Enteric neural progenitors are more efficient than brain-derived progenitors at generating neurons in the colon

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Findlay Q, Yap KK, Bergner AJ, Young HM, Stamp LA.
Enteric neural progenitors are more efficient than brain-derived progenitors at generating neurons in the colon. Am J Physiol Gastrointest Liver Physiol 307: G741–G748, 2014. First published August 14, 2014; doi:10.1152/ajpgi.00225.2014.—Gut motility disorders can result from an absent, damaged, or dysfunctional enteric nervous system (ENS). Cell therapy is an exciting prospect to treat these enteric neuropathies and restore gut motility. Previous studies have examined a variety of sources of stem/progenitor cells, but the ability of different sources of cells to generate enteric neurons has not been directly compared. It is important to identify the source of stem/progenitor cells that is best at colonizing the bowel and generating neurons following transplantation. The aim of this study was to compare the ability of central nervous system (CNS) progenitors and ENS progenitors to colonize the colon and differentiate into neurons. Genetically labeled CNS- and ENS-derived progenitors were cocultured with aneural explants of embryonic mouse colon for 1 or 2.5 wk to assess their migratory, proliferative, and differentiation capacities, and survival, in the embryonic gut environment. Both progenitor cell populations were transplanted in the postnatal colon of mice in vivo for 4 wk before they were analyzed for migration and differentiation using immunohistochemistry. ENS-derived progenitors migrated further than CNS-derived cells in both embryonic and postnatal gut environments. ENS-derived progenitors also gave rise to more neurons than their CNS-derived counterparts. Furthermore, neurons derived from ENS progenitors clustered together in ganglia, whereas CNS-derived neurons were mostly solitary. We conclude that, within the gut environment, ENS-derived progenitors show superior migration, proliferation, and neuronal differentiation compared with CNS progenitors.

neural crest; cell therapy; enteric neuron; central nervous system; enteric nervous system

THE ENTERIC NERVOUS SYSTEM (ENS) plays an important role in the control of gut motility, secretion, and blood flow. Defects in the ENS, enteric neuropathies, can result in motility disorders. Enteric neuropathies include congenital diseases such as Hirschsprung disease and acquired diseases such as achalasia and diabetic gastroparesis (15, 19, 33, 53, 55). Currently, the treatment for Hirschsprung disease involves surgical removal of the affected region of bowel (46, 65), but there are few effective treatments for many acquired enteric neuropathies (13, 23, 59).

There has been much recent interest in the transplantation of a stem or progenitor cell population into the gastrointestinal tract to treat enteric neuropathies (12, 27, 28, 30, 35, 61, 71). A variety of sources of stem/progenitor cells has been examined, including the ENS, the central nervous system (CNS), and skin, as well as embryonic and induced pluripotent stem cells (16, 21, 27, 28, 35, 37, 61, 67). It is essential that the source of stem/progenitor cells can be sufficiently expanded in vitro and subsequently generate mature, functional neurons of the appropriate phenotype. Resident neural progenitor cells have been isolated from fetal and postnatal rodent and human bowel, which can give rise to enteric neurons following coculture with embryonic or postnatal rodent gut explants or human gut muscle, or following transplantation in the postnatal rodent gut (2, 8, 9, 29, 34, 43, 47, 66, 70). It is well established that, when maintained under optimal conditions, CNS neural progenitors are capable of rapid clonal expansion in vitro, with a population doubling time of around 44 h (42). CNS-derived progenitors can also give rise to enteric neurons following transplantation in the stomach of mice (48, 49). Furthermore, in vitro, neurons derived from CNS progenitors show a variety of phenotypic similarities to enteric neurons following coculture with longitudinal muscle and myenteric plexus (36). Although ENS progenitors are more accessible than CNS progenitors for clinical applications (47, 72), clinical trials in which cells from fetal human brains are transplanted in the brains of patients with Parkinson’s disease have recently resumed (1). It is therefore possible that CNS progenitors could be a potential source of enteric neurons for patients with enteric neuropathies in the future. To date, a variety of sources of enteric neuron have been investigated, but different potential sources have not yet been directly compared in their ability to colonize recipient gut and differentiate into neurons.

In this study, we compared the abilities of ENS- and CNS-derived neural progenitors to generate enteric neurons following coculture with explants of embryonic gut or following transplantation in the colon of postnatal mice. Our data show that, compared with CNS-derived cells, ENS-derived progenitors maintain their proliferative capacity, migrate further, and undergo more efficient neuronal differentiation into ganglion-like clusters within the gut environment.

MATERIALS AND METHODS

Animals

Gut from wild-type C57BL/6 mice was used as recipients for both the in vitro aneural hindgut coculture [embryonic day (E) 11.5] and the postnatal (postnatal days 14–21) distal colon transplantation. CNS neural progenitors were isolated from NZ/Eg mice, where green fluorescent protein (GFP) is ubiquitously expressed in the nuclei of all cells. These knock-in mice are a modification of the Z/EG mouse, the in which the lacZ gene has been permanently excised and in its place a nuclear localization sequence inserted (45). ENS neural progenitors were isolated from either Wnt1-cre; EYFP mice or Ednrb-hKikGR mice (51). In Ednrb-hKikGR mice, the photocovertible protein KikumeGR (KikGR) is expressed under the control of an enteric-specific region of the Ednrb promoter. The result is the expression of the KikGR protein in all neural crest-derived cells within the gut (51). All animal studies were approved by the Anatomy

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and Neuroscience, Pathology, Pharmacology, and Physiology Animal Ethics Committee of the University of Melbourne.

Isolation and Culture of CNS and ENS Neural Progenitors

CNS progenitors were isolated from the cerebral cortex of E14.5 or postnatal days 1–4 NZ/EG mice. The tissue was dissociated and cultured in conditions that promote the formation of neurospheres (44). In each experiment, three or four embryos or postnatal mice were used. The cerebral cortices were removed, chopped finely with a scalpel blade, and digested with 1 ml of 500 µg/ml Trypsin in Hanks solution (Invitrogen, Mulgrave, VIC, Australia) for 10 min in a 37°C water bath, and then 1 ml of Trypsin inhibitor solution (Sigma-Aldrich, Castle Hill, NSW, Australia) was added for a further 5 min. Hanks balanced salt solution (8 ml, Sigma-Aldrich) was then added, and the cells were centrifuged for 5 min at 1,500 revolutions/min. The supernatant was removed, and 1 ml of Neurosphere Medium was added to the cell pellet and resuspended by gentle pipetting. The Neurosphere Medium contained 25 µg/ml insulin, 9.6 µg/ml putrescine dihydrochloride, 100 µg/ml apo-transferrin, 5.2 ng/ml selenium sodium salt, 6.3 ng/ml progestosterone, 10 ng/ml basic fibroblast growth factor, 4 µg/ml heparin sodium salt, 20 ng/ml epidermal growth factor, 2 mg/ml bovine serum albumin, 5 mM HEPES, and 0.60% d-glucose (all from Sigma-Aldrich) plus 100 µg/ml penicillin/streptomycin (Life Technologies, Mulgrave, VIC, Australia). The cells were plated at 50,000 cells/ml in 5 ml of Neurosphere Medium and incubated at 37°C and 5% CO2 for 10–14 days. ENS progenitors were isolated from the gut of E14.5 Wntl-cre;EYFP or Ednrb-hKikGR embryos and cultured as previously described (29). Briefly, the whole gut from stomach to anus was dissected and dissociated in 0.1% trypsin/EDTA (Life Technologies). Enhanced yellow fluorescent protein (EYFP)/hKikGR+ cells were isolated by flow cytometry and plated in round-bottom, low-attachment 96-well plates (Corning) at a density of 50,000 cells/ml in DMEM-F-12 containing 1% penicillin/streptomycin, 1× N-2 supplement, 1× B-27 supplement (all from Life Technologies), 1% L-glutamine (Sigma Aldrich), and 20 ng/ml epidermal growth factor and basic fibroblast growth factor (R&D Systems, Minneapolis, MN). Cell aggregation was promoted by brief centrifugation at 480 g for 3 min at 4°C. This resulted in the generation of ENS neurospheres that were relatively uniform in size; they were cocultured or grafted in vivo when they were ~200 µm in diameter. CNS neurospheres were variable in size, and so only CNS neurospheres that were similar in size to the ENS neurospheres were used.

In Vitro Cell Proliferation Assay

Three CNS or ENS neurospheres were plated on fibronectin-coated (20 µg/ml; Sigma Aldrich) 18-mm cover slips and cultured in the presence of the thymidine analog 5-ethyl-2’-deoxyuridine (EdU; 10 µM; Life Technologies) for 2 days. At the end of the culture period, preparations were fixed and processed for EdU according to the manufacturer’s instructions.

In Vitro Catenary Organ Culture

Aneural hindguts (portion caudal to the caecum removed before the arrival of neural crest-derived cells) were dissected from E11.5 embryos. The catenary cultures were set up as previously described (3, 24, 73). In brief, a V shape was cut out of small squares of black Millipore filter paper (type HA; Merck Millipore). The hindgut was then suspended across the V-shaped hollow and attached to the filter paper (Fig. 1A). Either E14.5 or postnatal CNS neurospheres or E14.5 ENS neurospheres were then attached to the paper, directly next to, and in contact with, the oral end of the hindgut explant. The catenary organ cocultures were then placed in Terasaki wells containing 20 µl of tissue culture medium (DMEM-F-12 containing 10% FCS). Cocultures were then cultured for 7–17 days in a humidified incubator at 37°C and 5% CO2, and medium was changed every 2 days. To assess proliferation, 10 µM EdU was added to the tissue culture medium for the 7-day culture period. At the end of the culture period, specimens were fixed and processed for immunohistochemistry.

In Vivo Transplantation of Neural Progenitors to the Postnatal Distal Colon

Recipient wild-type mice (2–3 wk) were anesthetized by subcutaneous injection of a mixture of 20 mg/kg xylazine and 100 mg/kg ketamine hydrochloride (Troy Laboratories, Sydney, NSW, Australia). A midabdominal incision was made, and the distal colon was exposed. Two neurospheres, dyed by a brief exposure to 0.1% trypan blue in phosphate buffer (PB), were transplanted in the external muscle layer of the distal colon as described previously (29). Four weeks after surgery, the recipient mice were killed by cervical dislocation, and the distal colon was removed.

Tissue Preparation

The postnatal distal colon was opened along the mesenteric border, stretched, and pinned on Sylgard-coated dishes. Both the stretched postnatal colon and intact embryonic gut explants were fixed in 4% paraformaldehyde in 0.1 M PB overnight at 4°C. The tissue was then washed in PB, and the mucosal layer of the postnatal colon was peeled away from the gut musculature before exposure to 1% Triton X-100 in PB for 30 min.

Immunohistochemistry

Both in vitro catenary organ cultures and in vivo specimens were processed for immunohistochemistry as whole mount preparations. The following primary antisera were used: rabbit anti-GFP (1:500; Molecular Probes/Life Technologies) or goat anti-GFP (1:400; Rockland Immunochemicals), rabbit anti-glial fibrillary acidic protein (GFAP, 1:1,000; Dako, Glostrup, Denmark), rabbit anti-S100β (1:1,000; Dako), rabbit anti-active caspase-3 (1:1,000; R&D Systems), human anti-Hu (1:5,000; gift of Vanda Lennon, Mayo Clinic, Rochester, MN), mouse anti-Tuj1 (1:2,000; Covance), and sheep anti-nNOS (1:2,000; gift of Piers Emson). After exposure to primary antibodies overnight at 4°C and washing with PB, specimens were incubated in secondary antibodies [donkey anti-rabbit FITC (1:200; Jackson ImmunoResearch), donkey anti-rabbit Alexa Fluor 594 (1:1,000; Molecular Probes), donkey anti-human Alexa Fluor 594 (1:400; Molecular Probes), donkey anti-sheep Alexa Fluor 647 (1:500; Molecular Probes), or donkey anti-mouse Alexa Fluor 594 (1:200; Molecular Probes)] for 2 h in the dark at room temperature. Specimens were washed before being mounted in Dako fluorescence mounting media (Dako). EdU incorporation was revealed using the Click-iT EdU Alexa Fluor 594 Imaging Kit (Life Technologies).

Measurement of Cell Migration, Proliferation and Differentiation, and Statistical Analysis

To determine the distance migrated along explants of embryonic colon by cells derived from CNS-derived neurospheres (from NZ/EG mice) or ENS-derived neurospheres (from Ednrb-hKikGR mice), a z-series of images were taken on a LSM 5 Pascal confocal microscope (Carl Zeiss, Jena, Germany). The images were projected, and the distance between the center of the most caudal GFP-positive (+), yellow fluorescent protein (YFP)+, or KikGR+ cell body and the edge of the nearest neurosphere was measured using the Image J software program (National Institutes of Health). To determine the area occupied by graft-derived cells in the postnatal colon, tile scans of whole mount preparations of recipient colon were taken using X5 objective program (National Institutes of Health). To determine the area occupied by graft-derived cells in the postnatal colon, tile scans of whole mount preparations of recipient colon were taken using X5 objective.
cells was measured using Image J software as previously described (7). Data collected from experiments were analyzed using either two-tailed t-test or one-way ANOVA followed by Tukey-Kramer post hoc test on GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). Results with difference of \( P < 0.05 \) were considered significant. All graphs were created with GraphPad Prism version 5.0.

**RESULTS**

Neurospheres were generated from the cerebral cortex of E14.5 and postnatal day 4 NZ/EG mice in which all cells express GFP in the nucleus; these neurospheres were termed CNS neurospheres. ENS neurospheres were generated from the gut of E14.5 Wnt1-cre;EYFP or Ednrb-hKikGR mice, in which all enteric neural crest-derived cells express the fluorescent protein YFP or KikGR, respectively (29, 51).

**Coculture Studies**

**Migration.** We first compared the ability of cells derived from CNS and ENS neurospheres to colonize explants of aneural hindgut. Segments of hindgut were removed from E11.5 wild-type C57BL/6 mice and were set up in organ cultures with E14.5 or postnatal CNS-derived or E14.5 ENS-derived neurospheres attached to the rostral/oral end of the gut explants. Migration distance was measured from the soma of the most caudal green fluorescent protein (GFP)-positive (+)/KikumeGR (KikGR)\(^+\) stem cells to the edge of the nearest neurosphere after 1 and 2.5 wk.\(B\) quantification of the distance (mean \( \pm \) SE) to the most caudal cells in the four different coculture groups. E14.5 CNS-derived stem cells migrated significantly further than other stem cell populations at either time point (one-way ANOVA, \( *P < 0.05 \)). E14.5 CNS 2.5 wk, neurospheres derived from the cerebral cortex of E14.5 NZ/EG embryos cocultured with aneural gut explants for 2.5 wk; P/N CNS 1 wk, neurospheres derived from the cerebral cortex of P1–4 NZ/EG mice cocultured with aneural gut explants for 1 wk; P/N CNS 2.5 wk, neurospheres derived from the cerebral cortex of P1–4 NZ/EG mice cocultured with aneural gut explants for 2.5 wk; E14.5 ENS 1 wk, neurospheres generated from the ENS of E14.5 mice and cocultured with aneural gut explants for 1 wk. C and D: representative confocal images of whole mount preparations of catenary cocultures showing migration of postnatal CNS-derived stem cells (C) and ENS-derived stem cells (D) along aneural gut explants and the location of the caudal-most cell (open arrow). Note CNS and ENS neurospheres of approximately equivalent size were used in catenary organ cultures and that GFP is only expressed in the nucleus of CNS-derived cells. Scale bar: 200 \( \mu \text{m} \).

Fig. 1. Migration of central nervous system (CNS)- and enteric nervous system (ENS)-derived stem cells in explants of embryonic aneural gut. A: embryonic day (E) 11.5 aneural hindguts from wild-type C57BL/6 mice were set up in catenary organ cultures with E14.5 or postnatal CNS-derived or E14.5 ENS-derived neurospheres attached to the rostral/oral end of the gut explants. Migration distance was measured from the soma of the most caudal green fluorescent protein (GFP)-positive (+)/KikumeGR (KikGR)\(^+\) stem cells to the edge of the nearest neurosphere after 1 and 2.5 wk. B: quantification of the distance (mean \( \pm \) SE) to the most caudal cells in the four different coculture groups. E14.5 CNS-derived stem cells migrated significantly further than other stem cell populations at either time point (one-way ANOVA, \( *P < 0.05 \)). E14.5 CNS 2.5 wk, neurospheres derived from the cerebral cortex of E14.5 NZ/EG embryos cocultured with aneural gut explants for 2.5 wk; P/N CNS 1 wk, neurospheres derived from the cerebral cortex of P1–4 NZ/EG mice cocultured with aneural gut explants for 1 wk; P/N CNS 2.5 wk, neurospheres derived from the cerebral cortex of P1–4 NZ/EG mice cocultured with aneural gut explants for 2.5 wk; E14.5 ENS 1 wk, neurospheres generated from the ENS of E14.5 mice and cocultured with aneural gut explants for 1 wk. C and D: representative confocal images of whole mount preparations of catenary cocultures showing migration of postnatal CNS-derived stem cells (C) and ENS-derived stem cells (D) along aneural gut explants and the location of the caudal-most cell (open arrow). Note CNS and ENS neurospheres of approximately equivalent size were used in catenary organ cultures and that GFP is only expressed in the nucleus of CNS-derived cells. Scale bar: 200 \( \mu \text{m} \).

Data collected from experiments were analyzed using either two-tailed t-test or one-way ANOVA followed by Tukey-Kramer post hoc test on GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). Results with difference of \( P < 0.05 \) were considered significant. All graphs were created with GraphPad Prism version 5.0.
ture: 3.6% of GFP+ cells were Tuj1+, n = 1,049 GFP+ cells in 12 explants). It was not possible to quantify neuronal differentiation of ENS-derived cells using Tuj1 because the ENS cells were in tight clusters and Tuj1 labels neurites, so it was frequently impossible to determine if an individual cell body was Tuj1+ or only surrounded by Tuj1+ neurites.

**Proliferation.** Proliferation plays an integral role in the colonization of the gastrointestinal tract with enteric neural progenitors during normal development (20). After 7 days in suspension culture, CNS and ENS neurospheres were plated on fibronectin-coated cover slips and cultured for a further 2 days in the presence of EdU. There was no significant difference in the proportion of CNS and ENS neural progenitors that had incorporated EdU (Fig. 3A). However, within the gut explants, a significantly greater proportion of ENS-derived progenitors incorporated EdU compared with CNS-derived progenitors (P < 0.05; Fig. 3B).

**Cell death.** There was no significant difference in the proportion of active caspase-3+ cells between CNS-derived (0.6%) and ENS-derived (0.8%) progenitors within gut explants (t-test; P > 0.05).

**In Vivo Studies**

CNS or ENS neurospheres generated from embryonic mice were transplanted in the distal colon of recipient 2- to 3-wk-old wild-type mice as described previously (29). Four weeks later, the recipients were killed, and the extent of migration and differenti-
ation was examined. Cells derived from ENS neurospheres migrated further than cells derived from E14.5 or postnatal CNS neurospheres ($P < 0.05$; Fig. 4); in fact, very few CNS-derived cells had migrated away from the transplant site (Fig. 5, E and I). In agreement with our earlier study (29), subpopulations of cells derived from embryonic ENS neurospheres expressed the pan-neuronal marker Hu or the glial marker S100B (Fig. 5, A–D). A subpopulation of cells derived from embryonic and postnatal CNS neurospheres expressed S100B or the glia and neural stem cell marker GFAP (Fig. 5, E–L). However, Hu-immunostained cells derived from E14.5 CNS neurospheres were only observed in one out of three recipients, and they were rare (Fig. 5, E–H). We also did not observe any Hu+ or Tuj1+ or nNOS+ cells among cells derived from postnatal CNS neurospheres ($n = 4$ recipients, 2 stained for Tuj1/nNOS and 2 for Hu/nNOS) despite strong Hu, Tuj1, and nNOS immunostaining in endogenous myenteric ganglia of the recipient.

DISCUSSION

Recent studies have shown that ENS progenitors are capable of migrating and differentiating into functional neurons following transplantation in the postnatal colon (29). Previous studies had also shown that CNS progenitors differentiate into nNOS neurons after transplantation in the stomach of mice (48, 49) and that, in vitro, coculture with longitudinal muscle and myenteric plexuses influences the phenotypic differentiation of neurons derived from CNS neurospheres (36). In this study, we directly compared the migratory and differentiation ability of these two neural progenitor populations in the pre- and postnatal colon. The media used to generate the CNS- and ENS-derived neurospheres had been previously used to expand these two progenitor populations (29, 44); although the two culture media had some differences in composition, we showed that CNS- and ENS-derived progenitors showed similar proliferation rates in vitro as judged by EdU incorporation.

We found that ENS-derived progenitors were superior to CNS-derived progenitors in their ability to colonize explants of aneural embryonic hindgut and the postnatal colon in vivo. The migration of neural crest cells in and along the developing gut has been shown to be dependent on the intrinsic properties of the neural crest-derived cells, factors produced by gut mesenchyme, interactions between migrating cells, and environmental factors (3–5, 10, 11, 17, 18, 50, 53, 74). In the current study, ENS progenitors were isolated from E14.5 gut and thus may

Fig. 5. Differentiation of E14.5 ENS- and E14.5 CNS-derived stem cells in the distal colon of wild-type C57BL/6 mice. A–D: representative confocal images of transplanted KikGR+ (green) ENS-derived stem cells in the distal colon of wild-type C57BL/6 mice after 4 wk. A: KikGR+ ENS-derived stem cells differentiated into Hu+ neurons (B, red) and S100B+ glia (C, cyan) in the distal colon of wild-type mice (D, merge). E–L: representative confocal images of transplanted GFP+ (green) CNS-derived stem cells in the distal colon of two wild-type C57BL/6 mice after 4 wk. GFP+ (green) embryonic CNS-derived stem cells gave rise to numerous S100B+ (G, cyan) and GFAP+ (K, cyan) glia. In one recipient, none of the CNS-derived cells showed detectable Hu immunostaining (F), and, in the second recipient, there was a small number of Hu+ neurons (J, arrows) in the distal colon. Scale bars: 100 μm.
have received some “priming” along their original route to and within the gut that gave them a competitive advantage over the CNS progenitors. For example, a recent study showed that exposure of vagal neural crest cells to retinoic acid resulted in the upregulation of Ret expression, the glial-derived neurotrophic factor receptor, and subsequently improved colonization of gut explants (62). Therefore, prior exposure to the migratory route to the gut and to the gut environment is likely to contribute to the difference between CNS and ENS progenitors in their ability to colonize the gut after isolation. Previous studies have reported that CNS-derived progenitors showed limited migration away from the injection site following transplantation in the gastric wall of adult mice (48, 49). We also observed very limited migration of CNS progenitors compared with ENS progenitors within the embryonic and postnatal gut. This is unlikely to be the result of an inherent inability of CNS progenitors to migrate, since they can migrate significant distances following transplantation in the mouse brain (14). Although the neuronal differentiation of CNS neurospheres in vitro is influenced by the presence of gut cells (36), it is possible that CNS progenitors respond only poorly, if at all, to some of the gut-derived cues that promote the migration of ENS progenitors. Future studies are required to determine whether CNS progenitors can be primed, for example, by culturing in the presence of gut-derived factors before transplantation in the gut (36), to give better migratory and neuronal differentiation responses in the gut environment. Experiments to compare migration and differentiation of ENS and CNS progenitors in the brain or other non-gut environments would also be informative.

Although trunk neural crest cells are poor at colonizing the gut (5), midbrain and hindbrain neural crest cells are capable of colonizing the gut and generating an ENS in chick-quail cocultures (75). It would therefore be interesting in future studies to examine the ability of neural progenitors derived from cranial neural crest-derived structures, such as the superior and jugular ganglia of the IXth and Xth nerves in which the neurons and glia are derived from the cranial neural crest (40), to generate enteric neurons.

Environmental factors as well as intrinsic factors, such as transcription factors, are known to influence cell fate specification during ENS development (22, 38, 39, 53, 60). Here we have shown that, although the CNS-derived progenitors are capable of giving rise to Hu+ and Tuj1+ neurons in the embryonic and postnatal gut environment, ENS-derived progenitors are significantly more efficient. When transplanted in the postnatal colon of mice, a subpopulation of CNS-derived cells expressed the glial markers, S100β and GFAP, but very few expressed the pan-neuronal markers, Hu and Tuj1, or the neuron subtype marker, nNOS. This suggests that the gut environment may selectively inhibit or delay neuronal differentiation of CNS-derived progenitors. This is in contrast to a previous study that showed CNS-derived progenitors gave rise to numerous neurons and glia following transplantation in the stomach of adult mice (48, 49). These differences might be because of differences in the environment between the stomach and the colon or because of differences in the properties of the CNS neurospheres used in the two studies. Because different enteric neuropathies affect different gut regions, future studies are required to examine systematically the abilities of different sources of neural progenitors to migrate and generate enteric neurons in different gut regions.

The ENS is composed of an interconnected network of ganglia. In the present study we found in explants of embryonic gut, neurons derived from ENS progenitors formed clusters, whereas neurons derived from CNS progenitors were mainly solitary. Our data suggest that the ability to cluster into ganglia is a cell-autonomous property of the progenitor populations, since the gut environment did not induce clustering of neurons derived from CNS progenitors.

It has been previously shown that both cell death and proliferation play important roles in the normal development of the ENS (20, 56, 68, 69). Proliferation is essential for the migration of ENS progenitors (63), and some of the genes associated with Hirschsprung disease play a role in proliferation (46). We have shown that there is no significant difference in the proportion of active caspase-3+ cells in gut explants between CNS-derived and ENS-derived progenitors. This indicates that increased cell death is not the mechanism by which CNS progenitors fail to colonize the pre- or postnatal bowel as efficiently as ENS progenitors. However, although there was no significant difference in the proportion of EdU+ cells in vitro, there were significantly less EdU+ CNS-derived cells within gut explants. This could indicate that factors within the gut inhibit CNS progenitor proliferation and/or CNS progenitors are unable to respond to signaling molecules secreted by the gut that drive ENS progenitor proliferation. The reduction in CNS progenitor proliferation could affect their ability to colonize the gut.

In a previous study, we showed that there is no significant difference in the ability of embryonic- and postnatal-derived ENS progenitors to migrate in vivo in the postnatal colon (29). In the current study, we compared the migratory and differentiation capacity of progenitors isolated from the brain of E14.5 and postnatal mice. Surprisingly, postnatal CNS progenitors had a tendency to migrate further in explants of embryonic gut compared with E14.5 CNS progenitors. Neuronal stem and progenitor cells are known to acquire both positional and temporal information (6, 25, 26, 31, 32, 41, 54, 57, 58, 64), and so it is possible that some of the signaling pathway components required for migration within the gut are not yet expressed by E14.5 CNS progenitors.

In conclusion, although CNS progenitor differentiation is affected by gut-derived factors (36), this study has demonstrated that ENS-derived progenitors are more efficient than CNS-derived progenitors at migration and neuronal differentiation in the pre- and postnatal mouse colon. Thus, ENS-derived progenitors are likely to be the preferable source for stem cell therapy to treat enteric neuropathies. Importantly, unlike CNS progenitors, patient-derived ENS progenitors can be readily obtained, using routine endoscopy techniques (47).

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