-low attachment culture plate (Corning, Kennebunk, ME) in conditioned medium (1:2 mix) to obtain the secondary neurospheres that were used in this study.

Compounds

5-HT₄ receptor agonist, RS67506 (Tocris Bioscience, Bristol, United Kingdom) was prepared as a stock solution in distilled water at $>10^3$ times the experimental concentration. Stock solutions were stored at -20°C and diluted in culture medium at the time of the experiment.

Formulation of RS67506-containing liposomal nano-carriers (RS-nanoparticles)

A solution of lipids was prepared with a 4:2:0.3 molar ratio of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC, Avanti Polar Lipids), cholesterol, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-mPEG2000). A thin film of the lipids was created under vacuum in the inner wall of a round bottom flask. Subsequently, this lipid bilayer was hydrated with an aqueous solution of RS67506 (1.1 mM). The resulting suspension of liposomes was subjected to probe sonication for 5 min and then extruded through a series of polycarbonate films (5 passes through 0.4 μm followed by 10 passes through 0.2 μm) to reduce their size to form unilamellar liposomes. Free drug was removed by running the sample through a PD-10 Sephadex column. Nanoparticles loaded with normal saline were used as control (CTRL-nanoparticles) in this study. Liposomal hydrodynamic diameter was determined by dynamic light scattering using Zetasizer ZS (Malvern, Worcestershire, UK). The zeta potential of the liposomes was determined by microelectrophoresis. The morphology of the liposomes was observed under transmission electron microscope. The liposomes were drop-coated on a formvar carbon-coated copper grid (Electron Microscopy Science, Hatfield, PA) and were negatively stained using 1.5% uranyl acetate. The samples were imaged under a JEOL 100-X transmission electron microscope (Peabody, MA) using an acceleration voltage of 80 MeV. Drug concentration at the final liposomal suspension was determined using high-pressure liquid chromatography (HPLC). The drug was released from the liposomes by adding a known

volume of the liposomes in a 1% Triton-X solution. The HPLC system consisted of a Waters LC (model 2487, Waters Corporation, Milford) equipped with a UV detector and a C18 column. The mobile phase consisted of methanol/water (1:1). The elution was performed using an isocratic flow rate of 1 ml/min.

Tissue preparation and immunohistochemistry

Cells and tissues were fixed in 4% paraformaldehyde. For whole-mount preparations of mouse LMMP, the distal colon was opened along the mesenteric border, pinned on Sylgard-coated plates, and fixed. The mucosa and submucosa were removed. For cryosections, tissues were transferred to 15% sucrose overnight at 4°C. The medium was changed to 7.5% gelatin containing 15% sucrose at 37°C for 1 hour and rapidly frozen at -50°C in methylbutane (Sigma). Frozen sections were cut at 12 µm thickness.

Immunohistochemistry was performed as previously described [40]. Cells or tissues were fixed, washed, and permeabilized with 0.1% Triton X-100 for 30 minutes. Primary antibodies included goat anti-Ret (1:20, Neuromics, Edina, MN), mouse anti-Tuj1 (1:100, Covance, Dedham, MA), mouse anti-Tuj1 conjugated Alexa Fluor 488 (1:400, BioLegend, Dedham, MA), rabbit anti-calretinin (1:200, Life Technologies, Carlsbad, CA), rabbit anti-CGRP (1:200, Peninsula Laboratories, San Carlos, CA), rabbit anti-nNOS (1:500, Santa Cruz Biotechnology, TX), rabbit anti-PGP9.5 (1:400, Cedarlane, Burlington, NC), rabbit anti-p75 (1:500, Promega, Madison, WI), and rabbit anti-S-100 (1:100, NeoMarkers, Fremont, CA). Secondary antibodies included donkey anti-goat Alexa Fluor 488, donkey anti-mouse Alexa Fluor 488 and 546, and donkey anti-rabbit Alexa Fluor 488 (Life Technologies). Cell nuclei were stained with DAPI (Vector Labs, Burlingame, CA). Images were captured with a Nikon 80i microscope, a Nikon AZ-100 microscope, or a Nikon A1R laser-scanning confocal microscope

ENSC proliferation assay

To passage enteric neurospheres, they were dissociated with Accutase at 37°C for 30 minutes with gentle pipetting. The cell suspension was passed through a 40 µm cell strainer and plated at 50,000 cells ml⁻¹ on glass-bottom chamber slides coated with 20 µg ml⁻¹ fibronectin (Biomedical Technologies, Ward Hill, MA). Cells were cultured for 7 days in proliferation medium containing EGF and FGF as described above and 10 µM EdU was added to the culture medium 24 hours prior to fixation. Secondary neurospheres were dissociated with Accutase and centrifuged (800 g for 2 minutes; Shandon Cytospin 3) onto a poly-L-lysine slide. Neural crest cells were visualized using p75 immunoreactivity with EdU incorporation detected using the ClickiT EdU Imaging Kit (Invitrogen). Ten random images were taken from each group and the number of p75+EdU- or p75+EdU+ cells counted using ImageJ software.

Measurement of neuronal differentiation, proliferation and neurite length

Following passage of secondary neurospheres as described above, cells were cultured for 7 days in NeuroCult NSC Differentiation Medium (StemCell Technologies) and 10 µM EdU was added to the culture medium 24 hours prior to fixation. Following staining with the neuronal marker, Tuj1, EdU incorporation was visualized using the ClickiT EdU Imaging

Kit (Invitrogen). Random images were taken from each culture condition and neuronal density measured quantitatively using ImageJ software. The number of Tuj1+EdU+ cells or Tuj1+EdU- cells were counted to evaluate neuronal proliferation. Neurite length was defined as the distance from the boundary of the cells to the tip of the longest branch of each identifiable neurite and was measured using ImageJ.

Ex vivo LMMP culture

LMMP was obtained from mouse colon and a 5×5 mm segment suspended as a catenary culture on filter paper in medium containing 10% fetal bovine serum (Life Technologies) and 100 U ml⁻¹ penicillin-streptomycin (Life Technologies). Cultures were maintained for 3-7 days in the presence of CTRL- or RS-nanoparticles, either with or without neurospheres. For neurosphere co-cultures, DsRed-expressing neurospheres were placed onto the LMMP and covered with 10 µL Matrigel (BD biosciences, CA, USA) to fix them in position. EdU (10 µM) was added to the medium 14 hours prior to fixation. Following cryosectioning, tissue was processed for immunohistochemistry. Cells were counted using ImageJ software to quantify neurogenesis among donor-derived and endogenous cells.

Preparation of Plurionics® F-127 (PF-127) thermosensitive hydrogel

Plurionics® is a member of the PEO-PPO-PEO triblock co-polymers. Plurionics® F-127 (PF-127, Sigma St Louis, MO) is a commercially available thermosensitive hydrogel. The aqueous solution of PF-127 at a concentration between 20-30% (wt.%) reversibly turns into a gel at room temperature [41]. The PF-127 (Sigma, St. Louis, MO) aqueous solutions were prepared by the cold method [42] by adding PF-127 slowly to cold (4°C) distilled water with gentle mixing until complete dissolution of the polymer. Stock concentration (28%) of PF-127 was stored at 4°C and cell suspension and compounds (liposomal nanoparticles, described above) were added to the stock to prepare the final concentration of PF-127 at 18-20% for this study.

Transplantation of ENSCs with RS-nanoparticles to mouse distal colon *in vivo*

Recipient 4-6-week-old C57BL/6 mice were anesthetized by isoflurane (Henry Schein Animal Health, Dublin, OH) inhalation. A peri-anal skin incision was made and the distal rectum exposed. A 50 µL suspension containing 1,000 cells µL⁻¹ in 18% PF-127 hydrogel containing either CTRL- or RS-nanoparticles was microinjected through a 30G needle into the wall of the distal colon under a dissecting microscope. At 1 and 14 days following injection, recipient mice were sacrificed and the distal colon removed. The extent of cell spread was determined using immunofluorescence imaging of wholemount preparations and area of cell coverage analyzed using ImageJ software as previously reported [7].

Neuronal differentiation and proliferation studies *in vivo*

Intraperitoneal injection of 50 mg kg⁻¹ EdU was performed 14 hours prior to sacrifice. The distal colon was removed and processed for immunohistochemistry using the pan-neuronal marker, PGP9.5. EdU incorporation was detected using the ClickiT EdU Imaging Kit (Invitrogen) and DsRed+PGP9.5+EdU+ or DsRed+PGP9.5+EdU- cells were counted using

ImageJ software to quantify the proportion of transplanted ENSCs-derived neurons that are actively proliferating.

Statistical analyses

Data are expressed as means $\pm$ SEM. Paired t-tests were used to evaluate the statistical significance between two groups. When more than two groups were compared, means were statistically compared by one-way ANOVA with Tukey's *post hoc* multiple-comparison tests of differences. Statistical significance was considered at $p < 0.05$.

Results

5-HT₄ receptor agonism significantly enhances neurogenesis in cultured ENSCs isolated from postnatal mouse intestine

ENSCs were isolated from the gastrointestinal tract of 2-4 week-old mice and expanded as enteric neurospheres in culture. Immunohistochemical characterization of neurospheres demonstrated that they contain neural crest-derived cells (Fig. 1A, p75; Fig. 1B, Ret), neurons (Fig. 1C, Tuj1), and glial cells (Fig. 1D, GFAP). Enteric neurospheres were dissociated and cultured in differentiation conditions in the presence or absence of the 5-HT₄ receptor agonist, RS67506. We found p75-immunoreactivity in $22.8 \pm 1.9\%$ of total cells in culture. Neuronal differentiation was determined by immunoreactivity to the pan-neuronal marker, Tuj1. In control conditions, $11.5 \pm 2.9\%$ of ENSCs gave rise to neurons, whereas this increased to $34.9 \pm 3.3\%$ ($p < 0.01$, $n=3$) in the presence of RS67506 (550 nM) (Fig. 1E). To determine whether the 5-HT₄ signaling-mediated increase in neuronal density was associated with increased proliferation of neuronal cell and/or enteric neural crest cells, incorporation of the thymidine analog, EdU, was measured. In the presence of RS67506, significantly more Tuj1+ neurons incorporated EdU as compared to control ($32.6 \pm 4.6\%$ vs $19.9 \pm 6.9\%$; Fig. 1F, $p < 0.05$, $n=3$). The percentage of proliferating undifferentiated neural crest cells (p75+) was also significantly increased in the presence of RS67506 ($40.7 \pm 5.3\%$ vs $18.8 \pm 3.7\%$; Fig. 1G, $p < 0.05$, $n=3$).

To test whether 5-HT₄ receptor agonism alters neuronal subtype specification, we examined neurotransmitter expression quantitatively using immunohistochemistry. We found the number of CGRP+ neurons was significantly increased in the presence of RS67506 ($43.6 \pm 0.3\%$ vs $29.9 \pm 1.8\%$; Fig. 1H, $p < 0.05$, $n=3$) whereas no difference was observed in the density of nNOS+ ($20.5 \pm 1.6\%$ vs $23.1 \pm 1.0\%$; Fig. 1I, $n=3$) or calretinin+ enteric neurons ($8.4 \pm 1.9\%$ vs $11.1 \pm 1.5\%$, $p=NS$, $n=3$). RS67506 also increased neurite length significantly ($435.3 \pm 29.4 \mu\text{m}$ vs $258.5 \pm 39.4 \mu\text{m}$; Fig. 1J, $p < 0.01$, $n=3$).

Production and characterization of RS-nanoparticles

The size, zeta potential and morphology of the liposomes was characterized. The carriers had an average hydrodynamic diameter of 156 nm (PDI: 0.354), as determined by dynamic light scattering (Fig. 2C), and a zeta potential of -39.6 mV based on microelectrophoresis (Fig. 2D). Imaging of the liposomes with transmission electron microscopy (TEM) showed round nano-carriers, with a diameter close to the diameter detected by dynamic light scattering (Fig. 2A,B). The amount of drug encapsulated in the nano-carriers was

determined by HPLC after releasing the drug by suspending the liposomes in a solution of the surfactant Triton-X. Drug concentration was adjusted as needed based on the requirements of the treatment.

RS-nanoparticles promote enteric neuronal proliferation and differentiation *in vitro*

To evaluate the effect of RS-nanoparticles on the capacity of ENSCs to proliferate and differentiate, primary enteric neurospheres were passaged on culture day 7 and re-plated to grow secondary neurospheres in medium containing either liposomal nanoparticles containing no drug (CTRL-nanoparticles) or RS-nanoparticles (550 nM) (Fig. 3A). After 7 days in culture (Fig. 3A, open arrow on Day 14), immunohistochemical analysis for p75+ and EdU was performed. The proportion of dividing neural crest-derived cells (EdU+p75+ cells / p75+ cells) within the secondary neurospheres was significantly increased in the presence of RS-nanoparticles compared to CTRL-nanoparticles (Fig. 3B, $12.4 \pm 2.4\%$ vs $5.9 \pm 1.5\%$, $p < 0.05$, $n = 4$). ENSCs were then cultured in differentiation conditions in the presence of CTRL- or RS-nanoparticles for 7 additional days (Fig. 3A). Immunoreactivity to Tuj1 showed that $19.1 \pm 1.4\%$ of ENSCs gave rise to neurons in medium containing CTRL-nanoparticles, whereas neuronal density increased to $26.6 \pm 3.4\%$ ($p < 0.05$, Fig. 3C) in the presence of RS-nanoparticles. Neuronal proliferation, determined by incorporation of EdU in Tuj1+ neurons (Fig. 3D, arrows), was also significantly increased in the presence of RS-nanoparticles compared to controls (Fig. 3E, $21.6 \pm 1.6\%$ vs $5.6 \pm 1.5\%$, $p < 0.001$, $n = 4$). Glial density, identified by immunoreactivity to S-100, was unchanged (Fig. 3F, $12.9 \pm 0.6\%$ vs $12.4 \pm 2.5\%$, $p = \text{NS}$, $n = 2$).

RS-nanoparticles increase neuronal differentiation and proliferation following transplantation to mouse LMMP *ex vivo*

To test whether addition of liposomal-containing factors can modify the microenvironment and optimize transplanted progenitor cells in the host gut milieu, postnatal DsRed+ ENSCs were co-cultured with LMMP obtained from 7 day-old wild-type mice. The LMMP was isolated from mouse gut and placed on filter paper containing a 3 mm rectangular window (Fig. 4A). A DsRed-expressing enteric neurosphere was placed on the LMMP and the co-culture preparations maintained in tissue culture medium in the presence of CTRL- or RS-nanoparticles (550 nM) for 7 days. During that time, neurofibers project extensively from the enteric neurospheres onto the LMMP (Fig. 4B). EdU (10 μM) was added to the culture medium 14 hours prior to the fixation. Co-culture preparations were processed for immunostaining for Tuj1 and EdU. The results demonstrate DsRed+ ENSCs migrating along the LMMP surface as well as projecting fibers within the muscle layer. Neuronal differentiation, determined by colocalization of DsRed and Tuj1 immunoreactivity (Fig. 4C, arrows), was significantly increased in the presence of RS-nanoparticles (Fig. 4E; $75.4 \pm 4.0\%$ vs $57.8 \pm 15.8\%$, $p < 0.05$, $n = 3$). Moreover, the percentage of proliferating neurons, detected by EdU incorporation into DsRed+/Tuj1+ cells (Fig. 4D, arrows), was markedly increased by the RS-nanoparticles (Fig. 4F; $28.0 \pm 7.7\%$ vs $15.1 \pm 2.9\%$, $p < 0.05$, $n = 3$).

To determine whether 5-HT₄ agonism had a similar effect on the host ENS, we examined cultured mouse LMMP in medium containing either control (empty)- or RS-nanoparticles for 3 days. EdU was added to the culture medium and the number of neurons incorporating

EdU was counted (Supplementary Figure 1A-D, arrows). We did not observe an increase in endogenous neuronal proliferation in the presence of the agonist (Supplementary Figure 1E, 0.22 ± 0.06 vs 0.28 ± 0.06 Hu+EdU+ cells per ganglia, $p=NS$).

ENSC delivery in hydrogel leads to an immediate and sustained increase in the area of cell coverage

Previous ENSC transplantation has demonstrated limited cell spreading using implantation of neurospheres [43]. To improve the area of cell coverage, we dissociated ENSCs and delivered them by microinjection in a thermosensitive hydrogel carrier in order to limit diffuse distribution of cells, to avoid leakage from puncture holes, and to maintain cells in proximity to co-injected nanoparticles. In a series of initial experiments, ENSCs were injected in hydrogel without added nanoparticles. At 1 and 14 days following ENSC transplantation, the area of cell engraftment was measured (Fig. 5A, dotted line) and the results compared to those following neurosphere implantation (Fig. 5B, dotted line). We found a dramatic increase in the area of cell coverage in the hydrogel group, as shown in Fig. 5C.

Proliferation and differentiation of transplanted ENSCs *in vivo* is significantly enhanced by RS-nanoparticles

Based on the positive effects of 5-HT₄ agonism *in vitro* and *ex vivo*, we next tested whether 5-HT₄ agonism can enhance the capacity of ENSCs to proliferate and differentiate following colonic transplantation *in vivo*. ENSCs were suspended in 18% PF-127 hydrogel on ice at a density of 1,000 cells per μ L without any additional compounds ($n=3$) or in the presence of CTRL- ($n=4$) or 550 nM RS-nanoparticles ($n=8$). The distal colon of 4 to 6 week-old C57BL/6 wild-type mice was exposed via a peri-anal skin incision and a 50 μ L cell suspension was injected using a 30G needle. Two weeks following transplantation, cell engraftment, neuronal differentiation and proliferation were evaluated immunohistochemically. Transplanted DsRed+ ENSCs were found in all ($n=15$) recipient colons, mainly distributed along the submucosal layer (Fig. 6A) with extensive spreading longitudinally. A subpopulation of injected DsRed+ cells also spread intramuscularly (Fig. 6B). These cells project fibers and differentiate into Tuj1+ neurons (Fig. 6A and B, arrows). Neuronal differentiation of transplanted ENSCs was determined by immunoreactivity to PGP9.5 (Fig. 6C-E). Synaptophysin expression was also seen in the transplanted ENSCs (Supplementary Figure 1F), supporting their functional integration. As shown in Fig. 6F, the proportion of PGP9.5+ neurons derived from DsRed+ transplanted ENSCs was significantly increased when co-injected with RS-nanoparticles ($74.3 \pm 1.3\%$) as compared to cell delivery with hydrogel alone ($48.0 \pm 5.5\%$) or delivery with CTRL-nanoparticles ($51.8 \pm 8.4\%$; $n=3$ per group). EdU was injected into recipient mice intraperitoneally to label dividing cells (Fig. 6C-E, arrows). As shown in Fig. 6G, we found a marked increase in neuronal proliferation with RS-nanoparticles ($17.4 \pm 3.5\%$) as compared to hydrogel alone ($5.0 \pm 1.4\%$) or with CTRL-nanoparticles ($7.2 \pm 0.6\%$; $n=3$ per group). Moreover, the length of projecting neurofibers extending from transplanted DsRed+ ENSCs was significantly longer when RS-nanoparticles were co-injected *in vivo* (Fig. 6H, $116.7 \pm 15.0 \mu$ m vs $74.3 \pm 6.9 \mu$ m, $p<0.05$, $n=3$).

Discussion

In this study, we isolate and culture ENSCs from postnatal mouse intestine and show that pharmacological stimulation of 5-HT₄ signaling augments their capacity to proliferate and differentiate *in vitro*. Encapsulation of the 5-HT₄ receptor agonist, RS67506, within liposomal nanoparticles effectively maintains this effect following ENSC transplantation *in vivo*. This represents a novel approach that enhances the otherwise limited proliferation and differentiation potential of ENSCs observed in prior transplantation studies [6, 9, 10].

ENSCs have been isolated from embryonic and postnatal rodent [4, 5, 7, 44] and humans [45], including patients with enteric neuropathies [46]. Most previous studies have demonstrated successful expansion of ENSCs as enteric “neurospheres.” In theory, dissociated intestinal cells are cultured in the appropriate conditions to allow neural progenitors to proliferate and other cell types to be eliminated spontaneously [13, 45, 46]. However, others have shown that enteric neurospheres consist of a heterogeneous population of cells, including neurons, glial cells, and non-neural crest-derived cells, such as smooth muscle and mesenchymal cells [8, 43]. We analyzed cell composition in our dissociated neurospheres and observed p75-immunoreactivity in up to 25% of cells, Tuj1+ neurons in 20%, and S-100+ glia in 12%. Since p75 labels ENSCs as well as glia, and Tuj1 labels neurons, that leaves approximately 60% other cell types. Several groups have used immunoselection using ENSC surface markers, such as p75, ret, and α 4 integrin [4, 44, 47, 48] to generate purer cultures and demonstrated their capacity for neuroglial differentiation and cell migration *ex vivo*. However, the prevalence of those cells in the postnatal gut is low (1-2% in adult rats [44], <5% in postnatal human colon [48]). Therefore, methods to optimize the isolation techniques and/or culture conditions are needed to obtain a large number of cells for future clinical application. Using unsorted gut-derived neurospheres can be readily achieved and would supply a robust number of cells in a reasonably short period, thus representing a clinically useful source of autologous cells to generate neurons and glia for transplantation.

Recent studies have shown that ENSCs can be transplanted to the gut wall *in vivo*, demonstrating successful engraftment, differentiation, migration, and possibly functional improvement [10-12], supporting their potential use for treating neurointestinal diseases [49, 50]. However, it has also been shown that functional improvement lasts only a short period (up to 1 week) [10, 11] and the migration and differentiation potency of the transplanted cells are limited [9, 12]. Previous studies have manipulated the stem cells using lentiviral vectors [51] or microenvironment modification by local injection of an anti-inflammatory drug [52] in order to enhance cell survival following transplantation. Micci et al. reported the limited benefit of inhibiting inflammation around the transplant site by local injection of a caspase-1 inhibitor, which resulted in increased cell survival at 1 week after transplantation into the gastric wall, but was followed by dramatically decreased cell numbers at 2-4 weeks post transplantation [52]. Overexpression of the anti-apoptotic gene, Bcl-2, in a lentiviral vector seems to ameliorate this problem, as Liu et al. observed a significant increase in cell survival, neuronal differentiation, and colonic muscle relaxation 8 weeks after transplantation of neuroepithelial stem cells into denervated rat colon [51]. While this approach may enhance the survival of transplanted cells, there is significant

concern regarding the delivery of genes commonly associated with human cancer. Developing a safe and efficient method of engineering ENSCs to optimize their success following transplantation is necessary.

Serotonin (5-hydroxytryptamine; 5-HT) is a hormone and neurotransmitter with a broad range of physiological functions. About 95% of 5-HT in the body is found in the gastrointestinal tract, primarily enterochromaffin (EC) cells in the mucosa, with a smaller pool in the ENS [53, 54]. 5-HT plays an important role in gastrointestinal motility. Serotonin is synthesized and released from EC cells in response to luminal pressure, stimulating mucosal sensory neurons and initiating peristaltic reflexes in the small intestine [55] and migrating motor complexes in the colon [56] via 5-HT₄ receptor signaling [57]. Serotonin is also a neurotransmitter of descending myenteric interneurons [58] and it appears that neuronal serotonin contributes to gut motility more significantly than that released from EC cells [16]. During ENS development, serotonin is required for differentiation of dopamine-, GABA-, and CGRP-expressing enteric neurons [16]. Moreover, stimulation of 5-HT₄ receptors protects cultured enteric neurons from apoptosis, enhances their development *in vitro* and induces enteric neurogenesis in adult mice *in vivo* [14]. Based on these observations, we hypothesized that serotonin signaling could improve the performance of ENSCs following transplantation into the intestine. Our results confirm that 5-HT₄ receptor agonism significantly enhances the ability of cultured ENSCs to proliferate and to differentiate into neurons, consistent with previous work from our lab and others [14, 17].

We found that the presence of 5-HT₄ receptor agonist influenced neuronal subtype specification. Specifically, the serotonin receptor agonism increased the density of CGRP-expressing neurons, whereas no difference was seen in the differentiation of nNOS- or calretinin-expressing neurons. Li et al. observed a reduction in nNOS- and CGRP-expressing subtypes in the gut of mice lacking neuronal 5-HT production [16], which is consistent with our observed change in CGRP expression. A proper balance of neurotransmitter expression and neuronal subtype differentiation is critically important to gut function. Some enteric neuropathies specifically affect subpopulations of excitatory or inhibitory motor neurons or possibly sensory neurons [3]. Understanding the factors that drive subtype specification will be important to achieving effective results following ENSC transplantation.

Several 5-HT₄ receptor agonists have been developed for the treatment of hypomotility disorders, including tegaserod, a partial agonist of 5-HT₄, and cisapride, a non-selective 5-HT₄ agonist. These drugs enhance gastric accommodation [59] and improve symptoms in patients with constipation-predominant irritable bowel syndrome or chronic constipation [60]. However, both drugs were removed from the market because of cardiovascular side effects [54]. Since their relative lack of receptor selectivity may have contributed, more selective agonists, such as prucalopride and mosapride, are currently drawing attention. To overcome the potential risks of systemic 5-HT₄ agonist administration, and also to achieve sustained drug delivery locally near the transplanted cells, we used nanoparticle drug delivery [61]. Nanoparticles, made of natural or synthetic polymer, typically in the range of 50-200 nm, can be used to encapsulate bioactive molecules. Liposomal nanoparticles, specifically, have been widely investigated and favorably used clinically as a drug carrier

due to their low toxicity and their physical structure, capable of entrapping both hydrophilic and hydrophobic drugs [24]. Liposomal encapsulation also works as a shield for the loaded drug, protecting against degradation and thus increasing drug stability. Natarajan et al. [62] encapsulated an intraocular pressure-lowering medicine, latanoprost, into egg-phosphatidylcholine liposomes. Following injection into the subconjunctiva of rabbit eyes, sustained release of the drug was observed and a low intraocular pressure was maintained for up to 90 days.

In our current study, we encapsulated a 5-HT₄ agonist into liposomal nanoparticles and found that these RS-nanoparticles significantly increase neuronal density and proliferation in both cultured ENSCs *in vitro* and transplanted ENSCs into colon *ex vivo*. In order to translate these observations into the *in vivo* transplantation model, we needed a mechanism to keep the nanoparticles in close proximity to the ENSCs so that they could exert their beneficial drug-mediated effect. We used a thermosensitive hydrogel to achieve this goal. Triblock co-polymers consisting of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) have been widely studied for various pharmaceutical and biomedical applications. Due to the difference in hydrophobicity between poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO) blocks, these copolymers are able to self-assemble into micellar structures in water. Pluronics[®] F-127 (PF-127; EO₁₀₀PO₇₀EO₁₀₀) is one member of this family of co-polymers and has been studied extensively as a DDS due to its biocompatibility and low cytotoxicity [63]. PF-127 is an FDA-approved polymer currently used in cosmetics and other products. The aqueous solution of PF-127 is viscous at or below room temperature (<25°C). Pharmaceutical agents can be formulated in the copolymer solution at lower temperature and injected into target tissue, where they form gels when warmed to physiological temperatures [26]. This unique characteristic and the low toxicity of PF-127 make it an excellent drug delivery vehicle at multiple application sites [41]. This gelation behavior also provides a good method for encapsulating cells for tissue-engineering applications. PF-127 has been used to encapsulate neural stem cells prior to their transplantation into a model of stroke [31, 32] and spinal cord injury [33], leading to increased survival of engrafted cells.

Given the stimulatory effect of serotonin signaling on enteric neurogenesis [14], we asked whether 5-HT₄ agonist-loaded nanoparticles promote neuronal proliferation of the host's endogenous ENS. Despite the positive effect on transplanted cells, we did not observe an effect on endogenous neurons. This could be due to the fact that cultured LMMP has been shown to stimulate neurogenesis [64], possibly due to the injury associated with harvesting the LMMP, thereby reducing the impact of any additional neuronal proliferation from serotonin receptor agonism. Secondly, our culture period was limited to 72 hours and it may take longer for host ENS cells to proliferate in response to serotonin signaling.

Recently it was proposed that stem cell preconditioning *in vitro*, combined with cell encapsulation, is a promising strategy to provide immune isolation, promote cell aggregation to enable cell-cell contact, and deliver mass transport of biomolecules, resulting in less cell apoptosis while maintaining biological functions of the cells and improved cell viability following transplantation *in vivo* [21]. Furthermore, several recent reports have revealed that the combination of hydrogel and encapsulated drug improves controlled drug release

[34-38]. Caicco et al. [38] observed that poly(lactic-co-glycolic) acid microsphere-encapsulated cyclosporine A (CsA) released from hyaluronan-methylcellulose hydrogel was prolonged for a period 3-4 weeks following epi-cortical delivery *in vivo*, whereas particulate CsA extended release to days from hours for solubilized CsA [38]. Nie et al. [36] formulated the anticancer drug, paclitaxel (PTX), into liposomal nanoparticles in PF-127 hydrogel to achieve the desired drug release rate. They found that drug release was significantly prolonged from liposomal-encapsulated PTX immobilized within PF-127 hydrogel compared to liposomal PTX alone or PTX in PF-127, leading to substantial cytotoxic benefit in a cancer cell line [36]. Liposomes may act as a reservoir for sustained drug release and PF-127 may enhance penetration of liposomal drugs to improve their efficacy.

In the current study, ENSCs were delivered by injection and spread longitudinally within the submucosa, with cells and fibers projecting into the muscle layer, similar to what we have observed following endoscopic delivery [20]. When co-delivered with RS-nanoparticles in PF-127 hydrogel, ENSCs exhibit markedly enhanced cell proliferation and neuronal differentiation. The success of this approach can be attributed to sustained release of 5-HT₄ agonist from liposomal nanoparticles, maintenance of spatial proximity of cells and particles when contained within the hydrogel, and the immune isolation afforded by hydrogel encapsulation. These findings support this novel approach that enhances the otherwise limited ENSC proliferation and differentiation previously reported [9, 10] and has the potential to significantly enhance the success of cell transplantation for treating neurointestinal diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. 5-HT₄ receptor agonism increases neuronal differentiation and proliferation in cultured ENSCs

ENSCs were isolated from postnatal mouse gut and expanded as enteric neurospheres, containing neural crest-derived cells (A, p75; B, Ret), neurons (C, Tuj1), and glial cells (D, GFAP). The addition of soluble 5-HT₄ receptor agonist (RS67506) to 2D-plated dissociated neurosphere cells significantly increases neuronal density (E), proliferation of neurons (F) and neural crest cells (G), and differentiation into CGRP-expressing neurons (H), but does not alter differentiation to nNOS+ neurons (I). RS67506 also increases neurite length (J).

*p<0.05, **p<0.01

NS, not significant

Figure 2. 5-HT₄ receptor agonist-loaded liposomes

Representative TEM images of the liposomes (A, B) show round and homogenous vesicles with an average diameter consistent with the hydrodynamic diameter as detected by dynamic light scattering (C). Representative Zeta-potential as determined by microelectrophoresis is shown (D). D, diameter; n, nanometers

Figure 3. RS-nanoparticles promote enteric neuronal proliferation and differentiation

RS67506-loaded nanoparticles were added to cultured ENSCs during the proliferation (A, 7-14 days) or differentiation (B, 14-21 days) phases. RS-nanoparticles significantly increase ENSC proliferation (B) and also promote neuronal differentiation (C) and proliferation (D, E), as shown by EdU incorporation into Tuj1+ enteric neurons (D, arrows). RS-nanoparticles have no effect on glial cell density (F). Scale bar 50 μ m (D).

* $p < 0.05$, *** $p < 0.001$

NS, not significant

Figure 4. RS-nanoparticles promote neuronal differentiation and proliferation of ENSCs following transplantation *ex vivo*

Mouse colonic LMMP was placed on filter paper and co-cultured with DsRed-expressing neurospheres (A, arrow). After 7 days, DsRed+ ENSCs migrate onto the LMMP (B) and give rise to neurons (C, arrows). Neuronal density is greater in the presence of RS-nanoparticles (E). EdU incorporation into DsRed+/Tuj1+ cells (D, arrows) is consistent with active neuronal proliferation, which is significantly greater in the presence of RS-nanoparticles (F). Scale bars 1 mm (A), 200 μ m (B).

* $p < 0.05$

Figure 5. ENSC delivery in hydrogel leads to immediate and sustained improvement in the area of cell coverage

DsRed+ ENSCs were injected with thermosensitive hydrogel, Pluronic® F-127 (PF-127), into wild-type mouse colon. Wholemout preparation of recipient colon 2 weeks following injection demonstrates that the area covered by transplanted cells (A, dotted line) is markedly increased at 1 and 14 days in the hydrogel group compared to neurosphere implantation (B, C). Scale bars 200 μ m (A, B).

NS, neurosphere; HGI, hydrogel injection

*p<0.05, **p<0.01

Figure 6. Transplanted ENSCs exhibit greater neuronal proliferation and differentiation in the presence of RS-nanoparticles *in vivo*

DsRed-expressing ENSCs were microinjected into the distal colon and, two weeks later, ENSC engraftment and neuronal differentiation are observed (A, B; arrows mark Tuj1+DsRed+ transplanted cells; arrowheads mark Tuj1+ endogenous host neurons). DsRed + ENSCs give rise to PGP9.5+ neurons that incorporate EdU administered to the recipient mice (C-E, arrows). Neuronal differentiation (F) and proliferation (G) of transplanted cells is markedly increased by RS-nanoparticles, which also increase the length of neuronal fibers projecting from the transplanted cells (H). Scale bars 500 μm (A), 50 μm (C-E).

* $p < 0.05$, compared to CTRL-nano; NS, not significant