The enteric nervous system (ENS) is the largest component of the autonomic nervous system, with neuron numbers surpassing those present in the spinal cord. The ENS has been called the 'second brain' given its autonomy, remarkable neurotransmitter diversity and complex cytoarchitecture. Defects in ENS development are responsible for many human disorders including Hirschsprung disease (HSCR). HSCR is caused by the developmental failure of ENS progenitors to migrate into the gastrointestinal tract, particularly the distal colon. Human ENS development remains poorly understood owing to the lack of an easily accessible model system. Here we demonstrate the efficient derivation and isolation of ENS progenitors from human pluripotent stem (PS) cells, and their further differentiation into functional enteric neurons. ENS precursors derived in vitro are capable of targeted migration in the developing chick embryo and extensive colonization of the adult mouse colon. The in vivo engraftment and migration of human PS-cell-derived ENS precursors rescue disease-related mortality in HSCR mice (Ednrbs-l/s-l), although the mechanism of action remains unclear. Finally, EDNRB-null mutant ENS precursors enable modelling of HSCR-related migration defects, and the identification of peptstatin A as a candidate therapeutic target. Our study establishes the first, to our knowledge, human PS-cell-based platform for the study of human ENS development, and presents cell- and drug-based strategies for the treatment of HSCR.
One key functional property of the ENC is the ability to migrate extensively and to colonize the gut\(^2\). Red fluorescent protein (RFP)-labelled, CD49D\(^+\)-purified (Fig. 1g) PS-cell-derived ENC precursors were injected into the developing chick embryo at the level of the vagal NC. Transplanted human cells migrated along the trunk of the embryo (Fig. 1h) and colonized the gut (22 out of 57 embryos injected; Fig. 1i). By contrast, stage-matched CNC or MNC precursors targeted cranial regions (CNC) or followed a trajectory along the dermis (MNC) (Extended Data Fig. 1i).

To address whether ES-cell-derived ENC precursors are capable of recreating ENS neuronal diversity, we maintained purified CD49D\(^+\) ENC precursors in 3D spheroids for 4 days followed by differentiation as adherent cultures in the presence of ascorbic acid and glial-cell-line-derived neurotrophic factor (GDNF) (Fig. 2a). The 3D spheroid step was required to retain high levels of SOX10–GFP expression (Fig. 2b). Replating of 3D spheroids under differentiation conditions yielded immature neurons expressing TUJ1 and the enteric precursor marker PHOX2A (day 20; Fig. 2b). Most PHOX2A\(^+\) cells were positive for TRKC (also known as NTRK3), a surface marker expressed in enteric neuron precursors\(^8\) suitable for enrichment for PHOX2A\(^+\) and ASCL1\(^+\) precursors (Extended Data Fig. 2a, b). Temporal expression analyses (Extended Data Fig. 2c–e) showed maintenance of ENC neuronal precursor marker expression by day 40 of differentiation (Fig. 2c, d), followed by an increase in the percentage of mature neurons by day 60 (Fig. 2e, f). In agreement with enteric neuron identity, we observed a broad range of neurotransmitter phenotypes including serotonin-positive (5-hydroxytryptamine, 5-HT\(^+\)), GABA\(^+\) (gamma-aminobutyric-acid-positive) and nitric-oxide-synthase-positive (NOS\(^+\)) neurons. The presence of these neurotransmitters in neurons derived from CD49D\(^+\)-purified NC precursors indicates ENC origin, since those neurotransmitters are not expressed in other NC lineages. Indeed, no 5-HT\(^+\) neurons were observed in parallel cultures derived from HOX-negative, CD49D\(^-\) cells (Extended Data Fig. 3a, b). CNC-derived precursors differentiated into tyrosine-hydroxylase-expressing neurons (Extended Data Fig. 3c), and gave rise to TRKB-positive rather than TRKC-positive precursors, suggesting enrichment for sympathetic neuron lineages (Extended Data Fig. 3d, e).

A major function of the ENS is the control of peristaltic gut movements. We probed the functionality of in-vitro-derived enteric neurons by assessing connectivity with smooth muscle cells (SMCs). ES-cell-derived SMCs were generated via a mesoderm intermediate after exposure to activin A and BMP4 in vitro\(^6\) and culture in the presence of TGF\(_{β}\) (Extended Data Fig. 4a). The resulting SMC progenitors expressed ISL1 and were immunoreactive for smooth muscle actin (SMA) (Extended Data Fig. 4b). For connectivity studies, an optogenetic reporter line (ref. 10) was used to allow for light-induced control of neuronal activity. Enteric neurons were derived from a human ES cell line expressing enhanced yellow fluorescent protein (eYFP)-tagged channelrhodopsin-2 (ChR2) under control of the human synapsin (SYN) promoter (Fig. 2g). GABA\(^+\) and 5-HT\(^+\) neurons in these co-cultures were closely associated with SMCs (Extended Data Fig. 4c). Interestingly, co-culture of day-25 neurons with SMCs triggered accelerated neuronal maturation, as illustrated by the increased expression of SYN–eYFP (Extended Data Fig. 4d). Conversely, ES-cell-derived SMCs also showed signs of accelerated maturation under co-culture conditions as illustrated by the expression of mature markers (MYH11 and acetylcholine receptor (AchR); Extended Data Fig. 4e) and the ability to contract in response to pharmacological stimulation (Supplementary Videos 1–6 and Extended Data Fig. 4f). While no spontaneous contractions were observed under co-culture conditions, a wave of SMC contractions could be triggered 5–10 s after light-mediated activation (10 Hz frequency) of SYN–ChR2–eYFP neurons (Fig. 2h, i and Supplementary Video 7). Notably, both light- and drug-induced SMC contractions

and induced pluripotent stem (iPS) cells; Extended Data Fig. 1d). Purified CD49D\(^+\) NC precursors, derived in the presence of retinoic acid, expressed HOXB2–HOXB5 indicative of vagal identity\(^9,10\), but not more caudal HOX transcripts such as HOXB9 (Fig. 1c). In further agreement with enteric identity, CD49D\(^+\), retinoic-acid-treated NC precursors expressed markers of early enteric NC (ENC) lineages\(^2\) including PAX3, EDNRB and RET (Fig. 1d and Extended Data Fig. 1e, f). Given the paucity of developmental data on human ENC development, we performed RNA sequencing (RNA-seq) analysis in ES-cell-derived ENC precursors, in CNCs (no retinoic acid), in melanocyte-biased\(^3\) NCs (MNCs) (Extended Data Fig. 1a), and in stage-matched CNC precursors\(^3\). Unsupervised clustering reliably segregated the transcriptomes of all PS-cell-derived NC populations away from CNC precursors and further subdivided the various NC sublineages (Fig. 1e). The most differentially expressed genes in the ENC compared to CNC lineage included general NC markers such as FOXD3 or TFFAP2a but also PAX3 and HOX genes related to the ENC lineage (Fig. 1f). CNCs and MNCs were also enriched in general NC markers but showed high levels of NEUROGI, ISLI or MLANA, TYR and DCT expression respectively, compatible with their subtype identity (Extended Data Fig. 1g, h). Direct comparison of the various NC lineages yielded novel candidate marker of human vagal NC/ENC lineage (Fig. 1f). A list of the top 200 enriched transcripts for each NC lineage is provided (Supplementary Tables 1–3).

Figure 2 | Differentiation of human ES-cell-derived ENC precursors into enteric neuron subtypes. a, Protocol for neuronal differentiation and maturation of ENC precursors. AA, ascobic acid; NB/N2/B27, neurobasal medium with N2 and B27 supplement. b, SOX10–GFP-expressing 3D spheroids from purified ENC precursors gave rise to TUJ1 and PHOX2A enteric neuron lineage. c, d, Phase-contrast and immunofluorescence images (c) and quantification (d) at day 40. ENC-derived cells express TRKC, ASCL1 and PHOX2A/B. PHOX2B expression was confirmed using H9 human ES-cell-based PHOX2B–GFP reporter line; n = 3 independent experiments. e, f, Immunofluorescence analysis (e) and quantification (f) for expression of diverse neurotransmitters. Cells were derived from FACS-purified, CD49D\(^+\) ENCs to ensure NC origin; n = 3 independent experiments. SST, somatostatin; TH, tyrosine hydroxylase. g, Light-stimulated activation of ENC-derived neurons expressing channelrhodopsin-2 (ChR2). h, Phase-contrast and live fluorescence images of human ES-cell-derived smooth muscle cells (SMCs) and ENC-derived neuron co-cultures subjected to light stimulation. i, Diagram representing extent of contraction of SMCs before and during light (450 nm) stimulation at increasing frequencies. Scale bars, 100\(μ\)m (b) and 50\(μ\)m (c, h). Data are mean ± s.e.m.
were slow and involved the movement of sheet-like structures, suggesting coordination among cells possibly via gap-junction-mediated coupling. These studies demonstrate functional connectivity between ES-cell-derived enteric neurons and SMCs. In vivo interactions of the ENS within the gut, however, are more complex and involve several cell types. As a first step in modelling those interactions in 3D, we used a tissue-engineering approach combining in vitro-derived human ENC precursors (CD49D+ day 15) with mouse primary intestinal tissue (Extended Data Fig. 5a). Using our previously established protocols to form organoid units, the recombined tissue constructs were seeded onto a scaffold and implanted onto the omentum of immunodeficient hosts for maturation in vivo. Human cells were readily detected within gut-like structures using the human-specific markers SC121 and synaptophysin. Importantly, cells were located both in epithelial and muscle layers (Extended Data Fig. 5b), showing their ability to interact with both target cell types.

Transplantation of PS-cell-derived precursors could yield novel therapeutic opportunities for ENS disorders such as HSCR. Children with HSCR are currently treated by surgical removal of the aganglionic portion of the gut. While life-saving, the surgery does not address dysfunc tion of the remaining gastrointestinal tract in surviving patients. Furthermore, therapeutic options in patients with total aganglionosis are limited. A major challenge in developing a cell therapy for HSCR is the need to repopulate the ENS over extensive distances. Previous studies tested the transplantation of a variety of candidate cell sources into the fetal or postnatal colon. Mouse fetal-derived ENC precursors resulted in the most promising data, with evidence of functional integration but limited in vivo migration. To assess the ability of human ES-cell-derived ENC precursors to migrate within the postnatal or adult colon (3–6 weeks of age), we performed orthotopic injections of CD49D+ RFP-labelled precursors into NOD-SCID-Il2rg−−/− (NSG) mice (Fig. 3a). Cells were injected into the wall of the caecum aiming for the muscle layer (Fig. 3a) and resulting in a well-defined deposit of RFP+ cells 1 h after injection (Extended Data Fig. 6a, left, and Fig. 3b, top). Notably, 2–4 weeks after transplantation, RFP+ cells had migrated extensively and repopulated the host colon over its entire length (Fig. 3b). The grafted ENC precursors formed clusters along the colon (Extended Data Fig. 6a, right) expressing TUJ1 (Fig. 3c). By contrast, stage-matched CNS and CNC precursors, grafted under identical conditions, showed limited migration (Extended Data Fig. 6b, c).

Given the extraordinary ability of the grafted cells to repopulate the host colon, we tested whether those cells could provide therapeutic benefit in an HSCR model. One widely used genetic model is the Ednrbs-l/s-l mouse. These mice develop a megacolon owing to aberrant peristalsis. As a consequence, mutant mice show high mortality by 4–6 weeks of age. Ednrbs-l/s-l mice were injected 2–3 weeks after birth with RFP+ human ES-cell-derived ENC precursors (treatment group) or with Matrigel vehicle (control group). Most control-injected animals (n = 6) died over a period of 4–5 weeks (Fig. 3d) with a megacolon-like pathology (Extended Data Fig. 6d). By contrast, all animals injected with ES-cell-derived ENC precursors (n = 6) survived (Fig. 3d). Grafted animals were assessed for graft survival and migration at 6–8 weeks of age. Whole-mount fluorescence imaging confirmed the migration of ES-cell-derived ENC precursors within the HSCR colon (Extended Data Fig. 6e, f). Preliminary studies showed a trend towards an improved gastrointestinal transit time in grafted versus the small subset of Matrigel-treated animals that survived beyond 6 weeks of treatment, as measured using carmine dye gavage (Extended Data Fig. 6g). Histological analyses at 6 weeks and 3 months after transplantation confirmed myenteric and submucosal localization of human cells in HSCR colon. While the location mimicked aspects of endogenous ENS, there was a preference towards the submucosal region (Fig. 3e and Extended Data Fig. 6h, top). Human cells were also detected in the distal colon (Extended Data Fig. 6h, bottom), where only few endogenous TUJ1+ cells were detected. Immunocytochemistry for SC121 and human-specific synaptophysin, not detected in Matrigel-injected animals (Extended Data Fig. 6i), confirmed human identity and presynaptic marker expression (Fig. 3f, right). In addition to neuronal cells (Extended Data Fig. 6h), we also observed human cells expressing glial markers such as GFAP (Extended Data Fig. 6j). Neuron-subtype-specific markers in SC121+ human cells included 5-HT, GABA and NOS (Fig. 3f and Extended Data Fig. 6k).

Our findings demonstrate that wild-type human PS-cell-derived ENC precursors can repopulate the colon of HSCR mice. However, HSCR-causing mutations often affect the migration of ENS precursor in a cell autonomous manner. Therefore, developing patient-matched cell therapies for HSCR may require complementary genetic or pharmacological strategies to overcome intrinsic migration defects of the transplanted cells. As causative genetic defects in HSCR patients are often not known or complex, gene correction before transplantation may not be possible. We therefore assessed whether human PS-cell-derived ENS precursors can be used to model HSCR and serve as a platform to screen for candidate compounds that could overcome disease-related migration defects. In a first step, we established isogenic ES cell lines with homozygous loss-of-function mutations in EDNRB using CRISPR/Cas-based gene-targeting techniques (Fig. 4a and Extended Data Fig. 7a, b). Loss-of-function mutations in EDNRB are a well-known genetic cause in a subset of HSCR patients. ENC precursors could be derived at comparable efficiencies from EDNRB-mutant and control lines (Extended Data Fig. 7c). However, CD49D+ ENC precursors from four EDNRB+−/− clones showed a notable migration defect when using the scratch assay to model aspects of the HSCR phenotype.
In *EDNRB*−/− ES-cell-derived NC precursors (Fig. 4e), Pepstatin A is a known inhibitor of acid proteases. Among potential pepstatin targets, we explored BACE2 because RNA-seq data showed upregulation in *EDNRB*−/− ES-cell-derived NC lineages (Extended Data Fig. 9a), and BACE2 had been recently shown to modulate the migration of NC derivatives in the developing zebrafish embryo. To address whether BACE2 mediates the pepstatin A effect, we tested structurally unrelated small molecules targeting BACE2. Exposure to the BACE inhibitor IV rescued the migration defect in the scratch assay similar to pepstatin A (Fig. 4f). Furthermore, BACE2 knockdown confirmed rescue of the migration defect in *EDNRB*−/− cells (Fig. 4g and Extended Data Fig. 9b). Finally, we tested whether pepstatin A exposure in *vivo* is sufficient to rescue the *in vivo* migration behaviour of *EDNRB*−/− ENC precursors (Fig. 4h). ENC precursors derived from *EDNRB*−/− cells exhibit a significant *in vivo* migration defect after transplantation into the adult colon (Fig. 4i, j). *EDNRB*-null precursors pre-treated with pepstatin A for 72 h before transplantation showed a significant rescue of *in vivo* migration (Fig. 4i, j and Extended Data Fig. 9c). Interestingly, wild-type-derived ENC precursors treated with pharmacological inhibitors of BACE showed migration defects *in vitro* and *in vivo* (Extended Data Fig. 9d–f), further supporting a role for BACE in human ENC migration and HSCR.

Our study describes an efficient strategy to derive and prospectively purify ENC precursors from human ES cells. In agreement with studies in model organisms, we demonstrate that human ES-cell-derived ENC gives rise to a broad range of neurotransmitter phenotypes characteristic of the ENS. The ability to model human ENS development *in vitro* should enable the large-scale production of specific human enteric neurons on demand. For example human PS-cell-derived enteric 5-HT neurons could serve as a tool to model gastrointestinal side effects of CNS-acting drugs such as Prozac.

We focused primarily on potential cell therapeutic applications of *ES-cell-derived ENC lineages in HSCR*. One of the most remarkable findings was the extensive *in vivo* migratory potential of human ENC precursors in the adult host colon. Future studies will have to define whether this ability is confined to early CD49D+ cells or maintained at later neurogenic stages. Similarly, it will be important to look at long-term efficacy and safety. While most of our *in vivo* studies in NSG mice (a total of 102 animals grafted) were limited to a 5–6-week survival period, animals analysed at 3–4 months after transplantation showed comparable *in vivo* properties without evidence of tumour formation or graft-related adverse effects. The therapeutic potential of the cells is illustrated by their ability to rescue *Ednrbs-l/s-l* mice. Future studies will be required to address mechanisms of the graft-mediated host rescue. The potential for widespread engraftment may eventually enable permanent, bona fide repair of the aganglionic portions of the gut. However, given the rapid action of the cells in preventing death of HSCR mice, it appears unlikely that rescue was mediated by functional integration of the cells. Future studies should also address alternative mechanisms such as cytokine release, immunomodulation or changes in barrier function for contributing to the therapeutic effect.

The identification of pepstatin A and BACE2 inhibition in rescuing HSCR-related migration defects represents proof-of-concept for the use of human ES-cell-derived ENC precursors in drug discovery. The mechanism of BACE2 inhibition on migration remains to be determined. Possible targets of BACE proteases include neuregulins and ErbB receptors previously implicated in NC migration. One obvious future direction is testing the therapeutic potential of BACE inhibitors in mouse models of HSCR. Such a strategy could enable the prevention of aganglionosis during pregnancy or enable repair of postnatal enteric neuron function. In the current study, we focused on combining pepstatin A as a neoadjuvant treatment to promote migration of *EDNRB*−/− ENC precursors. An important next question is whether cells pre-treated with pepstatin A are capable of rescuing lethality or other disease-associated phenotypes in HSCR mice. In conclusion, our work presents a powerful strategy to access human ENS lineages for exploring the second brain in human health and disease and for developing novel cell- and drug-based therapies for HSCR.

**Figure 4** | **EDNRB** signalling regulates human ENC precursor cell migration. **a**, *In vitro* HSCR disease modelling model. **b, c**, Representative images and quantification of scratch assay in RFP+/CD49D+ wild-type and *EDNRB*−/− human ES-cell-derived ENSCs (clones (C) 1–4); n = 3 independent experiments. **d**, Illustration of chemical screen. **e**, Dose-response of pepstatin A on migration of CD49D+ *EDNRB*−/− human ES-cell-derived ENSCs. **f**, Quantification of CD49D+ *EDNRB*−/− human ES-cell-derived ENC migration after treatment with pepstatin A (10μM) or BACE inhibitor (i.) IV (1μM); n = 3 independent experiments. **g**, Quantification of cell migration after BACE2 knockdown using a pool of five different short interfering RNAs (siRNAs) or four individual siRNAs; n = 3 independent experiments. **h**, Pepstatin A pre-transplantation treatment model. **i**, Whole-mount images of NSG colon transplanted with RFP+/CD49D+ purified wild-type and *EDNRB*−/− human ES-cell-derived ENC precursors with or without pepstatin A pre-treatment. **j**, Quantification of the fraction of animals with human cells present in colon at increasing distance from injection site (see Extended Data Fig. 9c); n ≥ 8 animals for each of the treatment groups. Scale bars, 200μm (b) and 1 cm (i). Data are mean ± s.e.m. ***P** < 0.01; ****P < 0.0001 (c, f and g; analysis of variance [ANOVA]; Dunnett test [compared to wild type]). P values in j are given numerically, log-rank (Mantel–Cox) test; n = 8 for each group.
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Culture of undifferentiated human ES cells. Human ES cell line H9 (WA-09) and derivatives (SOX16-GFP, SYN:GHR2-EYFP, SYN:EFYFP,PHOX2B-GFP, EF1::RFP Ednrb−/−) as well as two independent human iPS cell lines (healthy and familial dysautonomia, Sendai-based, OMSK (Cytotune)) were maintained on mouse embryonic fibroblasts (Global Stem) in knockout serum replacement (KSR; Life Technologies, 10828-028) containing human ES cell medium as described previously2. Cells were subjected to mycoplasma testing at monthly intervals and short tandem repeats (STR) profiled to confirm cell identity at the initiation of the study.

Neural crest induction. Human ES cells were plated on matrigel (BD Biosciences, 354234)–coated dishes (107 cells cm−2) in ES cell medium containing 10 mM FGF2 (R&D Systems, 233-FB-001MG/CF). Differentiation was initiated in KSR medium (knockout DMEM plus 15% KSR (Life Technologies, 10828-028), 1-glutamine (Life Technologies, 25030-081), NEAA (Life Technologies, 11140-050) containing LDN193189 (100 nM, Stemgent) and SB431542 (10 μM, Tocris). The KSR medium was gradually replaced with increasing amounts of N2 medium from day 4 to day 10 as described previously7. For CNC induction, cells were treated with 3 μM CHIR99021 (Tocris Bioscience, 4423) in addition to LDN193189 and SB431542 from day 2 to day 11. ENC differentiation involves additional treatment with retinoic acid (1 μM) from day 6 to day 11. For deriving MNCs, LDN193189 is replaced with BMP4 (10 nM, R&D, 314-BP) and EDN3 (10 nM, American Peptide company, 88-510B) from day 6 to day 11 (ref. 3). The differentiated cells are sorted for CD49D at day 11. CNS precursor control cells were generated by treatment with LDN193189 and SB431542 from day 0 to day 11 as previously described2. Throughout the manuscript, day 0 is the day the medium is switched from human ES cell medium to LDN193189 and SB431542 containing medium. Days of differentiation in text and figures refer to the number of days since the pluripotent stage (day 0).

FACS and immunofluorescence analysis. For immunofluorescence, the cells were fixed with 4% paraformaldehyde (Affymetrix-USB, 19943) for 20 min, then blocked and permeabilized using 1% bovine serum albumin (BSA) (Thermo Scientific, 23209) and 0.3% Triton X-100 (Sigma, T8787). The cells were then incubated in primary antibody solutions overnight at 4 °C and stained with fluorophore-conjugated secondary antibodies at room temperature for 1 h. The stained cells were then incubated with DAPI (1 ng ml−1, Sigma, D9542-5MG) and washed several times before imaging. For flow cytometry analysis, the cells are dissociated with Accutase (Innovative Cell Technologies, AT104) and fixed and permeabilized using BD Cytofix/Cytoperm (BD Bioscience, 554722) solution, then washed, blocked and permeabilized using BD Perm/Wash buffer (BD Bioscience, 554723) according to manufacturer’s instructions. The cells are then stained with primary (overnight at 4 °C) and secondary (30 min at room temperature) antibodies and analysed using a flow Cytometer (Flowjo software). A list of primary antibodies and working dilutions is provided in Supplementary Table 4. The PHOX2A antibody was provided by J.-F. Brunet (rabbit, 1:800 dilution).

In ovo transplants. Fertilized eggs (from Charles River Farms) were incubated at 37 °C for 50 h before injections. A total of 2 × 105 CD49D-sorted, RFP-labelled NC cells were injected into the intersomitic space of the vagal region of the embryos targeting a region between somite 2 and 6 (HH 14 embryo, 20–25 somite stage). The embryos were collected 36 h later for whole-mount epifluorescence and histological analyses.

Gene expression analysis. For RNA sequencing, total RNA was extracted using RNeasy RNA purification kit (Qiagen, 74106). For qRT–PCR assay, total RNA samples were reverse transcribed to cDNA using SuperScript II Reverse Transcriptase (Life Technologies, 18064-041). qRT–PCR reactions were set up using Quantitect SYBR Green PCR mix (Qiagen, 201418). Each data point represents three independent biological replicates.

In vitro differentiation of ENC to enteric neurons. ENC cells from the 11-day induction protocol were aggregated into 3D spheroids (5 million cells per well) in Neurobasal (NB) medium supplemented with l-glutamine (Gibco, 25030-164), 1-glutamine (Gibco, 25030-081), N2 (Stem Cell Technologies, 07156) and B27 (Life Technologies, 17004044) containing GDNF (25 ng ml−1), Peprotech, 450-10) and ascorbic acid (100 μM, Sigma, A8960-5G). The ENC progenitors were expandable in DMEM supplemented with 10% FBS.

Induction of SMCs. Mesoderm specification is carried out in STEMPRO-34 ( Gibco, 10639-011) medium. The ES cells are subjected to activin A treatment (100 ng ml−1, R&D, 338-AC-010) for 24 h followed by BMP4 treatment (10 ng ml−1, R&D, 314-BP) for 4 days2. The cells are then differentiated into SMC progenitors by treatment with PDGF-BB (5 ng ml−1, Peprotech, 100-14B), TGFβ3 (5 ng ml−1, R&D systems, 243-B3-200) and 10% FBS. The SMC progenitors are expandable in DMEM supplemented with 10% FBS.

ENC–SMC co-culture. The SMC progenitors were plated on PL/LO/FN–coated culture dishes (prepared as described previously2) 3 days before addition of ENC-derived neurons. The neurons were dissociated (using accutase, Innovative Cell Technologies, AT104) at day 30 of differentiation and plated onto the SMC monolayer cultures. The culture is maintained in neurobasal (NB) medium supplemented with 1-glutamine (Gibco, 25030-164), N2 (Stem Cell Technologies, 07156) and B27 (Life Technologies, 17004044) containing GDNF (25 ng ml−1, Peprotech, 450-10) and ascorbic acid (100 μM, Sigma, A8960-5G). Functional connectivity was assessed at 8–16 weeks of co-culture.

Pharmacological and optogenetic stimulations of co-cultured SMCs. SMCs and ENC-derived neuron co-cultures were subjected to acetylcholine chloride (50 μM, Sigma, A6625), carbamoylcholine chloride (10 μM, Sigma,C4382) and KCl (55 mM, Fisher Scientific, BP366–500) treatment, 3 months after initiating the co-culture. Optogenetic stimulations were achieved using a 450-nm pigtailed diode pumped solid state laser (OEM Laser, PSU-1150 LED, OEM Laser Systems, Inc.) achieving an illumination between 2 and 4 mW mm−2. The pulse width was 4 ms and stimulation frequencies ranged from 2 to 10 Hz. For the quantification of movement, images were assembled into a stack using Metamorph software and regions with high contrast were identified (labelled yellow in Supplementary Fig. 5). The movement of five representative high-contrast regions per field was automatically traced (Metamorph software). Data are presented in kinetograms (movement in pixels in x and y direction (distance) with respect to the previous frame).

Generation of chimaeric tissue-engineered colon. We used the previously described method for generation of tissue-engineered colon2. In brief, the donor colon tissue was collected and digested into organoid units using dispase (Life Technologies, 17105-041) and collagenase type 1 (Worthington, CLS-1). The organoid units were then mixed immediately (without any in vitro culture) with CD49D-purified human ES-cell-derived ENC precursors (day 15 of differentiation) and seeded onto biodegradable polyglycolic acid scaffolds (2-mm sheet thickness, 60 mg cm−2 bulk density; porosity >95%, Concordia Fibres) shaped into 2 mm long tubes with poly-i Lactide (PLLA) (Durect Corporation). The seeded scaffolds were then placed onto and wrapped in the greater omentum of the adult (>2 months old) NSG mice. Just before the implantation, these mice were irradiated with 350 Gy. The seeded scaffolds were differentiated into colon-like structures inside the omentum for 4 additional weeks before they were surgically removed for tissue analysis.

Transplantation of ENC precursors in adult colon. All mouse procedures were performed following NIH guidelines, and were approved by the local Institutional Animal Care and Use Committee (IACUC), the Institutional Biosafety Committee (IBC) as well as the Embryonic Stem Cell Research Committee (ESCRHO). We used 3–6-week-old male NSG (NOD.Cg-Il2rgtm1Wjl/SzJ) mice or 2–3-week-old Ednrb−/− (SSL/Lc) mice27 (n = 12, 6 male, 6 female) for these studies. Animal numbers were based on availability of homozygous hosts and on sufficient statistical power to detect large effects between treatment versus control (Ednrb−/+) as well as for demonstrating robustness of migration behaviour (NSG). Animals were randomly selected for the various treatment models (NSG and Ednrb−/+) but assuring for equal distribution of male/female ratio in each group (Ednrb−/+). All in vivo experiments were performed in a blinded manner. Animals were anaesthetized with isoflurane (1%) throughout the procedure, a small abdominal incision was made, and a sheet of wall musculature lifted and the caecum is exposed and exteriorized. Warm saline is used to keep the caecum moist. Then 20 μl of cell suspension (2–4 million REP−CD49D-purified human ES-cell-derived ENC precursors) in 70% Matrigel (BD Biosciences, 354234) in PBS or 20μl of 70% Matrigel in PBS only (control-grafted animals) were slowly injected into the caecum (targeting the muscle layer) using a 27-gauge needle. Use of 70% matrigel as carrier for cell injection assured that the cells stayed in place after the injection and prevented backflow into the peritoneum. After injection that needle was withdrawn, and a T-tip was placed over the injection site for 30 s to prevent bleeding. The caecum was returned to the abdominal cavity and the abdominal wall was closed using surgical tape. The surgical incision was closed using sterile wound clips. After wound closure animals were put on paper on top of their bedding and attended until conscious and preferably eating and drinking. The tissue was collected at different time points (ranging from two weeks to four months) after transplantation for histological analysis. Ednrb−/+ mice were immunosuppressed by daily injections of cyclosporine (10 mg kg−1 i.p. Sigma, 30024).

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Whole-mount fluorescence imaging and histology. The collected colon samples were fixed in 4% paraformaldehyde at 4 °C overnight before imaging. Imaging is performed using Maestro fluorescence imaging system (Cambridge Research and Instrumentation). The tissue samples were incubated in 30% sucrose (Fisher Scientific, BP220-1) solutions at 4 °C for 2 days, and then embedded in OCT (Fisher Scientific, NC9638938) and cryosectioned. The sections were then blocked with 1% BSA (Thermo Scientific, 23209) and permeabilized with 0.3% Triton X-100 (Sigma, T8787). The sections are then stained with primary antibody solution at 4 °C overnight and fluorophore-conjugated secondary antibody solutions at room temperature for 30 min. The stained sections were then incubated with DAPI (1 ng ml−1, Sigma, D9542-5MG) and washed several times before they were mounted with Vectashield Mounting Medium (vector, H1200) and imaged using fluorescent (Olympus IX70) or confocal microscopes (Zeiss SP5).

Total gastrointestinal transit time. Mice are gavaged with 0.3 ml of dye solution containing 6% carmine (Sigma, C1022-5G), 0.5% methylcellullose (Sigma, 274429-5G) and 0.9 NaCl using a #24 round-tip feeding needle. The needle was held inside the mouse oesophagus for a few seconds after gavage to prevent regurgitation.

After 1 h, the stool colour was monitored for gavaged mice every 10 min. For each mouse, total gastrointestinal transit time is between the time of gavage and the time when red stool is observed.

Gene targeting. The double nickase CRISPR/Cas9 system38 was used to target the EDRNB locus in EF1–RFP H9 human ES cells. Two guide RNA sequences were designed (using the CRISPR design tool; http://crispr.mit.edu/) to target the coding sequence with PAM targets ~20 base pairs apart (qRNA #1 target specific sequence: 5′-AAACGCTGAGCCGGGCGTGG-3′, RNA #2 target specific sequence: 5′-CCAGATGGCACGCGACGCCCAGG-3′). The cells were transfected with guide RNA constructs and GFP-fused Cas9-D10A nickase. The GFP-expressing cells were FACs purified 24 h later and plated in low density (150 cells cm−2) on mouse embryonic fibroblasts. The colonies were picked 7 days later and passaged twice before genomic DNA isolation and screening. The targeted region of EDRNB gene was PCR amplified (forward primer: 5′-AAACGCTGAGCCGGGCGTGG-3′, reverse primer: 5′-GTACGGCCCCAGGTCTCTC-3′) and cloned into Zero vector (Invitrogen, 450245). To ensure that both alleles (from each ES cell colony) are represented and sequenced, we picked 10 bacterial clones (for each ES cell clone) for plasmid purification and subsequent sequencing. The clones with bi-allelic nonsense mutations were expanded and differentiated for follow-up assays.

Migration assay. The ENC cells are plated on PO/LM/FN coated (prepared as described previously39) 96-well or 48-well culture plates (30,000 cm2). After 24 h, the culture lawn is scratched manually using a pipette tip. The cells are given an additional 24–48 h to migrate into the scratch area and fixed for imaging and quantification. The quantification is based on the percentage of the nuclei that are located in the scratch area after the migration period. The scratch area is defined using a reference well that was fixed immediately after scratching. Migration of cells was quantified using the open source data analysis software KNIME39 (http://knime.org) with the ‘quantification in ROI’ plug-in as described in detail elsewhere39.

Proliferation assay. To quantify proliferation, FACs-purified ENC cells were assayed using CyQUANT NF cell proliferation assay Kit (Life Technologies, C35006) according to manufacturer’s instructions. In brief, to generate a standard, cells were plated at various densities and stained using the fluorescent DNA binding dye reagent. Total fluorescence intensity was then measured using a plate reader (excitation at 485 nm and emission detection at 530 nm). After determining the linear range, the CD49D+ ENC precursors were plated (6,000 cell cm−2) and cells were assayed for lactate dehydrogenase (LDH) activity using CytoTox 96 cytotoxicity assay kit (Promega, G1780). In brief, the cells are plated in 96-well plates at 30,000 cm2. The supernatant and the cell lysate is collected 24 h later and assayed for LDH activity using a plate reader (490 nm absorbance). Viability is calculated by dividing the LDH signal of the lysate by total LDH signal (from lysate plus supernatant). The cells were cultured in neurobasal (NB) medium supplemented with l-glutamine (Gibco, 25030-164), N2 (Stem Cell Technologies, 07156) and B27 (Life Technologies, 17504044) containing CHIR99021 (3 μM, Tocris Bioscience, 4423) and FGF2 (10 nM, R&D Systems, 233-FB-001MG/CF) during the assay.

High-throughput screening. The chemical compound screening was performed using the Prestwick Chemical Library. The ENC cells were plated in 96-well plates (30,000 cm2) and scratched manually 24 h before addition of the compounds. The cells were treated with two concentrations of the compounds (10μM and 1μM). The plates were fixed 24 h later for total plate imaging. The compound scoring was based on their ability to promote filling of the scratch in 24 h. The compounds that showed toxic effects (based on marked reduction in cell numbers assessed by DAPI staining) were scored 0, compounds with no effects were scored 1, compounds with moderate effects were scored 2, and compounds with strong effects (that resulted in complete filling of the scratch area) were scored 3 and identified as hit compounds. The hits were further validated to ensure reproducibility. The cells were treated with various concentrations of the selected hit compound (pepstatin A) for dose response analysis. The optimal dose (10μM based on optimal response and viability) was used for follow-up experiments. For the pre-treatment experiments, cells were CD49D purified at day 11 and treated with pepstatin A from day 12 to day 15 followed by transplantation into the colon wall of NSG mice. The cells were cultured in neurobasal (NB) medium supplemented with l-glutamine (Gibco, 25030-164), N2 (Stem Cell Technologies, 07156) and B27 (Life Technologies, 17504044) containing CHIR99021 (3 μM, Tocris Bioscience, 4423) and FGF2 (10 nM, R&D Systems, 233-FB-001MG/CF) during the assay.

BACE2 inhibition and knockdown. To inhibit BACE2, the ENC precursors were treated with 1μM β-secretase inhibitor IV (CAS 797035-11-1, Calbiochem). To knockdown BACE2, cells were dissociated using accutase (Innovative Cell Technologies, AT104) and reverse-transfected (using Lipofectamine RNAiMAX-Life Technologies, 13778-150) with an siRNA pool (SMARTpool: ON-TARGETplus BACE2 siRNA, Dharmacon, L-003802-00-0005) or four different individual siRNAs (Dharmacon, LQ-003802-00-0002, 2nmol). The knockdown was confirmed by qRT–PCR measurement of BACE2 mRNA levels in cells transfected with the BACE2 siRNAs versus the control siRNA pool (ON-TARGETplus Non-targeting Pool, Dharmacon, D-001810-10-05). The transfected cells were scratched 24 h after plating and fixed 48 h later for migration quantification. The cells were cultured in neurobasal (NB) medium supplemented with l-glutamine (Gibco, 25030-164), N2 (Stem Cell Technologies, 07156) and B27 (Life Technologies, 17504044) containing CHIR99021 (3 μM, Tocris Bioscience, 4423) and FGF2 (10 nM, R&D Systems, 233-FB-001MG/CF) during the assay.

Statistical analysis. Data are presented as mean ± s.e.m. and were derived from at least three independent experiments. Data on replicates (n) is given in figure legends. Statistical analysis was performed using the Student’s t-test (comparing two groups) or ANOVA with Dunnett test (comparing multiple groups against control). Distribution of the raw data approximated normal distribution (Kolmogorov–Smirnov normality test) for data with sufficient number of replicates to test for normality. Survival analysis was performed using a log-rank (Mantel–Cox) test. Z-scores for primary hits were calculated as $Z = (x − μ) / σ$, in which x is the migration score value and μ is the mean migration score value, and σ is the standard deviation for all compounds and DMSO controls (n = 224).
Extended Data Figure 1 | Characterization of ES-cell-derived NC populations. a, Schematic illustration of CNC and MNC induction protocols. b, Flow cytometry for CD49D and SOX10–GFP in CNC and MNC cells. c, Immunofluorescence of unsorted and CD49D-sorted differentiated NC cells for SOX10. d, Flow cytometry for CD49D in ENCs derived from H9 human ES cells and control and familial dysautonomia (FD) human iPS cells. e, f, Representative immunofluorescence images and flow cytometry in ES-cell-derived ENC for enteric precursor lineage marker at day 11. g, List of the top 10 and selected additional (bold) most differentially expressed transcripts from the RNA-seq analysis of CNC compared to stage matched CNS precursors. h, Lists of the top 10 and selected additional (bold) most differentially expressed transcripts from the RNA-seq analysis of MNC compared to stage-matched CNS precursors. i, Distribution of CNC and MNC cells in developing chick embryos at 24–36 h after injection. Right, higher power image of the clusters of MNC cells in the developing surface ectoderm. NotoC, notochord; NT, neural tube; S, somite. Scale bars, 50 μm (c, i, middle), 25 μm (e, i, right) and 1 mm (i, left).
Extended Data Figure 2 | Characterization of human ES-cell-derived enteric neural lineages. a, Flow cytometry for TRKC and PHOX2A expression. b, Immunofluorescence for PHOX2A and ASCL1 for TRKC-positive and TRKC-negative ES-cell-derived ENC precursors. c, Time course qRT–PCR analysis of enteric lineage markers during more extended in vitro differentiation periods; n = 3 independent experiments. d, e, Flow cytometric quantification of enteric neuron precursor and neuronal markers in ENC-derived neurons at days 40 and 60 of differentiation; n = 3 independent experiments. Scale bars, 50 μm.
Extended Data Figure 3 | CNC gives rise to neurons enriched in autonomic lineage. a–c. Representative immunofluorescence images for expression of 5-HT, TUJ1 and tyrosine hydroxylase (TH) in CNC-derived neurons. In contrast to ENC-derived lineages, no serotonergic (5-HT+) neurons were detected under cranial conditions despite the presence of many TUJ1+ neurons and increased percentages of tyrosine-hydroxylase-positive cells. d, e. Flow cytometry for TRKB and TRKC under CNC and ENC conditions; n = 3 independent experiments. Scale bars, 50 μm.
Extended Data Figure 4 | Functional characterization of ES-cell-derived enteric neurons in co-culture with SMCs. a, Schematic illustration of SMC differentiation protocol. b, Immunofluorescence staining of SMC progenitors for SMA and ISL1. c, Association of various ENC-derived neuron subtypes with SMA+ cells. d, SYN-eGFP expression in ENC-derived neurons at 40 days of co-culture with SMCs and stage-matched neurons in the absence of SMCs. e, Monoculture of SMCs versus co-cultures of SMCs with ENC-derived neurons. Top, phase-contrast images showing morphological changes of SMCs in co-culture. Bottom, immunofluorescence staining of mature SMC markers MYH11 and AchR in monoculture of SMCs versus co-cultures of SMCs with ENC-derived neurons. f, Diagrams representing extent of contraction of SMC cultures. Arrows indicate the time of pharmacological stimulation. Scale bars, 50 μm (b, c, e) and 100 μm (d).
Extended Data Figure 5 | Generation of tissue-engineered colon using human ES-cell-derived ENC precursors. a, Schematic illustration for generation of tissue-engineered colon. b, Tissue engineered colon stained for human cytoplasmic marker SC121, TUJ1 and human-specific synaptophysin (hSyn). Dotted line shows approximate location of border between muscle and epithelial/submucosal-like layers. H&E, haematoxylin and eosin. Scale bars, 20μm (b, left and middle) and 40μm (b, right).
Extended Data Figure 6 | Characterization of transplanted human ES-cell-derived ENC precursors in adult colon of NSG and Ednrbs-l/s-l mice. a, Whole-mount microscopy of colon transplanted with RFP+ CD49D-purified ES-cell-derived ENC precursors to track RFP expression at injection site, at 1 h after transplantation to ensure that cells were injected at proper location (left), and at 2 weeks to show dispersal of the cells and congregation of subset of cells into distinct clusters (right). The dashed lines indicate the outer border of the intact colon tissue. b, c, Whole-mount fluorescence imaging and quantification of migration of grafted RFP+ ES-cell-derived CNS precursors and CD49D-purified CNC precursors inside the adult colon wall. d, Megacolon-like phenotype in control animals versus animals receiving ES-cell-derived ENC transplants. e, f, Whole-mount fluorescence imaging and quantification of migration of grafted RFP+ CD49D purified ES-cell-derived ENC precursors in colon of Ednrbs-l/s-l mice. g, Total gastrointestinal transit times by carmine dye gavage of Ednrbs-l/s-l mice grafted with RFP+ CD49D-purified ES-cell-derived ENC precursors versus Matrigel-only grafted mice; n = 3 for grafted animals, n = 2 for Matrigel group. Note that n = 2 for Matrigel group was due to the fact that nearly all Matrigel-injected animals died owing to their disease phenotype. h, Representative images of grafted ES-cell-derived ENC precursors at 3 months after transplantation into the colon of Ednrbs-l/s-l mice co-expressing TUJ1 and SC121. i, Immunofluorescence staining of cross sections of Ednrbs-l/s-l colons transplanted with Matrigel for SC121 and human-specific synaptophysin. j, Representative image of grafted ES-cell-derived ENC precursors at 3 months after transplantation into the colon of Ednrbs-l/s-l mice expressing human-specific GFAP (SC123). k, l, Representative images of grafted ES-cell-derived ENC precursors at 6 weeks after transplantation into the colon of NSG (wild type) and Ednrbs-l/s-l mice. The dashed lines indicate the border between submucosal and muscle layers. Scale bars, 200 μm (a), 1 cm (b, e) and 100 μm (h–l). AU, arbitrary units. P value for g is given numerically, t-test with Welch’s correction; n = 3 independent experiments.
Extended Data Figure 7 | Establishing and characterizing EDNRB-null human ES cell lines. a, Sequences of wild-type and Cas9-nickase induced bi-allelic nonsense mutations in targeted region of Ednrb−/− clones. b, Western blot analysis for EDNRB in ES-cell-derived ENC precursors showing lack of protein expression in the mutant clones C1–C4. c, EDNRB−/− human ES cells can be efficiently differentiated into CD49D+ human ES-cell-derived ENC based on CD49D expression (c) and expression of SOX10 (data not shown). d, Proliferation of EDNRB−/− human ES-cell-derived ENCs (day 11) versus wild-type-derived cells; n = 4 independent experiments. e, LDH activity measurement of cell viability in EDNRB−/− ES-cell-derived ENCs (day 11) versus wild-type-derived cells. *P < 0.05 (t-test; n = 3 independent experiments).

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Extended Data Figure 8 | Chemical screening for compounds that rescue migration of EDNRB−/− ES-cell-derived NC precursors.

a, Schematic illustration of the timeline and experimental steps involved in the chemical screening assay and migration scoring system.  
b, Example of a screening plate layout and locations of dimethylsulfoxide (DMSO) control wells.  
c, Migration scores of Prestwick library compounds and DMSO controls.  
d, Migration assay and scores for EDNRB−/− ES-cell-derived ENC precursors treated with primary hit compounds. Z-score for primary hit compounds in c is given numerically (compared to DMSO control; n = 224 technical replicates).
Extended Data Figure 9 | Pharmacological modulation of migration in human ES-cell-derived ENC precursors. a, BACE2 expression in the various human ES-cell-derived NC sublineages at day 11 as compared to stage-matched CNS precursors. b, qRT–PCR analysis to confirm knockdown of BACE2 in CD49D-purified EDNRB−/− ES-cell-derived ENC after siRNA transfection compared to control siRNA. c, Quantification of whole-mount images of the colon of NSG mice transplanted with RFP+ CD49D-purified wild-type and Ednrb−/− ES-cell-derived ENC precursors, with or without pepstatin A pre-treatment (compare to Fig. 4). d, Representative images of wild-type CD49D-purified ES-cell-derived ENC treated with EDN3 and BQ-788 (EDNRB inhibitor). e, Colon migration assay in wild-type ES-cell-derived ENC precursors after pre-treatment with BQ-788. f, Quantification of the data in e. AU, arbitrary unit. Scale bar, 1 cm.