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RESEARCH ARTICLE

SPECIAL ISSUE
THE EXTRACELLULAR ENVIRONMENT

Inhibition of chondroitin sulfate proteoglycans enhances cell migration following enteric neural stem cell transplantation

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ABSTRACT

Enteric nervous system development relies on migration, proliferation and differentiation of enteric neural crest-derived cells (ENCDCs), processes that are regulated by mesenchymal morphogens and the extracellular matrix (ECM). Hirschsprung disease (HD) is a congenital neurocristopathy in which abnormal gut motility results from loss of ENCDC-derived enteric ganglia in the colorectum. While enteric neural stem cell transplantation is a proposed therapy, current efforts are limited by poor cell migration in HD models. To address this, we investigated ECM-derived inhibitory cues on enteric neural stem cell transplantation efficiency. Immunohistochemistry of embryonic chick hindgut reveals dynamic chondroitin sulfate proteoglycan (CSPG) expression during development. *In vitro* assays show that CSPGs inhibit ENCDC migration, an effect reversed by chondroitinase ABC. Transplantation of enteric neurospheres into chick and mouse colon results in limited engraftment, but chondroitinase ABC significantly enhances migration of transplanted cells. Single-cell RNA sequencing of postnatal mouse neurospheres reveals high expression of CD44, a transmembrane glycoprotein that can act as a functional CSPG on the cell surface. Blocking CD44 function promotes neurosphere-derived ENCDC migration. Modulating inhibitory ECM components may enhance the success of regenerative therapies for HD.

KEY WORDS: Enteric nervous system, Neurosphere, Neural crest, Hirschsprung disease, CSPG, CD44, Chick, Mouse

INTRODUCTION

Development of the enteric nervous system (ENS) relies on reciprocal interactions between multipotent neural crest cells and the embryonic gut microenvironment. The ENS regulates essential gastrointestinal functions, including motility, absorption and secretion. Neural crest cells that form the majority of the ENS delaminate from the vagal neural tube, migrate to the foregut and undergo a cranio-caudal journey within the gut mesenchyme to form the enteric neurons and glial cells that comprise the ENS (Nagy and Goldstein, 2017). Migration of these enteric neural crest-derived cells (ENCDCs) is regulated by signals from the gut mesenchyme, including chemotactic and mitogenic factors such as glial-derived neurotrophic factor (GDNF) and endothelin 3 (Barlow et al., 2003; Nagy and Goldstein, 2006; Young et al., 2001). ENS development is also strongly influenced by the matrisome through which ENCDCs travel, in which they encounter extracellular matrix (ECM) proteins that either promote or inhibit their migration (Chevalier et al., 2016). The crucial role of the ECM is demonstrated by β1 integrins, which are expressed by ENCDCs and are indispensable for distal gut colonization, with conditional knockout leading to hindgut aganglionosis (Breau et al., 2006; Akbareian et al., 2013).

Studies on ENS development in mouse and chicken embryos have revealed the migration-promoting effects of several ECM proteins, including fibronectin (Breau et al., 2009), laminin (Nagy et al., 2009), tenascin-C (Akbareian et al., 2013) and collagen 18 (Nagy et al., 2018). In contrast, agrin (Nagy et al., 2018), collagen 6 (Soret et al., 2015) and versican (Dutt et al., 2006; Nagy et al., 2016) delay migration and promote premature neuronal differentiation. Interestingly, ENCDCs do not simply rely on microenvironmental signals during colonization, they also actively shape their environment by secreting ECM proteins, including tenascin-C and collagen 18, which promote their migration (Akbareian et al., 2013). Agrin, a heparan sulphate proteoglycan that inhibits cell migration, is also actively secreted by ENCDCs, but only after they undergo neuroglial differentiation (Nagy et al., 2018; Mueller et al., 2024).

Several studies have highlighted the importance of a developmental time window during which the hindgut is permissive for ENCDC colonization, and after which the microenvironment undergoes significant changes (Druckenbrod and Epstein, 2009). This change poses a barrier for regenerative cell therapies for Hirschsprung disease (HSCR), in which gut motility is impaired due to the absence of enteric ganglia along variable lengths of the colorectum (Montalva et al., 2023). Cell therapy studies aim to repopulate the aganglionic segment to restore gut motility. Enteric neurospheres derived from embryonic or postnatal intestine, as well as iPSC-derived neurospheres, contain progenitors with ENS-forming capacity upon transplantation (Majd et al., 2025; Pan et al., 2024; Rahman et al., 2024), but challenges such as poor cell migration have limited their success. Optimization of neurosphere culture conditions with growth factors, such as GDNF and retinoic acid, expands the number of progenitors and increases colonization after transplantation (McKeown et al., 2017), but the result remains insufficient for fully restoring gut motility to the aganglionic segment.

The role of the ECM in neuronal regeneration has been extensively studied in relation to traumatic spinal cord injury, where glial scar formation is accompanied by increased expression of chondroitin sulphate proteoglycans (CSPGs), which inhibit axonal growth and synaptic reconnection, making CSPGs a primary

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molecular barrier post-injury (Bradbury and Burnside, 2019; Kwok et al., 2008). Besides secreted CSPGs, CD44, a type I transmembrane glycoprotein, has been shown to undergo post-translational modification with chondroitin sulfate side chains to become a cell-surface CSPG. While unmodified CD44 primarily binds hyaluronic acid, chondroitin sulfate chains allow CD44 to bind other ECM components, such as fibronectin and collagen, having significant impact on cell adhesion and migration (Greenfield et al., 1999; Bajorath, 2000; Ponta et al., 2003). The role of CSPGs on ENCDC migration has not previously been explored. Leveraging our recent observation that inhibiting agrin expression in ENCDCs with lentiviral shRNA significantly increases their migration following transplantation into the colon (Mueller et al., 2024), we aimed to test the potential role of modulating CSPG expression on the success of ENS cell therapy.

RESULTS

Dynamic expression of CSPGs during hindgut ENS development

To investigate the spatial and temporal expression of CSPGs in ENS development, we immunostained consecutive transverse sections of the chicken hindgut between embryonic day (E) 5 and E9 using the pan-CSPG monoclonal antibody CS-56. Neuronal and glial differentiation and ENS formation were visualized using SOX10, TUJ1, NCADH and BFABP immunostaining, while smooth muscle development was demonstrated by alpha-smooth muscle actin (α-SMA) immunoreactivity (Fig. 1, Fig. S1). Prior to the arrival of enteric neural crest-derived cells (ENCDCs) in the hindgut at E5 (Fig. 1A), CSPGs are diffusely expressed throughout the mesenchyme (Fig. 1C‴), with no TUJ1+ enteric neurons (Fig. 1A′) or α-SMA expression present (Fig. 1A″). At E6, circular smooth muscle differentiation is marked by α-SMA immunoreactivity (Fig. 1B″). CSPG expression gradually changes from a uniform mesenchymal pattern to the inner mesenchymal regions adjacent to the gut epithelium (Fig. 1B‴; Fig. S2A). By E7, migrating ENCDCs reach the hindgut and are detected on either side of the circular muscle (Fig. 1C), forming the prospective myenteric and submucosal plexus regions (Fig. 1C′). At this developmental stage, CSPG expression is more restricted to the prospective submucosal layer of the gut (Fig. 1C‴). This redistribution coincides with active ENCDC migration and early ENS patterning. At later stages (E8-E9), ENCDC colonization of the hindgut is complete (Fig. 1D,E), and TUJ1 immunoreactive enteric ganglia are present on both sides of the circular muscle (Fig. 1D′,E′). The differentiation of the longitudinal muscle layer appears at E9 (Fig. 1E″). Throughout these stages, CSPG expression remains prominent in the submucosa and is gradually excluded from the myenteric region and neuronal plexuses (Fig. 1D‴,E‴).

To further characterize the molecular complexity of CSPGs expressed in the developing hindgut, we also examined the expression patterns of individual CSPG family members. Versican, a large CSPG bearing multiple chondroitin sulfate side chains, exhibits strong expression within the inner mesenchymal layer at E6 (Fig. S2B). In contrast, collagen IX, which contains a single chondroitin sulfate chain, was predominantly expressed in the mesenchyme adjacent to the epithelial basement membrane (Fig. S2C). At later developmental stages, both versican and collagen IX expression are restricted to the prospective submucosa (Fig. S2E,F,H,I), supporting the pan-CSPG staining pattern detected by anti-CS-56.

CSPGs inhibit GDNF-induced ENCDC migration *in vitro*

To determine the effect of CSPGs on ENCDC migration, we cultured E6 chick midgut explants in the presence of exogenous GDNF and compared ENCDC migration on fibronectin- or CSPG-coated surfaces. Migrating ENCDCs were visualized by immunofluorescence staining for HNK1 and TUJ1 (Fig. 2). On fibronectin-coated surfaces, GDNF induced robust ENCDC emigration from the explants, resulting in extensive radial migration of HNK1$^+$ cells away from the intestine (Fig. 2A). In contrast, migration was markedly reduced when explants were cultured on CSPG-coated substrates (Fig. 2B,C). Increasing the concentration of CSPGs from 1 µg/ml to 10 µg/ml significantly enhanced this inhibitory effect, demonstrating a dose-dependent restriction of ENCDC migration (Fig. 2C).

To determine whether CSPG-mediated inhibition was dependent on chondroitin sulfate side chains, CSPG-coated substrates were enzymatically pre-treated with chondroitinase ABC (chABC). In these cultures, GDNF-induced migration was restored (Fig. 2D,F). To examine the role of CSPGs in the presence of other ECM proteins, CSPGs and fibronectin were added in alternating stripes on tissue-culture dishes. Immunostaining with the CS-56 antibody specific for CSPGs confirms their relative position in the stripes. Note that the red stripes contain both fibronectin and CSPGs. We show that ENCDCs migrate normally in the presence of fibronectin alone, but their migration is inhibited by the addition of CSPGs (Fig. 2E). These *in vitro* results demonstrate that CSPGs act as potent, dose-dependent inhibitors of GDNF-induced ENCDC migration *in vitro*, and that enzymatic removal of chondroitin sulfate chains reverses this inhibitory effect.

Loss of CSPGs disrupts ENS patterning in hindgut explants

To determine how CSPGs influence ENCDC migration and early patterning of the hindgut ENS, E5 chick hindgut was placed in organotypic catenary culture (Nagy et al., 2016) for 3 days. Cross-sections from the mid-hindgut were immunolabeled for neurons (TUJ1), ENCDCs (SOX10), smooth muscle (α-SMA) and CSPGs. In control explants, committed TUJ1+ enteric neurons and migrating ENCDCs were localized concentrically within the gut mesenchyme forming the prospective submucosal plexus (Fig. 3A,B), with α-SMA immunoreactivity showing smooth muscle development during the culture period (Fig. 3C). CS-56 labeling revealed diffuse mesenchymal expression of CSPGs throughout the gut wall (Fig. 3D), consistent with the expression pattern observed in *in vivo* controls. Addition of chABC to the culture media resulted in a marked disruption of early ENS patterning, with ENCDCs and enteric neurons failing to maintain concentric organization and appearing ectopically within the gut mesenchyme (Fig. 3E,F). Although chABC treatment disrupts the formation of concentric enteric plexuses, it does not affect smooth muscle differentiation (Fig. 3G). Effective enzymatic digestion is confirmed by the loss of CS-56 immunoreactivity in chABC-treated explants (Fig. 3H).

Inhibiting CSPG expression enhances ENCDC migration following cell transplantation

Given that CSPGs inhibit ENCDC migration *in vitro* and that chABC treatment enhances cell migration while disrupting normal ENS patterning, we assessed integration and migration of transplanted ENCDCs in cultured embryonic gut in the presence and absence of CSPGs. To generate an effective and expandable cell source for transplantation, we employed a recently introduced avian enteric neurosphere protocol (Soós et al., 2025) to generate enteric neurospheres from E7 quail intestine. Enzymatically dissociated embryonic intestine maintained in culture for 7 days form compact spheroids with multiple distinct surface aggregates (Fig. 4A). Immunofluorescence analysis demonstrates that neuronal differentiation, marked by TUJ1 and Hu, is present in dense

Development (2026) 153, dev205625. doi:10.1242/dev.205625

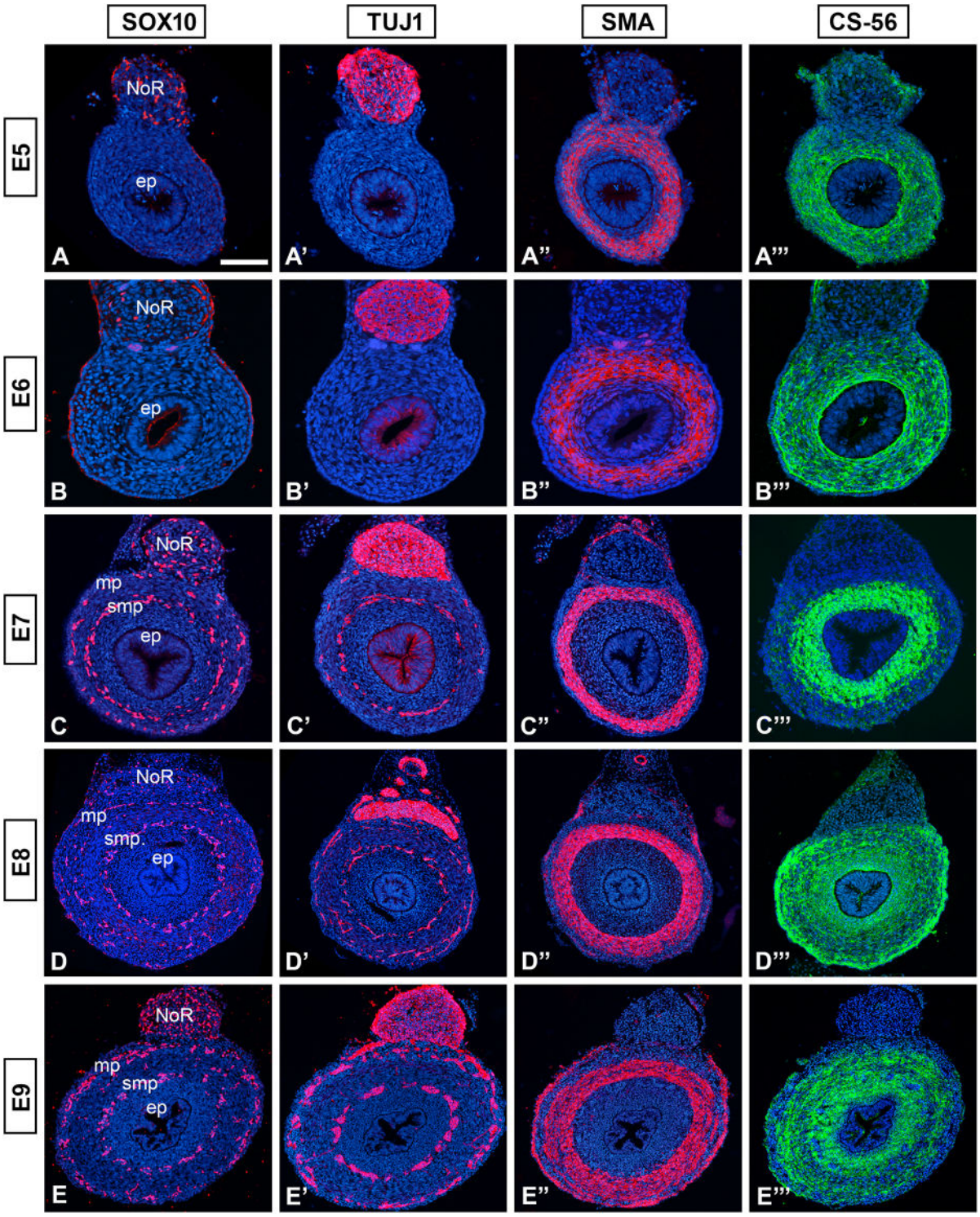


Fig. 1. Expression of chondroitin sulfate proteoglycans during hindgut enteric nervous system development. Cross-sections at the mid-hindgut level of embryonic day (E) 5-E9 chicken embryos were immunostained for enteric neural crest-derived cells (ENCDCs) (SOX10; A-E), neurons (TUJ1; A′-E′), smooth muscle cells (SMA; A″-E″) and chondroitin sulfate proteoglycans (CS-56; A‴-E‴) (*n*=6/stage). (A-E) Migrating ENCDCs give rise to (A′-E′) TUJ1-positive neurons of the hindgut enteric nervous system forming the prospective submucosal and myenteric plexuses. (A″-E″) ENCDC colonization in the hindgut coincides with the differentiation of the circular smooth muscle layer from E6, followed by the appearance of the longitudinal muscle of the developing gut wall starting from E9. (A‴-E‴) CSPGs show a dynamic and region-specific expression pattern during hindgut enteric nervous system development: diffuse mesenchymal expression is detected at E5-E6 that gradually shifts to the prospective submucosal layer of the gut wall starting from E7. Scale bar: 50 µm (A-A‴); 60 µm (B-B‴); 70 µm (C-C‴); 80 µm (D-D‴); 100 µm (E-E‴). ep, epithelium; mp, myenteric plexus; NoR, nerve of Remak; smp, submucosal plexus.

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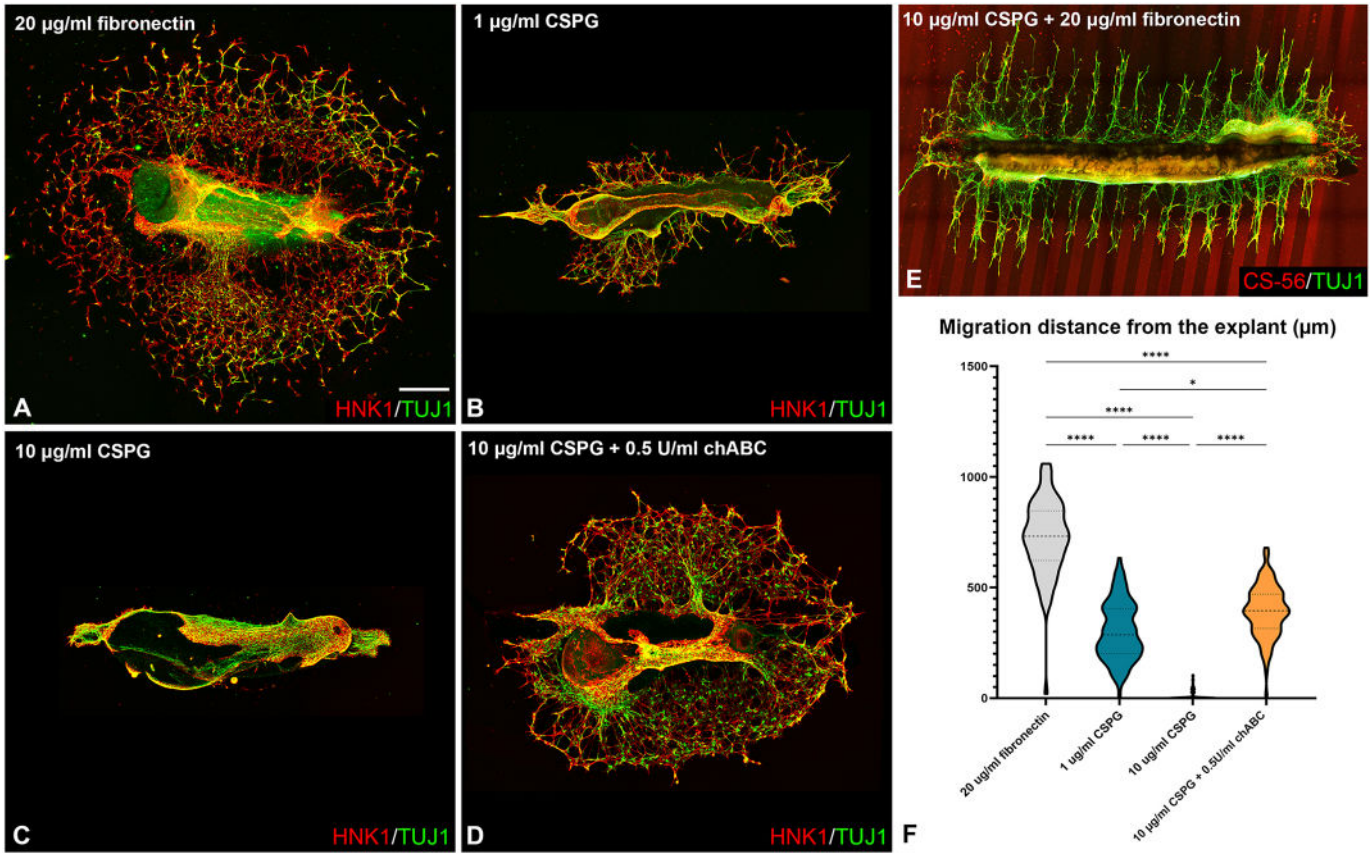


Fig. 2. Chondroitin sulfate proteoglycans inhibit enteric neural crest-derived cell migration *in vitro*. (A-D) To assess the effect of CSPGs on enteric neural crest-derived cell (ENCDC) migration, E6 midgut was cultured on a fibronectin- or CSPG-coated surface for 12 h in the presence of GDNF. Representative immunofluorescence images of migrating enteric neural crest-derived cells (ENCDCs) immunolabeled with HNK1 (red) and TUJ1 (green) to visualize neural crest cells and neurons, respectively. (A) Addition of exogeneous GDNF induces robust ENCDC migration on the fibronectin-coated surface. (B,C) In contrast, CSPGs markedly inhibit ENCDC migration, and this inhibitory effect becomes more pronounced with increasing CSPG concentrations. (D) Enzymatic pre-treatment with chABC efficiently rescues GDNF induced ENCDC migration. (E) On a surface coated with alternating stripes of fibronectin and fibronectin+CSPG proteins, ENCDCs show robust migration along stripes containing fibronectin alone, while the presence of CSPGs inhibit ENCDC migration. (F) Quantification of ENCDC migration distances from the explant (in µm) presented as violin plots. Migration is significantly reduced in the presence of 1 µg/ml and 10 µg/ml CSPG compared to fibronectin (P<0.0001), while enzymatic chABC treatment significantly increases ENCDC migration compared to CSPG-coated conditions. Statistical significance is indicated as follows: *P<0.05, ****P<0.0001. Scale bar: 200 µm.

clusters (neurospheres) at the spheroid surface (Fig. 4B,C insets), whereas the central spheroid core lacks neuronal marker expression (Fig. 4B,C). Higher magnification views confirmed that these peripheral clusters consist of densely packed neurons that are distinct from the inner spheroid compartment (Fig. 4C, inset).

To further characterize the neurospheres, PHOX2B (marker for neural crest cells and committed neurons) and SOX10 (neural crest and enteric glia marker) expression was assessed. PHOX2B+/SOX10− cells predominate within the surface aggregates, confirming their neuronal identity (Fig. 4D-F). In contrast, SOX10+/PHOX2B− cells were much less abundant. Of note, a small subset of cells co-expressed PHOX2B and SOX10, identifying a population of bipotent progenitor ENCDCs retained in culture (Fig. 4D-F, arrowheads). Quantification of neuronal (PHOX2B+), glial (SOX10+) and bipotent ENCDCs (PHOX2B+/SOX10+) is shown in Fig. 4G. These results indicate that, similar to chicken, the quail neurosphere culture system supports both neuronal differentiation and the maintenance of a limited pool of enteric progenitor cells. Immunostaining for CSPGs using the CS-56 antibody revealed diffuse CSPG expression in the HU+ neuronal aggregates (Fig. 4H), indicating that transplanted neurospheres contain CSPG-extracellular matrix. This finding suggests that enzymatic modulation of CSPGs may influence ENCDC behavior following tissue transplantation and provides a rationale for assessing the effects of CSPG removal on ENCDC migration and integration in *ex vivo* gut tissue.

To assess the role of CSPGs in an ENCDC transplantation model, enteric neurospheres generated from E7 quail intestine were transplanted into E15 chicken hindgut explants, maintained in *ex vivo* culture for 3 days under control or chABC-treated conditions and analyzed for the presence of integrated cells within the host tissue. Under control conditions, neurosphere-derived QCPN+ cells successfully integrated within the host gut wall but failed to migrate from the implantation site (Fig. 5A-C). QCPN monoclonal antibody is a quail cell-specific nuclear marker. In contrast, enzymatic treatment with 0.5 U/ml chondroitinase ABC markedly enhanced the migration of QCPN+ neurosphere-derived cells from the transplantation site (P<0.0001) (Fig. 5D-F). Loss of CS-56 immunoreactivity in treated explants confirmed effective enzymatic digestion of chondroitin sulphate proteoglycans (Fig. 5E). Notably, TUJ1+/QCPN+ transplanted neurons integrated into both host submucosal and myenteric plexuses following chondroitinase ABC treatment (Fig. 5F, inset). This suggests that CSPGs act as inhibitory extracellular cues that restrict the migration of transplanted ENCDCs

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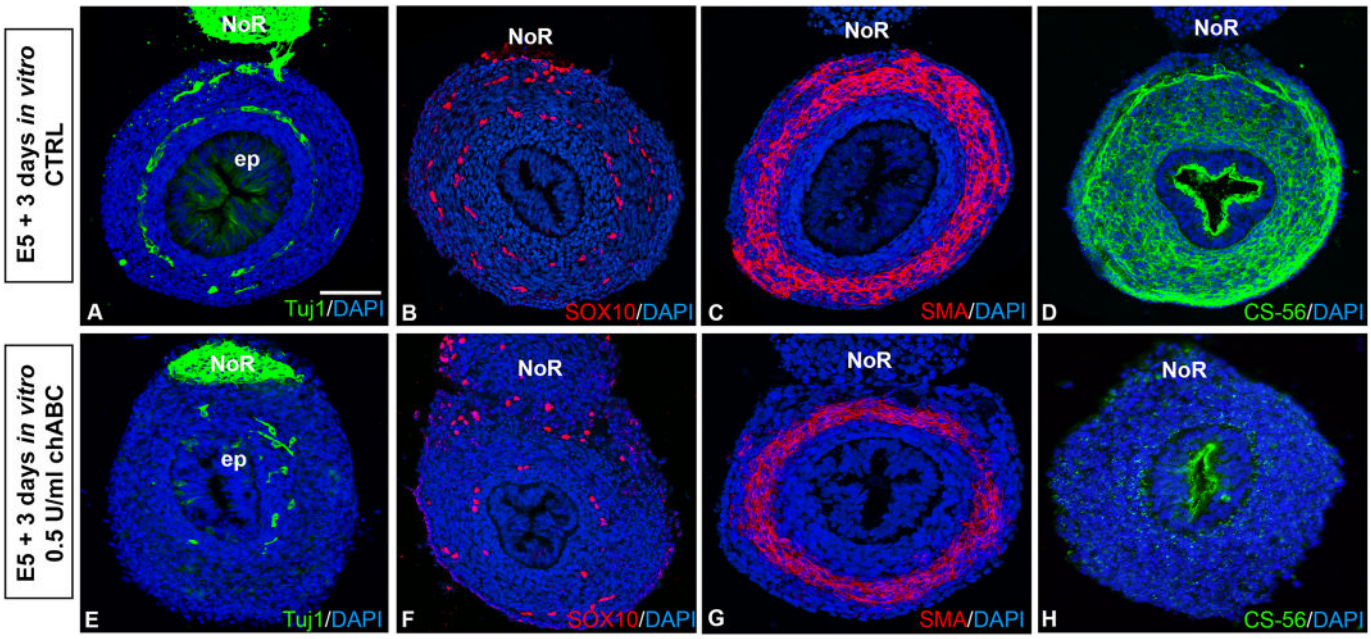


Fig. 3. Chondroitinase ABC treatment disrupts enteric nervous system patterning in the gut wall. E5 hindgut was cultured *in vitro* under control or enzymatic chondroitinase ABC-treated conditions for 3 days, and immunostained for the presence of neurons (TUJ1), smooth muscle (SMA) and chondroitin sulfate proteoglycans (CS-56). (A) Cross-sections of the explanted hindgut under control conditions, show the presence of cells immunoreactive for the early neuron marker Tuj1 forming the prospective submucosal plexus. (B) Enteric neural crest-derived cells migrate concentrically within the gut wall. (C,D) Explants show efficient smooth muscle differentiation (C), along with diffuse mesenchymal CSPG expression (D). (E,F) 0.5 U/ml enzymatic chondroitinase ABC treatment results in the disorganization of concentrically migrating enteric neural crest-derived cells within the gut wall (F), with Tuj1+ neurons losing plexus-like organization (E). (G) While chondroitinase ABC treatment disrupts concentric plexus formation, it does not influence smooth muscle differentiation. (H) Loss of CS-56 immunoreactivity confirms effective enzymatic digestion of chondroitin sulfate proteoglycans in chABC-treated explants. Scale bar: 75 µm. ep, epithelium; NoR, nerve of Remak.

within the gut wall and that their enzymatic removal enhances the dispersal of transplanted cells.

As chABC was present throughout the *ex vivo* culture period, the increased distribution of QCPN+ cells may reflect effects on both the donor neurosphere and the recipient gut wall. Quail neurospheres contain diffuse CS-56+ ECM (Fig. 4H), and digestion of CS/DS side chains may reduce neurosphere cohesion, thereby enabling emigration of donor-derived cells from the graft. In parallel, loss of CS-56 immunoreactivity in the host gut wall indicates that chABC also modifies the recipient ECM, which increases tissue permissiveness for donor-cell migration. In summary, distribution of ENSCs from the transplantation site can be the result of both increased cell release from the neurosphere and the permissiveness of the host intestinal wall.

CSPGs limit migration of mouse postnatal ENSCs

To extend our studies to mammals, we generated enteric neurospheres from *Wnt1*;tdT mice at both embryonic and postnatal stages. Previously, we demonstrated that ENSCs derived from embryonic enteric neurospheres are capable of extensive migration following transplantation into the embryonic avian intestine and can generate a complete ENS. In contrast, postnatally derived neurospheres exhibit very limited ENSC migration and a reduced capacity for ENS formation (Mueller et al., 2024), prompting us to test whether CSPG expression influences the migratory behavior of postnatal enteric progenitors. Neurospheres generated from the intestines of 6- to 12-week-old *Wnt1*;tdT mice (Fig. S3) were analyzed for the presence of members of the HSPG, CSPG and collagen-type ECM molecules. While enteric neurospheres show diffuse agrin and CS-56 expression (Fig. 6A,B), they do not express collagen IV (Fig. 6C). To test whether modulating the expression of CSPGs influences ENCDC migration, we cultured postnatal *Wnt1*;tdT neurospheres on fibronectin and treated with 0.5 U/ml chondroitinase ABC (chABC) for 3 days. Enzymatic degradation of CSPGs significantly enhanced the migration of neurosphere-derived cells compared with untreated controls (Fig. 6D-F). To further assess CSPG function in a tissue context, we performed *ex vivo* transplants, in which postnatal *Wnt1*;tdT neurospheres were delivered into LMMP preparations of wild-type mouse colon and maintained for 10 days. *Ex vivo* chABC treatment increased the migration of neurosphere-derived cells, although this trend did not reach statistical significance (P=0.08) (Fig. 6G-I).

In vivo engraftment of *Wnt1*;tdT neurospheres in aganglionic *Ednrb*$^{-/-}$ colon is associated with increased local CSPG expression

To determine the CSPG expression of transplanted ENS progenitors in an aganglionic environment *in vivo*, *Wnt1*;tdT-derived enteric neurospheres were microsurgically implanted into the rectal wall of *Ednrb*$^{-/-}$ mice through a trans-anal approach, and recipient colons were collected 1 week later (Fig. 7A). Consecutive longitudinal sections of the distal colon show successful engraftment of the *Wnt1*;tdT enteric neurospheres (Fig. 7B), with *Wnt1*;tdT^{+} cells emigrating from the graft into the host intestinal wall. Neurospheres were primarily detected within the muscularis layer of the aganglionic colon. Anti-CS-56 immunostaining showed enhanced CSPG immunoreactivity surrounding the transplanted neurosphere and the injection site (Fig. 7B,C).

Single-cell transcriptomics identifies a specific CSPG, CD44, as a significant inhibitor of ENCDC migration in postnatal neurospheres

CSPG expression was compared between embryonic progenitor cells and the progenitor cells in postnatal-derived neurospheres. Dimensional reduction was performed on postnatal-derived

Development (2026) 153, dev205625. doi:10.1242/dev.205625

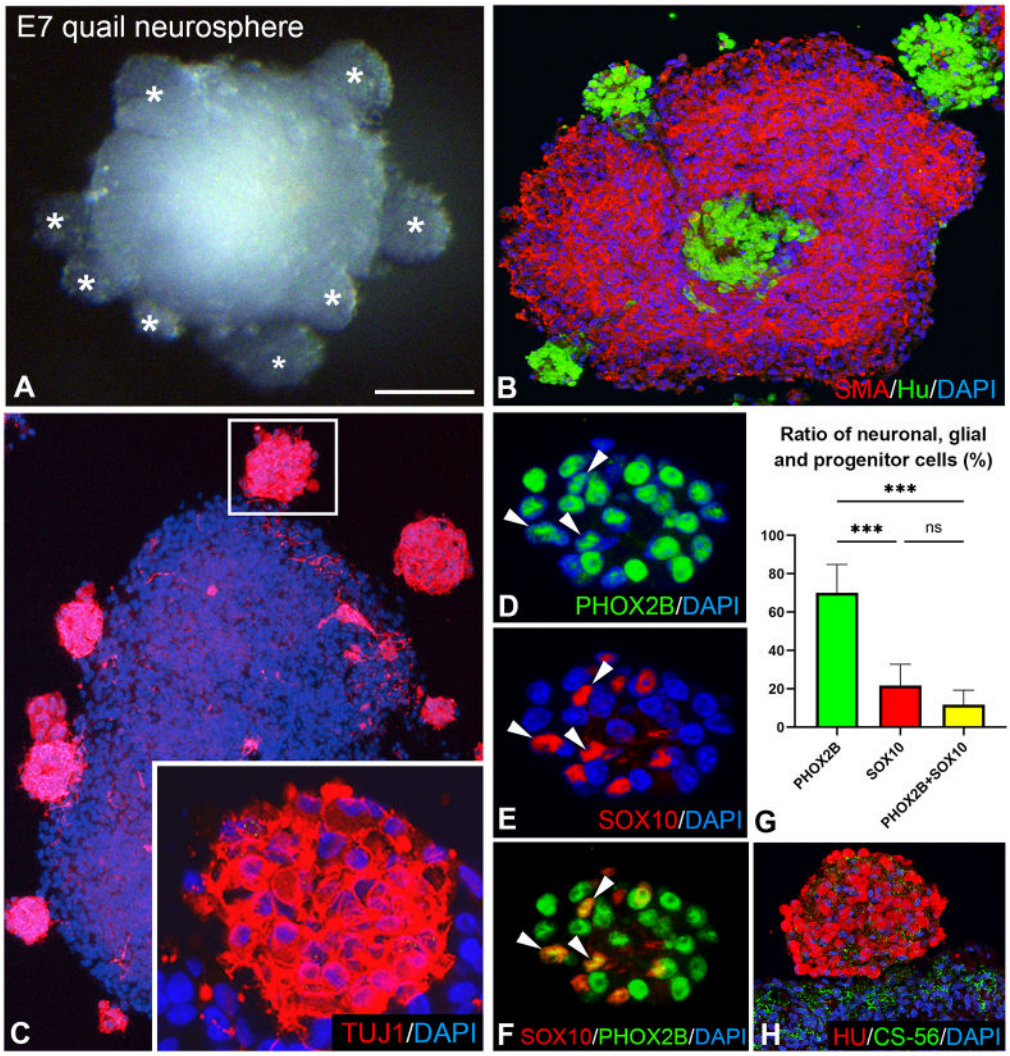


Fig. 4. Characterization of quail enteric neurospheres. (A) Neurospheres generated from embryonic day (E)7 quail intestine exhibit several surface aggregates (asterisks) after 7 days in culture. (B,C) Immunofluorescence characterization reveals that differentiating neurons indicated by Tuj1 (C) and Hu (B) immunoreactivity are restricted to the peripheral aggregates, which are associated with the smooth muscle core of the spheroid (outlined area is magnified in the inset). (D-F) While surface aggregates are predominantly committed to the neuronal lineage marked by PHOX2B expression, a few cells co-express SOX10 and PHOX2B, indicating the existence of bipotent progenitor enteric neural crest-derived cells (arrowheads). (G) The proportion of PHOX2B+ neurons, SOX10+ glia and PHOX2B+/SOX10+ progenitor enteric neural crest-derived cells. Data are mean±s.e.m. ***P<0.001 (one-way ANOVA followed by post-hoc comparisons with Tukey's test). (H) Immunostaining specific for chondroitin sulphate proteoglycans reveals diffuse expression associated with Hu+ neuronal aggregates. Scale bar: 120 µm (A,C); 100 µm (B); 25 µm (C, inset; D-F); 70 µm (H).

neurospheres from a dataset published by Guyer et al. (2023), revealing populations of glia (Fig. 8A), neurons (Fig. 8B), progenitor cells (Fig. 8C) and fibroblasts (Fig. 8D). The previously published E15.5 and E18.5 murine ENS scRNA-seq dataset (Morarach et al., 2021) (Fig. 8E) was restricted to clusters of progenitor cells based on their *Sox10* expression (Fig. 8F) This includes clusters 0, 2, 3 and 9, which we label 'embryonic progenitors' (Fig. 8G). Neurosphere sequencing data was restricted to progenitor cells based on their *Sox10 a*nd *Gfap* expression, resulting in clusters 0, 1, 2, 3, 4, 6, 8 and 14 being included in the analysis and labeled as 'neurosphere progenitors' (Fig. 8G). Differential gene expression analysis between the embryonic progenitors and postnatal neurosphere progenitors was performed, specifically examining the expression of different CSPGs (Fig. 8H). A specific CSPG, CD44 (CSPG8), had significantly higher levels of expression among postnatal neurosphere progenitor cells (Log2Fold change: 3.21). Additionally, only 1.4% of embryonic progenitors expressed CD44, whereas 98.2% of postnatal neurosphere progenitor cells expressed CD44 (Fig. 8I). Anti-CD44 immunostaining on postnatal neurospheres confirms the high expression of CD44 at the protein level (Fig. 8J). We hypothesized that CD44 expression by postnatal neurospheres may inhibit cell migration. *Wnt1*;tdT+ neurospheres derived from 4- to 12-week-old mice were cultured on fibronectin for 5 days. Treatment with a function-blocking CD44 monoclonal antibody significantly promoted ENCDC migration *in vitro* (Fig. 8K-M). To investigate CD44 function in a tissue-specific context, postnatal *Wnt1*;tdT neurospheres were transplanted *ex vivo* into wild-type mouse colon LMMP preparations and maintained for 48 h. Functional blocking of CD44 function during this *ex vivo* culture period significantly increased the migration of neurosphere-derived cells (Fig. 8N-P).

DISCUSSION

While stem cell-based therapy for Hirschsprung disease remains a promising approach, current strategies are hampered by limited migration of ENSCs following their delivery into the recipient postnatal gut wall. Mammalian and avian gut-derived ENSCs can be isolated and expanded as floating neurospheres. These cells can differentiate into multiple enteric neuronal subtypes capable of making functional neuromuscular connections with the colonic smooth muscle following their *in vivo* transplantation to the gut wall. However, the transplanted cells are not able to generate the extensive neuroglial network needed to restore gut function in large segments of colon (Cheng et al., 2017; Hotta et al., 2016, 2023; Lindley et al., 2008; Natarajan et al., 1999; Rahman et al., 2024; Soós et al., 2025). Most cell transplantation strategies have focused on the cell-autonomous properties of ENSCs while overlooking the recipient matrisome environment, which changes dramatically from its permissive state during embryonic gut development to a far less

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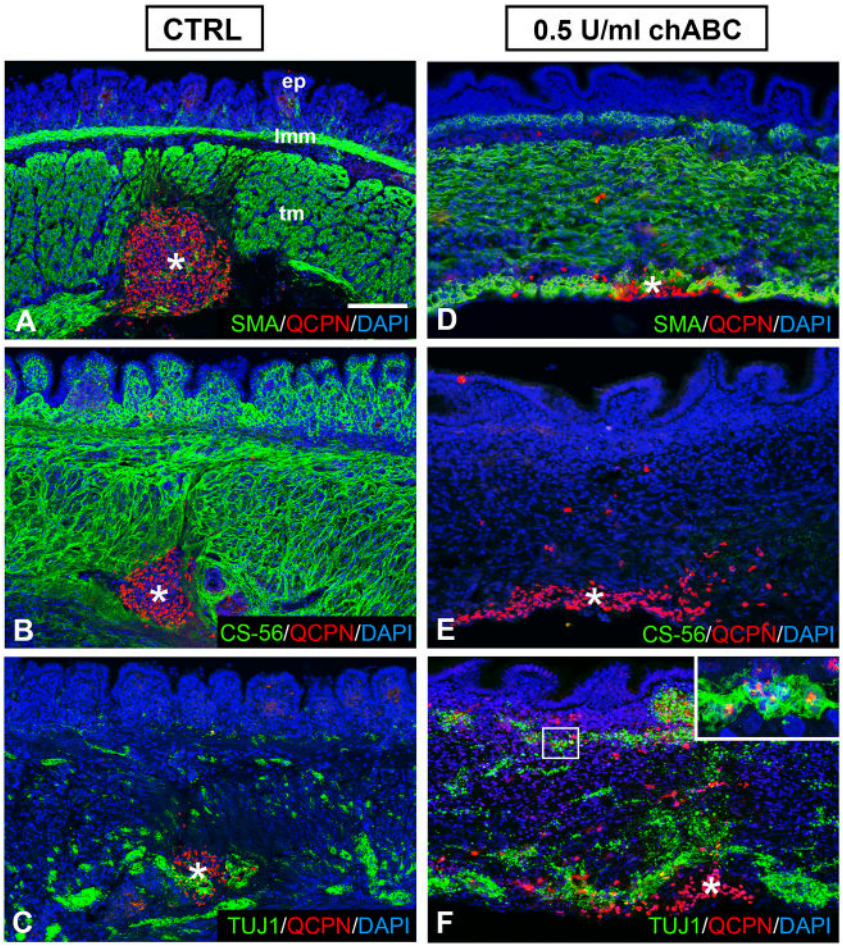


Fig. 5. Enzymatic chondroitinase ABC treatment significantly enhances cell migration following *ex vivo* neurosphere transplantation. Enteric neurospheres from E8 quail intestine were transplanted into E16 chicken hindgut and cultured for 3 days *ex vivo*. (A) Grafts cultured under control conditions show integration of neurosphere-derived QCPN+ cells within the gut wall. (B,C) Explants exhibit strong CS-56 immunoreactivity (B) and transplanted cells fail to migrate from the transplantation site (C). (D) In contrast, enzymatic treatment with 0.5 U/ml chABC results in the robust migration of neurosphere-derived cells. (E) Lack of CS-56 immunoreactivity confirms the effective enzymatic digestion of chondroitin sulphate proteoglycans in chABC-treated explants. (F) TUJ1+/QCPN+ transplanted neurons show increased migration and integration capacity within host submucosal and myenteric plexuses (outlined area is magnified in the inset). Migration of transplanted cells (123.30 µm±60.03), compared to control conditions (18.70 µm±14.47). Scale bar: 100 µm. ep, epithelium; lmm, lamina muscularis mucosae; tm, tunica muscularis. Area outlined in F is shown in the inset. Asterisks indicate the transplantation site.

permissive environment postnatally. This study aims to address this gap by identifying CSPGs as a potent inhibitor of ENCDC migration and showing that inhibiting its activity significantly enhances cell spread following transplantation, offering a novel matrisome-targeted strategy to enhance the success of regenerative cell therapy for Hirschsprung disease.

CSPGs are developmentally regulated ECM barriers to ENCDC migration

The ENS develops primarily from vagal neural tube-level NCCs that migrate from rostral to caudal along the gut and pattern radially to form two ganglionated plexuses of neurons and glia. These processes rely on reciprocal interactions between ENCDCs and their microenvironment that are essential for proper cell migration and differentiation. For example, when ENCDCs migrate through the gut mesenchyme, they are guided by ECM components such as laminin, fibronectin and collagen type I (Breau et al., 2009; Chevalier et al., 2016; Nagy et al., 2009; Newgreen and Hartley, 1995). As ENCDCs reach the proximal hindgut, the migratory wavefront cells produce collagen type XVIII, which is permissive for their migration. Once their migration ends, they secrete another heparan-sulphate proteoglycan-type basement membrane component, agrin, that inhibits ENCDC movement (Nagy et al., 2018). Tenascin C is also secreted by ENCDCs to promote their migration during ENS development (Akbareian et al., 2013). In contrast, collagen VI and agrin inhibit their locomotion (Nagy et al., 2018; Soret et al., 2015). However, the effect of ECM deposition in a 3D tissue environment is likely concentration and context dependent. For example, excessive collagen I deposition may increase extracellular matrix stiffness and thereby restrict ENCDC migration, as reported by Chevalier et al. (2016). This further underscores the importance of the spatial and temporal production of matrisome components, which is crucially important for proper ENS development.

Our analysis of the chick hindgut demonstrates that CSPGs represent another ECM component with a dynamic expression pattern and a key role in regulating ENCDC migration. Before ENCDC arrival, CSPGs are broadly present in the gut mesenchyme, then progressively redistribute and become enriched in the submucosal region as ENS colonization and plexus formation proceed. This spatial restriction is consistent with a developmental model in which CSPGs contribute to compartmentalization of ENCDCs and regulate the localization of the enteric plexuses in the outer region of the gut mesenchyme. Previously, we have shown that gut epithelium-derived Shh signaling induces the expression of CSPGs, such as versican and collagen type IX, in the surrounding mesenchyme (Nagy et al., 2016). Inhibition of Shh signaling with cyclopamine reduces CSPG expression and results in abnormal, large, ectopic ganglia, whereas overexpression of Shh leads to intestinal aganglionosis associated with increased and broad CSPG expression (Nagy et al., 2016). Similar to cyclopamine treatment, in *talpid2* and *talpid3* chicken mutants, where Hedgehog signaling is also disrupted, CSPG expression (specifically collagen type IX and versican) is significantly reduced or lost, causing improper ENCDC migration and abnormal ENS patterning (Brooks et al., 2021; Delalande et al., 2021). Alterations in ECM expression have also been noted in endothelin 3 mutant mice, where aganglionosis is

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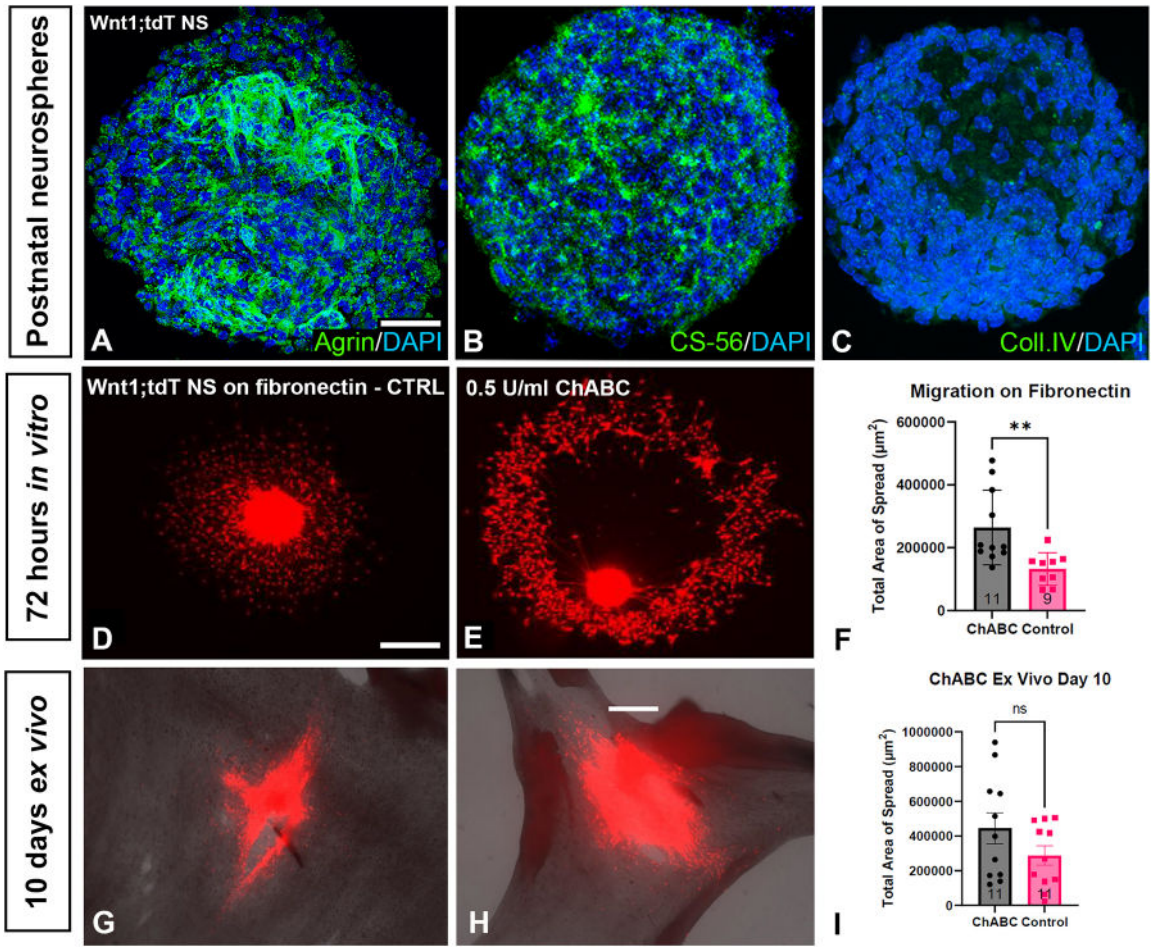


Fig. 6. CSPGs restrict the migration of mouse postnatal enteric neural stem cells. (A,B) Enteric neurospheres derived from postnatal *Wnt1*;tdT mouse intestines show diffuse expression of agrin (A) and CSPGs (B). (C) In contrast, collagen IV is absent from the neurospheres. (D,E) When cultured on fibronectin for 72 h *in vitro*, enzymatic chABC treatment significantly increases the migration of neurosphere-derived cells. (F,I) Bar graphs show the total area of spread under control and chABC-treated conditions. Data are mean±s.e.m. ***P*<0.01 (Mann–Whitney *U* test). (G,H) *Ex vivo* transplantation of postnatal-derived neurospheres onto LMMP tissue from wild-type mice colon results in increased migration of neurosphere-derived cells under enzymatic chABC treatment. Scale bars: 30 µm (A-C); 150 µm (D); 160 µm (E); 150 µm (G,H). Scale bar in A applies to A-C; scale bar in D also applies to E; scale bar in H also applies to G.

accompanied by increased proteoglycan deposition in the hindgut mesenchyme (Tennyson et al., 1990). These data support the idea that intestinal CSPGs are dynamic regulatory molecules that change over developmental time and actively direct ENCDC migration and patterning.

The importance of CSPGs in *in vitro* NCC migration is well known (Dutt et al., 2006; Kerr and Newgreen, 1997; Perissinotto et al., 2000), but their direct effect on ENCDC development has not been previously studied. Using *in vitro* migration assays, we demonstrate that CSPG-coated surfaces inhibit GDNF-induced ENCDC migration, and that treatment with chondroitinase (chABC) restores cell migration. Our catenary culture experiments reveal another important finding: removal of CSPGs during early gut development disrupts normal ENS patterning, leading to loss of concentric plexus organization, indicating that CSPGs contribute to the normal radial organization of the ENS. These results are consistent with observations following experimental spinal cord injury, where CSPGs are major components of the inhibitory ‘scar-like’ tissue that restricts axonal growth and neural tissue regeneration (Bradbury and Burnside, 2019; Bradbury et al., 2002; Davies et al., 1999; Moeendarbary et al., 2017).

CSPG degradation improves the spreading of transplanted neurosphere-derived cells

In quail-to-chick xenograft experiments, neurosphere-derived cells remain restricted to the implantation site under control conditions, whereas chABC treatment markedly increases their distribution into the host gut wall. These results support the hypothesis that inhibitory ECM components, rather than intrinsic limitations of donor cells, represent a major barrier to effective cell engraftment and colonization. Our mammalian data further strengthens the developmental niche hypothesis. Postnatal *Wnt1*;tdT mouse enteric neurosphere-derived cells show significantly increased migration both *in vitro* and following transplantation to *ex vivo* gut wall explants of wild-type mice following chABC treatment, suggesting that the degradation of neurosphere CSPGs enhances their migration. This finding is reminiscent of our prior work showing that downregulating the heparan sulphate proteoglycan type of agrin significantly improves the migration of transplanted cells following *ex vivo* and *in vivo* transplantation (Mueller et al., 2024).

Our findings also reveal an important limitation of broad CSPG degradation. Although chABC enhances the migration of transplanted neurosphere-derived cells, removal of CSPGs during early ENS

Development (2026) 153, dev205625. doi:10.1242/dev.205625

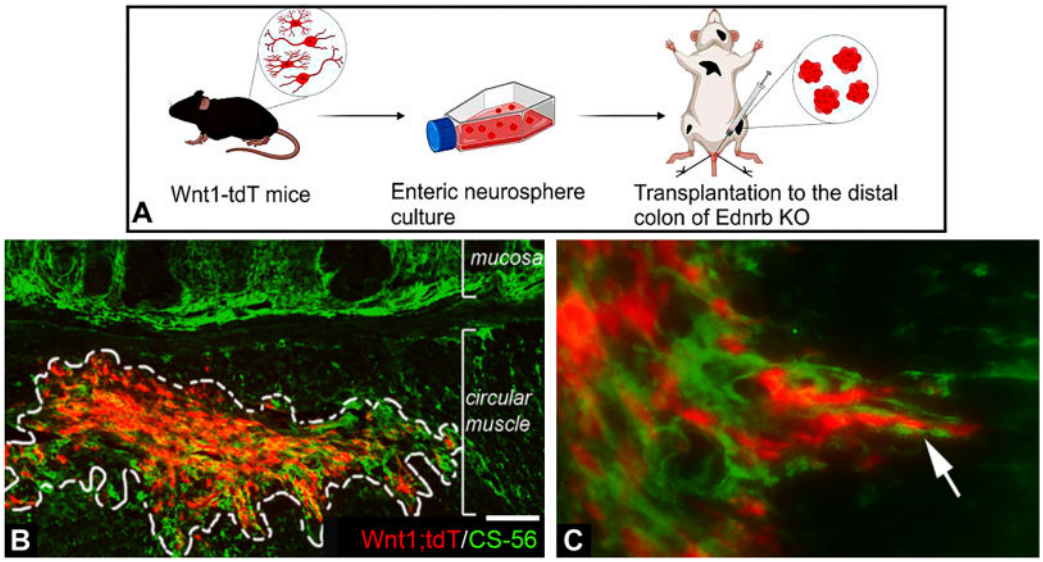


Fig. 7. *In vivo* transplantation of *Wnt1*;tdT neurospheres into the aganglionic distal colon of *Ednrb*$^{-/-}$ mice. (A) Schematic illustration of *in vivo* transplantation of *Wnt1*;tdT neurospheres into aganglionic colon of *Ednrb*$^{-/-}$ mice; the colon was fixed 1 week later. (B,C) Immunostaining of the longitudinal sectioned *Ednrb*$^{-/-}$ distal colon shows successful neurosphere engraftment (B, outlined with dashed line) in the inter-myenteric layer of the aganglionic region with emigration of transplanted *Wnt1*;tdT cells (C, arrow). (C) Higher magnification reveals that CSPG is highly expressed around the neurosphere. Scale bar: 60 µm (B); 20 µm (C).

development disrupts the concentric organization of enteric neurons and leads to ectopic distribution of the ENCDCs within the gut wall. Thus, CSPGs act not only as inhibitory barriers to ENCDC migration, but also as spatial guidance cues that pattern the gut wall and contribute to radial compartmentalization of the developing ENS. Consequently, chABC enzymatic digestion of CSPGs may enhance ENSC scattering while increasing the risk of ectopic enteric plexus formation.

It is important to specify that enzymatic chABC treatment removes both chondroitin and dermatan sulphate glycosaminoglycan side chains, but does not directly eliminate the CSPG core proteins. This suggests that CSPG core proteins, including versican, aggrecan and collagen IX, may also exert CS-independent effects on cell adhesion and cell migration.

CSPGs may also cooperate with other ENCDC inhibitory molecules, including agrin (Nagy et al., 2018), a heparan sulfate proteoglycan type of ECM. The expression of agrin after chABC treatment does not necessarily indicate that its inhibitory influence remains unchanged. Disruption of the CSPG-rich ECM may alter agrin expression or its spatial relationship with permissive matrix components. Therefore, according to this model, CSPGs function as ECM organizers that integrate multiple extracellular signals rather than acting solely as direct receptor ligands. We have previously shown that collagen XVIII and agrin exert opposing effects on ENCDC migration: collagen XVIII promotes wavefront migration, whereas agrin is expressed after neuroglial differentiation and restricts motility (Nagy et al., 2018; Mueller et al., 2024). Together with the present findings, this suggests that ENSC migration is regulated by the balance of permissive and inhibitory proteoglycan cues rather than by a single ECM component. This may also explain the mild effect of chABC in the adult mouse LMMP assay, since chABC digests CS/DS side chains but would not remove agrin or its heparan sulfate-dependent activity. Persistent agrin-mediated inhibition in both donor neurospheres and recipient tissue may therefore limit the response to CSPG digestion. Future studies comparing CSPG digestion, agrin inhibition and combined treatment will be needed to determine whether these pathways act independently, additively or synergistically to promote ENSC migration.

In addition, the sulfation patterns of the CSPGs can determine binding to multiple growth factors, including FGF, Wnts and members of the Hedgehog family (Domowicz et al., 2009; Ida et al., 2006). According to these studies, digestion of CSPGs may increase ENCDC motility by reducing inhibitory ECM interactions and simultaneously modifying local morphogen gradients required for normal enteric plexus organization. In the developing gut, where Sonic hedgehog-dependent mesenchymal patterning regulates the distribution of the CSPG-rich ECM (Nagy et al., 2016; Delalande et al., 2021), loss of CS/DS side chains consequently could disrupt the radial gradient of the gut-specific morphogens. This may explain why chABC treatment enhances migration and dispersal of transplanted cells, and disrupts normal concentric plexus formation. Other studies investigating neural stem cell development demonstrated that CSPGs can also regulate the maintenance of the progenitor stage, cell proliferation and differentiation (Sirko et al., 2007; Yamada et al., 2018; Hutchings et al., 2024). Removal of CS/DS side chains in embryonic gut may perturb both ENCDC migration and differentiation, while in the postnatal neurosphere transplantation experiments, CSPG inhibition appears to enhance donor-cell dispersal. Future studies need to investigate whether CSPGs directly regulate ENCDC proliferation, survival and enteric neuron or glial differentiation in addition to migration and patterning.

CD44 identifies a postnatal-associated inhibitory axis and provides a targeted alternative to global ECM digestion

By comparing postnatal-derived neurosphere progenitors (Guyer et al., 2023) with embryonic ENS progenitors (Morarach et al., 2021) and analyzing CSPG-associated genes, we identify CD44 (CSPG8) as a strikingly enriched feature of postnatal neurosphere progenitors, with near-ubiquitous expression in postnatal progenitors versus rare expression among embryonic progenitors. While primarily known as a hyaluronan receptor, CD44 can also be glycosylated with chondroitin sulfate chains, making it a functional CSPG on the cell surface (Henke et al., 1996; Jackson et al., 1995), and can interact with other ligands, including collagens and matrix metalloproteinases. The CD44 family comprises multiple isoforms, and changes in

Development (2026) 153, dev205625. doi:10.1242/dev.205625

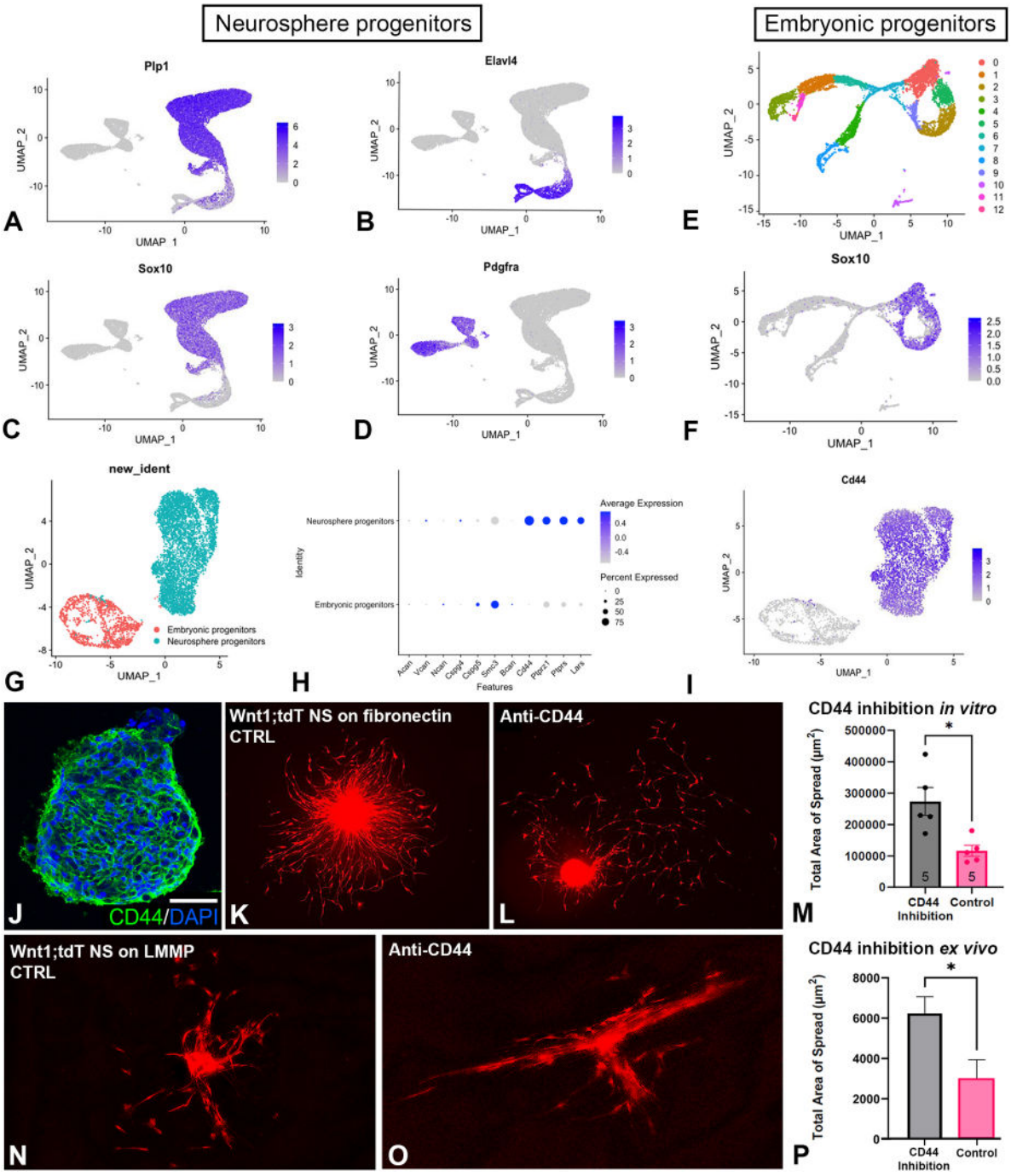


Fig. 8. Postnatal-derived mouse neurospheres differentially express CD44 compared to embryonic enteric neural crest-derived cells. (A-D) Single-cell RNA sequencing reveals specific glia (A), neuron (B), progenitor (C) and fibroblast (D) gene signatures in postnatal-derived neurospheres. (E-G) Cells selected based on *Sox10* and *Gfap* expression were labeled as neurosphere progenitors and compared with embryonic enteric nervous system progenitors using data published by Morarach et al. (2021). (H) CSPG gene expression is shown in a dot plot, with dot size corresponding to the percentage of cells in each cluster and color indicating relative gene expression. (I) Analysis of differentially expressed CSPG genes reveals a marked difference in CD44 expression: neurosphere progenitors express high transcript levels, while embryonic enteric neural crest-derived cells (ENCDCs) lack CD44 expression. (J) Immunofluorescence staining of postnatal neurospheres shows the diffuse expression of CD44. (K,L) Enteric neurospheres derived from postnatal *Wnt1*; tdT mouse intestines were cultured on fibronectin for 5 days *in vitro*. Blocking CD44 function with monoclonal antibodies significantly promotes neurosphere-derived ENCDC migration. (N,O) *Ex vivo* transplantation of postnatal-derived neurospheres onto LMMP tissue from wild-type mice colon results in increased migration of neurosphere-derived cells under anti-CD44 treated conditions. (M,P) Bar graphs show the total area of cell coverage in control and anti-CD44-treated cultures. Data are mean±s.e.m. **P*<0.05 (Mann–Whitney *U* test). Scale bar: 40 µm (J); 150 µm (K); 200 µm (L); 140 µm (N,O).

isoform expression correlate with altered adhesion, motility and metastatic behavior in cancer models: some isoforms promote strong matrix adhesion and others are associated with reduced cell-adhesion properties (Girbl et al., 2013; Klingbeil et al., 2009; Wong et al., 2003). Our *in vitro* and *ex vivo* experiments suggest that the inhibitory effects of CSPGs may be functionally linked to CD44. Although hyaluronan is the principal ligand for CD44, several CSPGs, including versican and aggrecan, can also interact with this

Development (2026) 153, dev205625. doi:10.1242/dev.205625

receptor (Kawashima et al., 2000). These interactions may induce CD44 clustering while the highly charged CSPGs can additionally regulate the organization of the ECM. In the developing gut, accumulation of CSPGs may restrict ENCDC migration through a complex mechanism where CSPGs form a non-permissive ECM that interferes with attachment to migration-supporting substrates such as fibronectin (Nagy et al., 2016), while their interaction with CD44 may alter cytoskeletal organization and the balance between adhesion and cell migration. Our results also show that functionally blocking CD44 with a monoclonal antibody significantly promotes migration of postnatal neurosphere-derived ENCDCs *in vitro* and *ex vivo*. This finding provides a potential molecular explanation for why postnatal-derived neurospheres, despite retaining ENSC identity, behave differently than embryonic progenitors. The postnatal progenitor state appears to be characterized by a restricted migration capability, which may be partially explained by increased CD44 expression. Importantly, treatment with chABC digests all CSPGs and disrupts normal ENS patterning in adult tissue; however, targeting a specific receptor pathway such as CD44 may enable a more selective release of migratory inhibition while minimizing broader perturbations of the ECM scaffold. Future experiments could test whether CD44 blockade improves migration and colonization in *in vivo* transplantation models, whether the effect generalizes across different ENSC sources (including iPSC-derived ENSCs) and whether ECM modulation combined with receptor inhibition yields additive or synergistic benefit.

While our study introduces enzymatic ECM modulation to ENSC transplantation to treat congenital neurointestinal diseases, several remaining questions need to be addressed. First, while our *ex vivo* data provide strong mechanistic support for CSPG inhibition, the long-term survival of transplanted ENCDCs, their long-distance migration, proper differentiation and functional rescue of gut motility will require extended transplantation studies with physiological assessments. Second, chABC treatment broadly affects CSPG side chains and may influence multiple other cell types, including smooth muscle, fibroblasts and immune cells, so the cellular specificity needs further analysis. Third, prior studies show that ENCDCs and the postnatal gut environment both contain many other inhibitory ECM proteins; how many of these need to be inhibited for successful cell transplantation remains an open question (Cheng et al., 2017). Our data add CSPGs and CD44-mediated interactions as actionable components of this niche limitation.

In summary, this study highlights the importance of ECM-derived inhibitory cues and emphasizes the concept that successful ENS regenerative therapy will require not only optimized ENSCs but also modification of recipient niche to generate a more embryonic-like permissive environment. A better understanding of how ENSC-ECM interactions influence embryonic and postnatal ENS development will enable advances in the development of cell therapy to treat Hirschsprung disease and other neurointestinal disorders.

MATERIALS AND METHODS

Animals

Fertilized White-Leghorn chicken (*Gallus gallus domesticus*) and quail *(Coturnix japonica domestica*) eggs were obtained from commercial breeders (Biovo, Mohacs, Hungary). Eggs were incubated at 37.5°C in a humidified hatching incubator (Heka Brutgerate, TS-7000C) and the age of the embryos was determined by the number of embryonic days (E). All animal experiments were approved by the Institutional Animal Care and Use Committee of Semmelweis University, Budapest, Hungary. Wnt1Cre/+ mice [B6.Cg-H2az2Tg(Wnt1-cre)11Rthg(Wnt1-GAL4)11Rth/JStock #009107] and R26R-tdT reporter mice [B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J; Stock #007914] were obtained from The Jackson Laboratory. Wnt1Cre/+ mice were crossed with R26R-tdT reporter mice to generate Wnt1-Cre+;R26-tdT mice (annotated as *Wnt1*;tdT). Mice studies were performed according to experimental protocols approved by the Institutional Animal Care and Use Committees of Massachusetts General Hospital. All mice were housed and bred at the Center for Comparative Medicine animal facility at Massachusetts General Hospital under specific pathogen-free conditions.

Histology

Following developmental staging, hindgut from E5-E10 chicken embryos or colon segments from older embryos were dissected. Samples were fixed in 4% paraformaldehyde (PFA) for 1 h at room temperature, rinsed thoroughly in phosphate-buffered saline (PBS) and cryoprotected in 15% sucrose at 4°C overnight. Tissues were then infiltrated with 7.5% gelatin (Sigma, G-2625) in 15% sucrose for 2 h at 37°C, embedded in the same gelatin medium, rapidly frozen at −50°C in 2-methylbutane (Sigma, 78-78-4) and stored at −80°C until cryosectioning. Sections (12 µm) were mounted on poly-L-lysine-coated slides (Sigma, P8920) and processed for immunohistochemistry.

Immunohistochemistry

For double immunofluorescence, sections were rehydrated in PBS at 37°C for 10 min and incubated with the following primary antibodies for 1 h at room temperature: anti-Tuj1 (1:200, Santa Cruz Biotechnology, sc-80016), anti-HuC/D (1:50, clone 16A11, Thermo Fisher Scientific, A-21271), anti-PHOX2b (1:200, R&D Systems, AF4940), anti-SOX10 (1:20, clone A-2, Santa Cruz Biotechnology, sc-365692), anti-SMA (1:200, clone: 1A4, Dako, M0851), anti-N-cadherin (cell culture supernatant, clone: 6B3, AB_528118), anti-BFABP (brain fatty acid binding protein, 1:50, Kurtz et al., 1994), anti-agrin (1:50; R&D Systems, AF550), anti-collagen IV (1:200, Santa Cruz Biotechnology, sc-29010), anti-CS-56 (1:1000, Sigma, C8035), anti-CD44 (Biolegend, 103065), anti-neurocan (1:100, R&D Systems, AF5800), anti-HNK1 (1:500, Thermo Fisher Scientific, MS-1163-P), anti-QCPN (cell culture supernatant, Developmental Studies Hybridoma Bank, DSHB, AB_531886), anti-N-cadherin (NCADH; 1:5, clone: 6B3, DSHB, AB_528118), anti-versican (cell culture supernatant, clone: 12C5, DSHB, AB_528503) and anti-collagen type IX (1:2, clone: 2B9, DSHB, AB_528170). All primary antibodies were diluted in PBS containing 1% bovine serum albumin (Sigma, A9647). Slides were washed in PBS for 15 min and incubated with Alexa Fluor-conjugated secondary antibodies: goat anti-mouse IgG 594, A-11005; goat anti-mouse IgM 488, A-10680; donkey anti-goat IgG 488, A-11055; goat anti-mouse IgM 594, A-21044 and goat anti-mouse IgG2b 488, A-21141 (all from Thermo Fisher Scientific). Secondary antibodies were diluted 1:200 in PBS, applied according to the isotype and species specificity of the primary antibody, and incubated for 45 min at room temperature. Cell nuclei were stained with 4,6-diamidino-2-phenylindole for 10 min (DAPI; Vector Labs) and sections were covered by aqueous Poly/Mount (Polyscience, 18606). Section images were recorded using a Nikon Eclipse E800 fluorescence microscope and Zeiss LSM 710 confocal microscope. Image processing, including tiling and merging pseudocolored immunofluorescent images used ZEISS ZEN Imaging proprietary software, ImageJ and Adobe Photoshop 7.0. Where acquisition settings were comparable, we performed a quantitative analysis of CSPG immunofluorescence intensity across stages using the raw image data. Specifically, CSPG signal was quantified in equivalent regions of interest, with background subtraction and identical analysis thresholds applied across samples.

Cell migration assay

To determine how chondroitin sulfate proteoglycans influence ENCDC migration, E6 chicken midgut segments were explanted on Petri dishes (CytoOne 60×15 mm, CC7682-3359) coated with 20 µg/ml fibronectin (Sigma, F1141), 1 µg/ml or 10 µg/ml chondroitin sulphate proteoglycans (CSPG, Sigma, CC117) for 1 h at 37°C (n=6/culture condition). One experimental condition included pretreatment of 10 µg/ml CSPG-coated culture dishes with 0.5 U/ml chondroitinase ABC (chABC from Proteus vulgaris, Sigma, C3667) for 30 min at 37°C. Cell migration was stimulated with 10 ng/ml glial cell-derived neurotrophic factor (GDNF, R&D Systems, 212-GD-010) diluted in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, GlutaMAX supplement, ThermoFisher

Development (2026) 153, dev205625. doi:10.1242/dev.205625

Scientific, 10565018). Explants were cultured *in vitro* for 12 h, at 37°C and 5% CO_2, fixed in 4% PFA for 20 min at room temperature and processed for immunohistochemistry.

For catenary cultures, E5 chicken midgut-hindgut were dissected and pinned down to silicone-coated tissue culture dishes and cultured for 72 h in DMEM or culture media containing 0.5 U/ml chondroitinase ABC enzyme (n=12/culture condition). Cultures were supplemented with fresh media every 24 h. After 3 days *in vitro*, explants were fixed in 4% PFA for 1 h at room temperature and processed for immunohistochemistry.

Generation of enteric neurospheres from quail

E8 quail intestines from the proximal midgut to the cloaca were dissected under sterile conditions, washed with PBS supplemented with 100 µg/ml penicillin-streptomycin (pen/strep; Gibco, 15140122) and pooled from 10 embryos for neurosphere generation. Tissues were enzymatically digested with dispase II (5 U/ml; StemCell Technologies, 07913) and 2.5 mg of collagenase IX (800 U/mg; Sigma, C9722-50MG) in NeuroCult Basal Medium (NBM; StemCell Technologies, 05700) for 45 min at 37.5°C, with gentle vortexing performed three times during the incubation. The cell suspension was filtered through a 40 µm cell strainer (Greiner, 542040), gently washed with NBM and completed to a final volume of 20 ml to stop the enzymatic activity. Cells were centrifuged at 500 ***g*** for 8 min and resuspended in NBM supplemented with NeuroCult Proliferation Supplement (mouse and rat, StemCell Technologies, 05701), 40 ng/ml epidermal growth factor (EGF; StemCell Technologies, 78006.1), 20 ng/ml basic fibroblast growth factor (bFGF; StemCell Technologies, 78003.1), 4.44 µg/ml (0.0004%) heparin (StemCell Technologies, 07980) and 40 ng/ml glial cell-derived neurotrophic factor (GDNF; R&D Systems, 212-GD-010). Neurosphere generation was initiated at a density of 6×10^5 live cells/ml on low-adherence tissue culture plates prepared by coating 6-well cell culture dishes (Thermo Scientific, 140675) with 2 ml of Anti-Adherence Rinsing Solution (StemCell Technologies, 07010) and incubated for 1 h at room temperature with gentle shaking at 80 rpm. Cultures were maintained for 7 days *in vitro*. Spheroids were fixed in 4% PFA for 30 min and processed for immunohistochemistry or transplanted into the muscular wall of chicken colon *ex vivo*.

Intestinal organ culture

To assess the colonization potential of enteric neurosphere derived ENSCs, *in vitro* cultured quail neurospheres were transplanted into the muscular wall of E16 chicken hindgut. For *ex vivo* catenary organ cultures the mid-hindgut section of E16 chicken embryos was longitudinally opened and pinned down to silicone-coated tissue culture dishes. Prior to grafting, the outer, smaller neurospheres were mechanically separated from the central mesenchymal core spheroid using fine tweezers under sterile conditions. Only these isolated outer neurospheres were collected and used for grafting; the central mesenchymal core spheroid was excluded from transplantation. An average of four to five enteric neurospheres were transplanted from the serosal surface of the explanted hindgut segments and cultured in DMEM or culture media containing 0.5 U/ml chondroitinase ABC enzyme (n=12/culture condition). To ensure effective enzymatic activity, the culture media was changed every 24 h. Explants were maintained for 3 days *ex vivo*, fixed in 4% PFA for 1 h at room temperature, serially sectioned and processed for immunohistochemistry. Cell migration was assessed with the Image J software, measuring the distance of QCPN+ cells from the transplantation site, which was determined by overlaying a circle with a diameter of 100 µm, representing for the average size of a neurosphere used for transplantation. Migration distance was measured from the edge of the circle, perpendicular to the centroid.

In vivo transplantation of *Wnt1*;tdT neurospheres to *Ednrb*$^{-/-}$ mice colon

Recipient 2-week-old *Ednrb*$^{-/-}$ mice were used for *in vivo* neurosphere transplantation experiments as described previously (Cheng et al., 2015; Mueller et al., 2024). Briefly, mice were anesthetized, and the distal colon was approached via a perianal skin incision. Two or three *Wnt1*;tdT neurospheres were implanted into the external muscle layer of the distal colon through a serosal incision (n=5). One week following the surgery, the recipient mice were euthanized using CO_2 asphyxiation, and the rectum was removed, cryosectioned and processed for anti-CSPG immunocytochemistry.

Generation of enteric neurospheres from mouse

Adult (4- to12-week-old) *Wnt1*;tdT mice were euthanized and their small intestine was removed from duodenum to terminal ileum. The longitudinal muscle-myenteric plexus (LMMP) layer was dissected from underlying tissue in PBS and digested for 60 min at 37°C in dispase (250 µg/ml; STEMCELL Technologies) and collagenase XI (1 mg/ml; Sigma-Aldrich). Following digestion, the cells were filtered through a 70 µm filter and resuspended at a density of 50,000 cells/ml with a total volume of 10 ml placed in 25 cm flasks (Corning). Neuroproliferation media contained Antibiotic-Antimycotic (100×, 1%; Gibco), B27 Supplement (1×; Gibco, Thermo Fisher Scientific), N-2 Supplement (1×; Gibco, Thermo Fisher Scientific), basic fibroblast growth factor (20 ng/ml; Stemcell Technologies), insulin-like growth factor 1 (20 ng/ml; Thermo Fisher Scientific), retinoic acid (75 ng/ml; Sigma Aldrich) and 2-mercaptoethanol (50 µmol/l; Gibco, Thermo Fisher Scientific) in equal parts Neurocult Mouse Basal Medium (Stemcell Technologies) and Dulbecco's Modified Eagle Medium (DMEM; Gibco, Thermo Fisher Scientific). These primary neurospheres were cultured at 37°C and 5% CO_2 for 10 days.

Primary neurospheres were then prepared for cell sorting. They were dissolved for 45 min into a single cell suspension using accutase (Stemcell Technologies), filtered through a 40 µm filter and stained with DAPI. Cell sorting was performed on the Massachusetts General Hospital campus by the Harvard Stem Cell Institute's Center for Regenerative Medicine Flow Cytometry Core, utilizing BD Biosciences FACS Aria sorting instruments. Cells were sorted for tdT positivity (indicating *Wnt1* expression) and DAPI negativity (indicating live cells). tdT+ cells were kept and manually counted with Trypan Blue to assess number and viability. TdT+ cells were then plated in a 96-well round-bottomed ultra-low attachment dish (Corning) at a density of 50,000 cells/ml. Fused, irregular or fragmented neurospheres were excluded and only similarly sized (∼100 µm diameter) neurospheres were used in control and treatment groups.

Neurosphere migration assay

Sorted tdT+ cells were plated in a 96-well dish at a concentration of 50,000 cells/ml and cultured for 72 h. Neurospheres utilized in the migration assay were therefore uniform in size and contained 10,000 cells. After 72 h, single neurospheres were transferred onto fibronectin-coated dishes (1:200 in PBS, Sigma Aldrich) and maintained for 10 days. Cultures were maintained in either DMEM-only, DMEM with chondroitinase ABC enzyme (0.5 U/ml; Sigma Aldrich) or DMEM with anti-CD44 monoclonal antibody (Mujal et al., 2016) (0.5 mg/ml, Thermo Scientific IM7) (n>9/culture condition).

Ex vivo LMMP transplantation and CD44 inhibition

Ex vivo enteric neurosphere transplantation was performed as previously described (Mueller et al., 2024). Briefly, colonic longitudinal muscle-myenteric plexus (LMMP) preparations were isolated from 4-week-old C57BL/6J mice. LMMPs were pinned in Sylgard-coated dishes, and two or three small pockets were created in the muscularis propria using fine forceps. Enteric neurospheres were transplanted into these pockets. Control explants (n=6) were cultured for 48 h in DMEM containing 5% fetal bovine serum and 1% antibiotic-antimycotic. For CD44 function-blocking experiments (n=6), anti-CD44 antibody was added to the culture medium at 10 µg/ml (anti-mouse/human CD44; BioLegend, 103065). After culture, tissues were fixed in 4% paraformaldehyde for 4-6 h and processed for imaging. Cell migration was assessed utilizing Image J software v1.53t (National Institutes of Health, Bethesda, MD, USA) using binary thresholding to determine total area covered by tdT+ cells.

Transcriptomic analysis

Published datasets (Guyer et al., 2023; Morarach et al., 2021) were utilized for differential gene expression analysis of CSPGs. Mouse embryonic datasets from Morarach et al. were downloaded from the NCBI Sequence Read Archive. Runs SRR11635571, SRR11635572 and SRR11635573 were obtained using the SRA Toolkit "fastq-dump" command. Genome alignment and feature-barcode matrix generation was performed with the

Cell Ranger "cellranger count" command on the Mass General Brigham ERISOne Research Computing Cluster. Subsequent analysis was performed in the R environment (RStudio Version 2023.03.0+386) with Seurat. The datasets were filtered by removing cells expressing a number of genes more than one standard deviation away from the mean and with greater than 10% mitochondrial genes. This filtration process removes low quality or dying cells, empty droplets, and doublets or multiplets from the dataset. Embryonic progenitors were selected based on high *Sox10* expression (clusters 0,2,5,9), labeled 'embryonic progenitors', and were included in further analyses.

The dataset on postnatal-derived neurospheres was provided by Guyer et al. (2023). The dataset was filtered as the authors describe in the original paper, with removal of cells expressing a number of genes more than one standard deviation away from the mean and removal of cells with greater than 10% mitochondrial genes. Progenitor cells were selected based on high *Sox10* and *Gfap* expression, with clusters 0, 1, 2, 3, 4, 6, 8 and 14 labeled 'neurosphere progenitors' and included in subsequent analyses.

The embryonic progenitor and neurosphere progenitor datasets were integrated using the "FindIntegrationAnchors" command in Seurat (Stuart et al., 2019). After integration, principle component analysis (PCA) was performed, neighbors were identified, and uniform manifold approximation and projection (UMAP) dimensional reduction was performed using the first 30 principal components. Clusters were identified using the "FindClusters" command with resolution=0.5 using the Louvain algorithm (Blondel et al., 2008). Differential gene expression analysis was performed using the R package DESeq2 (Love et al., 2014). All CSPG genes were evaluated in the differential expression analysis. Genes with log2 fold changes >0.5, percent expression >25% and adjusted *P*-value <0.001 were considered significant.

Statistical analysis

Statistical analyses were conducted using one-way ANOVA followed by post-hoc comparisons with Tukey's test or the Kruskal–Wallis test with a post-hoc Dunn's test, as appropriate (GraphPad Prism v10.5.0). *P*-values were adjusted using the Holm correction and values less than 0.05 were considered statistically significant, using the following significance thresholds: **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001. Error bars indicate the standard error of the mean (s.e.m.).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: E.S., J.L.M., A.M.G., N.N.; Formal analysis: R.B.T.; Funding acquisition: A.M.G.; Investigation: E.S., J.L.M., C.J., R.B.T., V.H., R.H., N.N.; Methodology: E.S., C.J., R.B.T., V.H., Á.S.; Supervision: A.M.G., N.N.; Validation: E.S., J.L.M., C.J., R.H., Á.S.; Visualization: E.S., J.L.M., C.J., R.B.T., V.H., Á.S., N.N.; Writing – original draft: E.S., J.L.M., A.M.G., N.N.; Writing – review & editing: A.M.G., N.N.

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Data and resource availability

All relevant data and details of resources can be found within the article and its supplementary information.

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