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EMBRYONIC GROWTH FACTOR CONDITIONING AND KNOCKDOWN OF THE AGRIN MOLECULE TO ENHANCE TISSUE INTEGRATION OF TRANSPLANTED ENTERIC NEUROSPHERES

PhD thesis

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LIST ABBREVIATIONS:

a.u.	Arbitrary units	hPSC	Human pluripotent stem cell
BFABP	Brain fatty acid-binding protein	HU	Hu neuronal RNA-binding protein marker
bFGF	Basic fibroblast growth factor	ICC	Interstitial cells of Cajal
BMP	Bone morphogenetic protein	IGF-1	Insulin-like growth factor 1
CAM	Chorioallantoic membrane	iPSC	Induced pluripotent stem cell
D0	Day of hatching / day 0	LMMP	Longitudinal muscle-myenteric plexus
ECM	Extracellular matrix	MOI	Multiplicity of infection
EDNRB	Endothelin receptor type B	NBM	NeuroCult basal medium
EGF	Epidermal growth factor	NCAM	Neural cell adhesion molecule
EdU	5-ethynyl-2'-deoxyuridine	NCC	Neural crest cell
ENCC	Enteric neural crest cell	NLB	Neurosphere-like body
ENCDC	Enteric neural crest-derived cell	nNOS	Neuronal nitric oxide synthase
ENS	Enteric nervous system	PBS	Phosphate-buffered saline
ENSC	Enteric neural stem cell	PCA	Principal component analysis
ET3	Endothelin-3	PFA	Paraformaldehyde
FGF2	Fibroblast growth factor 2	PHOX2B	Paired-like homeobox 2B
FN	Fibronectin	PLP1	Proteolipid protein 1
GDNF	Glial cell line-derived neurotrophic factor	RA	Retinoic acid
GFAP	Glial fibrillary acidic protein	RET	Rearranged during transfection receptor tyrosine kinase
GFP	Green fluorescent protein	SCP	Schwann cell precursor
GFRα1	GDNF family receptor alpha 1	SD	standard deviation
GWEN	GDNF, WNT11, endothelin-3, and Noggin	SEM	Standard error of the mean
HH	Hamburger-Hamilton stage	shRNA	Short hairpin RNA
HNK1	Human natural killer-1 antigen	SPF	Specific-pathogen-free
HSCR	Hirschsprung disease	tdT	tdTomato
HSP47	Heat shock protein 47	WNT	Wingless-related integration site signaling family

1. INTRODUCTION

1.1. Organization and function of the enteric nervous system

The enteric nervous system (ENS) is the largest division of the peripheral autonomic nervous system, containing approximately 400-600 million neurons in humans, more than all sympathetic and parasympathetic ganglia combined (Furness, 2012). Enteric neurons, together with enteric glial cells, are organized into two major plexuses: the myenteric (Auerbach's) plexus is located between the circular and longitudinal muscle layers throughout the intestinal tract, and the submucosal (Meissner's) plexus is present in the submucosa from the stomach to the rectum (Fig. 1A). Functionally, the myenteric plexus primarily regulates smooth muscle tone and coordinated motility patterns (e.g., peristalsis). In contrast, the submucosal plexus is strongly associated with epithelial secretion/absorption and with local regulation of blood flow. The two plexuses are interconnected, allowing sensory inputs and interneuronal circuits to coordinate mucosal function with motor output (Furness, 2012)(Fig. 1B,C). In the guinea pig ileum, 14 neuronal types have been identified, whereas 26 functionally and structurally defined neuronal classes have been described across the gastrointestinal tract as a whole. These classes are distinguished by morphology, electrophysiological properties, projections, and neurochemical coding, and include intrinsic sensory neurons, interneurons, and both excitatory and inhibitory motor neurons (Di Natale et al., 2026). The ENS operates with a high degree of autonomy, capable of reflexes like peristalsis independently of central input, earning it the nickname "the second brain". Although the ENS can generate reflex activity independently, it is extensively modulated by extrinsic sympathetic and parasympathetic inputs, which shape motility, secretion, and immune responses in response to physiological state. Bidirectional communication between ENS circuits and the central nervous system (CNS) underlies the gut-brain axis. It helps explain why stress, inflammation, and systemic disease can alter gastrointestinal function without structural obstruction (Furness, 2012). ENS neurons regulate gut motility, secretion, blood flow, and nutrient absorption by interpreting signals and orchestrating smooth muscle activity and glandular outputs (Furness, 2012). Enteric glial cells are not passive support cells; they contribute to neuronal homeostasis, synaptic signaling, epithelial barrier maintenance, and neuroimmune interactions in the gut wall. Their roles in injury responses

and inflammatory states make them relevant to both disease mechanisms and regenerative strategies targeting ENS repair (Schonkeren et al., 2022). Core ENS function is implemented through local reflex circuits in which intrinsic sensory neurons detect mechanical/chemical stimuli, interneurons integrate signals across segments, and motor neurons drive smooth muscle and secretory responses using distinct neurotransmitters (Furness, 2012).

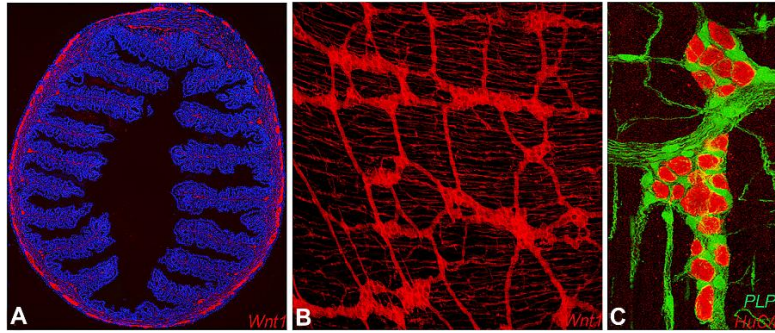


Figure 1. Enteric nervous system organization. (A) Cross-section of embryonic gut showing the distribution of *Wnt1*+ enteric neural crest-derived cells (ENCCs) in relation to the developed intestinal wall, with red nuclei counterstained with DAPI/blue. (B) Whole-mount immunostaining of the developed enteric network showing *Wnt1*+ ENCCs forming interconnected ganglionated strands within the gut wall. (C) Enteric ganglia, showing PLP1+ enteric glial cells in green and HuC/D+ enteric neurons in red. Adapted from Nagy and Goldstein, 2017.

1.2. Neural crest origins of the enteric nervous system

The enteric nervous system originates from the neural crest cells. The ENS is primarily derived from a specific embryonic cell type, the vagal neural crest cells (NCCs), which colonize the embryonic gut and differentiate into neurons and glial cells (Lin et al., 2026). NCCs are among the most intensively studied cell populations in the vertebrate embryo. They are a transient, multipotent, and highly migratory population of stem cell type that arises during early development and contributes to a wide array of tissues and organs. Because of their extensive developmental potential and their central importance in vertebrate morphogenesis, NCCs have often been described as a vertebrate innovation and, in some contexts, even as a “fourth germ layer”. Although this latter term is not literal in embryological classification, it reflects the extraordinary capacity of neural crest cells to

generate derivatives that span multiple tissue categories, including components of the peripheral nervous system, pigment cells, endocrine derivatives, and, in the cranial region, mesenchymal tissue such as cartilage, bone, and connective tissue (Adameyko and Fried, 2016; Bronner and Simões-Costa, 2016; Soldatov et al., 2019; Erickson et al., 2023).

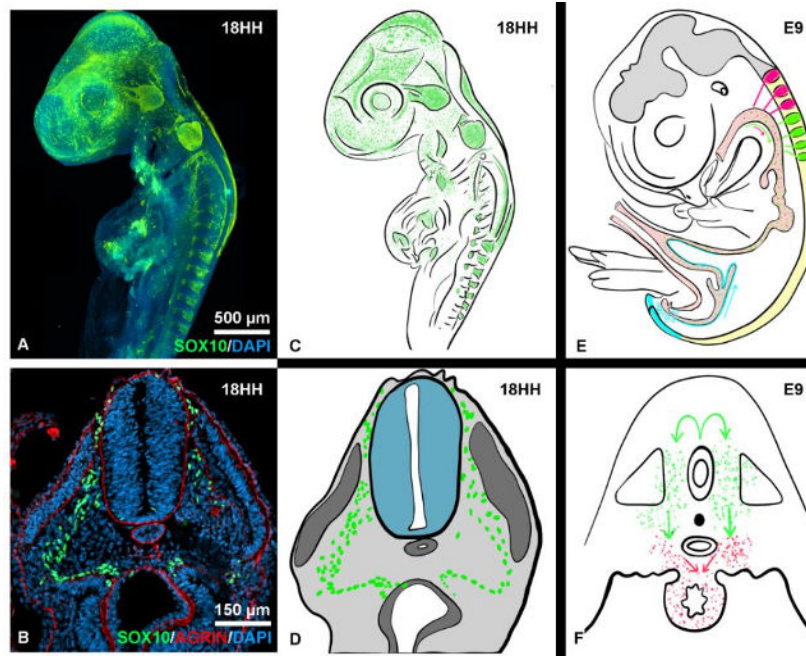


Figure 2. Neural crest cell migration, distribution, and receptor transition during embryonic development. (A) Whole-mount fluorescence image of a stage 18HH embryo showing the distribution of SOX10+ neural crest cells (green). (B) Cross-section of a stage 18HH embryo illustrating the migration pathways of neural crest cells (green). The extracellular matrix protein Agrin (red) acts as an inhibitory barrier to guide migration. Nuclei are counterstained (blue). (C, D) Schematic representations of the whole-mount embryo (A) and the transverse section (B), respectively, highlighting the distribution and migratory streams of SOX10+ NCCs. (E) Schematic illustration of an E9 embryo detailing specific neural crest subpopulations: cardiac neural crest (magenta), vagal neural crest (green), and sacral neural crest (turquoise). (F) Schematic model of neural crest migration in cross-section. Cells initially migrate (green arrows/dots) between the neural tube and through the somitic mesenchyme rich in retinoic acid. As neural crest cells progress ventrally toward the developing gut tube, they start to express the rearranged during transfection receptor tyrosine kinase (RET) receptor (indicated by the red dots/arrows). Author's own work.

Neural crest cells arise at the neural plate border, a specialized embryonic domain located between the neural and non-neural ectoderms during early neurulation. This border region is established through a combination of inductive signals and transcriptional interactions that specify a territory competent to generate neural crest progenitors. As neurulation proceeds and the neural folds elevate and fuse, prospective neural crest cells become positioned in the dorsal neural tube. From this location, they emerge as a distinct population that subsequently delaminates and begins migration (Bronner and Simões-Costa, 2016). NCCs arise from distinct axial levels of the neural tube and give rise to different derivatives. The vagal neural crest, originating from the level of somites 1-7, is the major source of ENCDCs. Cells from somites 1-3 include a cardiac neural crest population that contributes to the aorta-pulmonary septum, cardiac parasympathetic neurons, glia, and myocytes, as well as a ventrally migrating vagal population that forms enteric ganglia in the esophagus, stomach, and foregut. Neural crest cells from somites 4-7 join the rostral vagal population and together colonize the entire gastrointestinal tract, forming the intrinsic ENS. NCCs from the 7th somite level may also contribute to melanocytes and dorsal root ganglia. The trunk neural crest, arising between somites 8-28, does not form the intrinsic ENS but gives rise to sympathetic derivatives, including paravertebral and prevertebral ganglia, celiac and inferior mesenteric ganglia, and adrenal chromaffin cells. The sacral neural crest, originating caudal to somite 28, contributes mainly to the pelvic plexus and extrinsic hindgut innervation (Fig. 2). In birds, it may also contribute to the hindgut ENS, whereas in mammals, its contribution to the intrinsic ENS appears limited or absent (Yntema and Hammond, 1955; Le Douarin and Teillet, 1973; Burns and Le Douarin, 2001; Kuo and Erickson, 2011; Nagy et al., 2012; Yu et al., 2024).

The main part of the enteric nervous system (ENS) arises from vagal NCCs. During ontogeny, ENCCs give rise to two principal cellular components of the ENS: enteric neurons and enteric glia (Lake and Heuckeroth, 2013; Nagy and Goldstein, 2017; Bali and Grubišić, 2024; Gonzales and Gulbransen, 2025). A central developmental constraint is that ENCCs must expand and migrate rapidly enough to keep pace with intestinal growth (Newgreen et al., 1996; Simpson et al., 2007); if cell density falls below a critical threshold, the migratory front stalls, and distal regions remain uncolonized. Thus, ENS formation is not only a guidance problem but also a “population dynamics” challenge where proliferation, survival, and

migration are tightly coupled. This concept helps explain why genetic or environmental perturbations that slightly reduce ENCC proliferation and motility produce distal aganglionic segments during ENS development (Lake and Heuckeroth, 2013; Nagy and Goldstein, 2017). After entering the foregut, ENCCs advance caudally as a coherent migratory “wavefront,” with undifferentiated, proliferative cells concentrated at the leading edge and differentiating neurons and glia accumulating behind it. Efficient colonization, therefore, depends on both directional migration and sustained expansion of progenitors at the front as the intestine elongates. In the avian embryo, sacral neural crest cells also contribute to the distal ENS (Le Douarin and Teillet, 1973; Nagy et al., 2007; Halasy et al., 2023). In mice, the extent of sacral contribution remains debated and may be minimal, depending on the lineage-tracing strategy (Nagy and Goldstein, 2017; Yu et al., 2024). In addition to vagal and sacral NCCs, Schwann cell precursors (SCPs) that migrate along peripheral nerves have been shown to generate enteric neurons in the developing and postnatal gut. This nerve-associated pathway may serve as an additional reservoir of neural crest-derived progenitors, with region- and stage-specific relevance during development (Solovieva and Bronner, 2021; Kastriti et al., 2022). Recognizing these additional cell sources is essential when interpreting lineage-tracing studies and when designing cell-based strategies to rebuild enteric circuitry (Uesaka et al., 2015). This journey is remarkable in its scale: ENCCs migrate farther than any other neural crest population, taking over 25% of murine gestation (and ~3 weeks in humans) to complete gut colonization. During this process, ENCCs simultaneously proliferate, migrate, and differentiate into enteric neurons and glia, thereby contributing to the formation of the ENS (Burns and Goldstein, 2024). ENCCs typically migrate as interconnected chains or streams, maintaining frequent cell-cell contacts as they move through the gut mesenchyme (Broders-Bondon et al., 2016). Proliferation near the leading edge sustains a critical cellular density, enabling continuous forward advance as the intestine elongates (Newgreen et al., 1996; Conner et al., 2003). As regions become fully colonized, ENCCs progressively slow and differentiate, while the wavefront remains progenitor-rich and migratory (Stavely et al., 2023).

1.3. Molecular regulation of ENCC development

Critical molecular cues guide ENCC migration. The RET receptor expressed by ENCCs and its ligand GDNF (glial-derived neurotrophic factor), produced in the gut mesenchyme, generates a chemoattractive and proliferative function for ENCCs. Mutations in GDNF or its receptors (RET or GFR α 1) cause full-length aganglionosis of the colon. Concurrently, mesenchymal endothelin-3 (ET3) signaling synergizes with RET-GDNF by enhancing EDNRB+ ENCC proliferation and migration. Disruption of the EDNRB/ET3 pathway impairs ENCC colonization of the distal gut characteristic of Hirschsprung disease, a congenital neurocristopathy (Montalva et al., 2023). In normal development, GDNF is expressed in the gut mesenchyme ahead of the migrating crest cells and attracts RET-expressing ENCCs, while ET3/EDNRB signaling promotes their expansion and modulates migration timing (Nagy and Goldstein, 2006; Heanue and Pachnis, 2007; Amiel et al., 2008; Gazquez et al., 2016; Watanabe et al., 2017; Burns and Goldstein, 2024). The coordinated activity of these pathways ensures that neural crest-derived cells populate the entire bowel. In addition to RET-GDNF and EDNRB-ET3, other morphogens and signaling pathways - including SHH/Hedgehog, BMP/Noggin, Wnt, Notch, HOXA3, SOX10, Phox2b signaling, together with extracellular-matrix and retinoic acid-dependent cues - precisely control ENCC migration, radial patterning, proliferation, and differentiation into neurons and glia (Kang et al., 2021). ENCC colonization and patterning are shaped by the extracellular matrix (ECM), which also defines permissive versus restrictive migratory territories. SHH morphogen released from the gut epithelium regulates ENCC proliferation, differentiation, and migration, and has been linked to mesenchymal chondroitin sulfate proteoglycan expression that directly alters the migratory landscape (Fu et al., 2004; Nagy et al., 2016). Moreover, BMP4 is a gut mesenchymal morphogen that regulates the migration of enteric neural crest-derived cells (ENCCs) and influences the differentiation of both enteric neurons and glial cells. BMP signaling also contributes to the formation and patterning of enteric ganglia by supporting the proper distribution and connectivity of enteric neurons, a process associated with changes in neural cell adhesion molecule (NCAM) expression. In addition, BMP signaling has been shown to restrict migrating mouse ENS precursors to the outer layers of the developing gut wall (Chalazonitis et al., 2008; Chalazonitis et al., 2011; Kovács et al.,

2023). Non-canonical WNT signaling can also act region-specifically; for example, WNT11 expression in the avian cecal region has been shown to regulate colorectal colonization by preventing premature neuronal differentiation of migrating ENCCs (Nagy et al., 2021). Within the migratory microenvironment, ENCCs move along permissive extracellular matrix components, such as laminin and fibronectin. Still, their migration is strongly shaped by the biochemical and mechanical composition of the surrounding matrix. This is particularly important because ENCCs do not merely respond passively to the ECM; they actively remodel it as they advance through the gut wall. Tenascin-C is one example of this cell-matrix reciprocity: ENCCs produce tenascin-C, which promotes their migration and helps generate a supportive local migratory substrate (Akbarian et al., 2013). Similarly, ENCC-derived heparan sulfate proteoglycans regulate the migratory niche: collagen XVIII is enriched at the ENCC wavefront and acts as a permissive ECM component, whereas agrin appears later and is strongly inhibitory to ENCC migration, suggesting that dynamically changing ECM composition may help coordinate the transition from migration to ganglion formation (Nagy et al., 2018). In contrast, excessive collagen VI creates a less permissive matrix; increased collagen VI deposition slows ENCC migration, can interfere with fibronectin-mediated motility, and has been implicated in Hirschsprung disease pathogenesis, including the increased HSCR susceptibility associated with Down syndrome (Soret et al., 2015; Heuckeroth, 2015). These findings align with broader principles of neural crest migration, in which permissive ECM substrates, inhibitory proteoglycan-rich matrices, matrix stiffness, and proteolytic remodeling collectively determine migratory efficiency (Chevalier et al., 2021; Leonard and Taneyhill, 2020). Thus, ECM permissiveness provides a mechanistic rationale for the later chapters, which focus on improving ENCC migration and engraftment by modifying the intestinal niche and matrix environment.

1.4. Enteric neurocristopathies

Disruption of key developmental signaling pathways can lead to congenital intestinal disorders (enteric neurocristopathies), the most prominent example of which is Hirschsprung disease. For example, loss-of-function variants affecting the RET-GDNF or EDNRB-ET3

pathways can impair rostrocaudal colonization of the gut by enteric neural crest-derived cells (ENCCs), resulting in incomplete formation of the enteric nervous system (ENS).

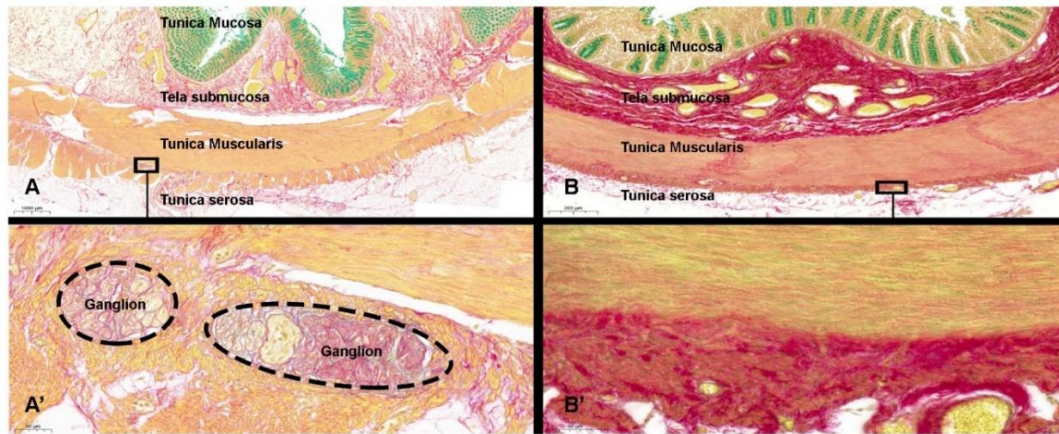


Figure 3. Histological comparison of a normal colon and biopsy from Hirschsprung's disease (Alcian green-Sirius red stain, 9 months old human). (A) Normal Colon (Low Magnification): Cross-section of a normally innervated colon. (A') A magnified view of the boxed region in (A) indicates the enteric ganglia of the *plexus myentericus* (Auerbach's plexus). (B) Section from the aganglionic colon. (B') A magnified view of the boxed region in (B). Enteric ganglia are absent in the myenteric region within the muscle layers and intestinal fibrosis phenotype with Sirius red staining showing more expressed collagens. Author's own work.

Hirschsprung disease (HSCR) is a typical congenital neurointestinal disorder characterized by the absence of ganglion cells in both the myenteric and submucosal plexuses, resulting in an aganglionic colon segment of variable length. HSCR occurs in approximately 1 in 5,000 live births and shows a male predominance (~3-5:1). Clinically, HSCR ranges from short-segment disease (most common) to long-segment disease and total colonic aganglionosis (Fig. 3); in general, earlier arrest of ENCC colonization results in longer aganglionic segments. Aganglionosis (most frequently affecting the rectosigmoid colon) causes tonic contraction of the affected bowel segment and functional intestinal obstruction in neonates (Wang et al., 2025). Diagnosis is confirmed by rectal biopsy demonstrating aganglionosis, often accompanied by hypertrophic nerve trunk. Definitive treatment is a pull-through procedure to remove the aganglionic bowel and restore continuity. Genetically, HSCR is

heterogeneous but frequently involves variants in RET or other components of the RET/GDNF and EDNRB/ET3 pathways that regulate ENCC migration, proliferation, and survival. Although surgery is often curative in relieving obstruction, many patients experience long-term complications, such as enterocolitis and constipation (Catto-Smith et al., 2007; Burns and Thapar, 2014; Montalva et al., 2023; Mueller et al., 2025). Similar disorder ENS phenotypes are intestinal neural dysplasia, hypoganglionosis, and ganglioneuroma (hyperganglionosis). Beyond HSCR, several pediatric motility disorders involve early-life ENS dysfunction and may share a particular vulnerability of inhibitory (nitrergic) motor pathways. Other conditions, such as achalasia and gastroparesis, are more commonly acquired in adults, but pediatric cases also do occur (Westfal and Goldstein, 2017).

1.5. Regenerative strategies for the enteric nervous system: stem cell transplantation

Because many enteric neuropathies result from missing or dysfunctional neurons, a compelling therapeutic strategy is cell replacement, i.e., introducing neural progenitors into the gut. Over the past two decades, research has increasingly focused on stem cell transplantation to repair the ENS (Burns et al., 2016). The original concept is to supply the aganglionic or bowel segment with new ENCCs that can engraft, migrate, and differentiate into the neurons and glia needed for normal gut function. Potential donor cell sources include gut-derived ENSCs/ENCCs (autologous or donor-derived) and pluripotent stem cell-derived ENS progenitors (Jevans et al., 2024). Gut-derived cells are attractive for autologous approaches but may vary with age, disease state, and yield. In contrast, human pluripotent stem cell (hPSC)-derived progenitors offer scalability and standardization but require stringent control of identity, maturation state, and safety (Stamp and Young, 2017). Proof-of-principle for this approach has come from preclinical models. Key efficacy benchmarks include robust engraftment, extensive migration/coverage of the target segment, differentiation into appropriate neuronal and glial lineages, and functional integration demonstrated by synaptic connectivity and improved motility assays (e.g., transit, contractility). Durable benefits and absence of aberrant growth are essential for translation (Hotta et al., 2013). For example, transplanting enteric neural stem/progenitor cells into aneural embryonic gut segments can generate new ganglia (Cheng et al., 2017). Human

pluripotent stem cell-derived enteric neural progenitors were transplanted into an aganglionic mouse colon, where they engrafted, differentiated, and improved functional outcomes (Fattahi et al., 2016; Majd et al., 2025). In parallel, gut-derived enteric neural progenitors, when expanded as neurospheres, can generate functional enteric neurons in the postnatal colon following transplantation (Hotta et al., 2013). Similarly, ENSC transplants in a mouse model of Hirschsprung's disease have been shown to partially restore gut motility (Rahman et al., 2024). These studies demonstrate the potential of cell therapy to address the root cause of enteric neuropathies by repopulating the enteric plexuses (Wilkinson et al., 2015; Montalva et al., 2023). Significant translational challenges include controlling graft identity and subtype output (e.g., ensuring appropriate inhibitory/excitatory balance), avoiding ectopic clustered growth, and addressing immune compatibility. For hPSCs, eliminating undifferentiated cells and establishing tumor-safety testing are central requirements (Liang et al., 2018; Ohkura et al., 2024). Long-term studies are also needed to assess the stability of graft-derived circuits and potential adverse remodeling (Stamp and Young, 2017).

A key question for the stem cell therapies is the optimal form for delivering cells. Neurospheres represent attractive candidates for ENS cell delivery. These *in vitro* structures are free-floating three-dimensional clusters of neural progenitor cells that have emerged as a promising vehicle for ENS transplantation (McCann et al., 2017). Neurospheres can be derived from enteric neural crest cells or dissociated ENS cells. In culture, cells form aggregates that can differentiate into enteric neurons and glia. Neurospheres can be heterogeneous structures and may contain non-neural cells and extracellular matrix, which limits dispersal and leads to localized engraftment rather than long-range repopulation. Optimizing sphere composition and the host microenvironment, therefore, is critical to achieve broad colonization of transplanted bowel segments (McKeown et al., 2017; Stavely et al., 2021). Neurospheres can be considered as pre-organized ganglion-like units that may integrate more rapidly into the host gut wall than an injected cell suspension (Burns et al., 2016). Neurospheres offer practical advantages for transplantation because their three-dimensional structure allows controlled delivery to specific regions of the bowel wall. Current experimental approaches include direct intramural injection, endoscopic delivery, and serosal application, all of which are being explored as potential strategies for future ENS

cell-replacement therapy. Each has trade-offs in targeting accuracy, spread, and feasibility of repeat dosing. Route and timing likely influence migration because the postnatal bowel is less permissive than embryonic tissue (Hotta et al., 2013; Hotta et al., 2023; Ohkura et al., 2024; Mueller et al., 2024). Early studies indicate that neurosphere-derived cells can undergo extensive migration and neuronal differentiation after transplantation, although efficiency varies (Almond et al., 2007; Hotta et al., 2013; McKeown et al., 2017; Mueller et al., 2024; Nagy et al., 2021).

1.6. Development of enteric neurosphere culture and transplantation approaches

The concept of culturing enteric neural stem cells into “neurospheres” was adapted from protocols for central nervous system stem cells to the ENS in the mid-2000s (Schäfer et al., 2009). Postnatal mouse gut-derived ENS progenitors were expanded *in vitro*, *engrafted*, and differentiated after transplantation into the recipient gut (Hotta et al., 2013). Earlier studies had already defined the main experimental criteria for identifying and testing enteric neural stem/progenitor cells. These criteria included whether the cells could form spheres in culture, give rise to multiple cell types, and integrate into tissue after transplantation (Almond et al., 2007). Metzger et al. (2009) achieved a significant milestone by isolating and expanding ENS progenitors from adult human intestine as neurosphere-like “enterospheres” (Metzger et al., 2009). In their pioneering study, small pieces of adult human gut tissue (both small and large intestine) were dissociated and cultured in stem cell media with growth factors, yielding floating “enterospheres” of proliferative cells. Enteric sphere cultures are typically supported by mitogens (commonly EGF and FGF2), with ENS-relevant cues, such as GDNF, used to promote survival, proliferation, and neuronal differentiation. It was also demonstrated that human enteric neurospheres could be passaged several times and induced to differentiate into neurons and glia. Importantly, when neurosphere-derived cells were implanted into embryonic gut explants or organotypic cultures, they integrated into the tissue and differentiated into multiple enteric lineages. This work provided the first proof that the adult human ENS harbors tissue stem cells capable of forming neurospheres and generating functional enteric neurons. Multiple groups demonstrated enteric neurosphere formation from various sources: fetal gut, postnatal pediatric gut, and even colonoscopic biopsies from

adults (Almond et al., 2007; Belkind-Gerson et al., 2013; Bondurand et al., 2003; Cheng et al., 2017; Hetz et al., 2014; Krishnamohan, 2013; Lindley et al., 2008; Metzger, Caldwell, et al., 2009; Rauch et al., 2006). Demonstrating sphere generation from minimally invasive sources (e.g., mucosal tissue/biopsies) was an essential translational step because it suggested a feasible route toward patient-derived cell sourcing and expansion (Belkind-Gerson et al., 2013). For example, Hetz et al. and others showed that neurosphere-like bodies derived from postnatal/adult bowel contain neural crest-derived progenitors that can engraft and generate neurons following transplantation, although outcomes vary with donor source and assay context (Cheng et al., 2017; Hetz et al., 2014; Hotta et al., 2013). In 2011, the paradigm of “enteric neural stem cells in a dish” was well established, with consistent protocols to expand ENS progenitors as free-floating spheroids (often called enteric neurosphere-like bodies, NLBs). Enteric neurospheres, therefore, became an important *in vitro* model for investigating enteric neurogenesis, progenitor differentiation, and cell-based transplantation approaches. (Burns et al., 2016). As the field advanced, greater attention was given to standardizing terminology, cell isolation procedures, culture conditions, and outcome measures, leading to expert recommendations for ENS stem cell preparation and for assessing transplantation success (Burns et al., 2016). Notably, comparative studies revealed that enteric neurosphere cells retained key properties of the ENS: they expressed typical neuronal and glial markers and could give rise to multiple neuronal subtypes. Newgreen and colleagues also noted that ENS-forming efficiency declined with the donor's age (Zhang et al., 2019).

More recently, researchers have derived enteric neural progenitors from human pluripotent stem cells and organized them into neurosphere structures for transplantation studies. These hPSC-derived neurospheres, when introduced into the colon of ENS-deficient mice, populated the gut and rescued functional deficits, heralding a possible cell therapy for Hirschsprung disease (Barber et al., 2019; Fattahi et al., 2016; Majd et al., 2025). Thus, in less than a decade, the ENS field went from the first isolation of enteric neurospheres to demonstrating therapeutic potential in animal models.

1.7. Methods and limitations of the enteric neurosphere technique

Unlike neurospheres from the central nervous system (which may consist mainly of neural stem cells), enteric neurospheres contain a mix of cell types. Both animal and human ENS spheres comprise neurons and glia (derived from neural crest), as well as other supporting cells such as fibroblasts, smooth muscle cells, and immune cells, in varying proportions. In other words, when gut tissue is cultured into neurospheres, not only do the neural crest-derived progenitors grow out, but also bystander cells from the gut wall become incorporated (Stavely et al., 2023). This can lead to a “spheroid” that includes a stromal core of non-neuronal cells. For example, recent analysis of postnatal human enteric neurospheres revealed neurons (TUJ1+) and enteric glia (GFAP+), reflecting the diverse cellular milieu of the gut (Navoly and McCann, 2021). The presence of mesenchymal cells means that neurospheres display a dense core of collagen-rich tissue produced by resident fibroblasts, which can influence the sphere’s structural integrity and the behavior of neural crest within it. This composition can limit their ability to disperse into host tissue after transplantation (Navoly and McCann, 2021). A significant limitation of the “neurosphere assay” is that sphere formation itself is not specific to neural crest/ENS cells; non-neural crest gut populations can also generate morphologically similar spheres. For this reason, many groups enrich ENS progenitors before sphere formation or prior to transplantation using markers such as p75 (human) or lineage reporters/markers in mice, to minimize accidental transplantation of non-therapeutic mesenchymal/ICC/smooth muscle populations (Binder et al., 2015). Thus, understanding and optimizing the cellular content of neurospheres is crucial. Transplantation of neurospheres into aganglionic gut has also revealed several important challenges. The success of these experiments is typically evaluated by assessing engraftment efficiency, migration distance, and coverage along the bowel, the balance between neuronal and glial differentiation, neuronal subtype specification, such as the generation of nitrergic motor neurons, and functional integration using motility assays and/or electrophysiological analyses. Ideally, donor-derived cells should exit the neurosphere, migrate extensively through the recipient gut wall, and contribute to the formation of functional enteric circuits. However, incomplete migration remains a major limitation. Donor cells often remain close

to the transplantation site, resulting in localized reinnervation rather than complete colonization of the aganglionic segment (Jevans et al., 2024; Majd et al., 2025; Mueller et al., 2024; W. Pan et al., 2024; Rahman et al., 2024). This limited migration may reflect the reduced permissiveness of the postnatal or diseased intestinal microenvironment. Contributing factors include the absence or reduced availability of developmental chemoattractants, the presence of inhibitory extracellular matrix components, and inflammation or fibrosis, which may create physical barriers to cell migration. In addition, immune compatibility, including differences between allogeneic and autologous grafts, as well as local inflammatory responses, can influence donor cell survival, differentiation, and integration (Kang et al., 2021; Mueller et al., 2024; Ohkura et al., 2024; W. Pan et al., 2024; Yu et al., 2024). These variables differ substantially between embryonic gut explants, immunodeficient mouse models, and clinically relevant postnatal bowel tissues (Navoly & McCann, 2021). Host tissue age is also an important determinant, as embryonic gut provides a more migration-permissive environment enriched in pro-migratory cues, such as GDNF and NCAM. In contrast, older tissue represents a less supportive niche for donor cell colonization (Nagy et al., 2020).

Recently, we demonstrated that adding exogenous GDNF to embryonic chick hindgut induces the formation of “ganglioneuromas” that can robustly populate an aneural hindgut upon transplantation. In the E6-E8 chick gut, high GDNF concentrations induced clusters of TUJ1+ neurons mixed with SOX10+ precursors to form on the gut surface, and when these clusters were transplanted into an aganglionic hindgut, they gave rise to an organized ENS network. However, the same GDNF stimulus applied to older gut (E9 chick) failed to generate such aggregates, reflecting a developmental decline in responsiveness. This suggests that timing and microenvironment are key factors for neurosphere transplantation: embryonic environment supports long-range ENS migration, whereas postnatal environments limit their spread within the gut wall (Nagy et al., 2020; W. Pan et al., 2024; Rahman et al., 2024).

Enteric neurosphere cultures have been established from several species, primarily rodents and humans. Mouse and rat intestines are commonly used due to their availability and the wealth of genetic tools in these models (Hotta, Cheng, Graham, Pan, Nagy, Belkind-Gerson,

et al., 2016). Human enteric neurospheres have been generated from all stages of life, fetal gut, neonatal surgical specimens, and adult intestinal biopsies, demonstrating the presence of ENS progenitors even in adult tissue (Binder et al., 2015). Multiple studies have shown that enteric neural stem cells (ENSCs), stem/progenitor cells prospectively isolated from the embryonic or postnatal enteric nervous system (ENS) of humans, mice, rabbits, and pigs, can be obtained by dissociating intestinal neuronal plexuses, then cultured and expanded *in vitro*, and transplanted into animal models of enteric neurocristopathies (Lindley et al., 2008; Metzger et al., 2009; Raghavan et al., 2013; Cheng et al., 2017; Hotta et al., 2016, 2023). In contrast, avian models (chick and quail), which are classic for developmental studies for neural crest and enteric nervous system, were for a long time not exploited for neurosphere cultures. Despite major advances in ENS progenitor culture and transplantation, efficient long-range migration and integration of donor-derived ENCCs remain limited, particularly in mature or less permissive intestinal environments. This thesis addresses this problem by using the avian gut as a developmental and transplantation model to investigate how neurosphere-like ENS progenitor aggregates interact with the intestinal microenvironment.

2. OBJECTIVES:

Enteric neurospheres generated from ENCCs have been shown to represent a promising stem cell source for regenerative therapy in HSCR. However, several challenges still need to be overcome before their successful application in stem cell-based therapy. Main limitations of neurosphere-based repair of the enteric nervous system include variable neurosphere composition and the microenvironment's poor permissiveness for long-range migration and integration. This project addresses both barriers by developing a model that generates more migration-competent enteric progenitors and modifies the neurosphere niche to better support their dispersal and incorporation into enteric networks.

2.1. Specific Aims:

2.1.1. **Specific Aim 1:** Establish novel avian-derived enteric neurosphere cultures by combining morphogens involved in the embryonic development of ENCCs, and characterize their progenitor identity, neuronal and glial differentiation potential, and migratory capacity after *ex vivo* colon transplantation.

2.1.2. **Specific Aim 2:** Modulate the neurosphere's extracellular matrix composition to increase cell dispersal and integration, and assess effects on migration distance, tissue coverage, lineage differentiation, and functional network formation.

3. MATERIALS AND METHODS

3.1. Ethics statement and experimental animals

3.1.1. Chicken embryos

White Leghorn-type specific-pathogen-free (SPF) chicken eggs (*Gallus domesticus*) were obtained from a commercial breeder (Biovo Ltd., Mohacs, Hungary). Transgenic mCherry chicken eggs were obtained from the Institute of Molecular Genetics of the Czech Academy of Sciences (Trefil et al., 2017). Eggs were incubated at 37.5 °C in a humidified hatching incubator (Heka Brutgerate, TS-7000C). Embryonic age was determined by embryonic day (E) and, where indicated, Hamburger-Hamilton (HH) staging. All chicken experiments were conducted in accordance with Semmelweis University Institutional Animal Care and Use Committee guidelines (nr.: 70/2012).

3.1.2. Mice

Mouse experiments were performed according to protocols approved by the Institutional Animal Care and Use Committees of Massachusetts General Hospital. *Wnt1*Cre/+ mice (B6.Cg-H2az2Tg(*Wnt1*-cre)11Rth Tg(*Wnt1*-GAL4)11Rth/J; Jackson Laboratory Stock #009107), R26R-tdTomato reporter mice (B6.Cg-Gt(*ROSA*)26Sortm14(*CAG*-tdTomato)Hze/J; Stock #007914), C57BL/6J mice (Stock #000664), and B6;129-*Ednrb*tm1Ywa/J mice (Stock #003295) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). *Wnt1*Cre/+ mice were crossed with R26R-tdT reporter mice to generate *Wnt1*-Cre+; R26-tdT mice (*Wnt1*;tdT). *Ednrb* heterozygotes were bred to generate *Ednrb* knockout mice (*Ednrb*-/-), which exhibit distal colonic aganglionosis. All mice were housed and bred under specific pathogen-free conditions at the Center for Comparative Medicine animal facility at Massachusetts General Hospital.

3.2. Generation and culture of enteric neurospheres

3.2.1. Chicken hindgut-derived neurospheres

Low-adherence tissue culture plates were prepared by coating 6-well cell culture dishes (Thermo Scientific, 140675) with 2 mL Anti-Adherence Rinsing Solution (STEMCELL Technologies, 07010) for 1 h at room temperature with gentle rocking (80 rpm). The colorectum of E12 and day-of-hatching (D0) chicken embryos was dissected under sterile

conditions, washed with PBS containing Penicillin-Streptomycin (100 µg/mL; Gibco, 15140122), and digested in 3 mL Digestion Enzyme Mix consisting of dispase II (5 U/mL; STEMCELL Technologies, 07913) and collagenase IX (2.5 mg; 800 U/mg; Sigma, C9722-50MG) dissolved in NeuroCult™ Basal Medium (NBM; STEMCELL Technologies, 05700). Digestion was performed for 45 min at 37.5 °C with gentle vortexing three times during incubation.

Following digestion, the cell suspension was filtered through a 40-µm cell strainer (Greiner, 542040) and washed with NBM to a final volume of 20 mL to stop enzymatic activity. Cells were centrifuged at $500 \times g$ for 8 min, the pellet was resuspended in growth-factor-supplemented medium, and cells were seeded at 6×10^5 cells/mL into prepared low-adherence plates. Neurospheres were cultured for 4-7 days at 37 °C and 5% CO₂ with 1:1 medium changes every second day.

3.2.2. GWEN supplementation to improve neurosphere quality

To model the contribution of cecum-associated mesenchymal cues and to improve neurosphere growth and lineage output, cultures were supplemented with a GWEN cocktail consisting of GDNF (10 ng/mL; R&D Systems, 212-GD-010), WNT11 (200 ng/mL; OriGene, TP761904), endothelin-3 (ET-3; 250 ng/mL; R&D Systems, 1162/100U), and Noggin (100 ng/mL; R&D Systems, 6997-NG-025), in addition to the base neurosphere medium (NBM + NeuroCult™ Proliferation Supplement (Mouse and Rat; STEMCELL Technologies, 05701), EGF 40 ng/mL (STEMCELL Technologies, 78006.1), bFGF 20 ng/mL (STEMCELL Technologies, 78003.1), and heparin 4.44 µg/mL (STEMCELL Technologies, 07980).

3.2.3. EdU labeling in chicken neurospheres

For proliferation assessment, EdU (10 µM; BaseClick) was added 5 h prior to fixation in 4% paraformaldehyde (PFA). EdU incorporation was detected using the Click-iT-based EdU Imaging Kit (BaseClick, BCK-EdU488IM100) according to the manufacturer's instructions.

3.2.4. Mouse enteric neurospheres

Mice were euthanized either at E11.5 or 3 weeks old, and the small intestine was carefully dissected and digested in dispase and collagenase XI, ensuring precise tissue processing for reliable results.

Neuroproliferation medium contained 1% penicillin/streptomycin (Thermo Fisher Scientific), B27 (1×; Gibco), N-2 (1×; Gibco), bFGF (20 ng/mL; STEMCELL Technologies), IGF-1 (20 ng/mL; Thermo Fisher Scientific), retinoic acid (75 ng/mL; Sigma-Aldrich), and 2-mercaptoethanol (50 μmol/L; Gibco) in equal parts NeuroCult Mouse Basal Medium (STEMCELL Technologies) and DMEM (Gibco). Primary neurospheres were cultured for 10 days at 37 °C and 5% CO₂.

3.3. Preparation of mouse neurospheres for migration and transplantation assays

3.3.1. Dissociation and fluorescence-activated cell sorting (FACS)

Primary mouse neurospheres were dissociated for 45 min using Accutase (STEMCELL Technologies) to generate a single-cell suspension, filtered through a 40-μm strainer, and stained with DAPI to exclude non-viable cells. FACS was performed using BD FACS Aria instruments (Harvard Stem Cell Institute Center for Regenerative Medicine Flow Cytometry Core, Massachusetts General Hospital). Cells were sorted for tdTomato positivity (*Wnt1* lineage) and DAPI negativity (live cells). Approximately 30% of cells were live and tdTomato-positive. Sorted tdT⁺ cells were manually counted with Trypan blue to determine yield and viability.

For reaggregation, tdT⁺ cells were plated into 96-well round-bottom ultra-low attachment plates (Corning) at 25,000 cells/mL for *ex vivo* and *in vivo* transplantation studies, and at 50,000 cells/mL for *in vitro* assays. Unless otherwise stated, reaggregated neurospheres were allowed to form for 72 h before downstream assays.

3.3.2. Agrin knockdown by lentiviral transduction

Immediately after sorting, tdT⁺ cells were transduced with either an agrin shRNA lentivirus (OriGene, TL512313V; 4 unique 29-mer agrin-targeting shRNAs; GFP reporter) or a scramble control shRNA lentivirus (OriGene, TR30021V; GFP reporter). Viruses were expanded and packaged at the MGH Vector Core Facility to a final concentration of 10¹² TU/mL. After testing multiple multiplicities of infection (MOI), an MOI of 90 was used for both viruses. Typical transduction efficiencies were 50-60% for agrin shRNA and 25-30% for scramble controls, with no overt toxicity observed. Cells were transduced for 72 h before assay or transplantation.

3.4. *In vitro* migration assays

3.4.1. Chicken neurosphere migration and neurite extension assay

To assess ENCDC migration from chicken neurospheres, cell aggregates were seeded onto Petri dishes coated with fibronectin (20 µg/mL; Thermo Fisher Scientific, 33010018) diluted in DMEM containing 1% Penicillin-Streptomycin. Emigration was stimulated using GDNF (10 ng/mL) diluted in DMEM and monitored for 24 h. Cultures were fixed in 4% PFA for 20 min, washed in PBS, and processed for immunocytochemistry.

Migration and neurite extension were quantified from TUJ1 and HNK1 immunostaining by measuring the longest neurite projection and/or the most distant cells from the spheroid border. Five measurements were recorded per spheroid (n = 15 spheroids total; 3 spheroids per group).

3.4.2. Mouse neurosphere migration, proliferation, and differentiation assays

For standardized reaggregation assays, sorted tdT⁺ cells were plated at 50,000 cells/mL (migration assay) or 12,500 cells/mL (proliferation/differentiation assay) in 96-well ultra-low attachment plates and cultured for 72 h, generating uniform neurospheres of ~10,000 cells (migration) or ~2,500 cells (proliferation/differentiation).

For migration assays, individual neurospheres were transferred to fibronectin-coated dishes (1:200 in PBS; Sigma-Aldrich) and cultured for 72 h in either DMEM alone or DMEM containing an agrin function-blocking antibody (20 µg/mL; MAB5204; Millipore). Migration area was quantified in ImageJ (v1.53t) by binary thresholding of tdT⁺ fluorescence to define the total area of cell spread.

Because differentiated preparations contain variable numbers of cell types, cell-type-specific migration was assessed using the octant method. Briefly, each image was divided into eight octants, and the distance from the neurosphere center to the farthest labeled cell in each octant was measured; the mean of the eight distances was used as the migration distance for that preparation.

3.4.3. Proliferation and differentiation readouts

For proliferation, EdU incorporation was assessed by counting tdT⁺ cells co-staining for EdU. For differentiation, tdT⁺ cells co-staining for glial (GFAP) or neuronal markers

(Calretinin, nNOS) were quantified in immunostained preparations (see Immunocytochemistry).

3.5. Organ culture and transplantation models

3.5.1. Chick-chick tissue recombination in collagen gel

For chick-mCherry chick recombination, E5 (HH25) chick midgut/hindgut was dissected from the ceca to the distal cloaca. The nerve of Remak and cloaca were removed. Proximal hindgut was recombined with mid-colon isolated from E12 and D0 mCherry chicken embryos while maintaining proximal-distal orientation. Recombinants were placed onto the surface of a collagen gel matrix. After 12 hours to allow for adhesion, a second collagen gel layer was applied to embed the tissues fully, and cultures were maintained for 72 hours.

3.5.2. Chicken neurosphere transplantation to the aganglionic chick hindgut and CAM culture

As described previously (Nagy et al., 2020), neurospheres were transplanted into the isolated preganglionic ceca + hindgut of E5 chick embryos. One neurosphere was placed into each of the two cecal buds. Recombinants were placed on a collagen gel matrix prepared from DMEM/F-12 (GlutaMAX™; Thermo Fisher Scientific, 10565018) supplemented with 1% Penicillin-Streptomycin and containing 1 mg/mL type I rat tail collagen (BD Biosciences, 354236), neutralized with 1 N NaOH. After 12 hours to allow integration, recombinants were transferred to the chorioallantoic membrane (CAM) of E9 host chick embryos and cultured for 8 days (n = 12 per group). They were then fixed and processed for immunofluorescence.

3.5.3. Chick-mouse chimeric CAM transplantation

To test cross-species integration and migration, preganglionic hindgut grafts were prepared from E5 (HH25) chick embryos by removing the cloacal region and the nerve of Remak using tungsten needles. Under stereomicroscope guidance, two *Wnt1*;tdT mouse-derived neurospheres (embryonic or adult origin) were implanted into each cecum. Recombinants were grafted onto the CAM of E9 (HH35) host chick embryos and cultured for 8 days. Explants were excised, fixed overnight in 4% PFA at 4 °C, embedded for cryosectioning, and the migration distance was assessed by identifying the farthest tdT⁺ cell away from the implantation site across multiple sections.

3.5.4. *Ex vivo* and *in vivo* transplantation in the mouse colon

Ex vivo transplants were performed onto colonic LMMP peeled from adult C57BL/6J mice (4-12 weeks) or 2-week-old *Ednrb*^{-/-} mice. After CO₂ asphyxiation, the colon was removed, opened longitudinally, washed in PBS, and the LMMP was peeled from the lamina propria under a dissecting microscope and pinned into a Sylgard-coated dish. For *Ednrb*^{-/-} mice, only the distal 2 cm was used to ensure the presence of aganglionosis. A small pocket was created in the muscularis propria, and a single sorted and transduced neurosphere was placed into the pocket. Tissues were cultured for 7 days in DMEM containing 5% FBS (Sigma-Aldrich) and 1% antibiotic-antimycotic (anti-anti; Gibco), then fixed for 4-6 h in 4% PFA and imaged.

For *in vivo* transplants, adult C57BL/6J mice were anesthetized with inhaled isoflurane (1-4%). After mini-laparotomy, the distal colon was exposed, and two subserosal pockets were created ~0.5 cm apart. One previously sorted and transduced neurosphere was placed in each pocket. Mice were recovered per protocol and euthanized by CO₂ asphyxiation 7 days after transplantation for tissue processing.

3.6. Tissue processing, immunostaining, and imaging

3.6.1. Cryosection preparation

For cryosections, tissues were fixed in 4% PFA for 1 h at room temperature, infiltrated with 15% sucrose overnight at 4 °C, then incubated in 7.5% gelatin (Sigma, G2625) in 15% sucrose for 1 h at 37 °C. Gelatin-impregnated tissues were rapidly frozen at -50 °C in 2-methylbutane (Sigma, M32631), and 12-μm cryosections were collected on poly-L-lysine-coated slides (Sigma, P8920).

3.6.2. Immunofluorescence on tissue sections

Frozen sections were rehydrated in PBS for 10 min at 37 °C and permeabilized in 0.1% Triton X-100 (Sigma, X100-5ML) in PBS. Sections were incubated with primary antibodies diluted in 1% PBS-BSA for 60 min (Table 1) in a humid chamber at room temperature. Alexa Fluor-conjugated secondary antibodies were applied according to host species and isotype (typically 1:200 in PBS for 45 min). For double labeling, sequential primary/secondary incubations were performed using isotype-specific secondary antibodies. Nuclei were counterstained with DAPI (Invitrogen, D1306), and sections were mounted using aqueous Poly-Aqua Mount (Polysciences) and stored at 4 °C.

Primary antibodies used are summarized in Table 1.

Antigen	Cell type/lineage	Catalog number	Source	Isotype
SOX10	Neural crest/glia	AF2864	Bio-Techne	Polyclonal goat IgG
S100B	Glia	RB087A1	NeoMarkers	Polyclonal rabbit IgG
Agrin	heparan sulfate proteoglycan	AF550	R&D Systems	Polyclonal Goat IgG
GFAP	Glia	ab53554	Abcam	Polyclonal Goat IgG
Calretinin	Neuron	18-0211	Invitrogen	Polyclonal rabbit IgG
nNOS	Neuron	61-7000	Invitrogen	Polyclonal rabbit IgG
B-FABP	Glia	(gift)	Thomas Müller (reported in Kurtz et al., 1994)	Polyclonal rabbit IgG
PHOX2B	Neural crest/neuron	AF4940	R&D Systems	Polyclonal goat IgG
HU (HuC/D)	Neuron	A-21271	Thermo Fisher Scientific	Mouse IgG2b
TUJ1 (β III-tubulin)	Neuron	SC-80016	Santa Cruz	Mouse IgG2a
HNK1	Neural crest-derived cell	MS-1163-P	Thermo Fisher Scientific	Mouse IgM
Desmin	Mesenchymal cell	MA5-13259	Acris (Germany)	Mouse IgG1

Collagen I	Mesenchymal cell	(gift)	Thomas F. Linsenmayer (Swasdison et al., 1992)	Mouse IgG2a
Hsp47	Mesenchymal cell	386023	Sigma	Mouse IgG2a
Calponin	Smooth muscle cell	CP-93	Sigma	Mouse IgG1

3.6.3. Whole-mount immunofluorescence of the mouse colon

For whole-mount staining, colons were fixed overnight in 4% PFA at 4 °C, permeabilized overnight at 4 °C in PBS containing 1% normal goat serum and 0.1% Triton X-100 (Sigma-Aldrich, 9036-19-5), then incubated overnight at 4 °C with anti-Agrin (1:50; R&D Systems, AF550) or Alexa Fluor 647-conjugated anti- β III-tubulin (Tuj1; 1:400; BioLegend). After PBS washes, Alexa Fluor 488 donkey anti-goat secondary antibody (1:100; Thermo Fisher Scientific, A11055) was applied for 1 h. Whole mounts were imaged on a Nikon SMZ25 fluorescence stereomicroscope equipped with a Prior L200/E unit.

3.6.4. Immunocytochemistry of cultured cells and neurosphere outgrowth

For staining of cultured cells, cells were fixed for 30 min in 4% PFA at 4 °C, permeabilized in 0.1% Triton X-100 for 20 min, washed in PBS, and blocked with 10% donkey serum for 1 h at room temperature. Primary antibodies (GFAP 1:500, Abcam ab53544; Calretinin 1:200, Invitrogen 18-0211; nNOS 1:400, Invitrogen 61-7000) were diluted in 10% donkey serum and incubated overnight at 4 °C. Secondary antibodies (donkey anti-goat IgG Alexa Fluor 555, 1:200; donkey anti-rabbit IgG Alexa Fluor 488, 1:200; Invitrogen) were applied for 2 h at room temperature. Nuclei were counterstained with DAPI. Images were obtained with a Keyence BZX-700 microscope and analyzed in ImageJ.

3.7. Microscopy and image processing

Fluorescence images were acquired using Nikon Eclipse E800 and Zeiss Axiovert 135 microscopes, and confocal images were acquired using Zeiss LSM 710/780/900 and Olympus FV3000/FV4000 systems, as appropriate for the experiment. Image processing

(including tiling, channel merging, and pseudocoloring) utilized manufacturer software (CellSens, ZEISS ZEN, NIS-Elements v5.02) and ImageJ (v1.53t/1.54c). Figure assembly and minor adjustments were performed in Adobe Photoshop CC 2020 when required.

3.8. Quantification and computational image analysis

3.8.1. Automated DAPI/Hu quantification of neurospheres

DAPI/Hu double-stained confocal images were analyzed using a custom Fiji macro (ImageJ 1.54c; Java 1.8.0_322). Neurospheres were identified as regions of interest (ROIs) by segmenting the Hu channel with inversion, thresholding, morphological filtering (20-30 px radius), and watershed separation, followed by particle counting (1,000-50,000 px²; circularity 0.5-1.0) and manual review. Within each ROI, nuclei were segmented from the DAPI channel using background subtraction (rolling = 30 px), thresholding (0-13 arbitrary units (a.u.)), median filtering (radius = 1 px), watershed separation, and particle counting (10-1,000 px²; circularity 0.3-1.0). Hu positivity was defined as the mean Hu-channel intensity greater than 10 a.u. Within each nucleus, counts were exported per neurosphere, and ROI/overlay images were saved for quality control.

3.8.2. Multichannel single-cell marker classification within neurospheres

A modified macro was used for multichannel quantification (PHOX2B, SOX10, and EdU). ROIs were drawn manually using the PHOX2B (green) channel. Images were background-subtracted (rolling = 30 px) and converted to 8-bit. DAPI+ nuclei were segmented by thresholding (0-50 a.u.), binarization, and watershed separation, retaining particles 0.001-1,000 px² (circularity 0.1-1.0). Mean nuclear fluorescence intensity was measured in PHOX2B (green), SOX10 (red), and EdU (far-red) channels, with thresholds set at 10 a.u. (green and far-red) and 30 a.u. (red) were used to classify nuclei as positive or negative. Results were exported to CSV, and overlay images were saved for quality control.

3.8.3. Statistical analysis

Statistical analyses were performed in GraphPad Prism (v10.6.1) and, where indicated, RStudio. Depending on the data distribution and experimental design, group comparisons were performed using either Kruskal-Wallis tests or one-way ANOVA with Dunn's or Tukey's post hoc tests, respectively. Pairwise comparisons were conducted using Mann-Whitney tests. For migration studies performed across multiple experimental batches,

unpaired two-sample t-tests were used when a single batch was analyzed, or two-way ANOVA was used to account for batch effects when multiple experiments were included. The Holm correction was applied when adjusting for multiple testing in multiple-comparison analyses. Statistical significance was defined as $p < 0.05$; error bars represent SEM.

3.9. Single-cell RNA-seq reanalysis

Published datasets by [Morarach et al., \(2021\)](#) and [Guyer et al., \(2023\)](#) were reanalyzed. Mouse embryonic datasets (E15.5 and E18.5) were downloaded from NCBI SRA (runs SRR11635571, SRR11635572, SRR11635573) using SRA Toolkit fastq-dump. Alignment and feature-barcode matrix generation were performed with Cell Ranger (cellranger count) on the Mass General Brigham ERISOne Research Computing Cluster. Downstream analysis was conducted in R (RStudio 2023.03.0+386) using the Seurat package. Quality control filtering excluded cells with gene counts >1 SD from the mean and cells with $>10\%$ mitochondrial reads. Embryonic progenitors were selected based on high Sox10 expression (clusters 0, 2, 5, 9). Postnatal neurosphere progenitor cells were selected based on high Sox10 and Gfap expression (clusters 0, 1, 2, 3, 4, 6, 8, 14). Datasets were integrated using FindIntegrationAnchors, followed by PCA, neighbor finding, UMAP dimensional reduction (first 30 PCs), and Louvain clustering (FindClusters, resolution 0.5). Differential gene expression used DESeq2; genes with log2 fold change >0.5 , percent expression $>25\%$, and adjusted $p < 0.001$ were considered significant and compared to the Gene Ontology ECM gene set (GO:0031012; 530 genes).

4. RESULTS

4.1. Cecal growth factors promote enteric neurosphere formation and hindgut colonization in the avian model

4.1.1. Distribution of SOX10+/PHOX2B+ enteric neural crest-derived cells (ENCDC) during development of the chicken hindgut.

In order to characterize the developmental dynamics of undifferentiated enteric neural crest-derived cells (ENCDCs) during avian hindgut ENS formation, we performed triple-immunofluorescence analysis of developing chicken hindgut using SOX10 and PHOX2B transcription factor-specific antibodies as ENCDC lineage markers, combined with either anti-B-FABP (brain-fatty acid binding protein) or Hu-specific (neuronal RNA-binding protein) antibodies to identify the early enteric glia and neurons, respectively (Fig. 4).

At E8, immunostaining of consecutive transverse cryosections of the hindgut revealed numerous SOX10+ ENCDCs within the developing enteric plexus, which co-express PHOX2B antigen (Fig. 4A, A2,A4,A5). A subset of SOX10+ cells co-expressed the cytoplasmic B-FABP antigen, identifying the enteric glial precursors (Fig. 4A1). Hu immunoreactive cells marking the early enteric neuronal cell population are distinct from SOX10+/PHOX2B+ ENCDCs (Fig. 4A3). By E12, SOX10+/PHOX2B+ cells were still present within enteric ganglia, but the Hu+ neuronal population clearly increased (Fig. 4B-B5). B-FABP+ cells are also present, consistent with continued maturation of glial-lineage (Fig. 4B1-B5). At hatching (D0), organization of enteric ganglia were dominated by differentiated compartments: PHOX2B+ neurons (with strong HU co-labeling clustered centrally) and SOX10+/B-FABP+ glial cells positioned peripherally, SOX10+/PHOX2B+ ENCDCs were seen also at the periphery of the ganglia (Fig. 4C1-C5).

Quantitative analysis revealed a developmental decline in the proportion of SOX10+/PHOX2B+ cells with increasing age. This population was most prominent at embryonic stages, with a mean of 17.91 ± 1.262 , and declined progressively by D0 to a mean of 0.7763 ± 0.3933 , indicating that the relative abundance of progenitor-associated ENS-lineage cells decreases significantly over the course of hindgut development ($P < 0.0001$). In parallel, the distribution of lineage markers shifted from populations associated with

immature or progenitor-like ENS states toward more differentiated neuronal and glial populations (diagram not shown).

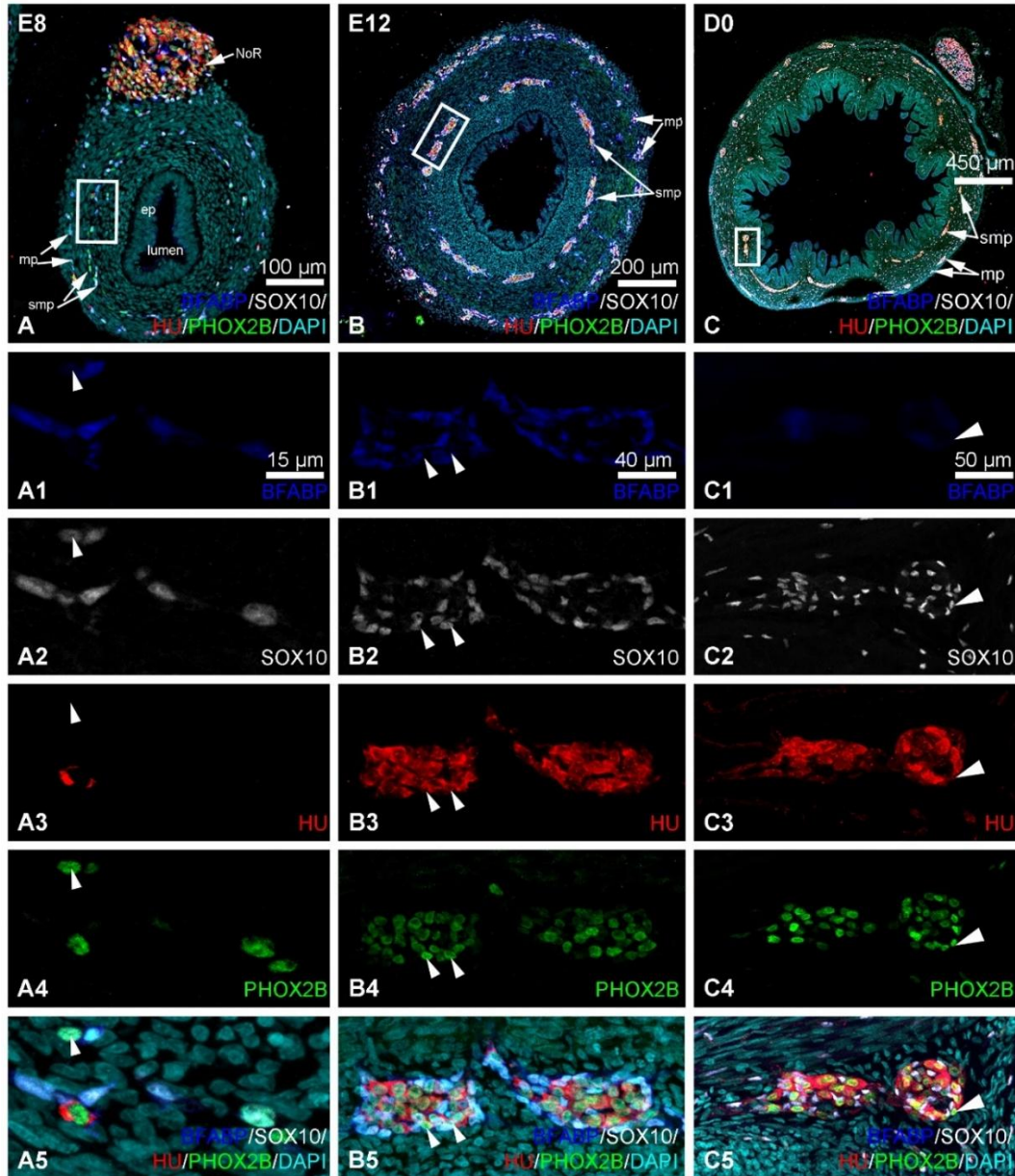


Figure 4. Characterization of developing enteric neural crest-derived cells (ENCDCs) during chicken hindgut development. Representative cross-sections of the chicken hindgut at embryonic day (E)8 (A), E12 (B), and day of hatching (D0; (C). The boxed area in (A) is shown at higher magnification in panels (A1-A5). Separate immunofluorescence channels show B-FABP (blue), SOX10 (white), HU (red), PHOX2B (green), and DAPI (cyan). The merged image illustrates the colocalization of SOX10+/PHOX2B+ ENCDCs within developing enteric ganglia. ENCDCs differentiate into PHOX2B+/HU+ enteric neurons, while B-FABP marks enteric glial precursors (A5-C5).

4.1.2. Stage-dependent ENS-forming capacity in chick gut recombination assays

To test whether ENCDCs from late developmental stages can migrate into an aneural hindgut environment and generate enteric ganglia, we established a novel intestinal recombination organ culture assay where preganglionic E5 wild-type (WT) ceca+hindgut tissue was recombined with either E12 or day 0 (D0) mCherry+ chicken colon and cultured in 3D collagen gel (Fig. 5A). After 12 h of culture, mCherry-derived cells from the E12 colon crossed the recombination border and colonized the WT hindgut. Cells from the D0 colon showed minimal invasion into the hindgut (Fig. 5B,E; arrows in B). Quantification confirmed a significantly greater migration distance of mCherry+ cells into the WT chicken embryo hindgut when mCherry+ recombinants contained E12 colon compared with D0 colon (Fig. 5A'; $P < 0.0001$, $n = 21$, Mann-Whitney test). After 72 hours of culture in 3D collagen gel, the explants were removed, embedded and serial-cryosectioned for immunocytochemistry studies. Double-immunofluorescence analysis demonstrated that E12 mCherry colon-derived cells contributed robustly to ENS formation within the proximal hindgut. In cross-sections, numerous mCherry+/TUJ1+ neurons were detected corresponding to the submucosal and myenteric plexuses (Fig. 5C; inset). High magnification of individual ganglia confirmed that HU+ enteric neurons within the ganglia were mCherry labeled (Fig. 5D). In contrast, recombinants generated using D0 mCherry colon failed to produce mCherry-derived enteric ganglia within the hindgut. Although occasional mCherry+ cells were present, these cells were primarily associated with TUJ1+ nerve fibers (Fig. 5F; arrows) and did not form enteric ganglia. Interestingly, ramified and spindle-shaped mCherry+ cells were disseminated throughout the mesenchyme of the hindgut (Fig. 5G; arrowheads). Double immunofluorescence confirmed that these cells express the differentiated smooth muscle marker calponin (Fig. 5G'; arrowheads), indicating a non-ENCDC origin.

In summary, tissue recombination assays demonstrate that E12 colon contains migratory ENCDC-derived progenitors capable of colonization of aneural hindgut and formation of enteric ganglia. In contrast, D0 colon-derived mCherry+ ENCDCs show markedly reduced colonization capacity and do not contribute to hindgut ENS.

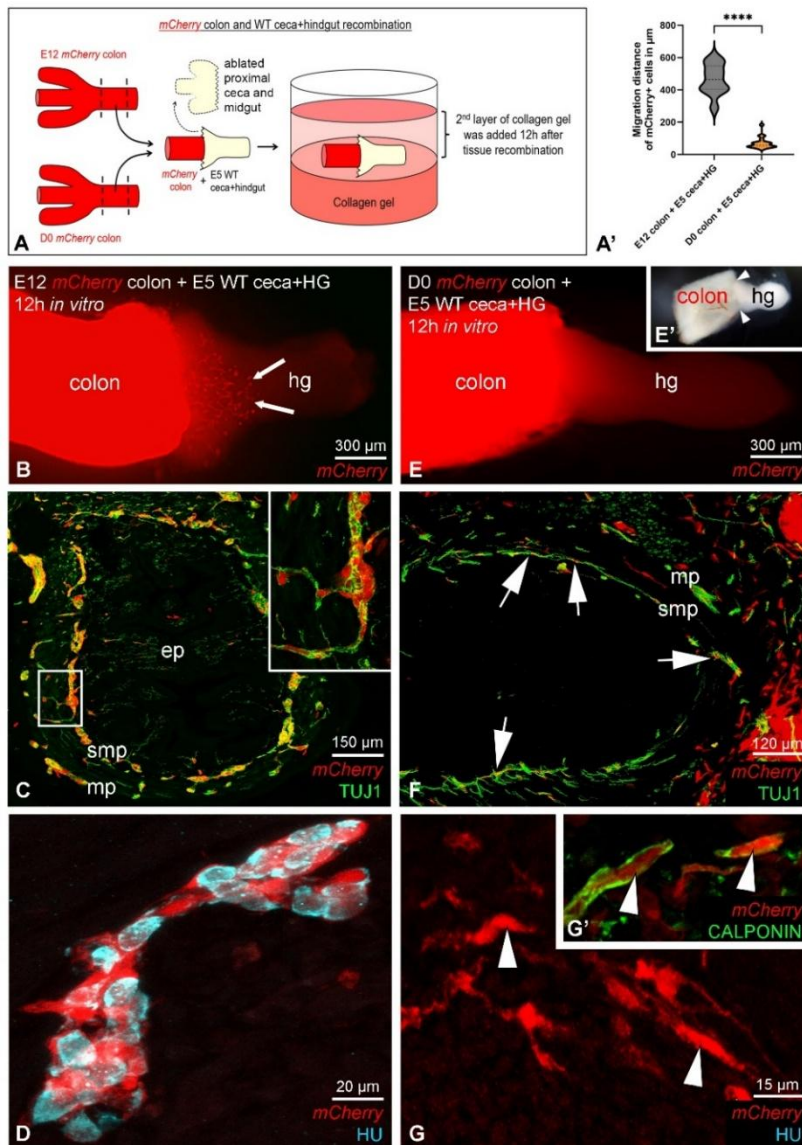


Figure 5. Chick-mCherry chick recombinants confirm the contribution of ENCDCs to the colon ENS.

Schematic illustration for intestinal organ culture assays to test the ENS forming ability of ENCDCs from late developmental stages (A). Quantification of mCherry+ cell migration into the cecal and hindgut regions after 12 h of culture, comparing E12 and D0 colons (A'). $P < 0.0001$, $n = 21$, Mann-Whitney test. Preganglionic E5 chicken hindgut was recombined with E12 (B) and D0 (E) mCherry chicken colon, cultured 72 h in collagen gel, and then examined by immunofluorescence. Brightfield image of the recombinants after 12 h incubation (E'). Arrows show the mCherry+ cells originating from E12 colon (B). The red

fluorescence reveals E12 mCherry chicken colon-derived TUJ1+ neurons in cross-section of the chicken proximal hindgut as they form the submucosal and myenteric plexus of the ENS (C). The outlined mCherry-derived TUJ1+ ganglia are magnified in the inset. Magnified view of a single submucosal ganglion: Immunofluorescence staining confirms that HU+ enteric neurons are of E12 mCherry colon origin (D). Double immunofluorescence with TUJ1 confirms that some of the mCherry+ cells in the chicken hindgut recombined with D0 mCherry colon are associated with the nerve fibers (arrows), but do not contribute to enteric ganglia in the hindgut (F). Few mCherry+ ramified and spindle-shaped cells are scattered throughout the aganglionic proximal hindgut ((G), arrowheads) and express smooth muscle cell specific calponin ((G'), arrowheads). *ep*, epithelium; *hg*, hindgut; *mp*, myenteric plexus; *smp*, submucosal plexus.

4.1.3. Generation of avian enteric neurospheres

To generate enteric neurospheres in avian embryos, dissociated embryonic chick gut cells from E8 transgenic mCherry embryos were cultured under low-adherence conditions for 7 days to promote three-dimensional aggregation and spheroid formation. Three experimental setups were performed. Experiment 1: control medium (CTRL); Experiment 2: culture media supplemented with GDNF, and Experiment 3: culture media supplemented with GDNF, WNT11, endothelin-3, and Noggin (GWEN). Under these *in vitro* conditions, dissociated embryonic gut cells formed free-floating spheroids (Fig. 6B-D) and differentiated to compact three-dimensional aggregates by day 7. These results demonstrate that embryonic chick gut-derived cells can be maintained *in vitro* in a three-dimensional format suitable for downstream phenotypic and functional analyses.

Immunophenotyping revealed that the spheroids contained PHOX2B⁺ cells, indicating retention of neuronal lineage identity within the aggregates (Fig. 6E-G). In addition, TUJ1⁺ fibers were detected within the spheroids, demonstrating that the cultures contained cells undergoing or maintaining neuronal differentiation. These results indicate that avian spheroids are not simply nonspecific aggregates of gut-derived cells, but instead preserved key components of the ENS lineage during spheroid formation. Under CTRL conditions, enteric spheroids contained few PHOX2B⁺/TUJ1⁺ neurons (Fig. 6E), and PHOX2B⁺ cells were not organized into aggregates. In contrast, addition of GDNF increased the formation and number of surface PHOX2B⁺/TUJ1⁺ aggregates (neurospheres) (Fig. 6F). Moreover, addition of GWEN supplement resulted in a pronounced phenotype, with large PHOX2B⁺/TUJ1⁺ neurospheres attached to the whole surface of the spheroids (Fig. 6G).

In summary, ENS-derived ENCDCs isolated from E8 chick embryos efficiently generated enteric neurospheres, with similar morphology and immunophenotype to mammalian counterparts (Hotta, Cheng, Graham, Nagy, et al., 2016; Hotta, Rahman, et al., 2023; McKeown et al., 2017).

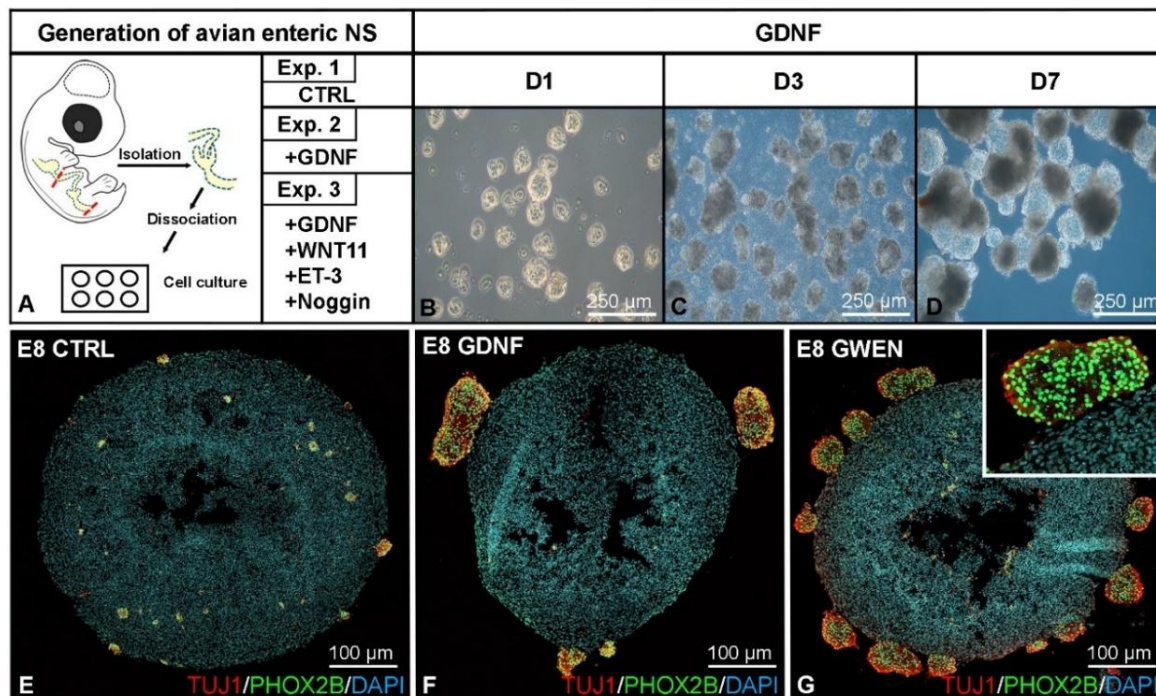


Figure 6. Isolation, characterization and differentiation of chicken enteric neurospheres. Schematic figure of avian enteric neurosphere generation (A). Brightfield images show examples of neurospheres generated from E8 ENSCs that emerged 1, 3, and 7 days in GDNF-containing medium (B-D). Representative immunofluorescence images of 7-day-old neurospheres generated from E8 chicken intestine (E-G). Samples were cultured under three different conditions: Exp.1: control medium ((E), CTRL), Exp. 2: GDNF containing cell culture media ((F), GDNF), and Exp.3: GWEN ((G), ceca-derived mesenchymal growth factors: GDNF, WNT11, ET-3, Noggin) supplemented media. Immunofluorescence of cross-sections confirms the presence of neurons expressing TUJ1, and neural crest cells expressing PHOX2B. Compared to CTRL and GDNF-only conditions, GWEN treatment resulted in a robust increase in TUJ1+/PHOX2B+ cell aggregates on the surface of the neurosphere and single Phox2b+ cells in the center of the aggregates. The inset shows a magnified view of a PHOX2B+/TUJ1+ aggregate, indicating enhanced differentiation of neural crest derivatives (E-G). Scale bar = 100 μ m. CTRL, control; NS, neurosphere.

To determine whether older developmental stages affect neurosphere formation and if ceca mesenchyme-derived growth factors promote this process, we dissociated E12 and D0 chicken colon tissue and cultured it in either control medium (CTRL) or medium supplemented with GWEN. In control conditions, E12 derived spheroids remained mostly compact and rounded, with only limited peripheral outgrowth and few small bright aggregates (neurospheres) visible on their surface (Fig. 7A). By contrast, GWEN-treated E12

spheroids showed a strong response, forming multiple neurospheres that emerged from and attached to the surface of the spheroid, suggesting enhanced expansion, organization of ENS-derived cells (Fig. 7C). This effect appeared to depend on developmental stage, because D0 spheroids exposed to GWEN remained largely compact and produced only few neurospheres (Fig. 7B).

Quantification supported that GWEN significantly increased neurosphere number in E12 cultures. E12 GWEN-treated spheroids producing an average of 5.875 neurospheres compared with 1.0 neurosphere in E12 control cultures (SEM = 0.2950 and 0.2673, respectively; $P < 0.0001$; Fig. 7D). The bar graph also shows that GWEN-induced neurosphere formation was much lower in D0 cultures than in E12 cultures, further supporting the idea that the response is strongest during the E12 developmental window. Immunofluorescence staining provided additional evidence for this effect: E12 control spheroids showed relatively limited TUJ1 signal, whereas GWEN-treated spheroids contained prominent peripheral neurospheres with strong TUJ1 expression and abundant HNK1 staining (Fig. 7E-F"). The merged images demonstrated HNK1+/TUJ1+ neurospheres forming on the surface of the GWEN-treated spheroids, indicating that GWEN promotes the formation of ENS-derived HNK1+ neural-crest cell-specific neurospheres most effectively in E12 spheroids (Fig. 7F-F").

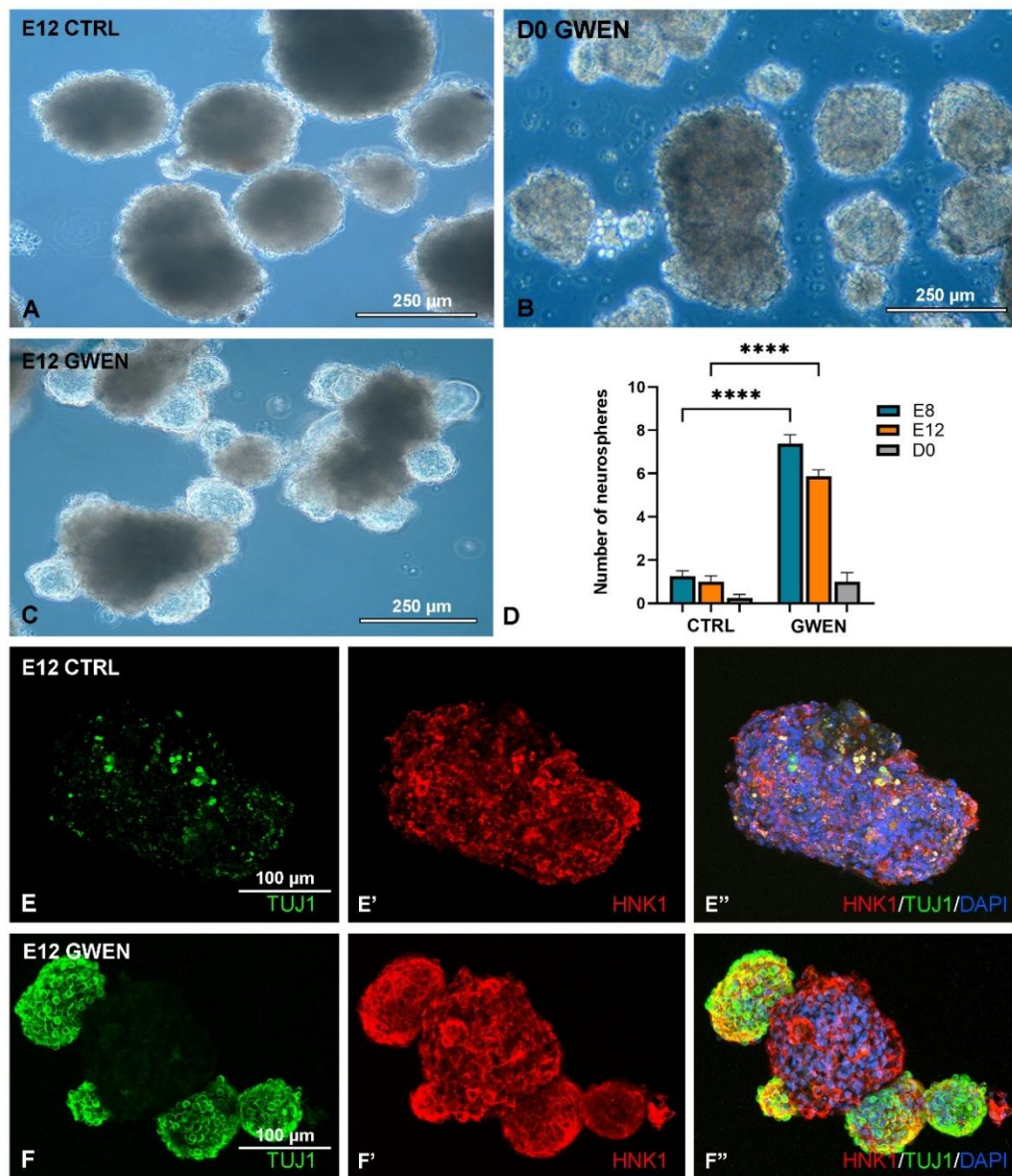


Figure 7. GWEN promotes ENS-derived neurosphere outgrowth and migration *in vitro*. Representative brightfield images of spheroids derived from E12 or D0 colonic tissue cultured in control (CTRL) cell culture media or with GWEN-supplement (A-C). E12 control (CTRL) spheroids show compact morphology (A). E12 GWEN-treated spheroids show the brighter cell aggregates (neurospheres) on their surface (B). D0 GWEN spheroids remain largely compact with no neurospheres (C). Quantification of the neurospheres on spheroids shows a significant increase in E12 GWEN-treated cultures compared to control and other developmental stages ($P < 0.0001$, $n = 8$, One-way ANOVA) (D). Immunofluorescence staining of E12 control (CTRL) and GWEN-treated neurospheres (E-F''). E12 CTRL spheroids exhibit compact organization with limited TUJ1+ immunoreactivity (E-E''). Addition of GWEN-supplement induced ENSCs to form HNK1+/TUJ1+ neurospheres on the surface of the spheroids (F-F'').

4.1.4. Ceca-mesenchyme derived growth factors promote enteric neurosphere formation and proliferation

To study whether individual mesenchymal-derived growth factors contribute to ENS-derived stem cell development and promote neurosphere formation, enteric neurospheres were generated from E8 chicken intestine and cultured for 7 days under control conditions or in medium supplemented with GWEN (Fig. 8A-F'''). Representative immunofluorescence images showed HU immunoreactive neuronal cells, PHOX2B+, SOX10+ neural crest-derived cells, and EdU+ proliferating cells within the neurospheres, demonstrating that the cultures contained differentiating and actively proliferating ENS-derived cell populations (Fig. 8A-F'''). Compared with control neurospheres addition of growth factor-treated cultures induced robust changes in the distribution and intensity of PHOX2B, SOX10, and EdU staining, suggesting that mesenchymal-derived signaling factors influence ENS-derived cell proliferation and neuronal/glial differentiation *in vitro* (Fig. 8A-F''').

Specifically, addition of GDNF increased the formation of HU+ neurospheres (Fig. 8B). These neurons co-express PHOX2B+ antigen (Fig. 8B'), whereas SOX10 immunoreactivity within these clusters remained low (Fig. 8B''). EdU incorporation was still observed within PHOX2B+ cells, supporting ongoing proliferation in ENS-derived populations under GDNF stimulation (Fig. 8B'''). In contrast, GWEN supplementation produced the most robust phenotype, with multiple HU+ neurospheres formed on the surface of the spheroids (Fig. 8C) co-expressing PHOX2B (Fig. 8C'). GWEN treatment increased the presence of SOX10+ cells within and around PHOX2B+ clusters (Fig. 8C''). These cell groups showed increased EdU activity (Fig. 8C'''), consistent with enhanced expansion/maintenance of proliferative SOX10+/PHOX2B+ neural crest-derived progenitors and SOX10+ enteric glial-lineage cells.

To evaluate the contribution of individual GWEN components, we next examined different combinations of growth factor supplements added to GDNF. Combination of GDNF+WNT11 supported formation of HU+/PHOX2B+ neuron clustering (Fig. 8D-D') but showed relatively low SOX10 labeling and limited EdU incorporation compared with GWEN (Fig. 8D''-D'''). In contrast, a combination of GDNF+ET-3 factors resulted in well-developed PHOX2B+ neuron clusters mixed with SOX10+ glial cells and widespread EdU+ nuclei

within these ENS-lineages (Fig. 8E'-E'''). Furthermore, the combination of GDNF+Noggin produced an intermediate pattern, with clear PHOX2B+/HU+ neurospheres (Fig. 8F-F'), weaker SOX10 immunofluorescence, and reduced EdU+ activity compared to GWEN or GDNF+ET-3 (Fig. 8F''-F''').

All together, these *in vitro* data show that while GDNF supports well the neurosphere formation, the combined mesenchyme-derived factor environment (GWEN) enriches SOX10+ populations and increases the PHOX2B+/SOX10+ progenitor compartment, consistent with enhanced neurosphere formation.

To determine whether the observed differences in neurosphere formation and phenotype across media conditions were associated with altered proliferative activity, proliferation was quantified during the neurosphere stage using EdU incorporation. Representative images showed EdU+ nuclei within PHOX2B+ and SOX10+ domains across all tested media conditions (Fig. 8A'''-F'''), indicating that proliferative cells were present within the developing neurospheres irrespective of treatment. However, clear differences were evident in both the abundance and spatial distribution of EdU labeling between conditions, suggesting that growth factor composition influenced not only overall cell proliferation but also the specific cellular compartments in which proliferation occurred. Quantitative analysis confirmed that the percentage of EdU+ cells differed significantly across conditions (Fig. 9C). These results indicate that media composition altered proliferative activity during avian neurosphere formation, as reflected in the number of neurospheres.

Because total proliferation alone does not indicate whether progenitor populations were being selectively expanded, proliferation was also compared within a progenitor-associated ENS compartment. For this purpose, the fraction of PHOX2B+/SOX10+/EdU+ triple-positive cells were quantified. This population was interpreted as proliferating cells within a progenitor-associated ENS lineage compartment, since PHOX2B and SOX10 coexpression marked cells retaining bipotent ENCDC identity. The proportion of triple-positive cells also differed significantly across different media conditions (Fig. 9F), demonstrating that growth factors exerted selective effects on the expansion of proliferating ENS progenitor-associated cells.

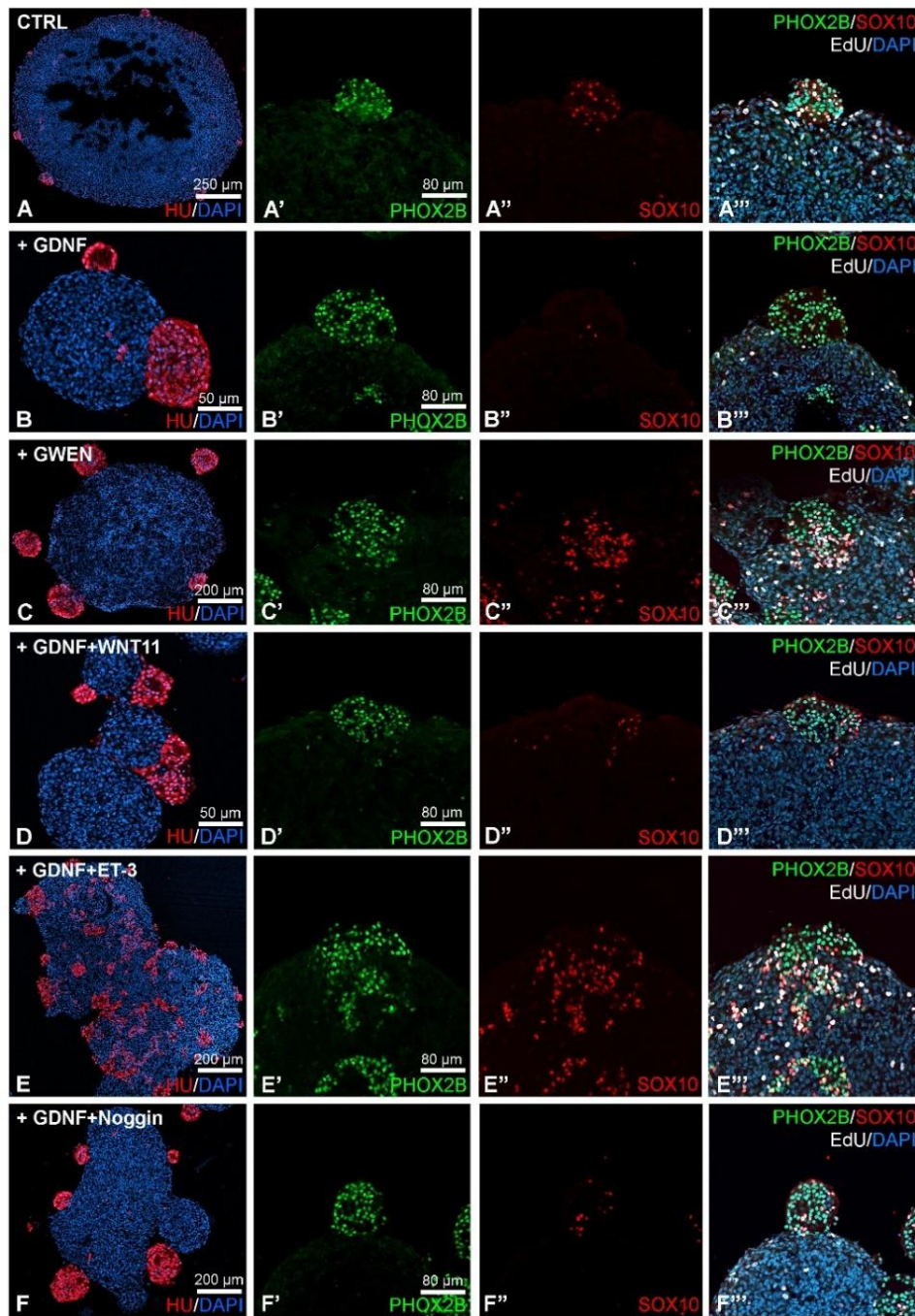


Figure 8. Mesenchymal derived growth factors promote neurosphere formation *in vitro*. Representative immunofluorescent images of 7-day-old neurospheres generated from E8 chicken intestine. Neuronal and glial differentiation were assessed by PHOX2B (green) and SOX10 (red) immunostaining, while proliferating cells were identified by EdU incorporation (grey). ENS-derived cells were maintained under control conditions (A-A'') or cultured with various growth factor supplements to compare the individual and combinatorial effects (C-C'') of GDNF, (B-B''), WNT11 (D-D''), ET-3 (E-E'') and Noggin (F-F'').

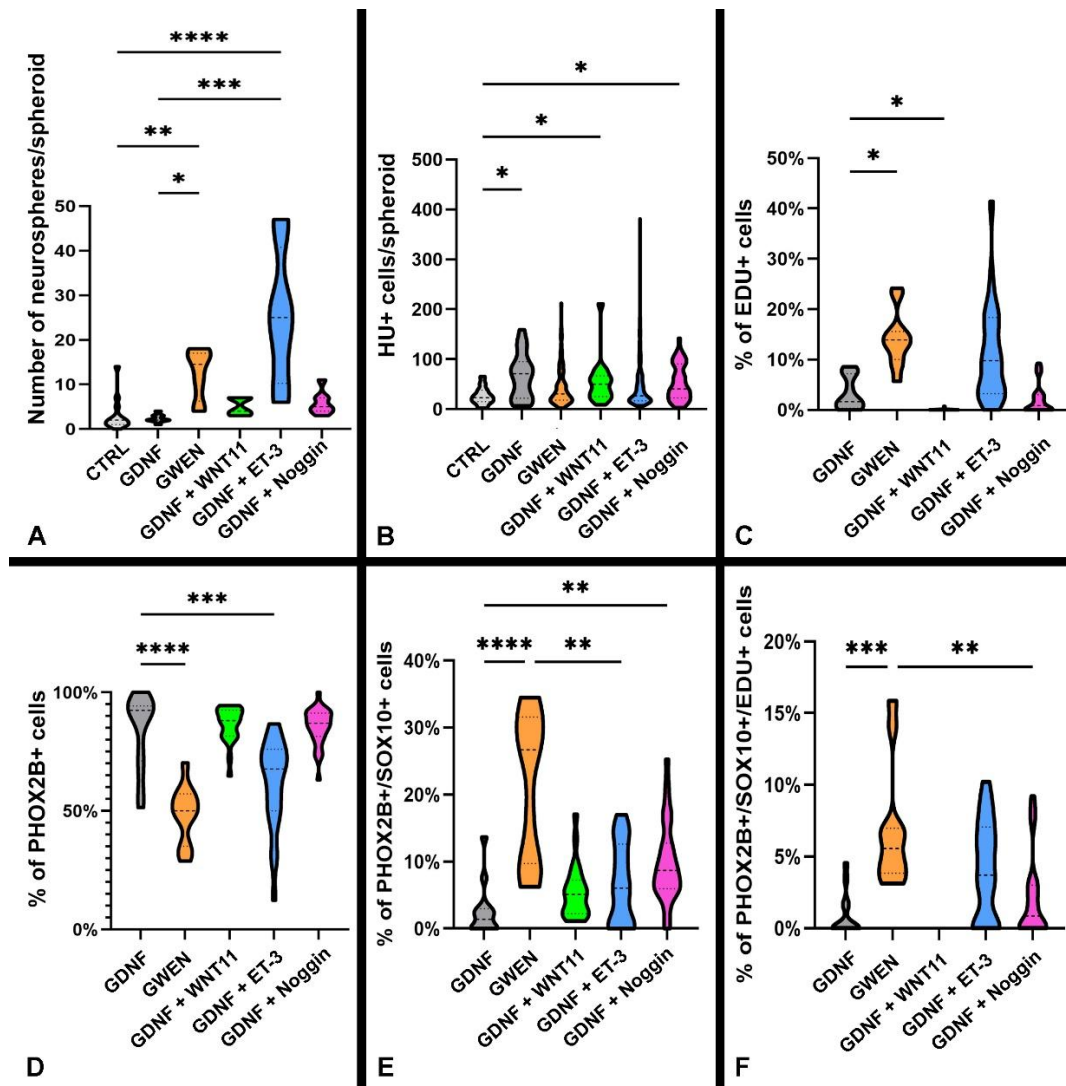


Figure 9. Effects of GDNF and co-factors on proliferation, differentiation, and neurogenesis of enteric neural progenitors within neurospheres. Quantitative analysis of neurosphere formation and cellular composition under different culture conditions: control (CTRL), GDNF alone, GWEN, GDNF + WNT11, GDNF + ET-3, and GDNF + Noggin (A). Number of HU+ (neuronal) cells per spheroid (B). Percentage of proliferating cells (EdU+) relative to total nuclei (DAPI) (C). Proportion of PHOX2B+ cells (% of DAPI), representing neuronal differentiation (D). Percentage of PHOX2B+/SOX10+ cells (% of DAPI), indicating bipotent progenitor populations (E). Percentage of proliferating PHOX2B+/SOX10+/EdU+ cells (% of DAPI), representing proliferating enteric neural progenitors (F). Violin plots show data distributions with medians and interquartile ranges. $P < 0.05$, $P < 0.01$, $P < 0.001$, $P < 0.0001$, $n = 8$, Kruskal-Wallis test.

4.1.5. GWEN-treated spheroids contain non-neuronal core and neuronal neurospheres

To determine the cellular composition of GWEN-derived spheroids, we performed double immunofluorescence staining using markers specific for neurons, mesenchyme, and smooth muscle (Fig. 10). These analyses showed that avian neurospheres were not composed exclusively of ENS neurons but instead formed heterogeneous three-dimensional structures containing multiple cell populations organized within the same spheroid (Fig. 10A-D”). The spheroid cores contained non-neuronal cells expressing calponin, consistent with smooth muscle-like differentiation, indicating that the cultures retained or generated mesenchymal derivatives alongside ENS-lineage cells (Fig. 10A-A”). Neuronal identity was evident from HU and TUJ1 immunoreactivity, which marked ENS-associated neuronal compartments within the spheroids and confirmed that GWEN-derived cultures supported neuronal differentiation or maintenance (Fig. 10A-B”).

Further staining revealed collagen I, desmin, and HSP47 expression within the spheroids, supporting the presence of stromal and mesenchymal-like components associated with extracellular matrix production, muscle-associated differentiation, and collagen-processing activity (Fig. 10B-D”). Together, these data demonstrate that GWEN promotes the formation of avian enteric neurospheres that contain ENS-derived neuronal populations as well as smooth muscle and stromal/mesenchymal components in the core spheroid, reflecting a more developmentally complex *in vitro* model of intestinal cell organization (Fig. 10A-D”).

4.1.6. Functional assessment of avian neurospheres *in vitro* and *ex vivo*

We next tested whether GWEN preconditioning improves avian neurosphere behavior in migration assays and transplantation models. Because insufficient donor-cell dispersal is a major limitation in ENS regenerative approaches, GWEN-treated neurospheres were assessed in both defined *in vitro* conditions and a preganglionic chick hindgut transplantation assay.

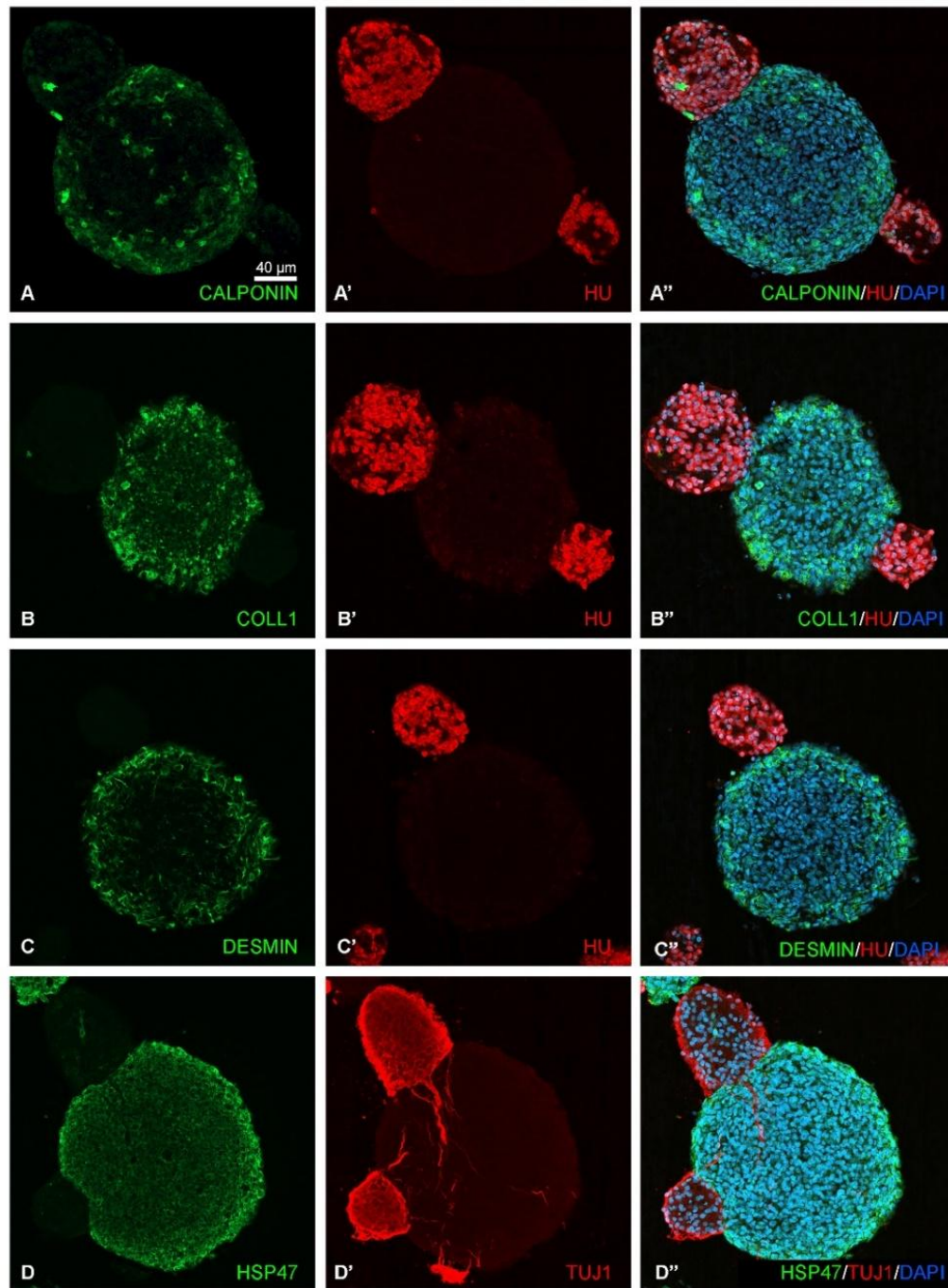


Figure 10. Spheroids contain heterogeneous cell populations expressing smooth muscle, mesenchymal, and neuronal markers. Double immunofluorescence staining of GWEN-treated E12 spheroids showing markers of non-neuronal and neuronal lineages (A-D''). Calponin (A-A'') labels smooth muscle cells, while collagen type I (B-B''), desmin (C-C''), and HSP47 (D-D'') highlight mesenchymal cells within the spheroid core. Co-staining with the pan-neuronal marker HU (A'-C'') or TUJ1 (D'-D'') reveals distinct neuronal compartments, referred to as neurospheres. Nuclei are counterstained with DAPI.

4.1.7. GWEN preconditioning increases neurosphere-derived ENCDC migration and neurite outgrowth on fibronectin

To investigate whether GWEN preconditioning improves the functional behavior of avian neurospheres on a defined extracellular matrix substrate, control and GWEN-treated neurospheres were plated on fibronectin and assessed for both cell migration and neurite extension. Under control conditions, neurospheres remained compact with reduced cell dispersal, with HNK1+ cells remaining closer to the spheroid body (Fig. 11A-A’). In contrast, GWEN-treated neurospheres exhibited a robust migratory response, with HNK1+ cells extending long processes from the neurosphere and forming a broader peripheral outgrowth zone (Fig. 11B-B’). These cultures also displayed extensive TUJ1+ neurite outgrowth, indicating that GWEN preconditioning enhanced both cell migration and neuronal process extension on fibronectin (Fig. 11A’,B’).

Quantitative analysis supported the representative imaging data, showing that GWEN-treated neurospheres had increased HNK1+ migration distance compared with control cultures (Fig. 11C). Measurement of neurite outgrowth length similarly demonstrated a significant increase in the GWEN group, consistent with enhanced neurogenic activity following GWEN exposure (Fig. 11D). Together, these findings indicate that GWEN preconditioning alters avian neurosphere behavior in a functionally relevant manner, promoting both the outward movement of ENS-associated cells and the extension of neuronal projections on a fibronectin substrate. Thus, the increased neurosphere formation observed under GWEN conditions is accompanied by improved migratory and neurite-forming capacity *in vitro*, suggesting that GWEN supports key cellular behaviors associated with ENS development and tissue colonization (Fig. 11A-D).

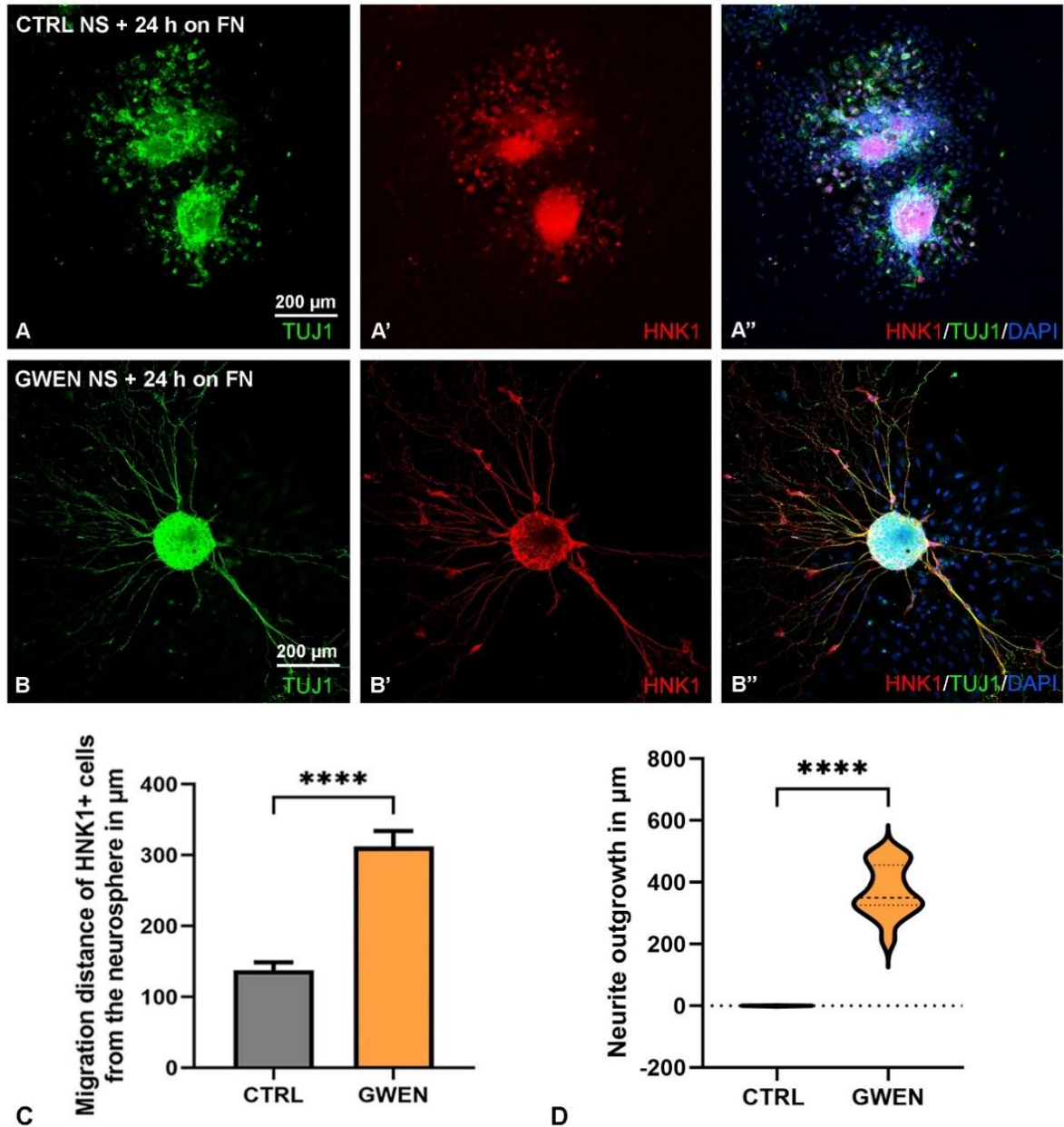


Figure 11. GWEN enhances neuronal differentiation and neurite extension from ENS-derived neurospheres. E12 control and GWEN neurospheres cultured on fibronectin (FN) coated surface for 24 h (A-B''). CTRL neurospheres show no neurite outgrowth and emigrated HNK1 immunoreactive cells (A-A''). GWEN-treated neurospheres exhibit robust radial neurite extension with overlapping TUJ1+ and HNK1+ projections extending from the core (B-B''). Nuclei are counterstained with DAPI. Quantification of HNK1+ cell migration distance from the neurosphere demonstrates significantly enhanced migration in GWEN-treated cultures compared to control (C) ($P < 0.0001$, $n = 15$, Mann-Whitney test). Violin plot showing increased neurite outgrowth (in μm) in GWEN-treated E12 neurospheres compared to controls ($P < 0.0001$, $n = 15$, Mann-Whitney test) (D).

4.1.8. GWEN-treated embryonic enteric neurospheres show enhanced migration, dispersal, and ENS formation after CAM transplantation into the preganglionic chick hindgut

We next asked whether the enhanced migratory and neurogenic behavior observed *in vitro* after GWEN treatment can be translated into improved donor-cell behavior and enhanced ENS-forming potency within the permissive embryonic intestinal tissue environment. To test this hypothesis, enteric neurospheres generated from E12 mCherry chicken intestine were implanted into the preganglionic ceca of E5 wild-type chick embryos and subsequently grafted onto the chorioallantoic membrane (CAM) of E8 host chick embryos (Fig. 12A-A'). One or two mCherry-positive neurospheres were inserted to each ceca bud and embedded in three-dimensional collagen to promote adherence and integration of the recombinant tissues (Fig. 12B). After 12 hours of organ culture, mCherry-positive cells were already observed emigrating from the implanted neurospheres and entering the surrounding ceca mesenchyme, indicating that donor-derived enteric neural crest-derived cells retained the capacity to respond to the host intestinal environment (Fig. 12B').

After 8 days of *in ovo* culture, longitudinal sections of CAM graft receiving control neurospheres showed limited donor-cell migration; mCherry-positive cells remaining restricted near the original transplantation site (Fig. 12C-C'). Although TUJ1+ nerve fibers were present in these grafts, the donor-derived mCherry signal was comparatively localized, suggesting that control neurospheres contributed only minimally to the ENS of the recipient hindgut (Fig. 12C'-C'') and did not organize into enteric ganglia.

CAM grafts from GWEN-treated embryonic neurospheres displayed robust mCherry+ cell migration throughout the hindgut, extending well beyond the implantation site and colonizing the gut mesenchyme (Fig. 12D-D'). These donor-derived cells were associated with neuron-specific TUJ1 expression and contributed to organized ENS-like structures within anatomical regions corresponding to the developing submucosal and myenteric plexuses (Fig. 12F-F'). Additionally, immunostaining for HU confirmed neuronal differentiation of donor-derived cells from GWEN-treated neurospheres, further supporting their contribution to enteric neuronal populations within the recipient hindgut (Fig. 12G-G').

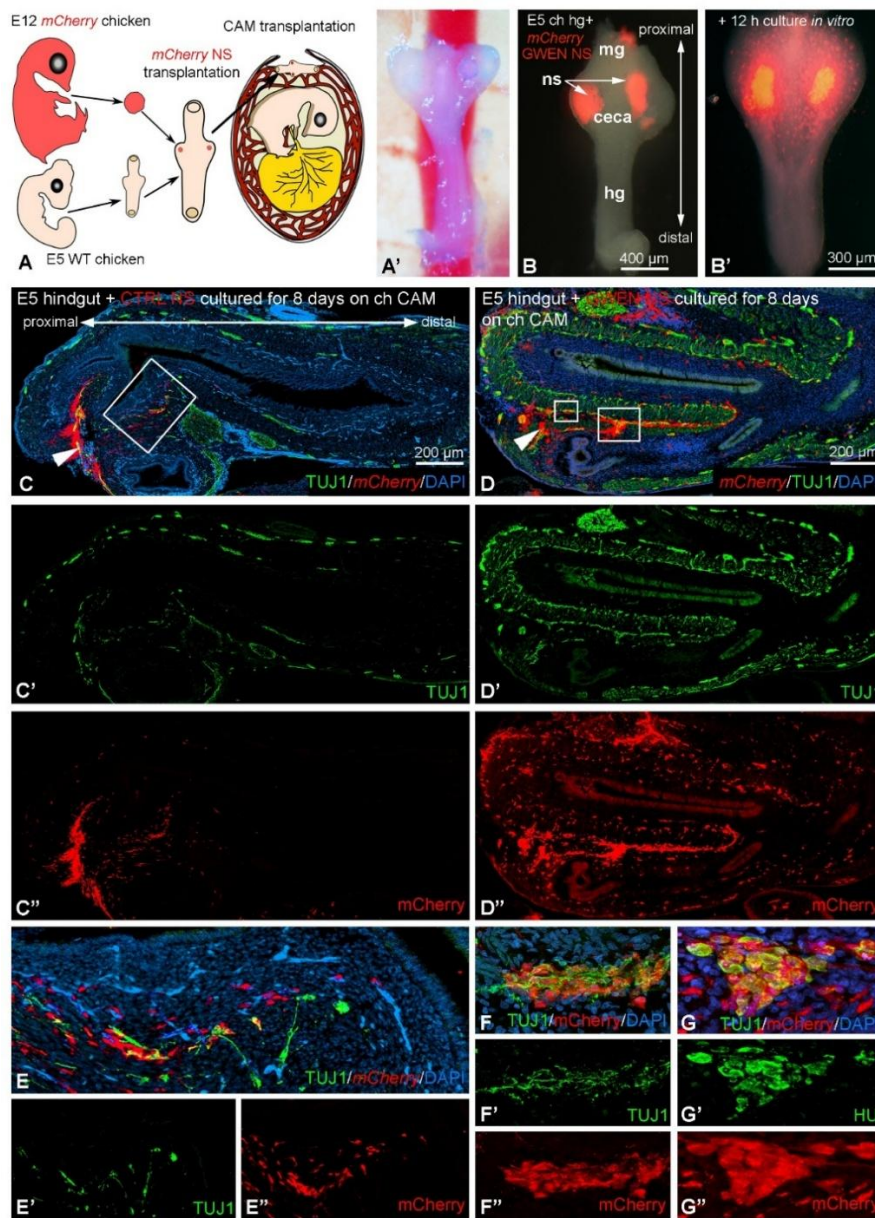


Figure 12. Chicken enteric neurospheres cultured in the presence of GWEN media supplement exhibit increased cellular migration and ENS formation following transplantation into the preganglionic hindgut.

Enteric neurospheres from E12 mCherry chicken intestine were implanted into E5 wild-type chicken ceca and then grafted onto the chorioallantoic membrane (CAM) of host E8 chick embryos (A). Wholemount image of the CAM graft (A'). One or two mCherry neurospheres were implanted into each ceca bud and cultured in a 3D collagen to ensure

the adherence of the recombinant tissues (B). After 12 h in culture, many mCherry expressing cells had emigrated from the neurospheres and colonized the ceca mesenchyme (B'). Neurospheres maintained in the presence of control media and cultured on the CAM for 8 days exhibit limited cell migration in CAM grafts. Arrowhead shows the site of neurosphere transplantation in longitudinal sections (C). Individual immunofluorescence images showing TUJ1+ nerve fibers (C',D',E') and mCherry expression (C'',D'',E''). Note that (E) represents a magnified view from the boxed area in (C). A similar experiment using embryonic neurospheres cultured in GWEN-media supplement (D-D'') reveals robust mCherry+ ENS migration throughout the hindgut to form submucosal and myenteric plexuses that express neuron-specific TUJ1 (F-F') and HU antigens (G-G'). hg: hindgut; mg: midgut; ns: neurosphere.

In summary, our CAM transplantation experiments show that GWEN conditioning improves the ability of avian enteric neurospheres to disseminate and migrate through embryonic hindgut tissue, and contribute neuronal elements to the ENS. Compared with control neurospheres, GWEN-treated neurospheres generated a broad distribution of mCherry+ donor-derived cells and a more substantial TUJ1+/HU+ neuronal contribution in the host hindgut (Fig. 12C-G”), control neurospheres show minimal tissue integration.

4.2. Agrin Inhibition in Enteric Neural Stem Cells Enhances Their Migration Following Colonic Transplantation

4.2.1. Comparative transcriptomic analysis identifies ECM-associated program enriched in postnatal neurosphere progenitors

It is widely accepted that neurospheres can be generated from mouse intestine, even from early postnatal stages, and these neurospheres express markers characteristic of neural crest cells and early neurons. However, when these neurospheres were transplanted into the aganglionic colon segment of a Hirschsprung disease mouse model, they showed a reduced migration pattern (Bondurand et al., 2003; Cheng et al., 2017; Hotta et al., 2013). Previously, we observed that neural crest cell migration is strongly influenced by extracellular matrix expression in both the recipient intestinal tissue and the neural crest cells forming the neurospheres (Akbareian et al., 2013; Chevalier et al., 2016; Nagy et al., 2018). To determine which matrix proteins differ between neurospheres isolated from the embryonic ENS, which show substantial cell migration, and postnatal enteric neurospheres, which display poor migration, we performed an RNA-seq analysis based on the evaluation of published transcriptomic datasets.

To identify extracellular matrix features that contribute to the reduced dispersal capacity of postnatal neurospheres, we compared ECM-associated transcript expression between embryonic enteric neural crest-derived cell progenitors (Morarach et al., 2021) and postnatal neurosphere-derived progenitors (Guyer et al., 2023) using previously published murine single-cell RNA sequencing datasets. First, combined E15.5 and E18.5 embryonic mouse ENS datasets from Morarach et al. were integrated and visualized by UMAP to define the major embryonic ENS cell populations included in the analysis (Fig. 13A-B). SOX10

expression was then used to identify progenitor-enriched embryonic clusters, because SOX10 marks enteric neural crest-derived progenitors and immature ENS-lineage cells. Clusters showing high SOX10 expression, including clusters 0, 2, 5, and 9, were therefore selected as the embryonic progenitor population for downstream comparison (Fig. 13C).

A parallel analysis was performed using a postnatal mouse neurosphere dataset generated from GFP-positive cells isolated from the small intestine of 12-16-week-old Plp1-GFP mice, published by Guyer et al. 2023. UMAP visualization of 15,426 GFP-positive neurosphere-derived cells showed multiple transcriptionally distinct cell clusters within the postnatal neurosphere cultures (Fig. 13D). Feature plots for SOX10 and GFAP were used to identify the progenitor-like population within these postnatal neurospheres (Fig. 13E-F). SOX10 was used to identify cells retaining an enteric neural crest/progenitor-like identity, while GFAP was included because postnatal enteric progenitor-like populations often overlap with glial-associated transcriptional states. Clusters co-enriched for SOX10 and GFAP were therefore classified as postnatal neurosphere progenitors and selected for comparison with the embryonic progenitor population (Fig. 13E-F).

Targeted differential expression analysis of ECM-associated transcripts revealed broad differences between embryonic ENS progenitors and postnatal neurosphere progenitors, identifying 89 ECM-related genes that were differentially expressed between the two populations (Fig. 13G). The dot plot illustrates both the proportion of cells expressing each ECM-associated transcript and the relative expression level within each progenitor group, allowing comparison of how broadly and strongly individual ECM genes are represented in embryonic versus postnatal progenitors (Fig. 13G). This analysis showed that the ECM transcriptome of postnatal neurosphere progenitors differs substantially from that of embryonic ENCDC progenitors, suggesting that postnatal neurospheres may carry a matrix-associated molecular profile that is less permissive for cell dispersal and migration.

Within this differentially expressed ECM gene set, several transcripts associated with matrix organization, cell-matrix interaction, or migratory restraint were enriched in postnatal neurosphere progenitors, whereas other ECM components and receptors were relatively reduced compared with embryonic ENCDC progenitors (Fig. 13G). Among these candidates, Agrin emerged as a prominent ECM-associated transcript enriched in the postnatal

neurosphere progenitor population. Ridge plot analysis confirmed that Agrin expression was higher in postnatal neurosphere progenitors than in embryonic progenitors and was detected across a larger fraction of postnatal cells (Fig. 13H). Because Agrin is a basement membrane-associated ECM component previously implicated in restricting ENCDC migration, its increased expression in postnatal neurosphere progenitors may reflect a more restrictive extracellular environment that contributes to the limited migration observed in postnatal neurosphere transplantation experiments.

Together, these analyses indicate that embryonic and postnatal mouse ENS progenitor populations differ substantially in their ECM-associated transcriptional programs. Embryonic SOX10-high progenitors display an ECM profile more consistent with a developmentally migratory state, whereas postnatal SOX10/GFAP-high neurosphere progenitors show increased expression of selected ECM-associated genes, including Agrin, that may contribute to reduced dispersal. These findings support the hypothesis that cell-intrinsic ECM remodeling programs in postnatal neurosphere progenitors create a less migration-permissive microenvironment, providing a potential molecular explanation for the reduced migratory performance of postnatal-derived neurospheres compared with embryonic ENS progenitors (Fig. 13A-H).

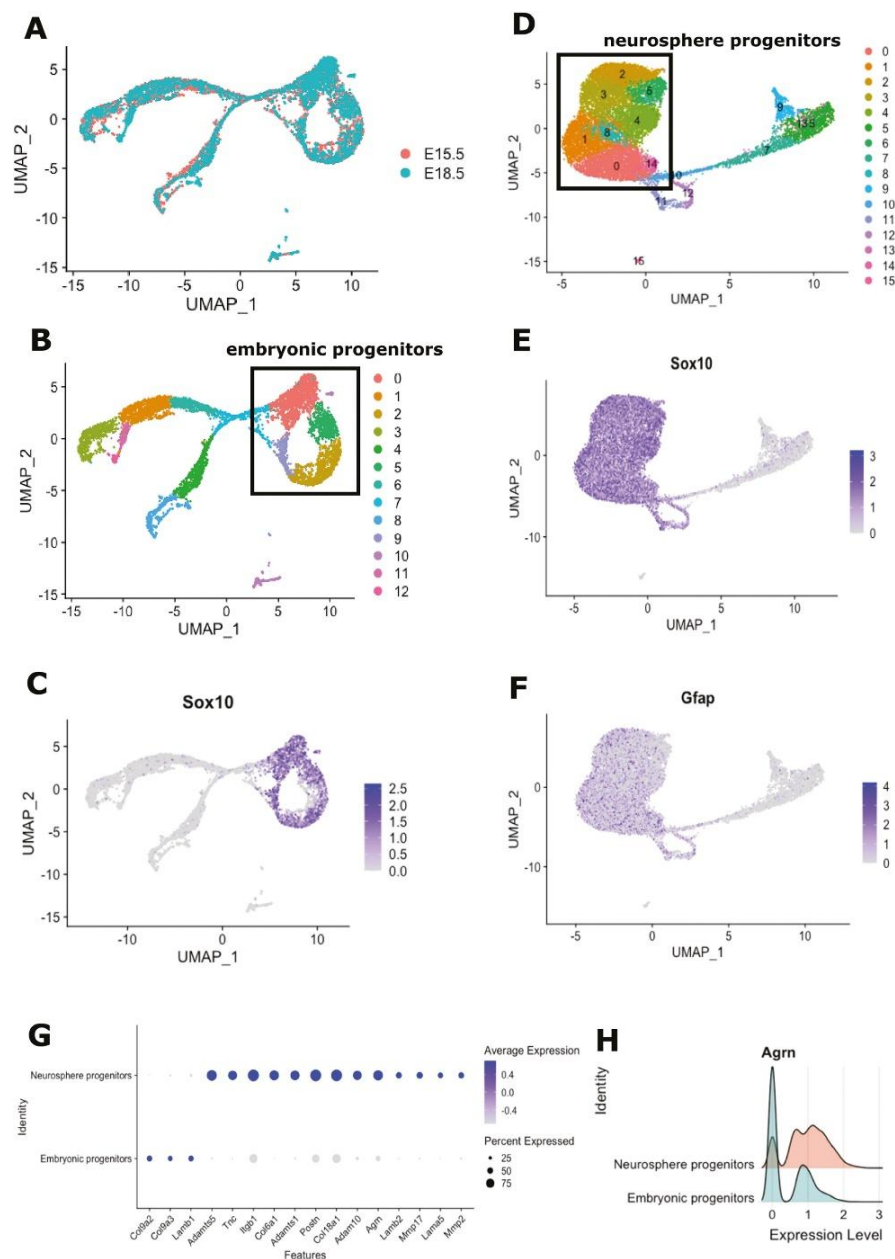


Figure 13. The ECM transcriptome is more permissive to cell migration in embryonic progenitors compared to postnatal-derived neurospheres.

Uniform Manifold Approximation and Projection (UMAP) shows combined E15.5 and E18.5 murine embryonic ENS datasets (A), using data published by Morarach et al (2021), with dimensional reduction performed (B). Feature plot of *Sox10* expression (C) used to select cells consistent with "embryonic progenitors" (clusters 0, 2, 5, 9 with high *Sox10* expression), which are included in subsequent analysis. UMAP of 15,426

GFP+ cells isolated from neurospheres generated from the small intestine of 12-16 week-old Plp1GFP mice (D), using data published by Guyer et al. (2023). Feature plots of *Sox10* (E) and *Gfap* (F) expression are shown to highlight progenitors. Clusters with high *Sox10* and *Gfap* are labeled "neurosphere progenitors" and selected for subsequent analysis. Expression of differentially expressed ECM genes between groups is shown on a dot plot (G), with dot size indicating the percentage of cells in each cluster with >0 transcripts detected, and color indicating the relative level of gene expression. A ridge plot of *agrin* expression in neurosphere progenitors and embryonic progenitors shows higher levels of *agrin* in the former (H).

4.2.2. Agrin expression is elevated in postnatal neurospheres and postnatal enteric ganglia

To validate the developmental upregulation of agrin at the protein level, we performed immunocytochemical analysis in embryonic and postnatal *Wnt1*;tdT transgenic mice to localize agrin, in which *Wnt1* neural crest-derived cells are labeled in red fluorescence. In postnatal longitudinal muscle-myenteric plexus preparations (LMMP), tdT-positive enteric ganglia and the connecting neural fiber network were clearly visible and showed intense agrin immunoreactivity, indicating that agrin protein is present within postnatal ENS-associated structures (Fig. 15A-B). These observations suggested that agrin is not only transcriptionally enriched in postnatal neurosphere-derived progenitors but is also detectable in the postnatal enteric neural microenvironment.

To compare agrin protein expression across developmental stages, neurospheres were generated from midgut segments of embryonic and postnatal *Wnt1*;tdT mice and stained under comparable conditions. Embryonic neurospheres showed low agrin immunoreactivity, with a weaker signal associated with tdT-positive neural crest-derived cells and surrounding neurosphere structures (Fig. 14G-G’'). In contrast, postnatal enteric neurospheres displayed prominent agrin staining, suggesting that postnatal ENS-derived neurospheres retain or acquire a more agrin-rich extracellular matrix environment (Fig. 15C-D). This difference was consistent with the single-cell transcriptomic analysis, which identified agrin as enriched in postnatal neurosphere progenitors compared with embryonic ENS progenitors.

Agrin immunoreactivity was also examined in postnatal gut tissue compartments that donor-derived cells would encounter during engraftment and dispersal. In these tissues, agrin signal was detected in regions associated with the enteric ganglia, neural fibers, and surrounding gut wall compartments, suggesting that the postnatal intestinal environment contains agrin-rich structures along potential migratory pathways (Fig. 15A-G). This distribution pattern is important because increased agrin deposition or accumulation in the postnatal gut may contribute to a less permissive extracellular environment for donor-cell migration after transplantation. By contrast, embryonic gut tissue and embryonic neurospheres showed lower

agrin immunoreactivity under the same staining conditions, consistent with a more migration-permissive developmental environment (Fig. 14A-G).

Quantitative analysis confirmed that the agrin protein signal was higher in postnatal neurospheres than in embryonic neurospheres (Fig. 14H). Together, these findings show that agrin enrichment is conserved at both the transcript and protein levels in postnatal ENS-derived neurospheres. Furthermore, the presence of high agrin immunoreactivity in postnatal neurospheres and in postnatal gut compartments encountered during migration supports the idea that agrin contributes to extracellular matrix features that restrict donor-cell integration into the host postnatal intestinal wall.

4.2.3. Postnatal neurospheres show high agrin expression and disperse poorly in a permissive embryonic host environment

To compare the colonization potency of postnatal (Fig. 15A-D) with embryonic ENCDCs (Fig. 15E, F) in a permissive host environment, adult+embryonic *Wnt1*;tdT mice-derived neurospheres were implanted into preganglionated embryonic chick ceca (Fig. 15) and cultured the recombinants on the chorioallantoic membrane (CAM) for 8 days. Postnatal neurospheres showed limited dispersal from the implantation site and retained agrin immunoreactivity within the graft-derived donor population (Fig. 15A-D). In contrast, embryonic donor neurospheres-derived cells migrated extensively in the same embryonic environment and generated enteric plexuses (Fig. 15E) but showed minimal donor-associated agrin staining (Fig. 15F). *Wnt1*;tdT+ cells were capable of glial differentiation based on s100 immunoreactivity (Fig. 15G), and HU+ neuronal differentiation (Fig. 15H). Quantification of migration confirmed these experimental findings: the distance of *Wnt1*;tdT mouse embryonic neurospheres was greater than that of postnatal neurospheres (Fig. 15I).

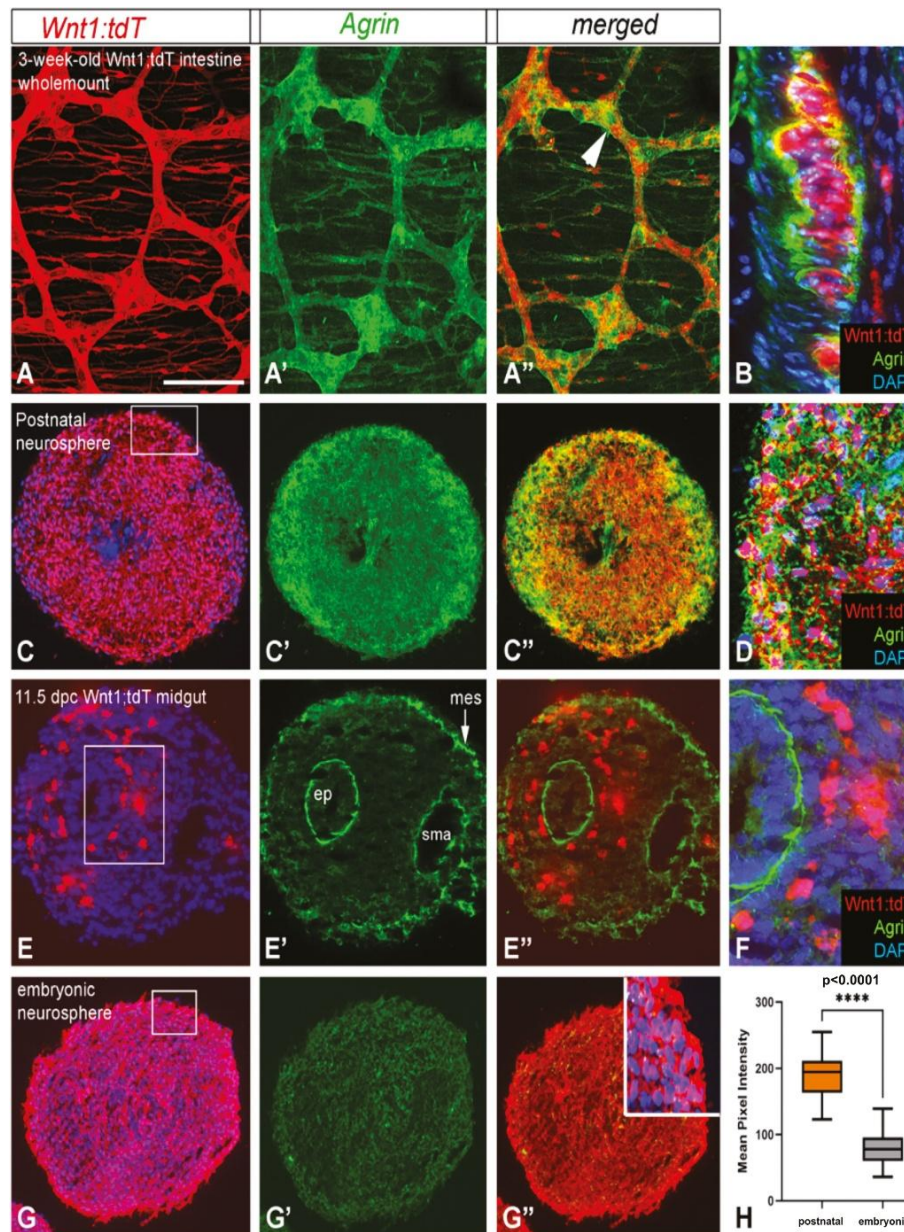


Figure 14. Agrin expression is significantly higher in postnatal as compared to embryonic neurospheres. Wholemount longitudinal muscle-myenteric plexus (LMMP) from 3-week old *Wnt1:tdT* mice reveals agrin expression in the myenteric plexus (A). Arrow in A'' indicates myenteric ganglia sectioned. Validation of agrin specificity to the enteric plexus of postnatal mice by labeling with *Wnt1:tdT* (B). Neurospheres derived from these mice show strong agrin expression (C). Boxed area magnified in D. In contrast, midgut from E11.5 *Wnt1:tdT* mice reveals markedly decreased agrin expression (E, boxed area magnified in (F), as do enteric neurospheres derived from that tissue (G, boxed area magnified in G'' inset). Quantification of mean pixel intensity of Agrin immunoreactive cells (H). Scale bar in A applies to all other panels: 150 μ m (A, C); 70 μ m (B); 45 μ m (D); 100 μ m (F); 145 μ m (G); 70 μ m (G, inset) ep, epithelium; mes, mesothelial cells; sma, superior mesenteric artery.

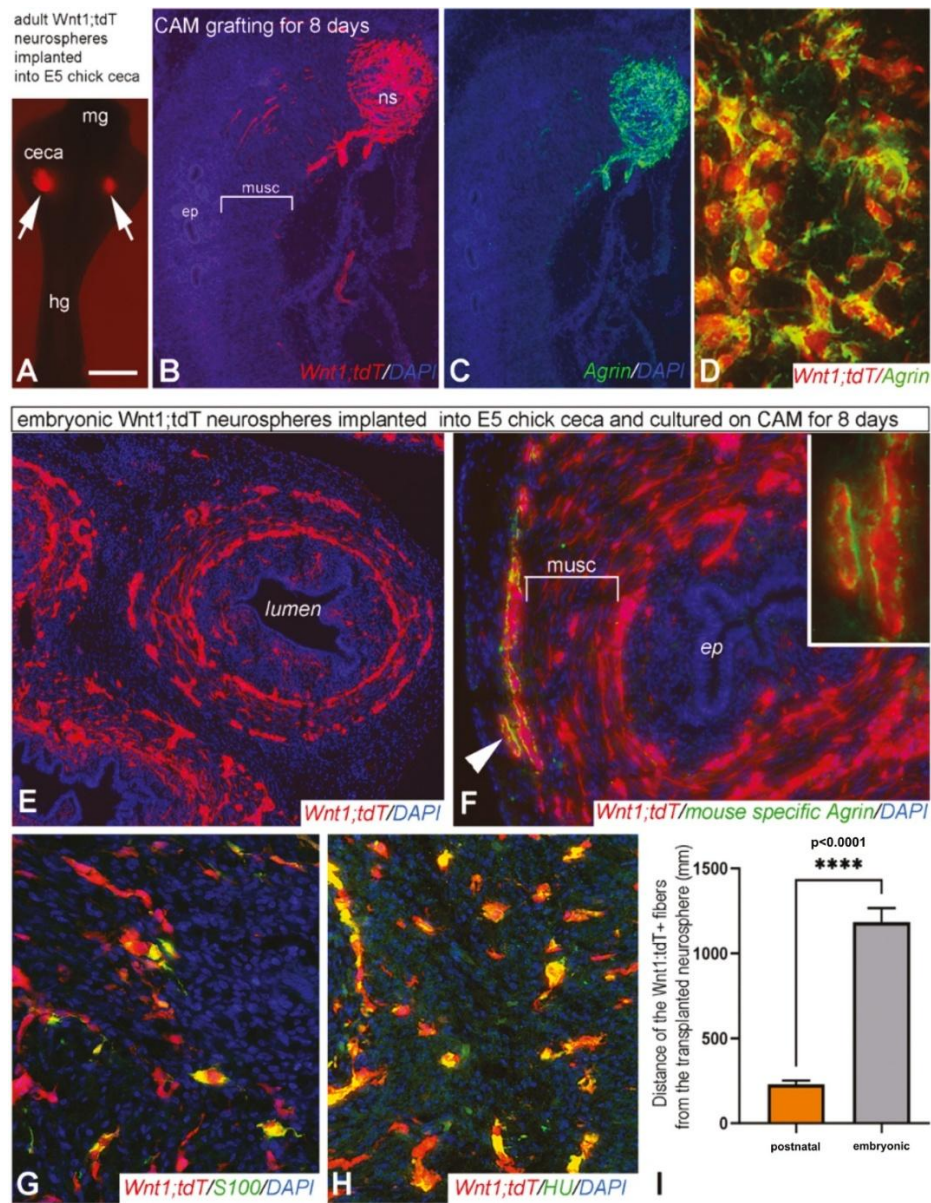


Figure 15. ENCDC migration is markedly diminished in postnatal neurospheres. Postnatal-derived neurospheres from 3-week old *Wnt1*;tdT mice were implanted into chick ceca and then grafted onto the CAM of host chick embryos (A). *Wnt1* + ENCDCs (B) express agrin (C) and exhibit limited cell migration (B). A higher power view reveals expression of agrin by *Wnt1*;tdT + cells (D). A similar experiment using embryonic neurospheres from E11.5 *Wnt1*;tdT mice (E) reveals minimal agrin expression (F) and robust tdT + ENCDC migration (E). Immunostaining of transplanted *Wnt1* + ENCDCs shows differentiation of transplanted cells into glia (S100+) (G) and neurons (HU+) (H). Quantification of the length (μ m) of *Wnt1*;tdT + fibers and cells migrating from the transplanted neurosphere (I). Scale bar in A applies to all other panels: 350 μ m (A), 175 μ m (B, C), 15 μ m (D), 150 μ m (E), 100 μ m (F), 50 μ m (G), 70 μ m (H). ep, epithelium; hg, hindgut; mg, midgut; musc, muscularis propria; ns, neurosphere; sma, superior mesenteric artery.

4.2.4. Matrix reprogramming by agrin inhibition improves postnatal neurosphere dispersal *in vitro*

We next tested whether acute agrin inhibition improves the *in vitro* behavior of postnatal neurosphere-derived cells. Because postnatal neurospheres exhibit restricted dispersal on fibronectin and in transplantation assays, we examined whether agrin function-blocking inhibition alters cell spread, cell-type-specific migration, proliferation, and neurite outgrowth.

4.2.5. Agrin inhibition enhances ENCDC dispersal from the postnatal neurosphere

To test whether agrin contributes functionally to the restricted dispersal of postnatal neurosphere-derived cells, *Wnt1*;tdT neurospheres were plated on fibronectin and treated with either a function-blocking anti-agrin antibody or control medium (Fig. 16A,B). In the presence of control media (Fig. 16A), *Wnt1*;tdT cells showed only limited emigration from the neurosphere, while agrin inhibition increased outward dispersal (Fig. 16B).

Quantification of total tdT⁺ cells spread area showed an increase after agrin blockade (Fig. 16C). These data indicate that agrin is inhibitory for ENCDC migration and agrin inhibition improves dispersal of postnatal neurosphere-derived cells on fibronectin *in vitro*.

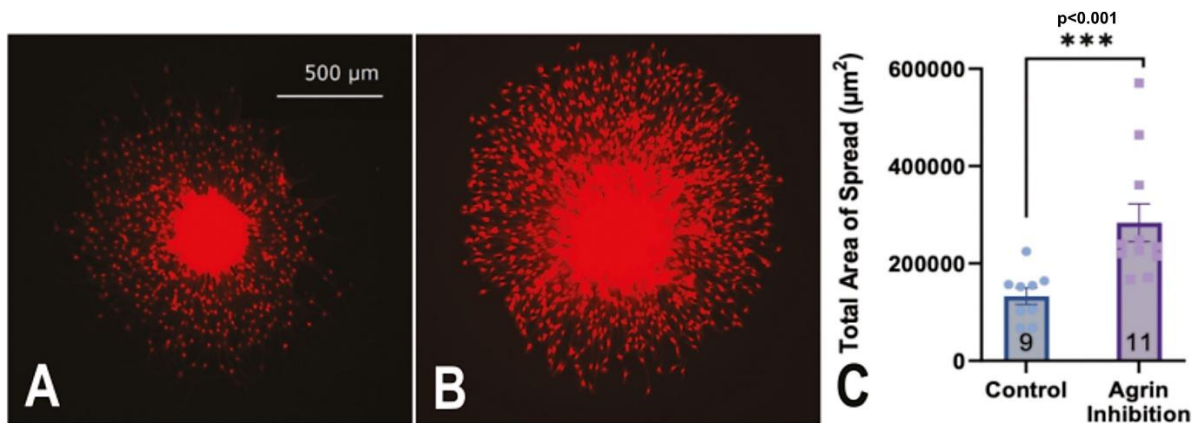


Figure 16. Agrin inhibition promotes cell migration from neurospheres *in vitro*. Enteric neurospheres derived from adult *Wnt1*;tdT mice were sorted for tdT expression and cultured on fibronectin for 72 hours. Control neurospheres show limited outward migration of cells from the neurosphere (A) as compared to those treated with an agrin function-blocking antibody (B). Bar graph of total area of cell spread (μm²) with SEM bars shows that agrin inhibition significantly enhances cell migration (P<0.001; CTRL n=9, mean=132,854 μm², Agrin inhibition n=11, mean=283,676 μm²) (C). Scale bar in A applies to panels A and B.

4.2.6. Agrin suppression using the shRNA method improves neurosphere dispersal *ex vivo*

We next tested whether donor-cell-intrinsic agrin suppression enhances neurosphere behavior in *ex vivo* colonic transplantation models. To do this, in collaboration with Allan Goldstein's lab (Harvard Medical School, Boston, USA), postnatal *Wnt1*-lineage neurosphere cells were sorted, transduced with agrin-targeting shRNA, reaggregated, and evaluated after transplantation into colonic tissue environments. To assess whether agrin-shRNA lentivirus suppression improves *Wnt1*;tdT-neurosphere derived donor-cell behavior in an adult mouse intestinal environment, control neurospheres (Fig. 17A) or agrin-suppressed neurospheres (Fig. 17B) were transplanted into *ex vivo* mice colonic longitudinal myenteric muscle plexus (LMMP) explants and cultured for 7 days. In wild-type (WT) colon, control neurospheres showed limited dissemination. In contrast, neurospheres transduced with agrin-shRNA lentivirus showed higher donor-cell dispersion (Fig. 17C). Agrin-suppressed neurospheres also showed increased dispersal in aganglionic *Ednrb*^{-/-} colon explants, although this effect did not reach statistical significance (Fig. 17D). The neuronal marker, TUJ1 immunostaining, confirmed the absence of enteric ganglia in *Ednrb*^{-/-} tissue (Fig. 17E).

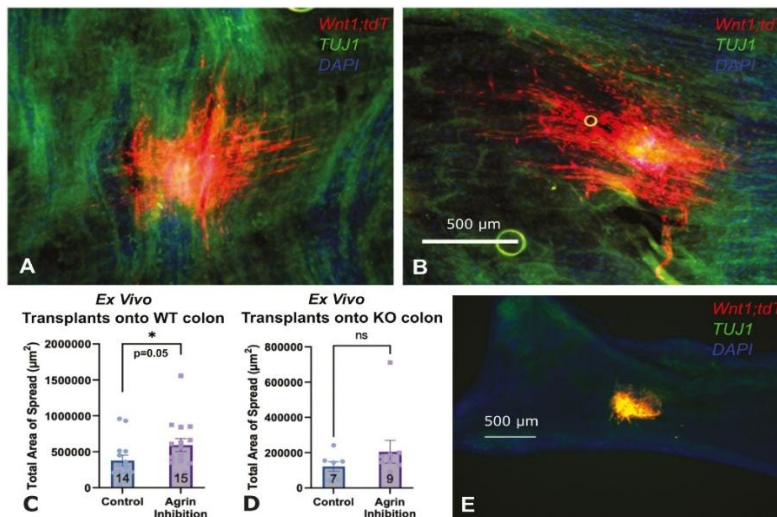


Figure 17. Agrin inhibition in neurospheres enhances cell migration following *ex vivo* transplantation.

Immunostaining of LMMP of wild-type mouse colon 7 days after *ex vivo* transplantation of adult *Wnt1*;tdT control neurospheres (A) shows significantly less migration of tdT-expressing cells as compared to transplantation

of neurospheres expressing agrin-shRNA lentivirus (B, C). *Ex vivo* transplantation of control neurospheres onto colonic tissue obtained from 2-week-old *Ednrb*^{-/-} mice reveals a trend toward decreased migration of tdT expressing cells as compared to transplantation of neurospheres expressing agrin-shRNA lentivirus (D). Immunostaining of *Wnt1* + transplanted cells on KO tissue for TUJ1 confirms aganglionosis (E).

5. DISCUSSION

Neural crest-derived cells provide the developmental basis for the ENS, and their capacity to migrate, proliferate, and differentiate along the gut is central to regenerative strategies for Hirschsprung disease. In this condition, enteric neural crest-derived cells (ENCDCs) fail to fully colonize the distal bowel, leaving an aganglionic segment that cannot support normal motility. Although surgery is essential and often lifesaving, it does not completely restore ENS function in all patients, as constipation, fecal incontinence, and enterocolitis may persist (Alhawaj, 2022; Ohkura et al., 2024). These limitations have encouraged the development of cell-based approaches in which ENS progenitors are expanded as enteric neurospheres and transplanted into aganglionic bowel to generate new neurons and glia (Ohkura et al., 2024; Rahman et al., 2024). This PhD research focuses mainly on establishing the neurosphere technique in the avian embryo model system and evaluating whether recapitulating the embryonic gut mesenchyme-derived culture conditions with a growth-factor mix could improve colonization and integration of ENCDCs. To leverage the results obtained from embryonic studies is particularly relevant because recent work using human ENS progenitors treated with GDNF and GDNF+RA has shown improved functional responses after transplantation into Hirschsprung disease patient-derived tissue (Jevans et al., 2024; Mueller et al., 2025; Han et al., 2026).

The chicken embryo is suitable for these experiments because it permits direct access to developing tissues, microsurgery, lineage tracing, and tissue recombination. These advantages allow ENS development and repair to be studied in a controlled developmental setting. Earlier avian experiments showed that vagal neural crest-derived ENCDCs have strong migratory and colonization capacity, making them a useful model for ENS replacement (Burns and Le Douarin, 2001; Nagy et al., 2012). In addition, the avian ceca provide a highly supportive environment for hindgut ENS formation (Nagy et al., 2021). Several developmental signals, including GDNF, WNT11, ET-3, and BMP4 antagonist Noggin, are enriched in the cecal region compared with other parts of the gut. This suggests that the cecal mesenchyme provides a molecular niche that promotes ENCDC proliferation, migration, and maintenance during colonization of the distal intestine (Hearn et al., 1998; Nagy et al., 2021; Kovács et al., 2023).

A key challenge in enteric neurosphere research is to define culture conditions that maintain progenitor potential while also supporting differentiation and integration after transplantation (Ohkura et al., 2024). Many early enteric neural stem cell (ENSC) culture protocols were adapted from protocols for central nervous system neurospheres. These methods included EGF and bFGF, even though these conditions were not specifically optimized for enteric progenitors. However, ENS-derived cells respond to growth factors in a stage-dependent and context-dependent manner. Some studies show that fetal and postnatal ENSCs can expand in the absence of EGF while maintaining glial and neurogenic potential (Bixby et al., 2002; Joseph et al., 2011). In contrast, others report that EGF and bFGF stimulate the proliferation of postnatal enteric progenitors (W.-K. Pan et al., 2016). These observations indicate that conditions that increase cell number are not necessarily optimal for producing neurospheres with high migratory or neurogenic capacity.

GDNF strongly supports embryonic enteric neurosphere growth but has limited effects on postnatal ENSC proliferation. WNT signaling can inhibit neuronal differentiation during embryonic ENS development, yet promote proliferation and neurogenesis in some postnatal ENSC cultures. ET-3 also acts in a stage-specific manner, stimulating ENCDC proliferation and delaying neuronal differentiation during early development, while having little effect in postnatal cultures (Hearn et al., 1998; Nagy and Goldstein, 2006; Rodríguez, 2017). Together, these findings suggest that an effective ENSC culture should not rely on a single growth factor or a generic neural stem cell protocol. Instead, it should reproduce the developmental environment that naturally supports ENCDC proliferation, migration, and hindgut colonization (Van Mechelen et al., 2025; Han et al., 2026).

GWEN Supplementation Enhances Enteric Neurosphere Formation and ENS Regenerative Capacity

For this reason, we have generated avian enteric neurospheres from wild-type and transgenic chicken embryos and analyzed their ENS-forming ability and tissue integration into the aganglionic colon. The central technical advance of our *in vitro* protocol was the use of the GWEN supplement, composed of GDNF, WNT11, ET-3, and Noggin (Soós et al., 2025). This growth factor combination was designed to mimic key features of the cecal signaling

environment. GDNF, WNT11, and ET-3, produced by the ceca mesenchyme, are associated with ENCDC proliferation, migration, and maintenance, while Noggin inhibits BMP4 signaling, which can influence progenitor behavior and differentiation. By combining these factors, GWEN creates a specific cell culture environment intended to preserve an ENCDC-like state and enhance the neurospheres ability to participate in ENS formation after transplantation. Enteric neurospheres have previously been generated from mammalian enteric progenitors, including mouse, rat, swine, and human tissues, demonstrating that ENS progenitors from different species can survive, expand, and form transplantable spheroid-like aggregates under defined culture conditions (Silva et al., 2008; Hotta et al., 2016; Cheng et al., 2017; Hotta et al., 2023; Mueller et al., 2024). However, our avian neurosphere system differs in that the forming spheroids are generated on the first day, on the second day of culturing, neurospheres form on the surface of the spheroids, which are enriched in ENS progenitors, containing PHOX2B+/SOX10+ cells; this is a significant difference compared to their mammalian counterparts. Under GDNF-only conditions, these avian neurospheres are broadly comparable to mammalian enteric neurosphere models, but GWEN supplementation significantly increases both neurosphere size and number, indicating enhanced cell expansion and aggregate formation. This allows the generation of cleaner, more homogeneous neurospheres enriched in ENS progenitor-like cells, providing a more consistent starting population for testing migration, differentiation, and ENS-forming capacity after transplantation.

While RA+GDNF treatment of human ENS progenitors has demonstrated important translational benefit in Hirschsprung disease patient-derived tissue (Jevans et al., 2024; Mueller et al., 2025), our GWEN approach provides a broader developmental cue combination by incorporating GDNF together with WNT11, ET-3, and Noggin, thereby better mimicking the cecal microenvironment that supports hindgut ENS colonization. The *in vitro* results show that GWEN improves both the generation and quality of avian enteric neurospheres. Compared with control conditions, GWEN increased neurosphere yield and promoted the formation of PHOX2B+/SOX10+ aggregates enriched in ENCDCs. This is important because these markers identify cells with ENS progenitor characteristics. GWEN-treated neurospheres also showed greater HNK1+ cell migration and stronger TUJ1+ neurite

outgrowth *in vitro*, indicating that the supplement enhanced behaviors directly relevant to ENS repair. The ability of cells to emigrate from the neurosphere and extend neurites is especially important because therapeutic success requires broad colonization, neurite formation, and ganglion differentiation in the aganglionic segment, rather than just survival at the implantation site.

The developmental stage of donor tissue is another major determinant of neurosphere potential (Zhang et al., 2019). Immunofluorescence analysis of hindgut tissue from E8 to D0 showed that undifferentiated SOX10+/PHOX2B+ ENCDCs are abundant during embryonic development but progressively decline with age. At E8, many ENCDCs co-expressed neural crest markers together with lineage-associated markers such as B-FABP and HU, suggesting that they retained bipotent or multipotent properties while beginning to acquire glial and neuronal identities. By D0, most cells had committed to either a neuronal or a glial lineage, leaving only a small population of multipotent progenitors. This developmental restriction explains why embryonic cells generally exhibit greater ENS-forming capacity than postnatal cells, underscoring the importance of donor age for regenerative applications.

The chick-mCherry chick recombination experiments supported this idea. E12-derived ENCDCs efficiently colonized and differentiated within E5 ceca+hindgut explants, whereas D0-derived cells failed to integrate into host tissue. These findings show that donor developmental stage strongly affects the cells' ability to respond to host signals and contribute to ENS formation. This also helps explain why postnatal ENSCs, despite being expandable and therapeutically promising, often show reduced regenerative capacity compared with embryonic progenitors (Cheng et al., 2017; Hotta et al., 2016; Zhang et al., 2019).

Our transplantation experiments provide strong evidence that GWEN improves neurosphere function. Only GWEN-treated neurospheres formed enteric ganglia after transplantation into preganglionic chick hindgut explants. These cells populated regions corresponding to both the submucosal and myenteric plexuses and expressed neuronal markers such as TUJ1 and HU, demonstrating neuronal differentiation and integration into the host gut. In contrast, control neurospheres contained fewer HNK1+ cells, had a more rounded structure with a dense mesenchymal core, and contributed only weakly to the host ENS. Therefore, GWEN

appears to improve several essential steps in ENS repair: progenitor enrichment, cell migration, neurite extension, neuronal differentiation, and ganglion formation.

These findings are relevant for Hirschsprung disease because cell therapy must rebuild an organized ENS network rather than deliver neural cells into the bowel wall (Jevans et al., 2024; Rahman et al., 2024). Neurospheres that survive but fail to migrate or differentiate appropriately are unlikely to restore function across a long aganglionic segment. The avian model provides a suitable system for testing the stem cell properties of neurospheres before translation into human patients (Jevans et al., 2024; Ohkura et al., 2024). It allows direct assessment of whether neurospheres can leave the graft, enter host tissue, form ganglion-like structures, and populate the correct plexus regions. In this context, the chicken embryo provides a cost-effective and accessible experimental model for refining ENS regenerative strategies. In the future, it would be valuable to test whether GWEN supplementation can similarly enhance the expansion, migration, and ENS-forming capacity of human stem cell-derived enteric progenitors. Applying this approach also to human iPSC-derived ENS progenitors would help determine whether the developmental benefits observed in avian neurospheres can be translated into clinically relevant cell sources for Hirschsprung disease therapy.

Extracellular Matrix Regulation of Enteric Neurospheres

Although the neurosphere technique is the focus, interactions between stem cells and their extracellular environment also influence transplantation outcomes (Mueller et al., 2024). ENCDCs actively remodel their extracellular matrix as they migrate through the gut (Akbareian et al., 2013; Anderson, 2010; Nagy et al., 2016, 2018), and Hirschsprung disease involves not only the absence of ganglia but also changes in the surrounding matrix (Parikh et al., 1992, 1994; He et al., 2023). This matters because transplanted cells must migrate through a host environment that may be less permissive than the embryonic gut. In addition, donor neurospheres may express extracellular matrix molecules that either promote or restrict migration.

Transcriptomic comparison between migrating embryonic ENCDC progenitors and postnatal enteric neurospheres identified 89 differentially expressed extracellular matrix-associated

genes. Among these, agrin was strongly overexpressed in postnatal neurospheres. This is important because agrin is normally absent from the migratory wavefront and is instead associated with trailing ENCDCs that have completed migration and begun neuroglial differentiation (Nagy et al., 2018; Stavelly et al., 2023). Agrin, therefore, appears to mark a less migratory, more settled cellular state, and its overexpression may contribute to the weaker migration of postnatal neurospheres.

Our agrin inhibition experiments support this interpretation. In collaboration with the Goldstein laboratory (Mueller et al., 2024), using a function-blocking antibody and an agrin-targeting shRNA lentiviral vector, agrin downregulation significantly increased ENCDC migration from neurospheres *in vitro*, *ex vivo*, and *in vivo*. Embryonic neurospheres, which expressed little or no agrin, migrated farther than agrin expressing postnatal neurospheres after transplantation into avian embryonic hindgut. In aganglionic *Ednrb*^{-/-} mouse colon cultures, agrin inhibition had the same positive effect. The mechanism by which agrin limits migration remains unclear. However, dystroglycan is a reasonable candidate because ENCDCs and enteric ganglia express it and can bind agrin and other extracellular matrix proteins. One hypothesis is that ENCDCs secrete agrin after reaching their destination, and agrin then signals through dystroglycan to reduce migration (Nagy et al., 2018). This hypothesis must be tested in future studies.

One biological limitation of this neurosphere work is that small intestines were isolated, these experiments should be interpreted as proof-of-principle experiments for testing, rather than as a fully region-matched preclinical model of colonic ENS replacement.

Future work should determine whether GWEN-treated neurospheres with the inhibition of agrin can establish long-term synaptic connectivity, integrate into functional enteric circuits, restore coordinated motility, and be adapted to human ENS progenitors, including those derived from induced pluripotent stem cells. By combining developmentally informed neurosphere culture with selective modulation of inhibitory matrisome signals, regenerative therapy for Hirschsprung disease may move closer to broad colonization and meaningful functional repair of aganglionic bowel (Jevans et al., 2024; Rahman et al., 2024).

6. CONCLUSIONS

My PhD work addressed two major limitations of enteric neurosphere-based therapy for Hirschsprung disease.

1. Specific Aim 1 was achieved by establishing an avian enteric neurosphere model from transgenic mCherry-labeled chick gut tissue and by using embryonic developmental signals to improve the quality of donor cells. We demonstrated that the combination of GDNF, WNT11, endothelin-3, and Noggin (GWEN medium) increased neurosphere formation and supported the maintenance of enteric progenitors. GWEN-treated neurospheres after transplantation colonized the host hindgut and formed enteric ganglion-like structures in the myenteric and submucosal plexuses. These results show that ceca-derived developmental signals can generate migration-competent enteric progenitors and improve the ENS-forming capacity of avian neurospheres.
2. Specific Aim 2 was addressed by modifying the extracellular matrix composition of enteric neurospheres, with particular focus on agrin, an ECM molecule highly expressed in postnatal neurospheres and associated with reduced cell migration. Agrin inhibition, using either a blocking antibody or shRNA-mediated silencing, increased ENS-derived stem cell dispersal and intestinal tissue integration *in vitro* and *ex vivo*.

Future directions: Our findings demonstrate that efficient ENS repair requires both developmentally optimized donor cells and reduction of inhibitory extracellular matrix signals that limit their migration after transplantation. The project supports a combined therapeutic strategy in which enteric neurospheres are first generated under GWEN treatment and then modified to overcome extracellular matrix barriers within the recipient bowel. Although further studies are needed to prove long-term survival, synaptic integration, functional motility recovery, and applicability to human or iPSC-derived cells, these results provide an important experimental foundation for improving regenerative therapy for Hirschsprung disease.

7. SUMMARY

The enteric nervous system (ENS), which develops from enteric neural crest-derived cells (ENCDCs), controls essential gastrointestinal functions. ENCDCs isolated as enteric neurospheres from embryonic or postnatal gut tissue, are being explored as a regenerative therapy for Hirschsprung disease (HSCR), where enteric neurons and glia are absent from affected bowel segments. However, therapeutic success remains limited by poor ENSC migration and incomplete colonization after transplantation.

We hypothesized that improving neurosphere culture conditions by adding embryonic pro-migratory signals while reducing inhibitory extracellular matrix (ECM) cues would enhance ENSC transplantation. First, we developed a chick enteric neurosphere culture system using mCherry-labeled embryonic gut and optimized it with cecum-specific developmental factors: GDNF, WNT11, endothelin-3, and the BMP inhibitor Noggin, collectively termed GWEN. GWEN treatment significantly increased neurosphere number and size and produced mixed cell populations containing SOX10⁺ glial precursors, PHOX2B⁺/HU⁺ neurons, and SOX10⁺/PHOX2B⁺ bipotent precursors. These neurospheres showed improved migration and neurite outgrowth on fibronectin in response to GDNF. In *ex vivo* gut recombination assays combined with chorioallantoic membrane transplantation, GWEN-treated mCherry⁺ neurospheres migrated extensively and formed enteric ganglia within host hindgut tissue.

Second, to reduce inhibitory signals in the postnatal gut environment, we compared transcriptomes of embryonic ENCDCs and postnatal-derived neurospheres and identified ECM-related genes with altered expression. Agrin, a heparan sulfate proteoglycan previously associated with reduced ENCDC migration, was strongly upregulated in postnatal neurospheres compared with embryonic neurospheres. Blocking agrin with a monoclonal antibody or reducing its expression using lentiviral shRNA significantly improved postnatal ENSC spread after intestinal transplantation, both *ex vivo* and *in vivo*.

Together, these findings support a dual strategy to improve enteric neurosphere transplantation for congenital neurointestinal disorders: enhancing ENSC migratory potential with gut-specific developmental growth factors and reducing inhibitory ECM components such as agrin. This approach may improve ENSC migration, tissue colonization, and integration into the host gut wall.

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