Transplanted progenitors generate functional enteric neurons in the postnatal colon

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Cell therapy has the potential to treat gastrointestinal motility disorders caused by diseases of the enteric nervous system. Many studies have demonstrated that various stem/progenitor cells can give rise to functional neurons in the embryonic gut; however, it is not yet known whether transplanted neural progenitor cells can migrate, proliferate, and generate functional neurons in the postnatal bowel in vivo. We transplanted neurospheres generated from fetal and postnatal intestinal neural crest–derived cells into the colon of postnatal mice. The neurosphere-derived cells migrated, proliferated, and generated neurons and glial cells that formed ganglion-like clusters within the recipient colon. Graft-derived neurons exhibited morphological, neurochemical, and electrophysiological characteristics similar to those of enteric neurons; they received synaptic inputs; and their neurites projected to muscle layers and the enteric ganglia of the recipient mice. These findings show that transplanted enteric neural progenitor cells can generate functional enteric neurons in the postnatal bowel and advance the notion that cell therapy is a promising strategy for enteric neuropathies.

Introduction
The enteric nervous system (ENS) plays an important role in regulating a number of gut functions including motility (1, 2). Enteric neuropathies, which result from diseased, damaged, or congenitally absent enteric neurons, cause motility disorders, most of which are poorly managed by current treatments (3). Cell-based therapies have potential for the treatment of enteric neuropathies by replacing diseased neurons (for example, in gastroparesis or achalasia) or by generating enteric neurons in regions that entirely lack an ENS due to developmental defects (as in Hirschsprung disease) (4–12). Cell-based therapies also hold promise for the treatment of the injured or diseased CNS, but cell therapy for enteric neuropathies is likely to be less complicated because of accessibility and the potential of expanding stem/progenitor cells from healthy regions of the intestine for transplantation into diseased regions of the same patient (13).

Many studies have demonstrated the ability of a variety of sources of stem/progenitor cells to give rise to enteric neurons in the embryonic gut (14–18). For example, enteric neural stem/progenitor cells isolated from postnatal human bowel migrate within the embryonic chick or mouse gut and differentiate into neurons and glial cells (13, 19). However, it is essential that cell therapy to treat enteric neuropathies be carried out postnatally in infants, children, or adults, as diagnosis only occurs after birth. During development, the structure of the gut wall changes dramatically from undifferentiated mesenchyme to a highly organized, concentric-layered structure of differentiated cells (20–24). It is unknown whether the fully differentiated gut wall is permissive for migration of neural progenitor cells. Furthermore, molecules produced by the gut mesenchyme are essential for the normal development of the ENS (12, 25–27), but it is unclear whether these factors are expressed at sufficient levels in the postnatal bowel to permit the development of enteric neurons from progenitors. Previous studies have transplanted CNS neural stem cells, ENS stem/progenitor cells, or ENS cell lines into the gut of postnatal animals in vivo (4, 28–33) or grown cocultures between stem/progenitor cells and the muscle of postnatal human gut (13), but the extent of migration, and whether the graft-derived neurons have the electrophysiological properties of enteric neurons and are incorporated into the neuronal circuitry, have not been determined.

In the present study, we generated neurospheres (NSs) from enteric neural crest–derived progenitors isolated from the fetal and postnatal gut and transplanted them into the postnatal mouse colon in vivo. Although there are a number of possible sources of enteric neurons (4, 5, 7, 8, 10, 11, 13, 16, 34–39), enteric neural crest–derived ENS progenitors were chosen, as they are likely to be the most clinically relevant source of cells, are readily accessible (13), and can give rise to enteric neurons in the embryonic gut or when cocultured with colonic muscle from infants (13, 14, 18). We showed that after transplantation into the colon of postnatal mice, ENS progenitors proliferated; migrated extensively and differentiated into neurons with the neurochemical, morphological, and electrophysiological characteristics of enteric neurons; and received synaptic inputs.

Results
Formation of NSs from dissociated fetal and postnatal gut. Previous studies have shown that all neural crest–derived cells in the gut express KikGR in embryonic EdnrbKik mice (40) and EGFP in embryonic RetTGM mice (41). Although KikGR is a photoconvertible protein that can be converted from green to red by the presence of UV light, we did not exploit this property in the current study, and the native green fluorescence was used to identify neural crest–derived cells. EdnrbKik- or RetTGM-positive cells were isolated by FACS from freshly dissociated gut of E13.5/E14.5 or P4 EdnrbKik or RetTGM
mice, aggregated by gentle centrifugation and then cultured. After 7 days, NS-like bodies up to 250 μm in diameter had formed (Figure 1E, inset). To characterize NSs derived from dissociated fetal gut (fNSs; E13.5/E14.5) or postnatal gut (pNSs; P4) in vitro, NSs were grown on fibronectin-coated coverslips for 2 days, fixed, and processed for immunohistochemistry. Many cells emigrated from the fNS and pNS, and most of the cells within and surrounding the explanted NSs showed immunoreactivity for the neural crest cell marker Sox10 (Figure 1A, B, and D). A subpopulation of cells (arrows) expressed the neuronal marker Tuj1 (Figure 1C and D). Prominent Tuj1+ neurites projected from subpopulations of cells within and surrounding the NSs (Figure 1C and D).

NS-derived cells migrate and project nerve fibers after transplantation into the postnatal colon. 2 fNSs or pNSs were transplanted into the external muscle layers of the distal colon of 2- to 3-week-old wild-type mice. Recipient colons into which fNSs had been transplanted were examined 1, 2, 3, 4, 8, 12, or 16 weeks after surgery; recipient colons into which pNSs had been transplanted were examined 4 weeks after surgery only. Graft-derived cells and neurites were present within 94% (62 of 66) of recipients into which fNSs were transplanted, including in 3 of 3 allowed to survive for 16 weeks after surgery, and in 92% (33 of 36) of recipients into which pNSs were transplanted. Graft-derived cells were present circumferential, oral, and anal to the transplantation site, and there was no obvious preference in the direction in which the cells had migrated (Figure 1E). Numerous graft-derived neurites were also observed extending in all directions beyond the graft-derived cell bodies. There was no obvious difference in the migration of cells from NSs derived from EdnrbKik- and RetTGM-positive mice, but as there was KikGR, but not EGFP, expression in the neurites of graft-derived cells, most of the experiments were performed using NSs derived from EdnrbKik mice.

There was a time-dependent increase in the area occupied by cells and fibers derived from fNSs up to 6–8 weeks after surgery, when graft-derived cells occupied an area of around 11 mm² (Figure 1F). Fibers were observed up to 9.8 mm from the transplanted...
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and fibers showed immunoreactivity to neuronal NOS (Figure 5, A–F), choline acetyltransferase (ChAT; Figure 5, G–I), vesicular acetylcholine transporter (VACHT; data not shown), and calretinin and calbindin (Supplemental Figure 3). Moreover, some of the graft-derived NO neurons possessed flattened, lamellar dendrites (Figure 5, A–C), similar to the dendritic morphology of NO enteric neurons in the mature intestine (43). The proportions of Hu+ neurons derived from fNSs that expressed ChAT or NOS were determined in 3 recipients 4 weeks after transplantation. ChAT+ and NOS+ neurons accounted for 52.5% ± 2.4% and 49.0% ± 15.0% of graft-derived Hu+ neurons, respectively, similar to the proportions of ChAT+ and NOS+ endogenous myenteric neurons in the distal colon adjacent to the transplant site (54.6% ± 4.8% and 38.2% ± 2.3%, respectively). These findings showed that, within the environment of postnatal colon, fNS- and pNS-derived cells differentiated into neurons with appropriate neurochemical and morphological phenotypes, and in the correct proportions.

No evidence of inappropriate differentiation or tumor formation. Interstitial cells of Cajal (ICCs) are found closely associated with enteric neurons (44–46), but do not arise from the neural crest (47, 48). ICCs can be identified by the expression of the receptor tyrosine kinase Kit (49, 50). We examined whether any fNS-derived cells expressed Kit. Endogenous Kit+ ICCs of the recipient were found to be closely associated with graft-derived ganglion-like clusters, but no graft-derived Kit+ cells were observed (Figure 5f), which suggests that grafted fNS-derived cells had not differentiated into ICCs in vivo.

We also examined whether fNS-derived cells formed tumors, as some neural crest stem/progenitor cells have the potential to form neuroblastomas (51). No unusual mass formations were observed at or near the transplant sites within the gut wall of any of the recipient mice into which fNSs (n = 66) or pNSs (n = 36) were transplanted. At 16 weeks after transplantation of fNSs, we also dissected the adrenal glands, lumbar sympathetic ganglia, and lymph nodes from the recipient mice, cut cryosections, and examined whether any graft-derived cells were present at these sites (n = 3).

The adrenal medulla and sympathetic chain ganglia are common primary and secondary sites of neuroblastoma (51). No tumor formation or fNS-derived cells were detected at any of these sites. Hence, we found no evidence that fNS-derived cells form tumors or emigrate from the gut to invade distant tissues.

Transplanted fNS- and pNS-derived cells differentiate into neurons with appropriate electrophysiology. To examine whether graft-derived neurons are electrically active and receive synaptic inputs, intracellular recordings of graft-derived neurons were performed using electrodes containing biocytin to confirm that the impaled neurons were graft derived (Figure 6, A–D and G–I). Successful intracellular recordings were made from neurons in 13 of 17 preparations containing fNS-derived cells and in 12 of 14 preparations containing pNS-derived cells. All electrophysiological experiments were performed 3–5 weeks after NS implantation. Depolarizing step currents triggered 1–7 action potentials (APs) at the beginning of the pulse in both fNS- and pNS-derived neurons (Table 1 and Figure 6, E and J). In response to a single stimulus, the recorded graft-derived cells displayed 1 or multiple (between 2 and 10) fast excitatory postsynaptic potentials (fEPSPs; Figure 6, F and K). Trains of stimuli did not reveal any slow synaptic potentials. Spontaneous activity in these neurons was abolished by the nicotinic receptor antagonist hexamethonium (200 μM, n = 2; data not shown). The durations and amplitudes of evoked fEPSPs and the number of fEPSPs in groups elicited by single-pulse stimulations were also reduced by hexamethonium (200 μM, n = 2; data not shown). The resting membrane potential and input resistance of graft-derived neurons (Table 1) were similar to those recently reported for P0 and P10 enteric neurons (52), but the neurons were more depolarized than adult enteric neurons (53).

Post-hoc examination of the impaled, biocytin-filled neurons revealed that they possessed lamellar and/or filamentous dendrites (Figure 6, B and G), and most possessed a single, long axon-like process that projected within the plane of the myenteric plexus to the circular muscle, and/or ended in an expansion bulb.
Together, these results showed that ENS progenitor cells isolated from the fetal and postnatal mouse gut differentiated into neurons with appropriate electrophysiological properties within the environment of postnatal gut.

**Discussion**

If cell therapy is to be used to treat gastrointestinal motility disorders, it is crucial to determine whether neural stem/progenitors can migrate and differentiate into neurons with the appropriate neurochemical and electrophysiological properties after transplantation into the postnatal gut in vivo. In the present study, we showed that NSs generated from neural crest–derived cells from both the fetal and the postnatal gut survived, proliferated, migrated, and differentiated into glial cells and a range of neuron subtypes that exhibited neurochemical, morphological, and electrophysiological characteristics similar to those of resident enteric neurons.

The wall of the embryonic gut consists largely of undifferentiated mesenchyme at the time of migration of neural crest–derived cells. The present study showed that NSs generated from neural crest–derived cells from both the fetal and the postnatal gut survived, proliferated, migrated, and differentiated into glial cells and a range of neuron subtypes that exhibited neurochemical, morphological, and electrophysiological characteristics similar to those of resident enteric neurons.

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The neural circuits controlling motility consist of a variety of subtypes of enteric neurons, including intrinsic sensory neurons, interneurons, cholinergic motor neurons that mediate contraction of the gut wall, and inhibitory motor neurons that mediate relaxation and express NOS (56, 57). For cell therapy for motility disorders, all neuron subtypes will need to be generated from transplanted progenitors and form appropriate connections with each other and with muscle cells. In the present study, grafted ENS progenitor cells gave rise to neurons possessing neurochemical and morphological features similar to those of normal enteric neurons. For example, graft-derived neurons expressed a number of neurotransmitters characteristic of normal enteric neurons.

**Figure 5**

Phenotypes of neurons and neurites derived from fNSs or pNSs within whole-mount preparations of recipient colon. (A–C) Ganglion-like cluster of cells derived from a pNS. A subpopulation of graft-derived Hu+ cells in the cluster showed NOS immunoreactivity (asterisks); some of the NOS+ neurons had lamellar dendrites (arrows), characteristic of NOS enteric neurons. (D–F) Neurites arising from a transplanted fNS within the circular muscle layer. Some of the varicosities of graft-derived neurites showed NOS immunoreactivity (arrows). (G–I) Ganglion-like cluster of cells derived from a fNS. A subpopulation of graft-derived Hu+ cells in the cluster showed ChAT immunoreactivity (asterisks). (J) The processes (arrows) of Kit+ ICCs (asterisks) were closely associated with clusters of graft-derived cells, but none of the graft-derived cells showed Kit immunostaining. Scale bars: 10 μm.
of markers characteristic of subtypes of enteric neurons in mice, humans, and other species, including ChAT, VACHt, NOS, calbindin, and calretinin (43, 58–60). These markers are not, however, expressed exclusively by neurons in the ENS, but are also expressed by some classes of neurons elsewhere in the nervous system. Importantly, most of the graft-derived NOS neurons possessed lamellar dendrites, which is notable because enteric NOS neurons possess lamellar dendrites that are the sites of many of their synaptic inputs (61, 62). The proportions of graft-derived neurons expressing NOS and ChAT were similar to those of myenteric neurons in the neighboring region of distal colon. This is reassuring for the generation of an ENS in the aganglionic region of patients with Hirschsprung disease, in which all neuron subtypes will need to be generated (63); however, for enteric neuropathies such as achalasias, in which there is degeneration of specific classes of enteric neurons (3, 56), manipulation of the cells prior to transplantation is likely to be required to bias the differentiation of cells to particular neuron subtypes.

Our study showed that transplanted ENS progenitor-derived cells migrate and settle in locations similar to those occupied by neural crest–derived cells during normal development. Furthermore, varicose, graft-derived neurites were present in the muscle layers and formed close associations with myenteric neurons of the recipient and with other graft-derived neurons. Thus, cues must exist in the postnatal gut that graft-derived neurites can use to navigate to specific targets. However, graft-derived fibers were
likely that submucosal ganglia are also essential for a fully functional enteric nervous system (ENS). They are located in the submucosal plexus (66, 67). It is therefore highly likely that sensory neurons involved in the circuits controlling motility are transplanted into animal models of enteric neuropathies such as Hirschsprung disease (13), and their use will allow immune responses and the ethical issues associated with some sources of stem/progenitor cells (10). It remains to be determined whether cells derived from NSs generated from the adult gut have post-transplantation properties similar to those of NS- and pNS-derived cells.

Our data lay the foundation for studies in which ENS progenitors are transplanted into animal models of enteric neuropathies to determine whether graft-derived neurons ameliorate the motility defects. In preliminary studies, we transplanted genetically labeled (EdnrbKik) NSs into the aganglionic region of colon of postnatal sl/sl mice, a mouse model of Hirschsprung disease (71). However, very few transplanted cells survived beyond 1 week due to immunological rejection, as the EdnrbKik mice are on a different genetic background from sl/sl mice. As a result, detailed studies have not been possible, and we encountered limitations in cell survival and differentiation.

Table 1

<table>
<thead>
<tr>
<th>Property</th>
<th>fNS derived</th>
<th>pNS derived</th>
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<tbody>
<tr>
<td>Resting membrane potential (mV)</td>
<td>~27 ± 4 (n = 9)</td>
<td>~36 (n = 1)</td>
</tr>
<tr>
<td>Input resistance (MΩ)</td>
<td>231 ± 30 (n = 11)</td>
<td>316 ± 33 (n = 5)</td>
</tr>
<tr>
<td>AP amplitude (mV)</td>
<td>43 ± 4 (n = 6)</td>
<td>33 ± 4 (n = 6)</td>
</tr>
<tr>
<td>AP duration (ms)</td>
<td>1.3 ± 0.1 (n = 6)</td>
<td>1.4 ± 0.2 (n = 6)</td>
</tr>
<tr>
<td>Maximum APs (no.)</td>
<td>3 ± 1 (n = 6)</td>
<td>3 ± 1 (n = 6)</td>
</tr>
<tr>
<td>AP threshold (pA)</td>
<td>123 ± 10 (n = 6)</td>
<td>78 ± 12 (n = 6)</td>
</tr>
<tr>
<td>Neurons with spontaneous fEPSPs (%)</td>
<td>74 (17 of 23)</td>
<td>82 (31 of 38)</td>
</tr>
<tr>
<td>Neurons with evoked fEPSPs (%)</td>
<td>100 (23 of 23)</td>
<td>100 (23 of 23)</td>
</tr>
<tr>
<td>Evoked fEPSP maximum amplitude (mV)</td>
<td>30 ± 2 (n = 23)</td>
<td>27 ± 2 (n = 23)</td>
</tr>
<tr>
<td>Evoked fEPSP total duration (ms)</td>
<td>57 ± 5 (n = 23)</td>
<td>83 ± 13 (n = 23)</td>
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For evoked fEPSPs, membrane potential was held at ~100 mV. AP duration was calculated as time to decay from maximum amplitude to half amplitude (APD50). Because AH neurons are not generated from transplanted progenitor cells in the postnatal colon, or because neurons do not develop AH characteristics until 3–4 weeks after transplantation, when all of the electrophysiological experiments were performed.

The neural circuitry controlling motility involves precise connectivity among different functional classes of neurons (26, 70). Although we showed that graft-derived neurons projected to the correct gut layers, it remains to be determined whether each of the different neurochemical types of graft-derived neurons formed appropriate synaptic connections. Also important is that graft-derived neurons do not make incorrect synaptic connections. However, while no gut motility studies were performed, the recipient animals survived and did not exhibit any obvious signs of motility defects, such as stool retention, which would be suggestive of inappropriate circuitry.

There were no significant differences in the behavior of cells derived from fNSs and pNSs. This is important because patient-derived cells are an accessible source of cells to treat congenital motility disorders such as Hirschsprung disease (13), and their use will allow immune responses and the ethical issues associated with some sources of stem/progenitor cells (10). It remains to be determined whether cells derived from NSs generated from the adult gut have post-transplantation properties similar to those of fNS- and pNS-derived cells.

This is the first study to report the electrophysiological properties of neurons generated from neural progenitor cells transplanted into the gut. During ENS development, some cells express pan-neuronal markers, but do not have the electrophysiological properties of neurons (68). However, graft-derived neurons in the postnatal colon had electrophysiological properties similar to those of mature, functional enteric neurons. Importantly, the presence of fEPSPs showed that the graft-derived neurons had integrated into the neuronal circuitry, although we were unable to determine whether the synaptic inputs arose from other graft-derived neurons, from the recipient’s neurons, or both. There are 2 main electrophysiological classes of myenteric neurons in the ENS, S neurons and AH neurons (53, 69). S neurons are uniaxonal and show monophasic repolarization after an AP and fEPSPs in response to fiber tract stimulation. In contrast, AH neurons show a biphasic repolarization and a slow afterhyperpolarizing potential after an AP and rarely display fEPSPs (53). In the current study, only a single electrophysiological class of neuron was observed, possessing properties very similar to S neurons (53), in the mouse distal colon. Although AH neurons account for about 20% of neurons in the mouse distal colon (53), we did not encounter graft-derived neurons with AH-type electrophysiology. This might be because AH neurons are not generated from transplanted progenitor cells in the postnatal colon, or because neurons do not develop AH characteristics until 3–4 weeks after transplantation, when all of the electrophysiological experiments were performed.
have had to be postponed until the Ednrb\textsubscript{Kik} mice are backcrossed onto the same genetic background as the s/s mice. Nonetheless, we performed some preliminary experiments in which NSs were generated from N4 backcrossed mice and implanted into the aganglionic region of s/s mice. After 4 weeks, graft-derived cells were present, some of which had migrated away from the transplant site and formed clusters of Hu\textsuperscript{a} cells (Supplemental Figure 4, A and B), and graft-derived neurites were abundant in the circular muscle layer. Furthermore, electrophysiological recordings from 2 briefly impaled graft-derived neurons revealed EPSPs (Supplemental Figure 4C). These preliminary data showed that cells transplanted into the aganglionic region survived and migrated in the absence of endogenous enteric neurons and that graft-derived neurons received synaptic inputs. Our findings of immunological rejection after transplantation of cells between mouse strains strongly suggest that patient-derived cells will be the best source of enteric neurons to transplant into patients with enteric neuropathies. Furthermore, although our data using postnatal donor and recipient mice support the idea that cell therapy might be used to treat pediatric enteric neuropathies, additional studies in which cells isolated from the adult mouse gut are transplanted into adult mice are required to demonstrate proof of principle that cell therapy might also be used to treat adult enteric neuropathies.

In conclusion, the ability of ENS stem/progenitor cells to proliferate, migrate extensively, differentiate into neurons of the appropriate phenotype, associate closely with endogenous enteric neurons, and incorporate into the neuronal circuitry in postnatal colon suggests that cell therapy to replace the diseased ENS in postnatal mice. After 4 weeks, graft-derived cells were present, some of which had migrated away from the transplant site and formed clusters of Hu\textsuperscript{a} cells (Supplemental Figure 4, A and B), and graft-derived neurites were abundant in the circular muscle layer. Furthermore, electrophysiological recordings from 2 briefly impaled graft-derived neurons revealed EPSPs (Supplemental Figure 4C). These preliminary data showed that cells transplanted into the aganglionic region survived and migrated in the absence of endogenous enteric neurons and that graft-derived neurons received synaptic inputs. Our findings of immunological rejection after transplantation of cells between mouse strains strongly suggest that patient-derived cells will be the best source of enteric neurons to transplant into patients with enteric neuropathies. Furthermore, although our data using postnatal donor and recipient mice support the idea that cell therapy might be used to treat pediatric enteric neuropathies, additional studies in which cells isolated from the adult mouse gut are transplanted into adult mice are required to demonstrate proof of principle that cell therapy might also be used to treat adult enteric neuropathies.

In vivo transplantation of Ednrb\textsubscript{Kik}- or Ret\textsubscript{TGM}-positive stem/progenitor cell NSs to the colon of postnatal mice. Recipient wild-type mice (2–3 weeks of age) were anesthetized by subcutaneous injection of a mixture of 20 mg/kg xylazine (Troy Laboratories) and 100 mg/kg ketamine hydrochloride (Troy Laboratories). A midabdominal incision was made, and the distal colon was exposed. 2 or 3 NSs, dyed by brief exposure to 0.1% trypan blue in PB, were transplanted into the external muscle layer of the distal colon. At 1, 2, 4, 8, 12, or 16 weeks after surgery, recipient mice were killed by cervical dislocation, and the distal colon was removed.

**Fixation and tissue preparation.** For whole-mount preparations, the distal colon was opened along the mesenteric border, pinned, stretched on Sylgard-coated dishes, and fixed in 4% paraformaldehyde in 0.1M phosphate buffer (PB) overnight. The tissue was then washed and the mucosa removed. For cryosections, the distal colon was opened along the mesenteric border, loosely pinned to a Sylgard dish, and then fixed in 4% paraformaldehyde in 0.1M PB overnight. The tissue was washed and transfected to 5% sucrose in 0.1M PB, then 1:1 OCT/sucrose, and then transfected to cryomold containing OCT (Tissue-Tek). Frozen sections (12 μm thick) were cut transversely on a cryostat.

**Immunohistochemistry.** Cells or tissues were fixed overnight in 4% paraformaldehyde in 0.1M PB at 4°C, exposed to 0.1% Triton X-100 for 30 minutes, and then exposed to primary and secondary antibodies (Tables 2 and 3). Note that an antibody was not used to reveal KikGR-expressing cells; only the native KikGR protein was observed. Preparations were viewed on a confocal microscope. Segments of control intestine (lacking transplanted cells) did not contain any fluorescent cells in the external muscle layers.

**EdU studies.** For 5 consecutive days after transplantation of NSs, mice were injected intraperitoneally with 50 mg/kg EdU. 4 weeks later, mice were killed by cervical dislocation, and the colon was dissected, pinned out, and
fixed overnight in 4% formaldehyde at 4°C. The mucosa was removed, and Edu was detected using the Click-IT Edu Imaging Kit (Invitrogen) according to the manufacturer’s instructions. The alkyne group in this detection reaction, which covalently binds the alkylene group associated with the incorporated Edu, was coupled to Alexa Fluor 647. The preparations were also processed for immunohistochemistry using a human anti-Hu.

Measurement of area occupied by graft-derived cells. To determine the area occupied by graft-derived cells plus fibers or by only cells, tile scans of whole-mount preparations of recipient colon were taken using ×5 or ×10 objectives on a confocal microscope. The total area occupied by graft-derived cells plus fibers, or cells only, in each preparation was measured using Image J software.

Electrophysiology. Segments of distal colon were removed and immediately placed in physiological saline (118 mM NaCl, 25 mM NaHCO3, 11 mM t-glucose, 4.8 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 1.0 mM NaH2PO4, 2.5 μM nicardipine, and 1 μM hyoscine) bubbled with 95% O2 and 5% CO2. The region of distal colon containing EdnbRαβ-positive grafted cells was cut along the mesenteric border and pinned flat, mucosa side up, in an organ bath lined with a silicone elastomer (Sylgard 184; Dow Corning). The mucosa and submucosa were dissected and removed from the underlying smooth muscle and myenteric plexus layers. The preparation was continually superfused with physiological saline (33°C–34°C) and left to equilibrate for 1 hour.

Standard intracellular recording methods (52) were used to impale and record from EdnbRαβ-positive grafted cells. Intracellular microelectrodes (100–200 MΩ) containing 1 M KCl and 2% biocytin (Sigma-Aldrich) were used. Electrical stimuli of a single pulse (0.4–1.8 mA) or trains of stimuli (3, 10, or 15 pulses) was applied via a focal stimulating electrode positioned on interganglionic fiber tracts about 200 μm oral to the impaled cell region to determine whether the grafted cells display synaptic potentials. The excitability of the grafted cells was examined by holding the membrane potential at –60 mV and applying depolarizing current pulses (500 ms duration) in 10-pA increments over a range of 50–300 pA. Input resistance of the grafted cells was measured from hyperpolarizing current pulses (500 ms, 10-pA increments, 100–300 pA). After electrophysiology, the preparations were fixed in 4% formaldehyde and processed to reveal the impaired neurons (Streptavidin Alexa Fluor 594, 1:200; Invitrogen). Hyoscine and hexamethonium (Sigma-Aldrich) and were prepared as stock solutions dissolved in distilled water, and diluted to their final concentrations before usage in experiments. The amplitude of all the peaks (or maximum peak amplitude, where indicated) and total duration of the stimulated fEPSP complexes were measured. The number of APs triggered by depolarizing current pulses was counted. The amplitude of APs was measured, and the duration of an AP was measured as the time to decay from maximum amplitude to half amplitude. Statistics. Data are displayed as mean ± SEM and were analyzed using 2-tailed t tests. A P value less than 0.05 was considered significant.

Study approval. All studies were approved by the Anatomy and Neuroscience, Pathology, Pharmacology, and Physiology Animal Ethics Committee of the University of Melbourne (ethics ID 0911131).

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