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Exploring Schwann cell precursor-derived enteric neurogenesis in post-embryonic development and regeneration

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Exploring Schwann cell precursor-derived enteric neurogenesis in post-embryonic development and regeneration

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Molecular, Cellular, and Integrative Physiology

by

Wael Noor El-Nachef

2019

ABSTRACT OF THE DISSERTATION

Exploring Schwann cell precursor-derived enteric neurogenesis
in post-embryonic development and regeneration

by

Wael Noor El-Nachef

Doctor of Philosophy in Molecular, Cellular and Integrative Physiology
University of California, Los Angeles, 2019
Professor Marianne Bronner, Co-Chair
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The enteric nervous system (ENS) is essential for normal gastrointestinal function, and defects in the ENS define several difficult to treat conditions ranging from Hirschsprung disease to esophageal achalasia. While the developmental origin of the ENS from neural crest cells is well understood, there is conflicting evidence regarding postnatal enteric neurogenesis and neuronal homeostasis. Using zebrafish as a model due to its simplified ENS which is amenable to live-imaging, we sought to explore the origin of enteric neurons that arise in post-embryonic life in both normal development and upon injury, and tested effects of the $5HT_4$ receptor agonist, prucalopride, in this process.

To assess for putative resident neuronal precursors, we performed in situ hybridization, immunohistochemistry, and confocal imaging of transgenic lines (live larvae and dissected adult intestine). To detect post-embryonic enteric neurogenesis, we photoconverted enteric neurons within the intestines of a Phox2b-kaede transgenic line such that all resident neurons turned from green to red. We then performed live time-lapse imaging to assess for the emergence of new, green-only neurons. In other experiments, resident enteric neurons were removed using two-photon laser ablation. To follow the potential contribution of neural crest-derived cells to the gut, lineage tracing was performed with neural tube injections of a lipophilic dye as well as with an inducible Sox10-Cre transgenic line. Lastly, post-embryonic zebrafish were exposed to prucalopride to test this drug's effect on enteric neurogenesis both during normal development and after injury.

Our results suggest that the post-embryonic zebrafish intestine lacks resident neurogenic precursors, and indeed appears to have no enteric glia. Despite this, enteric neurogenesis persists post-embryonically both during normal development and after injury. Our data suggest that new enteric neurons arise from trunk neural crest-derived Schwann cell precursors (SCPs) that migrate along nerves to the intestine. Prucalopride increases enteric neurogenesis in normal development as well as after injury if exposure occurs prior to injury. This study provides evidence that SCPs originating from the trunk neural crest mediate this neurogenesis in post-embryonic life and can be pharmacologically manipulated, which could have significant clinical implications for several enteric neuropathies.

This dissertation of Wael Noor El-Nachef is approved.

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2019

DEDICATION

This dissertation is in many ways a result of my uniquely nurturing family: my father, who imparted scientific curiosity; my mother, who instilled the importance of grit; my gastroenterologist sister; and my basic scientist brother. But most of all, this study would not have been possible without the unconditional love and support of my wife, Corine, to whom this dissertation is dedicated.

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Chapters 2-5 are a version of “De novo neurogenesis in the post-embryonic zebrafish enteric nervous system from Schwann cell precursors rather than resident cell types”, which is a manuscript that has been submitted.

Chapter 6 is a version of “No more extrapolation: whole adult zebrafish intestine imaging to quantitate all enteric neurons”, which is a manuscript in preparation.

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Chapter 1

Established and emerging concepts of enteric nervous system development and homeostasis

Introduction:

The enteric nervous system (ENS) is composed of as many neurons as the spinal cord and is responsible for mediating crucial functions of the gastrointestinal tract, including motility, afferent "sensing", and secretion[1]. The importance of the ENS is underscored by its designation as both the "second brain" given its size and complexity of its function[2,3], as well as the "first brain" given its probable evolutionary predating of the central nervous system (CNS)[4]. The ENS has many characteristics that suggest it can function independently of the CNS—indeed, isolated intestine which has been placed into a physiologic bath can be elicited to exhibit contractions[1]. This is due to the complex and intricate intrinsic reflex circuits that are mediated by interneurons within the intestine. Although all the necessary components for ENS function are located within the intestine, the function of the ENS in vivo operates with significant innervation and modulation from the central and peripheral nervous systems[5,6], and interfering with this modulation can have significant clinical implications[7].

ENS organization and cellular components:

In amniotes, the ENS is divided into histologically distinct plexuses and extends essentially uninterrupted from the proximal intestine through the rectum[1]. The most prominent plexuses are the submucosal (Meissner) plexus, located between the mucosa and the smooth muscle layers, and the myenteric (Auerbach) plexus, located between the inner circular smooth muscle layer and the outer longitudinal smooth muscle layer. The network formed by these plexuses interconnects ganglia composed of enteric neurons and enteric glia. The number of cells within enteric ganglia is variable, and it is unclear what dictates their composition[8]; however, ganglia of the myenteric plexus are generally larger than those of the submucosal plexus. Enteric neurons are classified by their neuronal subtype, which is defined using a "chemical code", i.e. a molecular signature consisting of a combination of peptide/protein markers[5]. While several

enteric neuronal subtypes have been identified, distinguishing a specific functional subtype often requires the determination of the presence or absence of multiple markers within the chemical code. Practically, though, these neurons are often categorized as excitatory motor neurons, inhibitory motor neurons, intrinsic primary afferent ("sensing") neurons, interneurons, and secretomotor/vasodilator neurons. Although enteric glia outnumber enteric neurons 7:1 in humans and have been classified into groups based on morphology, histologic location, and expression patterns of various glial markers[9], no functional significance based on these groupings of enteric glia has yet been elucidated in vivo[8].

Development of the ENS:

During evolution, the neural crest became a unique feature of vertebrates[10]. These cells are multipotent progenitors that are highly migratory and give rise to diverse derivatives throughout the body[11]. Neural crest derivatives include osteocytes of the facial skeleton, the aorticopulmonary septum, neurons of the peripheral nervous system, and melanocytes[11]. Early during embryogenesis, the neural crest arises from the neural plate border, located between the neural and non-neural ectoderm. As development progresses and the neural ectoderm forms the neural tube, neural crest cells undergo epithelial-to-mesenchymal transition (EMT) and delaminate from the neural tube. Neural crest subpopulations are divided along the body axis regions typically as cranial, vagal, trunk, and sacral neural crest. The timing of delamination from the neural tube, the migration trajectory, and ultimate cell differentiation fates are dictated by this regionalization. Notably, the vagal crest was originally recognized as the neural crest population that gives rise to enteric neurons and enteric glia[12].

After delaminating and emigrating from the neural tube, the vagal neural crest invades the foregut and begins its colonization of the gastrointestinal tract in a general rostral to caudal

migration[13]. However, in some species, such as mice, there is evidence that some of these cells perform transmesenteric migration through a region corresponding to a folded portion of the proximal hindgut[14]. Enteric vagal neural crest cells proliferate as they migrate, and a proportion of the daughter cells cease migration and differentiate into neurons and glia. As vagal neural crest cells differentiate into enteric neurons, they downregulate Sox10 expression and express Phox2b and other neuronal differentiation markers[15]; in contrast, enteric glia maintain Sox10 expression and upregulate GFAP, S100B, and PLP1[16]. Colonization of the entire gut by enteric vagal crest precursors is complete by gestational week 7 in humans and 3 days post fertilization (dpf) in zebrafish[17].

Enteric neuropathies and human disease:

Hirschsprung disease (HD) is a congenital neurocristopathy that in many ways is the quintessential enteric neuropathy. HD is most commonly described as a failure of the enteric vagal neural crest to successfully colonize the entire intestinal tract, leading to a segment of intestine that is devoid of enteric neurons ("aganglionic")[18]. The vast majority of HD cases involve the distal colon; however, rare cases involve the entire colon[19] or extend into the small intestine[19]. Several culprit genetic lesions have been identified, with mutations in the RET oncogene being the best described and most commonly identified mutation[15]. However, about half of HD cases are not caused by a known genetic defect[20], and there is evidence that environmental and drug exposures can produce HD-like phenotypes[21,22]. Without intervention, HD is not compatible with life as it clinically presents as a functional obstruction of the large intestine[18]. Currently, the only therapeutic approach is surgical resection of the aganglionic segment of intestine. Although surgery is life-saving, post-surgical HD patients nonetheless frequently suffer from chronic sequelae such as fecal incontinence, constipation, and HD-associated enterocolitis, and often score lower on quality of life indices[18].

Enteric neuropathies may also present later in life as acquired conditions of adulthood, and diseases relevant to each segment of the gastrointestinal tract can be involved. A notable example is esophageal achalasia, typically characterized by a preferential loss of inhibitory motor neurons in the lower esophageal sphincter[23]. The pathogenesis of esophageal achalasia is unclear, but current evidence supports an autoimmune mediated destruction of neurons that is thought to be triggered by post-viral molecular mimicry[23]. Treatments for esophageal achalasia are evolving, but none return the esophageal innervation to baseline and instead focus on mechanically disrupting the lower esophageal sphincter (myotomy) to allow gravity-dependent passage of swallowed materials[24]. Although surgical and endoscopic techniques are generally successful at accomplishing myotomy, these invasive interventions are associated with procedure-related risks and post-myotomy gastroesophageal reflux[24].

Other acquired enteric neuropathies may include gastroparesis, which is often a complication of diabetes mellitus[25]. The etiology of diabetic gastroparesis is unclear, but evidence suggests loss of neurons and/or interstitial cells of Cajal likely play a role[26,27]. Aside from dietary changes, strict control of blood glucose, and palliative measures, there are no effective and safe treatments specific for gastroparesis[28]. Furthermore, decreases in enteric neuron numbers are thought to underly the pathogenesis of some forms of small intestine bacterial overgrowth[29,30] and slow transit constipation (colon)[31]. The ENS may also be involved in irritable bowel syndrome[32] and intestinal barrier homeostasis[33], though the evidence for this is in early stages.

Hypotheses and controversies regarding ENS homeostasis:

In humans, the intestine undergoes significant growth from birth to childhood, nearly doubling in length by age 5 (239 cm to 424 cm)[34]. Autopsy studies suggest that this growth continues

proportionally with overall growth into the adult stage, with the intestine reaching up to roughly 700 cm[35]. Furthermore, unlike the CNS, the ENS is frequently exposed to infection, inflammation, and mechanical stress given its location within the intestine[36]. These observations suggest that there is likely to be a need for continuous ENS neurogenesis throughout life to increase numbers and/or replace lost enteric neurons.

Surprisingly, the question of postnatal enteric neurogenesis is controversial, with several studies reporting conflicting results. Earlier groups investigated for the possibility that enteric glia are the source enteric neurogenesis in the postnatal period, as enteric glia de-differentiate into neurogenic precursors in vitro[37]. One group which tested this hypothesis in vivo using a murine inducible Cre transgenic line driven by the GFAP promoter found no evidence of enteric glia-derived enteric neurogenesis under several conditions, including injury models[37]. Admittedly, the GFAP inducible Cre had a relatively low recombination rate (~5%), which may have limited the sensitivity of the study design. However, another research group, which employed a murine inducible Cre transgenic line driven by the Sox10 promoter, did find evidence of postnatal enteric neurogenesis[38]. Of note, this neurogenesis was modest and was detected in only one of their injury models (topical application of the detergent benzalkonium chloride). Moreover, as Sox10 is not specific to only enteric glia[11], the identity and origin of these neurogenic precursors was not definitively demonstrated.

A more recent study provided yet another incongruent conclusion—that postnatal enteric neurogenesis occurs rapidly, with total neuronal turnover measured in days/weeks[39]. Notably, this study reported the ability to detect common proliferation markers such as Ki67 within enteric neurons, which other groups have been unable to demonstrate in the past. The study concluded that enteric neurogenesis arises from vagal crest-derived resident enteric neuronal precursors

which are interestingly Sox10-negative. These findings are surprising, and we await independent replication of these results.

Lastly, postnatal enteric neurogenesis has also been assessed in the context of pharmacologic exposures. Most notably, $5HT_4$ receptor agonists have been found to promote enteric neurogenesis in a guinea pig injury model (colonic transection followed by re-anastomosis)[40] as well as in adult mice[41,42]. In one study[41], enteric neurogenesis was detected only in mice receiving a $5HT_4$ receptor agonists, but not in control mice. Moreover, the newly born enteric neurons initially appeared outside of enteric ganglia but then migrated over time into the ganglia. These findings could suggest that $5HT_4$ receptor agonism mediated its enteric neurogenic effect through a resident progenitor, but the available evidence is inconclusive as a gut-extrinsic source was not assessed. Alternatively, the newly born extra-ganglionic enteric neurons could have initially arisen from outside of the intestine as precursors that migrate into the gut prior to differentiating into enteric neurons.

The contribution of Schwann cell precursors to enteric neurogenesis:

Just as the neural crest represents a multipotent and highly migratory source of precursor cells during early embryogenesis, there is increasing evidence that neural crest-derived stem cells, termed “Schwann cell precursors” (SCPs), similarly contribute to neural crest derivatives in later stages, including postnatal and adult stages[43]. SCPs arise from traditional neural crest cells and reside along peripheral nerves where they serve as a reservoir of precursor cells to accommodate continued growth and injury[6,44]. The range of SCP-derived cell types, like the embryonic neural crest, is wide and includes parasympathetic neurons[45], melanocytes[46], and cardiomyocytes[47]. SCPs share neural crest cell markers such as Sox10 and p75, but are distinguished by their expression of cadherin 19 (CDH19) and desert hedgehog (Dhh)[48].

With respect to the ENS, evidence for an SCP-contribution has been found in multiple vertebrate species. Using an indelible Dhh promoter driven Cre in the murine model, investigators found SCPs contributed approximately 20% of hindgut neurons, and that these contributions are required for maintenance of ENS integrity in the postnatal period[49]. The authors of this study provide evidence that SCPs migrate along extrinsic nerves towards the intestine; after gut invasion, these cells express enteric neuronal differentiation markers RET and Phox2b, with a majority of these eventually restricted to calretinin-expressing neuronal subtypes. In chick, SCPs contribute nearly half of foregut neurons and are dependent on Neuregulin-1/ErbB3 signaling[50].

While chick and mice exhibit SCP-derived enteric neurogenesis that complements the enteric neurogenesis arising from the vagal neural crest, the lamprey presents an interesting problem: as a basal vertebrate, the lamprey lacks a vagal crest (hence the absence of a jaw) but possess enteric neurons. Our group has studied the origin of the enteric neurons in lamprey and found that they originate from the trunk neural crest and migrate along extrinsic nerves to invade the gut, where they differentiate into neurons[51]. This process of providing the intestine with neurons appears to be homologous to SCP-derived enteric neurogenesis, raising the possibility that SCP-like cells were the initial evolutionary strategy for providing the intestine with neurons. With the advent of the vagal neural crest, SCPs then appear to have been repurposed to supplying enteric neurons in later stages of development. However, the evolutionary development of SCPs is still under investigation[52].

The zebrafish model in the study of ENS development and homeostasis:

While best studied in chick and mouse, development of the enteric nervous system is largely conserved across jawed vertebrates including teleosts such as zebrafish[17,53]. As in chick and mouse, zebrafish vagal neural crest cells enter the foregut and migrate rostrocaudally to colonize its entire length. Zebrafish offer several advantages for studying ENS development and maturation. First, they are amenable to live-imaging techniques that allow direct visualization in vivo of cell behavior within the context of the entire organism. Second, zebrafish have a simplified ENS compared with that of chick and mouse, with two streams of vagal neural crest cells migrating along the left and right sides of the intestine, facilitating imaging studies. Third, a variety of transgenic lines are available to label particular cell types of interest. Fourth, zebrafish are amenable to experimental manipulation and highly accessible to drug treatment. Lastly, the relatively rapid rate of development in zebrafish facilitates time-lapse microscopy—the colonization of the intestine by the enteric vagal crest is complete by 3 dpf, and the intestine is functional such that zebrafish begin eating at 5 dpf[17].

The simplified zebrafish ENS compared with amniotes makes it a highly tractable model in which to examine the complex nature of post-embryonic enteric neurogenesis. Importantly, the zebrafish ENS develops in a homologous manner to humans during embryogenesis, and the intestine is anatomically and functionally segmented similarly to human small intestine and colon[54]. Of note, the $5HT_4$ receptor arose early in evolution[55,56], and 82% of disease-related human genes have a zebrafish homologue[57]; thus, the zebrafish is an ideal system in which to explore fundamental features of post-embryonic enteric neurogenesis.

Chapter 2

Putative resident neuronal precursors are absent in the zebrafish intestine

Introduction:

Previous studies have hypothesized that enteric neurogenesis is maintained postnatally, arising either from resident enteric neuronal precursors or enteric glia[37,38,40–42]. Studies investigating these cell types as potential neuronal precursors employed rodent models; however, it is unclear whether the zebrafish intestine possesses these cell types. Confirming their presence or absence in the zebrafish intestine would be an essential first step in assessing their role in enteric neurogenesis in later developmental stages.

Sox10 is an early neural crest marker important for differentiation of nearly every neural crest lineage with the exception of cartilage, which instead uses its paralog Sox9[11]. Sox10 marks early migratory neural crest cells and is retained after differentiation by enteric glia as well as melanocytes, but lost from enteric neurons[15]. During embryogenesis, vagal neural crest cells expressing Sox10 delaminate from the neural tube, invade the foregut, and migrate in a generally rostral to caudal fashion along the intestine[13,58]. In chick and murine models, these enteric vagal neural crest cells proliferate as they migrate, and a proportion of the daughter cells cease migration and differentiate into neurons and glia. As vagal neural crest cells differentiate into enteric neurons, they downregulate Sox10 expression and express Phox2b and other neuronal differentiation markers[15]; in contrast, enteric glia maintain Sox10 expression and upregulate GFAP, S100B, and PLP1[16,59]. In zebrafish, enteric vagal neural crest cells complete their colonization of the hindgut by 3 days post fertilization (dfp)[17].

Therefore, Sox10 marks both enteric vagal crest neuronal precursors as well as enteric glia, and absence of Sox10 in the post-embryonic intestine would suggest that these cell types are not involved in enteric neurogenesis in later developmental stages.

Materials and methods:

Transgenic lines

Zebrafish (*danio rerio*) were maintained at 28°C, with adults on a 13-hour light/11-hour dark cycle. All zebrafish work was completed in compliance with the California Institute of Technology Institutional Animal Care and Use Committee. Transgenic lines used in this study were the Phox2b-kaede line[60], the sox10-mRFP line[61], and the sox10:GAL4-UAS-Cre[62] ("indelible Sox10-Cre") line which was crossed with the ubi-switch reporter line[62]. All lines were within an ABWT background.

In situ hybridization (ISH)

Embryos and larvae underwent hybridization as previously described[63], with the following changes: samples were stored in ethanol and digestion was performed with 1 mg/mL collagenase 1a [Sigma C9891] (5 min, 12 min, and 20 min for 2 dpf, 3.5 dpf, and 5 dpf, respectively) prior to proteinase K digestion (12 min, 14 min, and 16 min for 2 dpf, 3.5 dpf, and 5 dpf, respectively). All imaging of ISH specimens was performed on a Zeiss Imager.M2 with an ApoTome.2 module.

Immunohistochemistry (IHC)

Our whole-mount IHC staining of embryos and larvae protocol was adapted from a prior study[64] and was performed by fixation in 4% PFA in PB overnight at 4°C, then washing in 1x PBS, followed by incubation in 0.5x PBS for 30 minutes. Samples were then placed in blocking solution (2% goat serum, 1% BSA, 1% DMSO, 0.1% Triton X-100, and 0.05% Tween in 1x PBS) for two hours at room temperature. Samples were then incubated in primary antibody diluted in blocking solution overnight at room temperature and washed for 2-3 hours in 1x PBS

plus 0.1% Triton X-100. Then, samples were incubated overnight in secondary antibody diluted in blocking solution plus DAPI [1:1000; ThermoFisher Scientific D1306] overnight at room temperature and washed for 2-3 hours in 1x PBS plus 0.1% Triton X-100. Samples were then mounted in RIMS[65] to achieve optical clearing.

For histologic sections, cryosections were collected at 10µm thickness. Blocking and antibody incubation occurred the same as with wholemount samples, except that antibody incubations occurred at 4°C and samples were mounted with Fluormount-G [ThermoFisher Scientific, 00-4958-02].

The primary antibodies used in this study were mouse anti-HuC/D IgG2b [1:200; ThermoFisher Scientific A21271], mouse anti-mCherry IgG1 [1:200; Clontech Living Colors 632543], rabbit anti-GFAP IgG [1:200; Genetex GTX 128741]. The secondary antibodies used in this study were goat anti-mouse IgG2b 647 [1:500; ThermoFisher Scientific A21242], goat anti-mouse IgG1 568 [1:500. ThermoFisher Scientific A21124], goat anti-rabbit IgG 647 [1:500; ThermoFisher Scientific A21134]). All imaging of IHC specimens was performed on the Zeiss LSM 800 confocal microscope and figures produced with ImageJ software [National Institutes of Health].

Adult intestine wholemount imaging

Adult zebrafish intestine was procured as previously described[66]. Intestine was then cut open longitudinally, fixed in 4% PFA in PB overnight at 4°C, washed in 1x PBS, incubated in DAPI 1:1000 for 2 hours at room temperature, washed in 1x PBS, then incubated in RIMS[65] for 2 days at 4°C. The intestine was then mounted onto a slide in RIMS and imaged with the LSM 800 confocal microscope.

Live-imaging

Live zebrafish larvae were anesthetized with tricaine and mounted within chamber slides using 1.2% low-melt agarose prepared in embryo water[67]. Additional embryo water was added after solidification of the agarose. All live-imaging was performed on a Zeiss LSM 800 confocal microscope with the incubator set at 28°C. 2D projections of Z-stacks were produced using Imaris software [Bitplane]. All other live-images were produced with ImageJ software.

Results:

In search of resident neuronal progenitors in the larval zebrafish intestine, we first performed in situ hybridization (ISH) for Sox10 and the enteric glial marker PLP1a. Surprisingly, while Sox10 signal was identified as expected on migrating vagal neural crest cells within the intestine at embryonic stages, it was down-regulated in the intestines of 3.5 dpf and 5 dpf larvae. Furthermore, PLP1a transcripts also were absent in the intestine at all stages examined [Figure 1b], albeit present in other parts of the peripheral nervous system.

We next performed live-imaging using transgenic line Sox10-mRFP crossed with Phox2b-kaede, in which Sox10 labels neural crest-derived cells in red and Phox2b labels enteric neuronal precursors and neurons in green. As expected, Sox10 labelled cells were observed migrating along the intestine at 2 dpf, with only sparse Phox2b co-expression in the proximal foregut. In contrast, by 3.5 dpf, Sox10 was no longer expressed within the intestine (though Sox10-positive melanocytes were identified dorsolateral to the intestine). By 5 dpf, a conspicuous neuronal plexus expressing Phox2b-kaede had formed but no Sox10 expression was observed in the intestine [Figure 1c, Supplement 1]. Together, these results show that from 3.5 dpf onward, Sox10-expressing cells appear to be absent from the zebrafish intestine and

confirm our in situ hybridization data suggesting that there are no Sox10 expressing cells resident in the intestine at 3.5 dpf and 5 dpf.

Next, we employed an indelible Cre transgenic line, Tg(sox10:GAL4-UAS-Cre;ubi-Switch) which permanently labels all Sox10-derived lineages with mCherry. This line is a double transgenic line in which the Sox10 promoter drives expression of Cre recombinase. Upon activation of Sox10 expression, eGFP is excised, thus permanently labeling Sox10-derived cells with mCherry. Fish were euthanized and fixed at 5 dpf and then immunostained for the neuronal marker HuC/D and the Cre reporter mCherry. The results show that all Cre labelled cells co-localized with HuC/D, but no Cre-labelled cells were HuC/D-negative [Figure 2] suggesting that 1) all Sox10-derived cells within the intestine have differentiated into enteric neurons by this stage, and 2) there are no remaining Sox10-derived cells (i.e. either resident precursors or glia) in the post-embryonic intestine. Of note, at this developmental stage, all Phox2b-kaede expressing cells co-localize with HuC/D [Supplement 2], indicating that these cells are differentiated neurons.

An antibody to GFAP has previously been used as a marker to suggest the presence of enteric glia in zebrafish intestine[68,69]. Therefore, we performed immunohistochemistry on 5 dpf Phox2b-kaede larvae sections using an antibody against zebrafish GFAP. As shown previously, we found GFAP immunoreactivity within the intestine; however, the GFAP appeared to be associated with cell processes but absent from cell bodies within the intestine [Figure 2b]. These findings likely reflect projections from extrinsic fibers but not resident cells within the intestine.

Lastly, to determine if enteric gliogenesis occurs later in development, we performed wholemount imaging of RIMS[65]-cleared adult zebrafish intestine from the Sox10-mRFP x Phox2b-kaede line. While numerous Phox2b-kaede cells were present within the muscularis, no Sox10 cell bodies were identified, though RFP-positive signal corresponding to cell projections was observed [Figure 2c].

Discussion:

Taken together, these data suggest that vagal neural crest-derived cells within the zebrafish intestine all differentiate into neurons, with apparent absence of resident glial or progenitors. There is conflicting evidence in the literature regarding the presence of enteric glia in zebrafish. Some authors have reported GFAP immunoreactivity in the intestine[68,70], leading them to conclude the presence of enteric glia. However, the observed immunoreactivity is fibrillary and likely to reflect projections from extrinsic glia or other cells types, as no cell bodies are evident. Furthermore, S100β, a common enteric glial marker which exhibits nuclear expression, failed to reveal enteric glia in the zebrafish[71].

Other studies have raised questions about the functional importance of glia in the mammalian ENS. For example, in male mice from which all enteric glia were genetically ablated, no obvious differences were observed in intestinal motility or predilection to enterocolitis[72]. As zebrafish have a functional intestine despite the apparent absence of enteric glia, when and why enteric glia evolved represents an interesting question for future study. Considering that humans likely possess hundreds of millions of enteric glia[9], clarifying their functional significance carries broad implications in gastroenterology and may be aided by investigating their evolutionary development.

In conclusion, the zebrafish intestine does not appear to possess putative resident neuronal precursors; thus, if enteric neurogenesis persists in post-embryonic stages, gut-extrinsic sources of neurogenesis should be evaluated.

Figure 1.

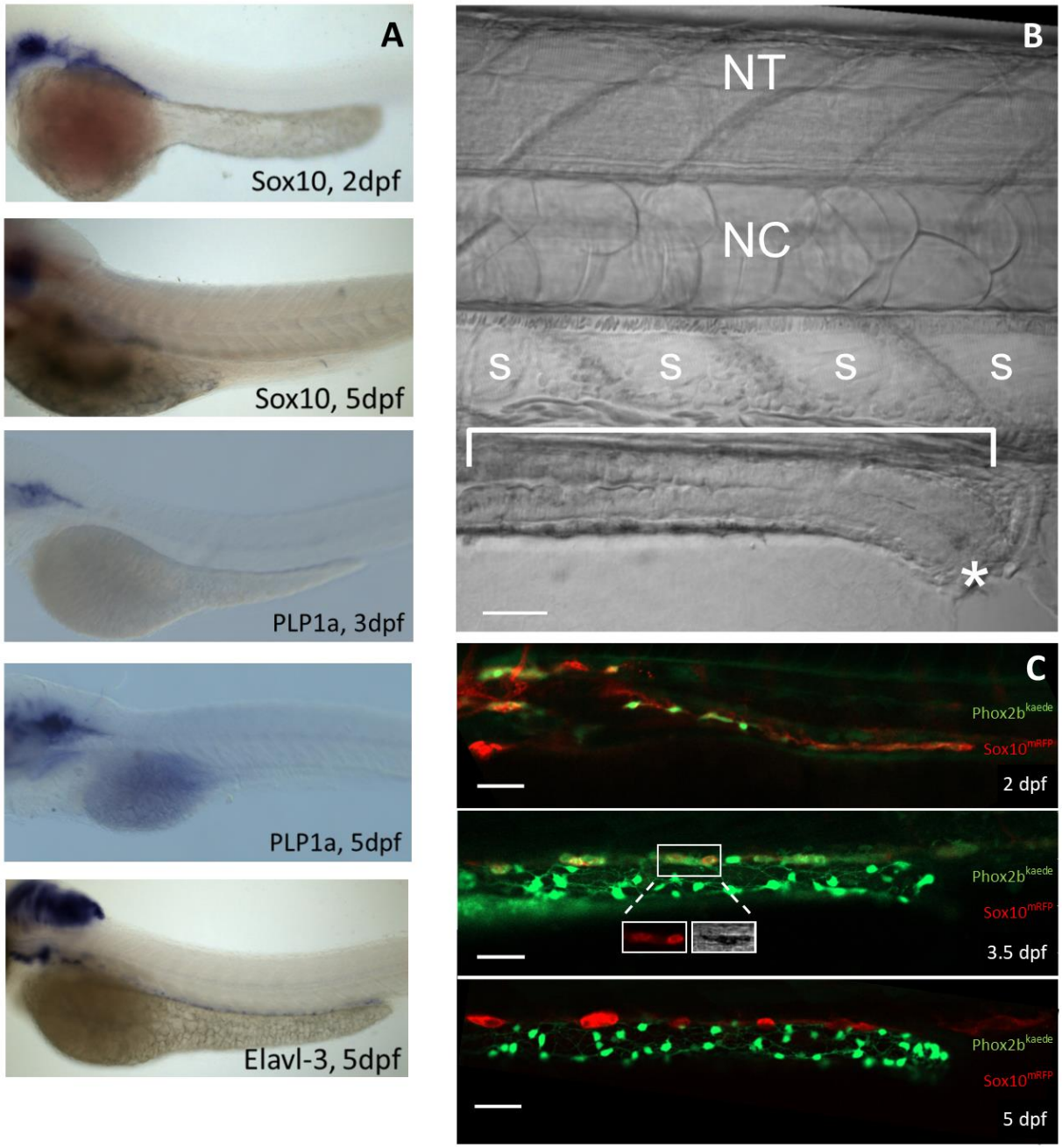


Figure 1: Resident neuronal progenitors are absent in the post-embryonic intestine.

1A) ISH of neurogenic progenitors and enteric glia are absent in the post-embryonic intestine. Sox10 is detected at 2 dpf as a stream in the midgut that does not yet extend to the hindgut. By 3.5 dpf, Sox10 is no longer detected by ISH within the intestine, and remains so at 5 dpf. The enteric glial marker PLP1a was not detected within the intestine in all above stages. In contrast, the neuronal marker Elavl-3 was evident with this protocol at 5 dpf and served as positive control.

1B) Anatomic orientation; fluorescent-microscopy images are oriented in this manner unless otherwise stated. The intestine is located ventrally (bracket) and extends anterior (left) to posterior (right), ending at the anus (star). A row of polygonal somites (s) are arranged dorsal to the intestine. The notochord (NC) and neural tube (NT, not visible in this image) are located dorsally.

1C) Live imaging of Phox2b-kaede x Sox10-mRFP fish are consistent with ISH results: a migrating chain of Sox10 cells is observed in the midgut that does not yet extend to the hindgut at 2 dpf, but then Sox10 expression ceases at 3.5 and 5 dpf. A few Sox10-expressing cells are seen dorsolateral to the intestine are consistent with melanocytes, as supported by visible pigment in TPMT (inset, 3.5 dpf panel).

All scale bars = 50 um

Figure 2.

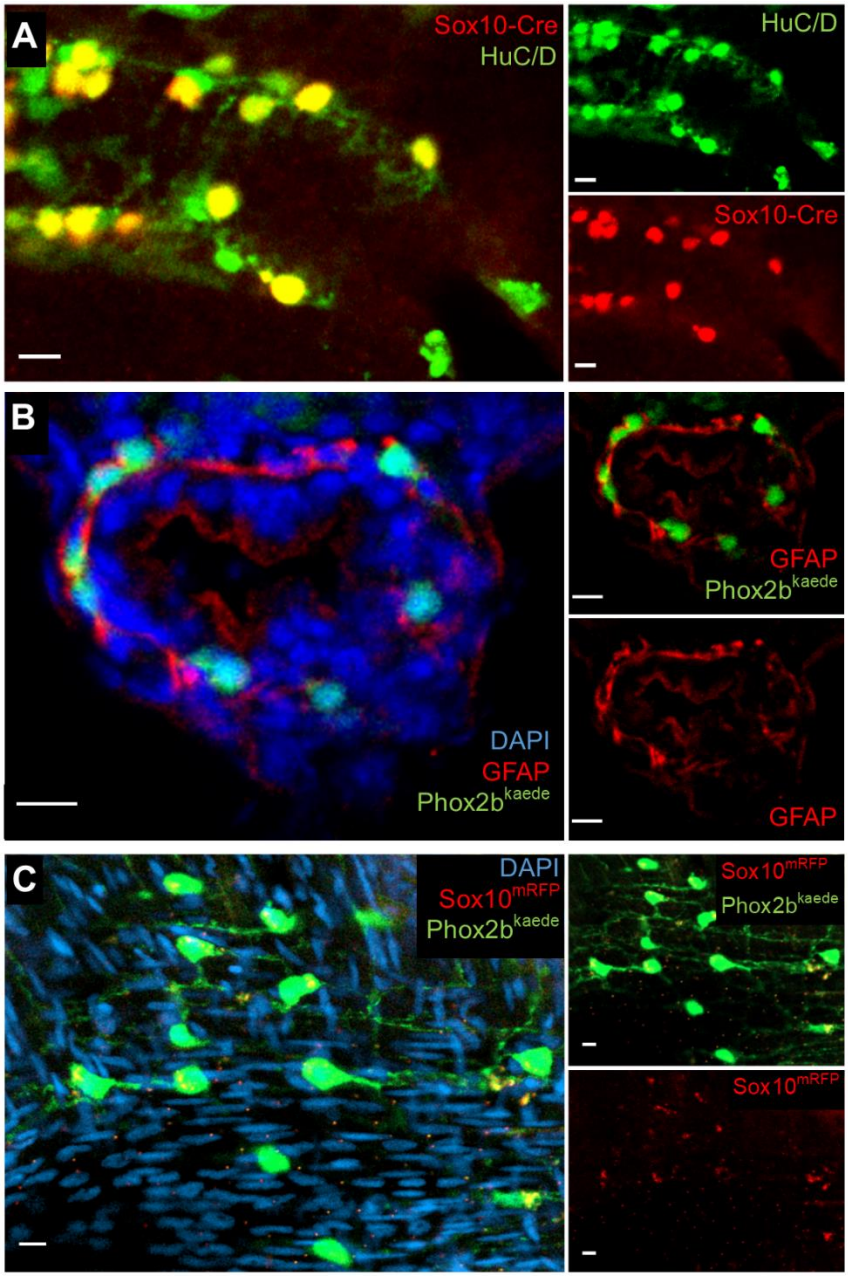


Figure 2: Further assays in larvae and adult support an absence of resident neuronal progenitors and enteric glia in the intestine.

1A) Lineage tracing with an indelible Sox10-Cre line suggests enteric neurons are the sole fate of enteric vagal crest cells. At 5 dpf, fish were fixed and underwent IHC for the Cre reporter, mCherry, and the neuronal marker HuC/D. All Cre-labelled cells co-localized with HuC/D, and no Cre-positive, HuC/D-negative cells were observed.

1B) IHC with GFAP does not clearly demonstrate convincing enteric glial cell bodies. Phox2b-kaede fish were fixed at 5 dpf, and axially sectioned for IHC for GFAP, a glial marker with cytosolic expression. Imaging of the endogenous Phox2b-kaede signal in concert with the GFAP IHC revealed a fibrillary pattern of GFAP closely associating with enteric neurons and other cells, which likely represents projections from extrinsic glia.

1C) Whole-mount imaging of adult zebrafish intestine suggests that enteric glia and resident neuronal progenitors do not form later in development. Adult intestine from Phox2b-kaede x Sox10-mRFP fish that underwent optical clearing with RIMS revealed numerous enteric neurons, but no cell bodies expressing Sox10. Extrinsic glial projections are suggested by a fibrillary pattern of Sox10 expression.

All scale bars = 10 um

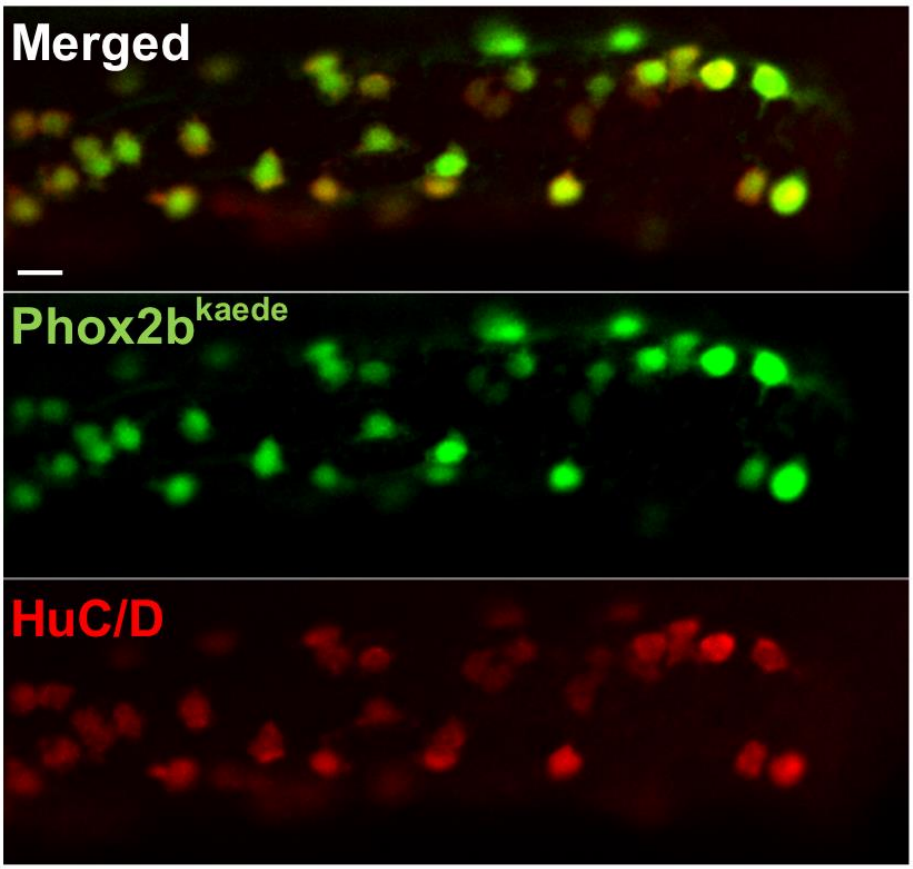
Supplement 1.

Video available online.

Supplement 1: Sox10 expression at 5dpf likely corresponds to melanocytes

A 2D projection of a z-stack collected at 5 dpf in Phox2b-kaede x Sox10-mRFP fish reveals a linearly arrange collection of Sox10-expressing cells. However, 3-dimensional assessment indicates that these cells are located dorsolateral to the intestine and likely correspond to melanocytes which reside in this location.

Supplement 2.



Supplement 2: HuC/D co-localizes with all Phox2b-kaede enteric neurons at 5 dpf.

Phox2b-kaede fish were fixed at 5 dpf and underwent IHC for HuC/D, and imaged for the endogenous kaede fluorescence and HuC/D. All Phox2b-kaede cells co-localized with HuC/D, indicating that at this stage, Phox2b represents differentiated enteric neurons.

Scale bar = 10 um

Chapter 3

Enteric neurogenesis persists in post-embryonic stages in both normal development and after injury

Introduction:

Given the continued growth of the intestine through adulthood[34,35] and the susceptibility of the ENS to injury[36], we and others[36–42,49] have hypothesized that enteric neurogenesis persists in post-embryonic stages. Prior studies investigating postnatal enteric neurogenesis utilized assays such as incorporation of thymidine analogs to assess for proliferation[37–39,41], and these studies reported conflicting results. Experiments of proliferation using thymidine analogs are challenging as the timing and frequency of dosing, as well as the duration of time between dosing and assay readout, must be chosen in the absence of knowledge of when or if enteric neurogenesis occurs. Furthermore, enteric neuronal precursors theoretically may have proliferated long before the thymidine analog is injected, and growth or injury could be a cue for these cells to migrate and differentiate rather than proliferate.

To address these methodological problems, we turned to the zebrafish model as its relatively rapid development facilitates live-imaging experiments. By assessing for enteric neurogenesis in real-time, the question of enteric neurogenesis can be directly assessed. And, as discussed in Chapter 1, the highly tractable zebrafish intestine appears to lack putative resident neuronal precursors; thus, if enteric neurogenesis persists in post-embryonic stages, this would suggest a gut-extrinsic source of these de novo neurons.

Materials and methods:

Transgenic lines

Zebrafish (danio rerio) were maintained at 28°C, with adults on a 13-hour light/11-hour dark cycle. All zebrafish work was completed in compliance with the California Institute of Technology Institutional Animal Care and Use Committee. The transgenic line used in this study was the photoconvertible Phox2b-kaede line[60]. All lines were within an ABWT background.

Photoconversion

Adapting a previously described protocol[73], we photoconverted all enteric neurons of Phox2b-kaede fish at 4.5 dpf using a Zeiss LSM 800 confocal microscope. Full thickness photoconversion was confirmed by post-conversion imaging through the full z-stack in all fish.

Two-photon cell ablation

Adapting a previously described protocol[74], we ablated 10 enteric neurons within the distal hindgut (i.e. corresponding to the last two somite lengths of hindgut) of Pho2b-kaede fish at 4.5 dpf using a Zeiss LSM 710 confocal microscope with two-photon laser ablation.

Live-imaging

Live zebrafish larvae were anesthetized with tricaine and mounted within chamber slides using 1.2% low-melt agarose prepared in embryo water[67]. Additional embryo water was added after solidification of the agarose. All live-imaging was performed on a Zeiss LSM 800 confocal microscope with the incubator set at 28°C. For time-lapse experiments, z-stacks were collected every 4 minutes with a duration of 8 to 10 hours. Videos and 2D projections of Z-stacks were produced using Imaris software. All other live-images were produced with ImageJ software.

Results:

To test for the persistence of enteric neurogenesis in the post-embryonic period, we employed the photoconvertible Phox2b-kaede line which upon exposure to 405 nm wavelength of light converts from green to red. We photoconverted all kaede-labelled cells within the 4.5 dpf intestine, after the vagal neural crest has completely colonized the intestine, such that all

neurons that were initially in the green fluorescent conformation [Figure 3a] were converted to red [Figure 3b]. At 5 dpf, these fish were re-imaged.

Interestingly, we noted the appearance of Phox2b+ cells that only had green fluorescence [Figure 3c], suggesting they were newly born enteric neurons that did not arise from pre-existing Phox2b cells. To further validate this, we performed a 10 hour live time-lapse imaging experiment after photoconversion of all Phox2b-kaede cells and captured the emergence of de novo, green-only Phox2b-kaede enteric neurons [Figure 3d, Supplement 3].

Next, we examined whether loss of existing enteric neurons enhanced neurogenesis. Using the Phox2b-kaede line, we conducted two-photon laser ablation of 10 Phox2b-kaede cells in the distal hindgut of 4.5 dpf zebrafish [Figure 4a-b], and immediately photoconverted the remaining cells as described above. Upon re-imaging at 5dpf, we again detected de novo enteric neurons [Figure 4c]. Time-lapse imaging over 8 hours in a 4.5 dpf Phox2b-kaede fish that underwent laser injury of 10 distal hindgut enteric neurons followed by photoconversion of all remaining enteric neurons revealed an injured neuron involuting and then being replaced by an emerging Phox2b-kaede de novo cell that appeared to actively migrate and extend projections to nearby neurons [Figure 4d, Supplement 4].

Discussion:

By applying live-imaging techniques, we directly observed the emergence of de novo enteric neurons in the post-embryonic period, thus providing compelling evidence of ongoing enteric neurogenesis. Photoconversion of all enteric neurons allows us to effectively “time stamp” pre-existing enteric neurons and also assess for the potential possibility that pre-existing Phox2b cells may proliferate. However, as de novo enteric neurons exhibited only green fluorescence,

this would suggest that they did not divide from pre-existing Phox2b cells as they would be partially red fluorescent in that scenario.

Importantly, we also developed an injury model that is highly specific to enteric neurons with little collateral damage to surrounding cells and structures, thus allowing for the assessment of enteric neurogenesis in the context of neuronal loss. Prior studies in other models employed non-specific injuries such as the topical application of toxic chemicals[38,42] or full thickness transection and re-anastomosis[40], all of which would damage several other cell types.

In the absence of putative resident neuronal precursors in the intestine (as discussed in Chapter 1), the persistence of enteric neurogenesis in the post-embryonic period would imply that the origin of these de novo neurons arise from a gut-extrinsic source.

Figure 3.

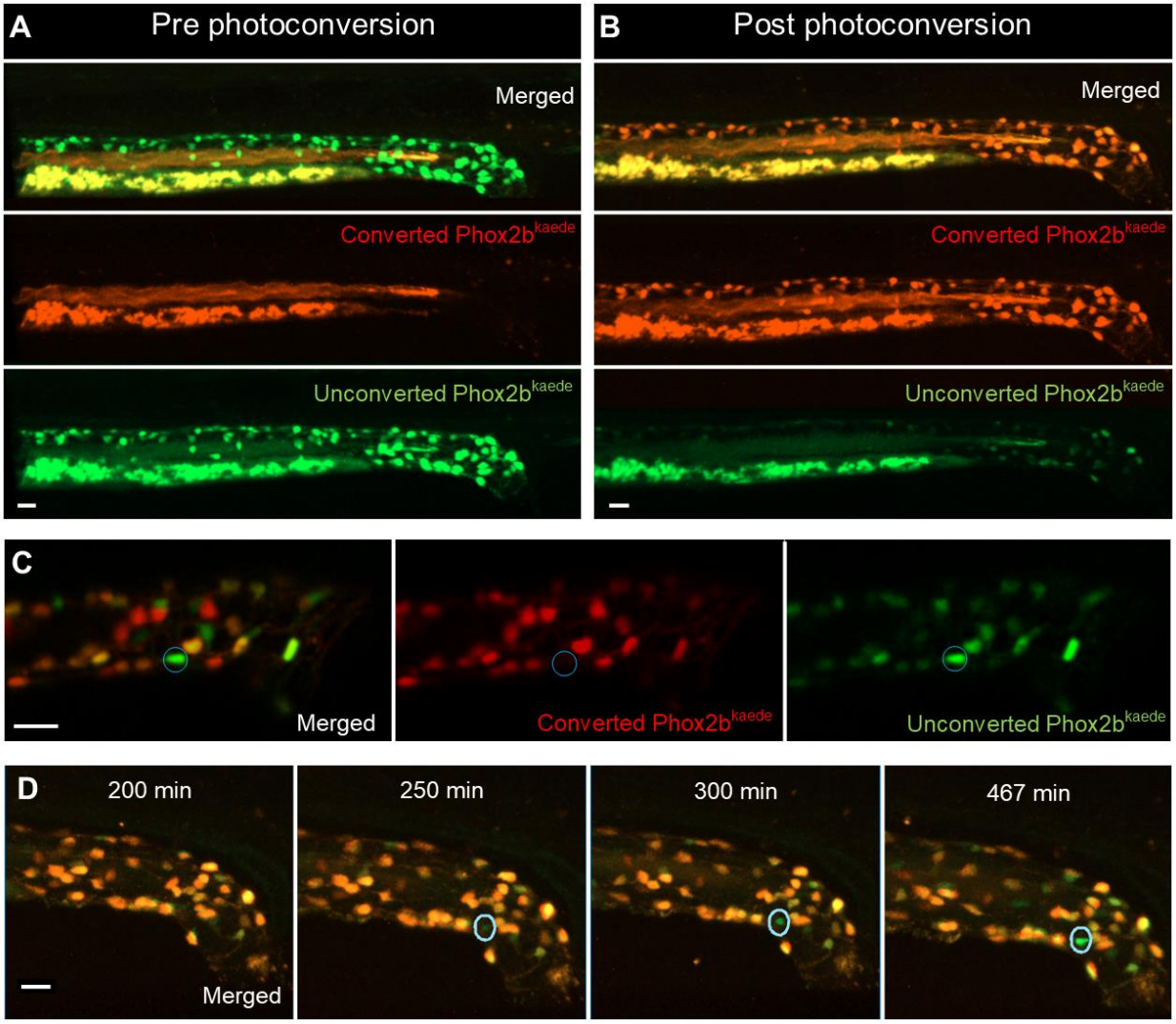


Figure 3: Enteric neurogenesis persists in the post-embryonic development despite an absence of resident neuronal precursors.

3A-B) 2-dimensional projection of z-stack from a 4.5 dpf Phox2b-kaede fish demonstrates green fluorescent enteric neurons, but no red fluorescent cells (3A). Yolk and intraluminal mucous exhibit expected autofluorescence in both channels. After photoconversion of all Phox2b-kaede

neurons in the gut, all enteric neurons fluoresce red, though some retain decreased green fluorescence (3B).

3C) Live imaging 12 hours after photoconversion at 4.5 dpf reveals the appearance of green fluorescent enteric neurons in the intestine with no red fluorescence, indicating that these neurons did not arise from pre-existing red fluorescent Phox2b-kaede cells.

3D) Live 2D projection of a 10-hour time-lapse after photoconversion at 4.5 dpf detects the emergence of de novo enteric neurons, as indicated by the gradual appearance of a green-only neuron in a region of the intestine that was originally not occupied by a neuron.

All scale bars = 20 um

Figure 4.

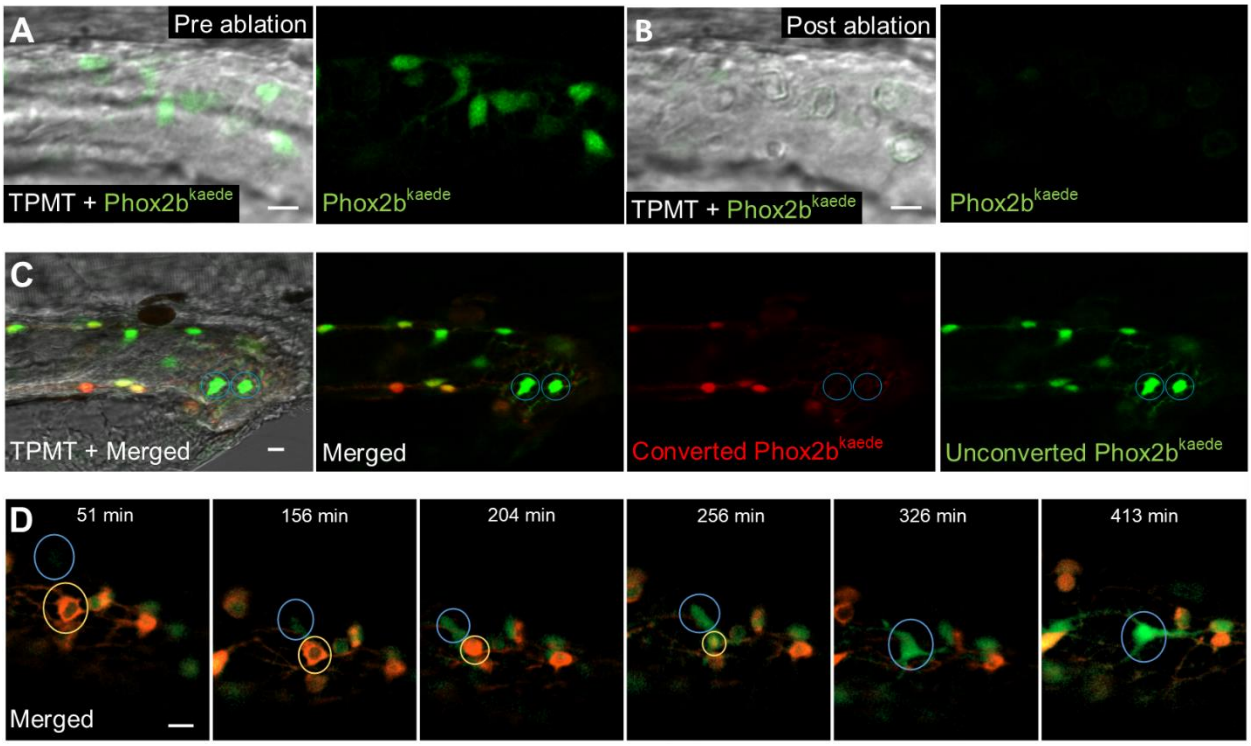


Figure 4: De novo enteric neurons replace ablated neurons in a post-embryonic injury model.

4A-B) Prior to 2-photon laser ablation, Phox2b-kaede enteric neurons are clearly visualized within the hindgut (A). After ablation, these neurons are no longer present in the hindgut, and TPMT reveals the injury site to be restricted to the neuron location (B).

4C) At 4.5 dpf, fish underwent laser ablation of 10 enteric neurons within the distal hindgut, followed by photoconversion of all remaining enteric neurons within the whole length of the gut. Live-imaging was performed 12 hours later, and detected multiple de novo, green fluorescent-only enteric neurons in the hindgut.

4D) 8-hour time-lapse of a fish at 4.5 dpf that underwent focal injury (but not complete ablation) of enteric neurons followed by pan-gut photoconversion reveals the involution of an injured neuron that is replaced by a de novo, green fluorescent-only enteric neuron. The new neuron initially appears very faintly at the dorsal-most aspect of the intestine but increases in intensity as it migrates to replace the involuted neuron and extends projections to neighboring neurons.

All scale bars = 10 um

Supplement 3.

Video available online

Supplement 3: Video of the live time-lapse experiment from Figure 3D depicts the gradual appearance of a de novo enteric neuron in a portion of the hindgut that initially did not contain an enteric neuron. The de novo enteric neuron appears to make contact with neighboring cells.

Supplement 4.

Video available online.

Supplement 4: Video of the live time-lapse experiment from Figure 4D depicts the appearance of a de novo enteric neuron that initially appears very faintly in the dorsal periphery of the intestine. As an injured enteric neuron involutes, it is replaced by the migrating de novo enteric neuron, which gradually increases Phox2b-kaede expression and extends projections to nearby enteric neurons.

Chapter 4:

Lineage tracing supports a trunk neural crest origin of post-embryonic enteric neurogenesis

Introduction:

The evolutionarily basal lamprey lacks a vagal neural crest yet has enteric neurons. Our group has demonstrated that the lamprey's enteric neurons originate from the trunk neural crest and migrate along extrinsic nerve to reach the intestine[51]. Other groups have shown that a similar process of enteric neurogenesis is present in chick[50] and murine[49] models in the form of Schwann cell precursors (SCPs). SCPs are neural crest-derived stem cells that serve as a source of precursor cells to contribute neural crest derivatives in later stages of development[43]. With the advent of the vagal neural crest in jawed vertebrates which supplanted the trunk crest as the initial source of enteric neurons, SCPs may have been repurposed for de novo enteric neurogenesis as a means to supplement additional enteric neurons to accommodate continued growth during post-embryonic development as well as regeneration after injury.

It is unknown if SCP-derived enteric neurogenesis is conserved in teleosts such as zebrafish. Given that new Phox2b neurons apparently do not arise from existing neurons (Chapter 3) and there do not appear to be progenitors/glia in the intestine at this stage (Chapter 2), we hypothesized that these de novo neurons may arise from sources extrinsic to the gut, such as trunk crest-derived SCPs. To investigate this hypothesis, we applied two lineage tracing strategies: neural tube injection with a lipophilic dye, and genetic lineage tracing with an inducible Cre transgenic line.

Methods:

Transgenic lines

Zebrafish (danio rerio) were maintained at 28°C, with adults on a 13-hour light/11-hour dark cycle. All zebrafish work was completed in compliance with the California Institute of Technology Institutional Animal Care and Use Committee. Transgenic lines used in this study

were the Phox2b-kaede line[60] and the cmlc:GFP-sox10:ERT2-Cre ("inducible Sox10-Cre") line[75] which was crossed with the ubi-switch reporter line[62]. All lines were within an ABWT background.

Immunohistochemistry (IHC)

Our whole-mount IHC staining of embryos and larvae protocol was adapted from a prior study[64] and was performed by fixation in 4% PFA in PB overnight at 4°C, then washing in 1x PBS, followed by incubation in 0.5x PBS for 30 minutes. Samples were then placed in blocking solution (2% goat serum, 1% BSA, 1% DMSO, 0.1% Triton X-100, and 0.05% Tween in 1x PBS) for two hours at room temperature. Samples were then incubated in primary antibody diluted in blocking solution overnight at room temperature and washed for 2-3 hours in 1x PBS plus 0.1% Triton X-100. Then, samples were incubated overnight in secondary antibody diluted in blocking solution plus DAPI [1:1000; ThermoFisher Scientific D1306] overnight at room temperature and washed for 2-3 hours in 1x PBS plus 0.1% Triton X-100. Samples were then mounted in RIMS[65] to achieve optical clearing.

The primary antibodies used in this study were mouse anti-HuC/D IgG2b [1:200; ThermoFisher Scientific A21271] and mouse anti-mCherry IgG1 [1:200; Clontech Living Colors 632543]. The secondary antibodies used in this study were goat anti-mouse IgG2b 647 [1:500; ThermoFisher Scientific A21242] and goat anti-mouse IgG1 568 [1:500. ThermoFisher Scientific A21124]. All imaging of IHC specimens was performed on the Zeiss LSM 800 confocal microscope and figures produced with ImageJ software [National Institutes of Health].

Lipophilic dye neural tube fills

The far-red lipophilic dye DiIC18(5)-DS [ThermoFisher Scientific D12730] was prepared according to manufacturer's instructions and injections were performed by adapting a previously described protocol[76]. Briefly, 2.3 nL of dye was injected at approximately 30 hpf into the open anterior neuropore using a needle prepared from a glass capillary affixed to a microinjector [Nanoliter 2000, World Precision Instruments]. Imaging at 6 dpf was performed with a Zeiss LSM 800 confocal microscope.

Live-imaging

Live zebrafish larvae were anesthetized with tricaine and mounted within chamber slides using 1.2% low-melt agarose prepared in embryo water[67]. Additional embryo water was added after solidification of the agarose. All live-imaging was performed on a Zeiss LSM 800 confocal microscope with the incubator set at 28°C. Figures of live-images were produced with ImageJ software.

Cell counting and statistics

Cell counting was performed manually using ImageJ software. Statistics were performed using Graphpad Prism 8 [Graphpad Software, Inc.] using Student t-test for 2 group comparisons and a P-value <0.05 indicating statistical significance.

Results:

To explore the possibility that these come from the trunk spinal cord from which some Schwann cell precursors arise, we performed lineage tracing with the lipophilic dye DiIC18(5)-DS, which fluoresces in the far-red wavelength. To this end, we injected dye into the neural tube of Phox2b-kaede embryos at approximately 30 hours post fertilization (hpf), after the vagal neural crest has completed emigration from the neural tube [Supplement 5]. Live-imaging of injected

fish at 6 dpf revealed numerous dye-labelled enteric neurons: of 30 DiI-labeled fish, 15 had Phox2b-kaede enteric neurons that co-localized with the dye (mean: 4.60 dye-labelled enteric neurons per fish, SD: 2.29) [Figure 5a-b]. There was no statistically significant difference in the distribution of dye-labelled enteric neurons within the foregut, midgut, or hindgut. Given that trunk neural crest cells migrate from the neural tube during this time frame, these findings suggest that trunk, but not vagal, neural crest-derived cells are the source of these new enteric neurons.

Next, we performed genetic lineage tracing using transgenic approaches with the inducible Sox10-Cre line, Sox10ERT2 x ubi:switch. Cells expressing Sox10 during the induction period are permanently labelled with the reporter, mCherry. Zebrafish were induced at 3.5 dpf (after vagal crest has completed intestinal colonization and Sox10 expression is no longer observed in the intestine) for a total of 16 hours and then fixed at 5.5 dpf [Supplement 6]. These fish then underwent immunostaining using neuronal marker HuC/D and the Cre reporter, mCherry. While this line only labels a subset of Sox10 expressing cells, the results revealed enteric neurons that co-localized with the Cre reporter [Figure 5c-d]. Of the 34 induced fish, 11 exhibited Cre-labelled enteric neurons (mean: 1.63 Cre-labelled enteric neurons per fish, SD: 0.67). These results confirm that these neurons arose from a neural crest-derived source external to the intestine.

Discussion:

Taken together, our two lineage tracing experiments support the intriguing possibility that trunk crest-derived neural crest stem cells, likely to be Schwann cell precursors that migrate along nerves from the spinal cord to the intestines, are a source of post-embryonic enteric neurogenesis. As cells labelled with dye would indicate a trunk neural tube origin, and Cre-

labelled cells would indicate migratory neural crest (Sox10) identity, these experiments also provide evidence that SCP-derived enteric neurogenesis is conserved in teleosts such as zebrafish.

Other studies investigating SCP-derived enteric neurogenesis used transgenic markers and IHC to identify SCPs and located these cells on nerves extrinsic to the gut[49,50]; however, the proximal origin of these cells was not confirmed. As the zebrafish is externally fertilized, they are readily accessible to experimental interventions during embryogenesis. Due to these advantageous characteristics, we were able to perform neural tube injections of lipophilic dye during a specific developmental time point that would mark pre-migratory trunk neural crest but not vagal neural crest (which at that time has already delaminated).

The timing of SCP migration from the neural tube to final tissue targets, as well as the dynamics of this migration, are areas for future study. Nonetheless, our current results strengthen our understanding of fundamental features of SCP-derived enteric neurogenesis.

Figure 5.

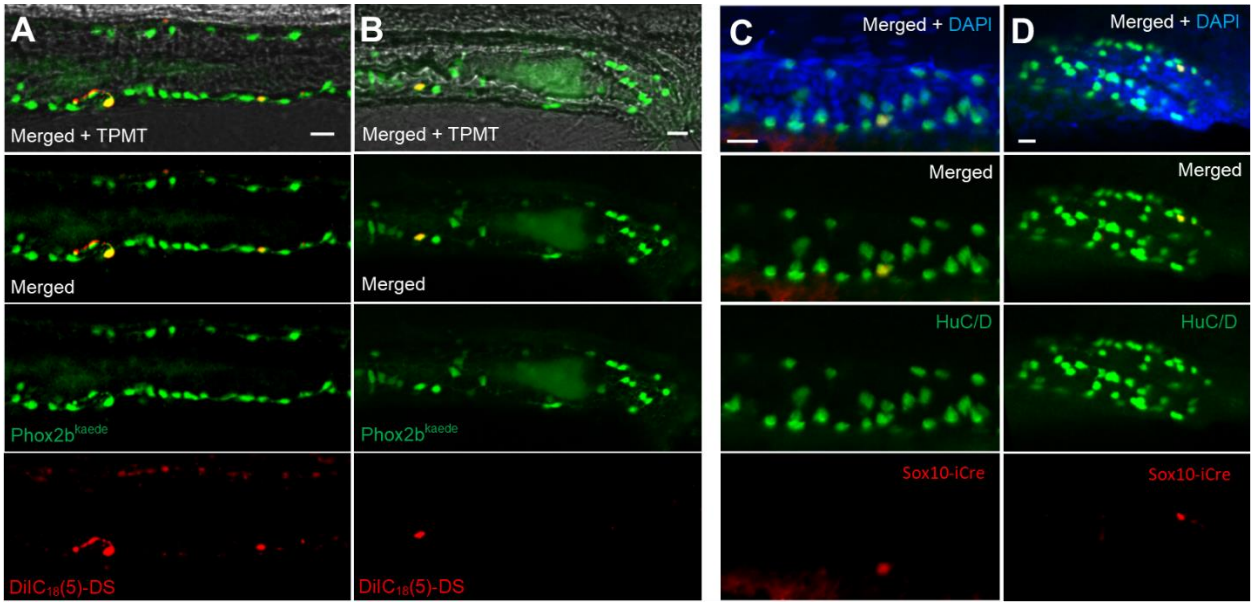


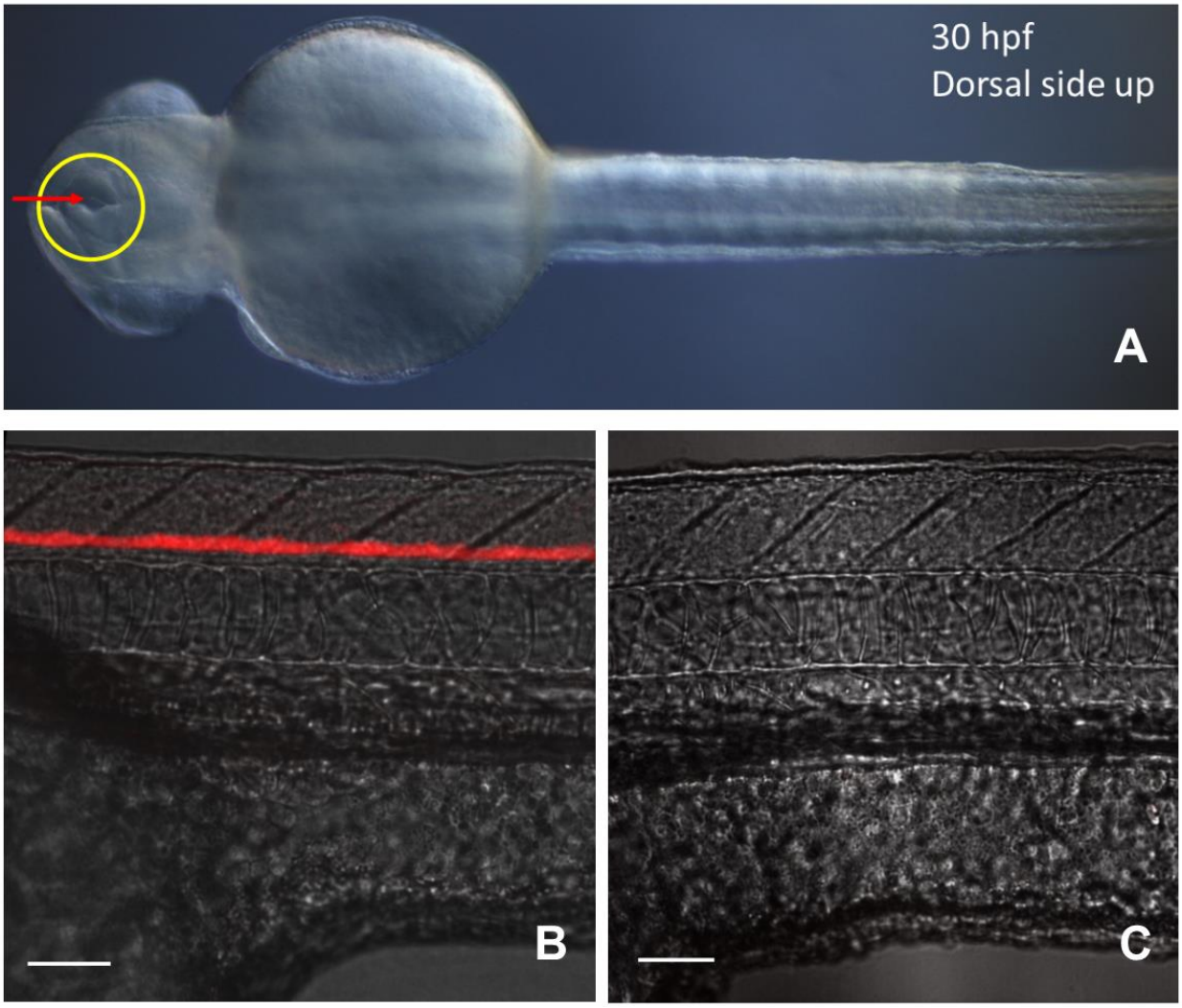
Figure 5: Lineage tracing demonstrates a trunk neural crest origin of post-embryonic neurogenesis.

5A-B) Phox2b-kaede embryos underwent neural tube injections of a far-red lipophilic dye at 30 hpf, after vagal crest has delaminated from the neural tube. Live images at 6 dpf of the midgut (5A) and hindgut (5B) demonstrate Phox2b-kaede enteric neurons that co-localize with the dye, indicating their trunk origin.

5C-D) Fish from the inducible Sox10-Cre line were exposed to 4-OHT at 3.5 dpf and underwent IHC for the Cre reporter, mCherry, and the neuronal marker, HuC/D at 5.5 dpf, with Cre labelled enteric neurons observed in the midgut (5C) and hindgut (5D). As Cre induction occurred after Sox10 is no longer present within the intestine, these results support a trunk neural crest origin of these enteric neurons.

All scale bars = 20 um

Supplement 5.

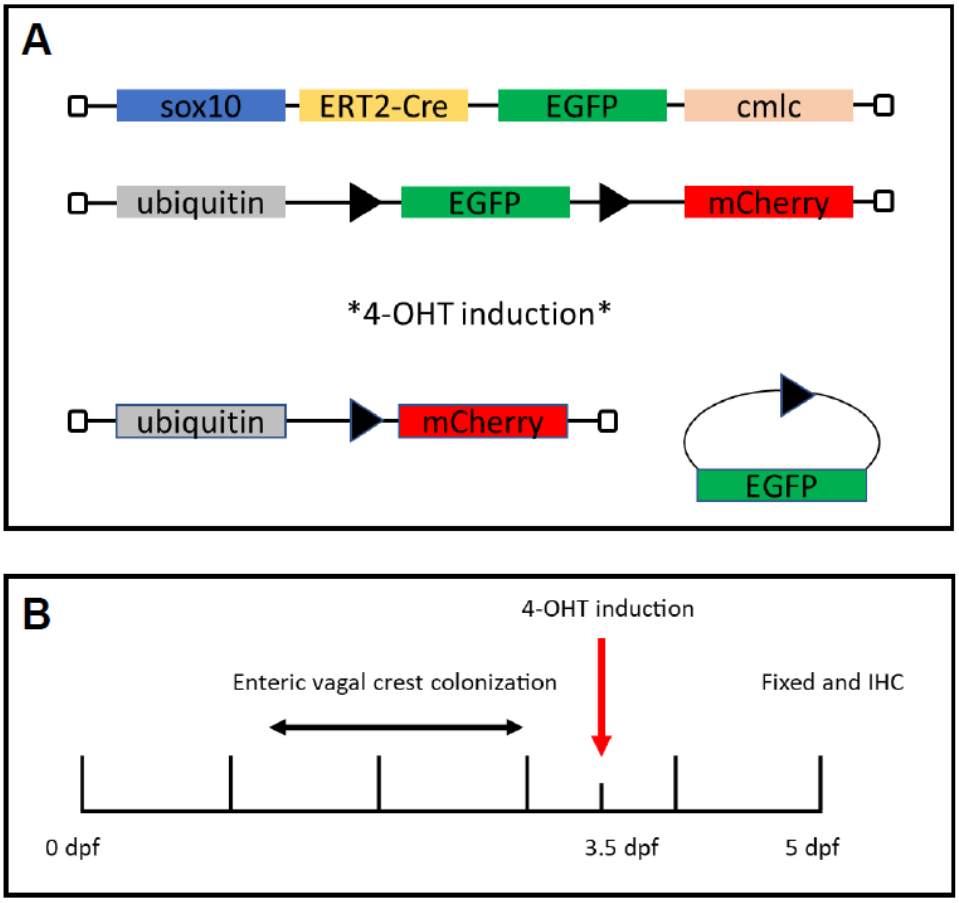


Supplement 5: Neural tube injection of lipophilic dye

S5A) Schematic of lipophilic dye injection at 30 hpf. The anterior neuropore (yellow circle) is open at this time point, allowing insertion of a dye-filled capillary in the trajectory depicted by the arrow.

S5B-C) 1-hour post injection, a dye-colored stripe is present indicating successful neural tube fill. Control fish that were not injected did not exhibit far-red fluorescence.

Supplement 6.



Supplement 6: Experimental approach for transgenic lineage tracing

S6A) The inducible Sox10-Cre line was crossed with the reporter, ubi:switch. After exposure to the induction agent, 4-OHT, Cre is activated, cleaves the loxP sites specifically in cells expressing Sox10 at the time of induction, leading to those cells permanently being labelled by mCherry.

S6B) Schematic of the induction protocol for Figure 5C-D. Fish were exposed to 4-OHT at 3.5 dpf for 16 hours, when Sox10 is no longer expressed in the intestine. Cells labelled during this time are thus gut-extrinsic Sox10-expressing cells.

Chapter 5

$5HT_4$ receptor agonism promotes increased post-embryonic enteric neurogenesis in normal development and after injury

Introduction:

$5HT_4$ receptor ($5HT_4R$) agonists have been employed clinically in the United States for the treatment of constipation as they have a pro-motility effect[77,78]. However, earlier $5HT_4R$ agonists such as tegaserod have restricted use in the United States due to nonspecific binding to off-target sites resulting in significant cardiovascular side effects[77,79]. $5HT_4$ receptors are present on enteric neurons, but it is unclear if intestinal epithelial cells also possess these receptors[80,81]. Along with pro-motility effects, multiple groups have demonstrated that this drug class promotes enteric neurogenesis in the adult stage of development. Indeed, previous studies in rodents have shown that postnatal enteric neurogenesis occurs after exposure to $5HT_4R$ agonists[40–42]. In one study, adult mice exposed to tegaserod exhibited enteric neurogenesis, while control mice did not[41]. In another study, gerbils that had undergone colonic resection and re-anastomosis demonstrated increased enteric neurogenesis at the anastomotic site when treated with mosapride[40]. Recently, the highly specific $5HT_4R$ agonist prucalopride has been approved for clinical use in the United States[82] to treat slow transit constipation as this drug stimulates pro-motility activity of enteric neurons[77]; however, this drug has not yet been evaluated with respect to enteric neurogenesis.

Methods:

Transgenic lines

Zebrafish (danio rerio) were maintained at 28°C, with adults on a 13-hour light/11-hour dark cycle. All zebrafish work was completed in compliance with the California Institute of Technology Institutional Animal Care and Use Committee. Transgenic lines used in this study were the photoconvertible Phox2b-kaede line[60] and the HuC:GCaMP6 transgenic line[83]. All lines were within an ABWT background, with the exception of the HuC:GCaMP6 line, which was backcrossed onto the pigmentless “casper” line[84].

Photoconversion

Adapting a previously described protocol[73], we photoconverted all enteric neurons of Phox2b-kaede fish at 4.5 dpf using a Zeiss LSM 800 confocal microscope. Full thickness photoconversion was confirmed by post-conversion imaging through the full z-stack in all fish.

Two-photon cell ablation

Adapting a previously described protocol[74], we ablated 10 enteric neurons within the distal hindgut (i.e. corresponding to the last two somite lengths of hindgut) of Pho2b-kaede fish at 4.5 dpf using a Zeiss LSM 710 confocal microscope with two-photon laser ablation.

Drug exposure

Prucalopride [Millipore Sigma SML1371] was prepared at 10 uM and 100 uM in DMSO, and GR113808 [Millipore Sigma G5918] was prepared at 10 uM in DMSO and exposure occurred at 4.5 dpf through 5.5 dpf, unless otherwise stated. 4-OHT [Millipore Sigma H7904] was prepared at 20 uM in ethanol and exposure occurred at 3.5 dpf for a total of 16 hours. Controls in the prucalopride/GR113808 and 4-OHT experiments were exposed to equal volumes of DMSO or ethanol, respectively.

Live-imaging

Live zebrafish larvae were anesthetized with tricaine and mounted within chamber slides using 1.2% low-melt agarose prepared in embryo water[67]. Additional embryo water was added after solidification of the agarose. All live-imaging was performed on a Zeiss LSM 800 confocal microscope with the incubator set at 28°C. For time-lapse experiments, z-stacks were collected every 4 minutes with a duration of 8 to 10 hours. Videos and 2D projections of Z-stacks were

produced using Imaris software [Bitplane]. All other live-images were produced with ImageJ software.

For the functional assay, a continuous video was collected after placing the mounted larvae in the microscope's heated incubator chamber for 30 minutes. Then, a baseline video was collected for 3 minutes in the z-plane corresponding to the mid-depth of the intestine, followed by addition of prucalopride in DMSO for a final concentration of 10 uM, GR113808 in DMSO for a final concentration of 10 uM, or DMSO alone to the individual fish's chamber, and then a 15-minute video was immediately collected. An "expulsive contraction" was defined as a contraction resulting in the expulsion of autofluorescent intraluminal mucous out of the hindgut and into the external environment. Videos were produced using ImageJ software.

Cell counting and statistics

Cell counting was performed manually using ImageJ software. In non-ablated fish, cell counts were within the hindgut corresponding to the last four somite lengths of hindgut. In the cell ablation experiments, cell counts were within the distal hindgut corresponding to the last two somite lengths of hindgut (i.e. within the field of ablation). Statistics were performed using Graphpad Prism 8 [Graphpad Software, Inc.] using Student t-test for 2 group comparisons and 1-way ANOVA for >2 group comparisons, with a P-value <0.05 indicating statistical significance.

Results:

Using zebrafish transgenic line HuC:H2B-GCaMP635, we found that fish exposed to 10 uM prucalopride exhibited significantly increased hindgut contractions resulting in increased intraluminal expulsion at 5 dpf compared to controls (mean: 4 vs 0.5 expulsive contractions; p <0.001) and to fish exposed to the $5HT_4R$ antagonist GR113808 (mean: 4 vs 0.67 expulsive contractions, p <0.001) [Figure 6a-b, Supplement 7a-b]. Notably, these contractions appeared

to be associated with increased GCaMP activity in enteric neurons, suggesting neuronally-mediated contractions. The results from this functional assay demonstrate that $5HT_4R$ signaling in zebrafish is active at these drug concentrations.

To assess the effects of prucalopride on post-embryonic enteric neurogenesis, we utilized the Phox2b-kaede line and photoconverted all enteric neurons at 4.5 dpf. Cohorts of these fish were then exposed to 10 uM prucalopride, 100uM prucalopride, or DMSO [Supplement 8A]. Live-imaging was performed at 5 dpf. The results show that both prucalopride treated cohorts possessed significantly more de novo enteric neurons (green-only) in the hindgut compared to controls ($p<0.05$) [Figure 7a]. As there was no difference in de novo enteric neuron numbers between the 10 uM and 100 uM cohorts, 10 uM of prucalopride was the dose used in subsequent experiments.

We next tested the effect of the $5HT_4R$ antagonist GR113808 using the same experimental design and found that GR113808 treated fish had fewer de novo enteric neurons than the control cohort that was statistically significant, though the absolute difference was modest (4.5 neurons, $p<0.05$) [Figure 7b].

To assess if $5HT_4R$ agonism is involved in enteric neurogenesis after injury, we performed two-photon laser ablation of enteric neurons in the hindgut of Phox2b-kaede fish at 4.5 dpf followed by photoconversion of all enteric neurons within the intestine. Subsequently, one cohort of these fish were treated with 10 uM prucalopride and a control group was treated with DMSO [Supplement 8B]. Fish were re-imaged at 5 dpf, and de novo enteric neurons within the distal hindgut were counted; no significant difference was noted between the two groups [Figure 7c].

We next repeated this experiment but treated one cohort of fish with 10 uM prucalopride for 24 hours prior to laser ablation and photoconversion at 4.5 dpf [Supplement 8C]. Compared to controls pre-treated with DMSO, pre-treatment with prucalopride resulted in significantly more ($p<0.05$) de novo enteric neurons after injury [Figure 7d].

Discussion:

These results indicate that prucalopride promotes not only intestinal motility, but also post-embryonic enteric neurogenesis. As demonstrated in previous Chapters, these new neurons do not appear to arise from resident Phox2b-kaede expressing cells or glia/progenitors. Furthermore, these findings suggest that exposure to prucalopride prior to injury promotes regeneration of enteric neurons, whereas a short course of treatment after injury has no effect on neurogenesis.

Our simplified motility assay to assess prucalopride's effect on the zebrafish intestine is easily accessible without the need for custom cameras or imaging processing programs, contrasting with other gastrointestinal motility studies in zebrafish[85–87]. The results demonstrate a functionally significant assay that is intuitively translatable to clinical end-points. The $5HT_4R$ antagonist GR113808 did not decrease expulsive contractions compared to controls and only modestly decreased post-embryonic enteric neurogenesis, which may indicate compensatory or redundant mechanisms for these processes, such as BDNF-promoted enteric neurogenesis[88]. While pre-treatment with prucalopride promoted enteric neurogenesis after injury, treatment after injury did not. This may suggest a period of recovery or a longer treatment duration is required to promote enteric neuronal regeneration, but further investigation is required.

As SCP-derived enteric neurogenesis is conserved in mammals, our demonstration that this source of enteric neurogenesis may be amenable to pharmacologic manipulation deepens the rationale for further exploration into $5HT_4R$-based therapies for human enteric neuropathies. Other $5HT_4R$ agonists such as mosapride (not available in the United States) and tegaserod (limited use in the United States due to off-target side effects)[79] have previously supported the role of this signaling pathway in enteric neurogenesis. Our study is the first to employ prucalopride, a highly specific $5HT_4R$ agonist that has recently been approved for use in the United States[82]. Prior studies suggested $5HT_4R$ agonism mediated its enteric neurogenic effect through a resident progenitor, but the evidence was inconclusive as a gut-extrinsic source was not assessed. Indeed, in one of these studies, de novo enteric neurons were first detected outside of enteric ganglia and then appeared to migrate within the ganglia[41], which has lead us and others[49] to hypothesize that these observations are consistent with SCP-derived enteric neurogenesis.

The mechanism of $5HT_4$ receptor agonism promoted enteric neurogenesis is unknown. Complicating this topic, the distribution of these receptors is controversial, with one study claiming that, within the intestine, they are only present on enteric neurons[80], while another group reporting expression within enteric neurons and intestinal epithelial cells[81]. Whether $5HT_4R$ agonists bind directly to SCPs or act on SCPs indirectly and the cellular signaling mechanism are areas for future investigation.

Figure 6.

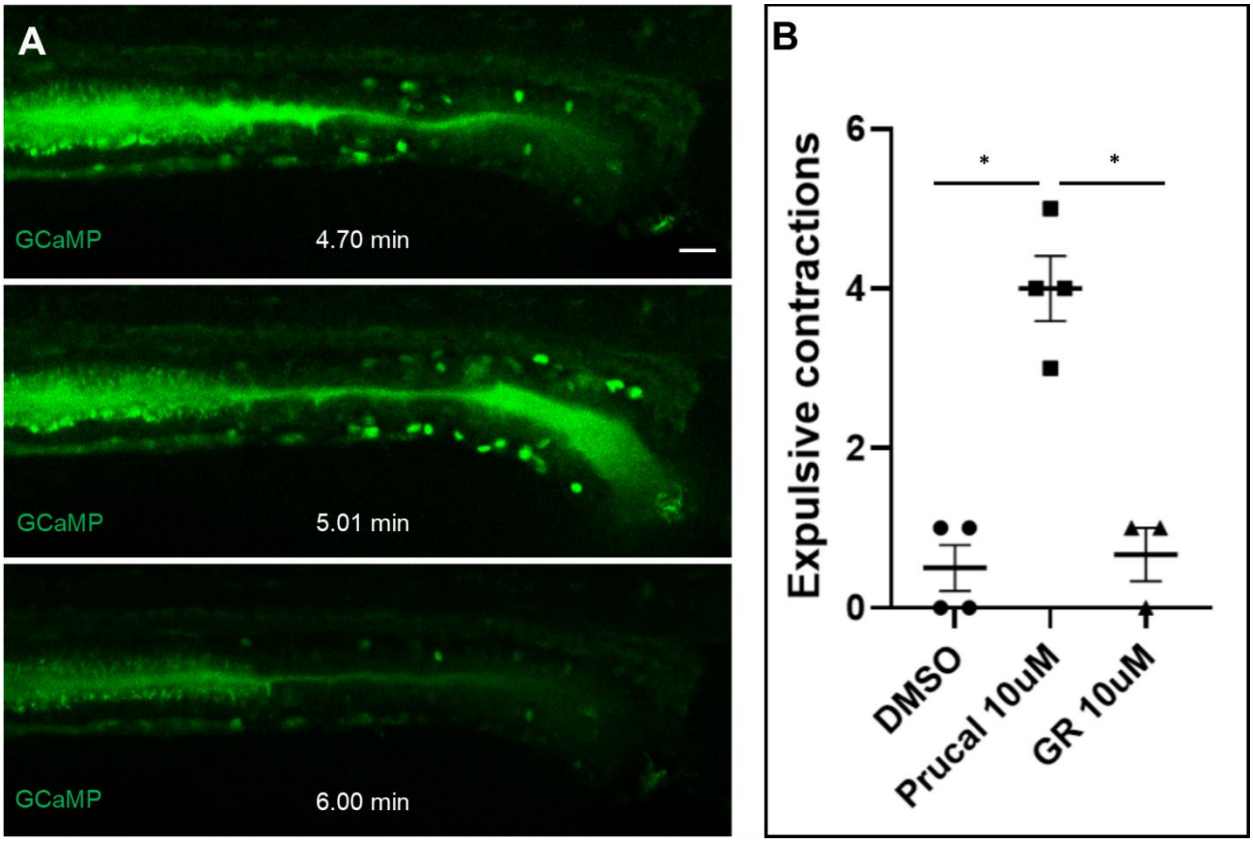


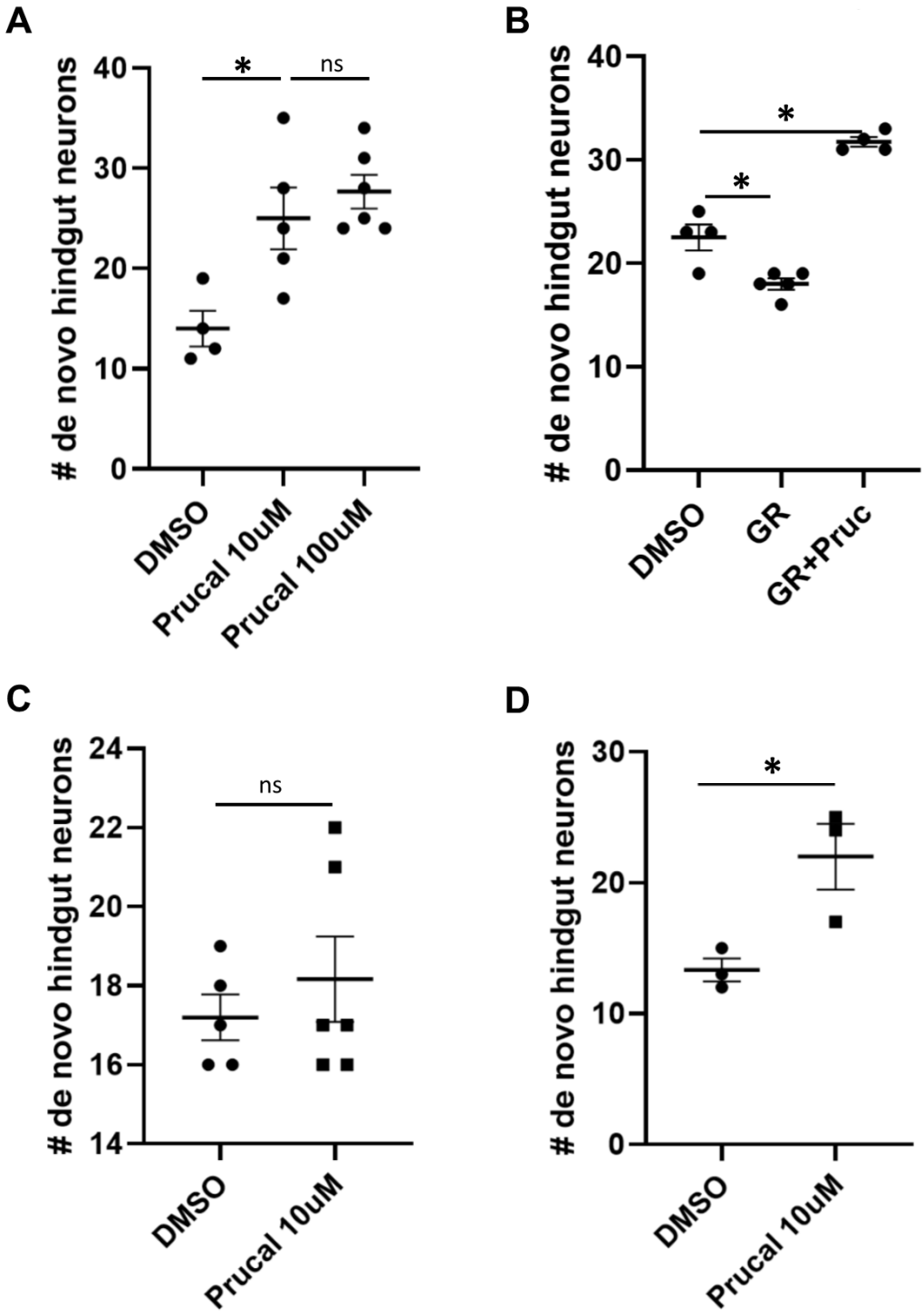
Figure 6: Prucalopride is active in the zebrafish and increases intestinal motility.

6A): Stills from a video of a live HuC-H2B GCaMP6 fish exposed to 10 uM prucalopride at 5 dpf reveals increased intestinal motility, as measured by expulsive contractions of autofluorescent intraluminal mucous into the external environment. Increased GCaMP signal was observed in association with expulsive contractions, suggesting neuronally-mediated motility.

Scale bar = 20 um

6B): Compared to controls and fish exposed to the 5HT4R antagonist, GR113808, fish exposed to prucalopride exhibited significantly more expulsive contractions.

Figure 7.


A
de novo hindgut neurons
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30
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Prucal 10uM
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DMSO
Prucal 10uM

Figure 7: Prucalopride promotes enteric neurogenesis in normal development and injury.

7A) After photoconversion of all enteric neurons at 4.5 dpf, cohorts of Phox2b-kaede fish were exposed to 10 uM prucalopride (N=5), 100 uM prucalopride (N=6), or DMSO (N=4) for 12 hours and then live-imaged at 5 dpf. The number of de novo hindgut neurons was significantly higher in fish treated with prucalopride.

7B) Under the same experimental design as 7A, fish were exposed to 10 uM GR113808 (N=5), 10 uM GR113808 plus 10 uM prucalopride (N=4), or DMSO (N=4) for 12 hours and then live-imaged at 5 dpf. The number of de novo hindgut neurons was significantly lower in fish treated with GR113808 compared to control but did not neutralize the effect of prucalopride exposure.

7C) At 4.5 dpf, Phox2b-kaede fish underwent laser ablation of 10 distal hindgut enteric neurons and then photoconversion of all enteric neurons. Cohorts were exposed to 10 uM prucalopride (N=6) or DMSO (N=5) for 12 hours and then live-imaged at 5 dpf. There was no difference in de novo distal hindgut neurons between these two groups.

7D) Under similar experimental design as 7C, fish were instead exposed to prucalopride 10 uM (N=3) or DMSO (N=3) at 3.5 dpf for 12 hours, and then underwent cell ablation and photoconversion at 4.5 dpf. At 5 dpf, live-imaging revealed significantly more distal hindgut neurons in fish pre-treated with prucalopride.

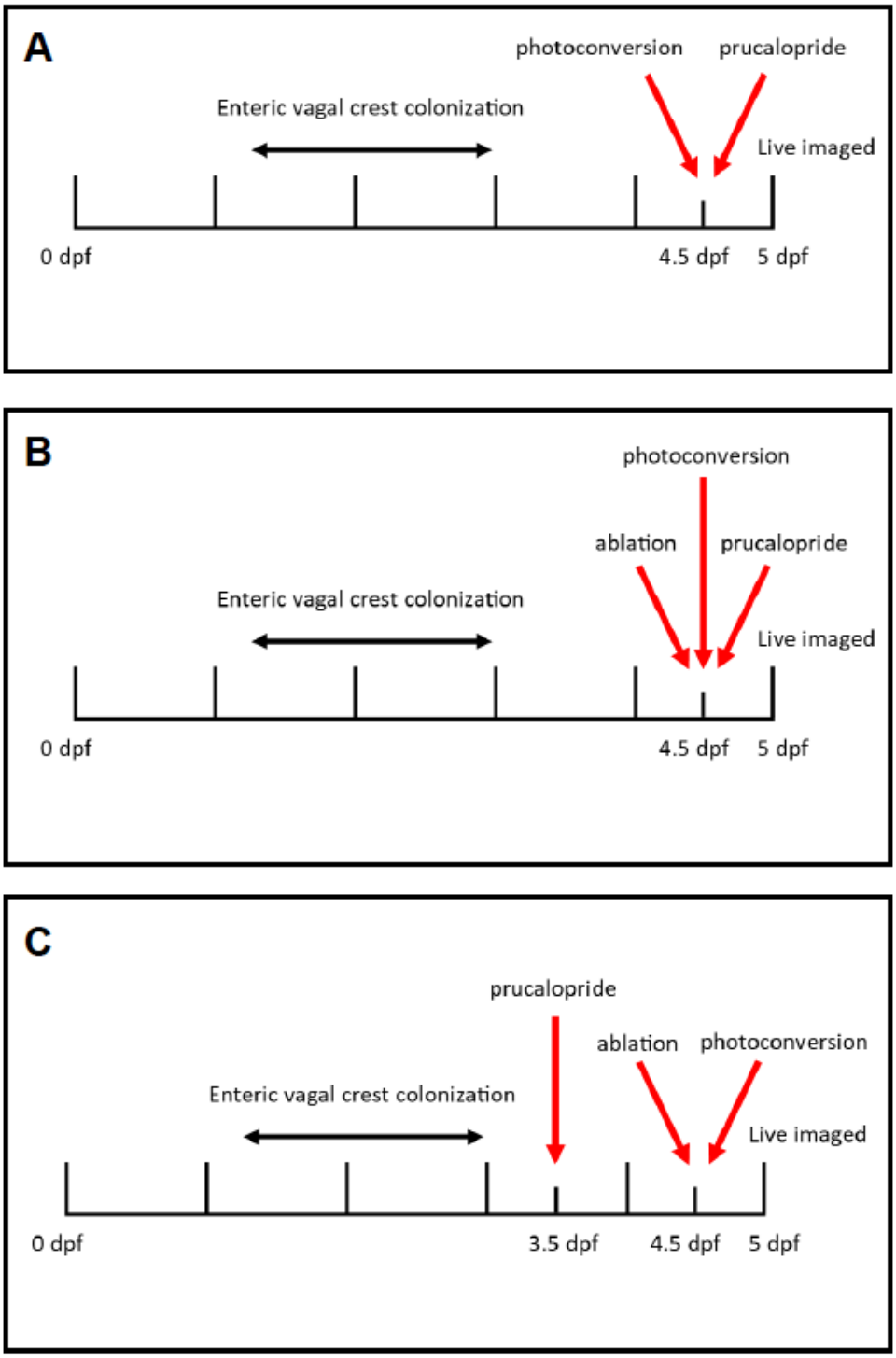
Supplement 7.

Videos available online.

Supplement 7: Live videos of expulsive contractions

Video of a 5 dpf HuC-H2B GCaMP6 fish exposed to DMSO reveals low baseline motility over a 15 min time frame (S7A). In contrast, multiple expulsive contractions are observed with exposure to 10 uM prucalopride (S7B).

Supplement 8.



Supplement 8: Experimental design of drug exposure, photoconversion, and laser ablation

S8A-C: Schematic of the protocols for Figures 7A, C, and D depicting the timing of drug exposure, photoconversion, and laser ablation.

Chapter 6

Summary and future directions

Summary:

In this study, we provide evidence that zebrafish enteric neurogenesis persists in the post-embryonic intestine both during normal development and after ablation of enteric neurons despite an apparent absence of enteric glia and/or Sox10-derived resident progenitors. Rather, lineage tracing experiments support the intriguing possibility that trunk crest-derived neural crest stem cells, likely to be Schwann cell precursors that migrate along nerves from the spinal cord to the intestines, are a source of this post-embryonic enteric neurogenesis. Along with the expected pro-motility effect, we also demonstrated that $5HT_4$ receptor agonism with prucalopride increased post-embryonic neurogenesis in normal development and appeared to promote regeneration of enteric neurons if the exposure occurred prior to injury. Altogether, our results reveal fundamental features of post-embryonic ENS development and regeneration in the zebrafish, pointing toward potential therapeutic strategies to promote Schwann cell precursor-derived enteric neurogenesis in the treatment of enteric neuropathies.

Future directions:

Nevertheless, this study reveals several future avenues of investigation. Firstly, the evolutionary development of enteric glia is a particularly interesting question as it appears that teleosts do not possess this cell type yet have a well-functioning intestinal tract. Of note, enteric glia have not been reported to be present in the lamprey. Adding to the perplexity is the finding that ablating enteric glia in adult mice had an underwhelming effect on intestinal motility and did not affect intestinal barrier integrity[72], which highlights the field's gap of knowledge regarding the functional role of enteric glia. Given that enteric glia outnumber neurons 7:1 in the human intestine[9], it would be reasonable to assume that the hundreds of millions of enteric glia have some functionally significant role. We suspect that enteric glia may have arisen when

vertebrates became terrestrial, which may help elucidate their functional role, but clearly further study is required to investigate this issue.

Secondly, while our lineage tracing of SCPs supports the hypothesis that these cells arise from the neural tube, the timing of migration, cues for migration, and migration dynamics are as of yet undetermined. Further research is required to determine these features and will be assisted by the creation of a transgenic line specific to SCPs. Candidate markers for SCPs in zebrafish include desert hedgehog (Dhh) and cadherin 19 (CDH19)[48], as demonstrated in other animal models. While an SCP reporter line would be useful for early migration studies, these cells downregulate Dhh and upregulate neuronal markers as they enter the intestine[49]. Thus, transgenic lines which permanently label these cells, such as the Cre-loxP system, may be beneficial for lineage tracing during later migration.

Thirdly, while this study and others have demonstrated increased enteric neurogenesis with $5HT_4$ receptor agonist treatment, the details of the mechanism underlying this effect are unknown. For example, it is unclear if SCPs have $5HT_4$ receptors that bind the drug directly, or if the drug binds other cells that then signal to SCPs to migrate and differentiate. Moreover, it has been speculated that the $5HT_4$ receptor serves as a co-receptor to RET[89], but this has not been directly investigated. Thus, the cellular response to $5HT_4$ receptor agonists is an area of research that is ripe for further study. Furthermore, the $5HT_4$ receptor antagonist GR113808 did not decrease expulsive contracts compared to controls and only modestly decreased post-embryonic enteric neurogenesis, which may indicate compensatory or redundant mechanisms for these processes, such as BDNF-promoted enteric neurogenesis[88]. Thus, it may be helpful to investigate a dose-response relationship of $5HT_4$ receptor antagonists in motility and enteric neurogenesis.

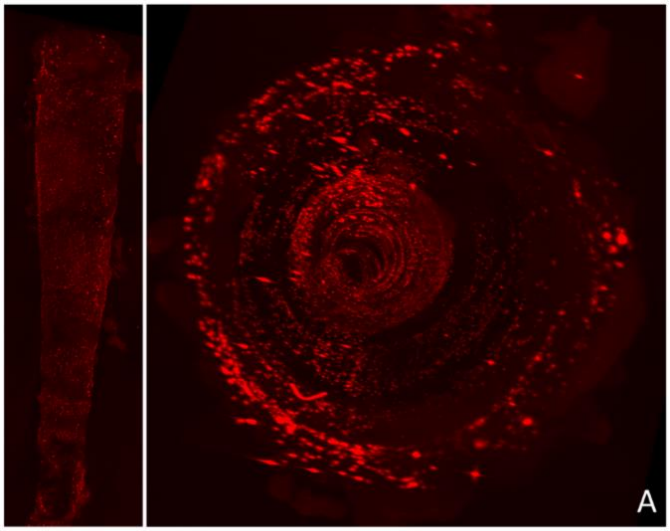
Additionally, our injury model utilizing 2-photon laser ablation of individual enteric neurons presents many opportunities for future investigations. For example, it is unclear if loss of 10 distal hindgut neurons results in functional changes in motility or response to the pro-motility effect of prucalopride, which we could explore with our expulsive contraction assay. Moreover, it remains to be determined if the enteric neurogenesis observed after injury utilizes the same program as that seen during normal development, or if there is a distinct response by SCPs to the injury.

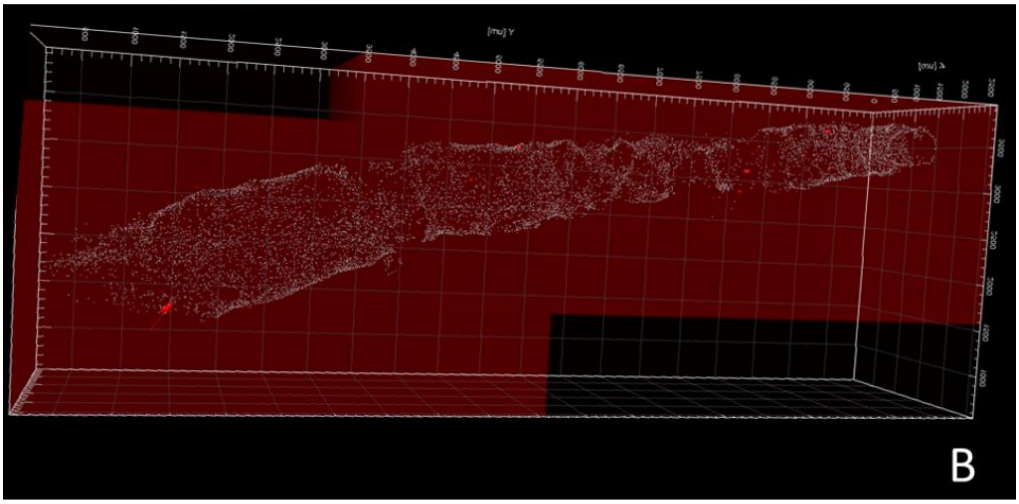
Lastly, applying these findings to later developmental stages, specifically to adult zebrafish, would be beneficial for their translational potential to adult human diseases. A fundamental problem in the assessment of the pathogenesis of enteric neuropathies in humans has been the practice of quantifying the enteric neurons from a small sample of the gastrointestinal tract and then extrapolating to the whole organ segment. As the distribution of the ENS is patchy and heterogenous within each segment of the gastrointestinal tract, this extrapolation method yields variable results that lead to inconsistent conclusions between studies[26,27]. Indeed, a position statement authored by several leaders in enteric neuropathies called for investigators to cease quantifying the ENS by extrapolation[90]. In light of this expert panel's recommendation, the very basis of several enteric neuropathies now appears to be in question. To address this, we utilize the advantage of the small scale of the adult zebrafish intestine, which permits whole organ imaging and thus quantification of all enteric neurons within the entire intestinal tract. To accomplish this, the dissected gut is fixed as a straight cylindrical tube, undergoes whole-mount IHC against the neuronal marker HuC/D, optically cleared with RIMS[65], mounted within agarose prepared in RIMS, and then imaged using a lightsheet microscope. The 3-dimensional image of the whole gut is then processed using Imaris software, allowing for automated quantification of

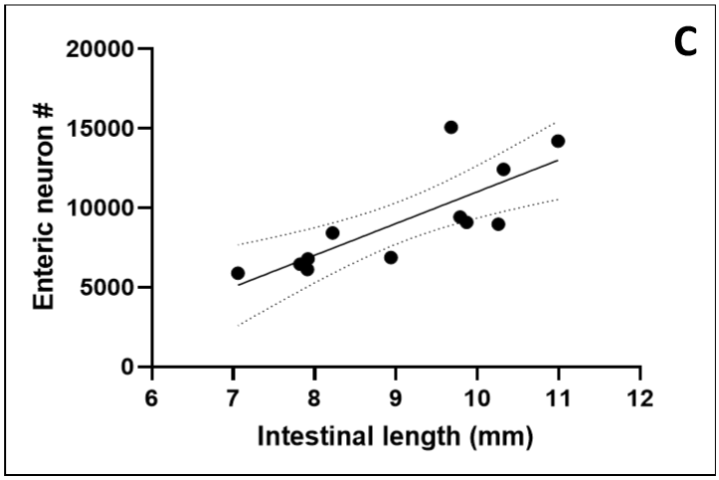
all enteric neurons. In our pilot study employing this method, we find that enteric neuron number is proportional to intestinal length, which, while being intuitive, is a novel finding. With this new assay of universal enteric neuron quantification, we plan to assess the role of SCP-derived enteric neurogenesis in normal development and in models of enteric neuropathy, such as diabetes mellitus.

In conclusion, the zebrafish model enabled us to perform compelling studies into the nature of enteric neurogenesis, revealing that this neurogenesis persists in the post-embryonic period in both normal development and after injury. Schwann cell precursors provide enteric neurons in this time period and appear to originate from the trunk neural crest. The $5HT_4$ receptor agonist prucalopride, which was recently approved for use in the United States, promotes this enteric neurogenesis. Establishing these fundamental features of ENS homeostasis with relation to a recently approved therapeutic agent has significant clinical applications for several human enteric neuropathies, for which there are currently few safe, effective, and non-invasive therapeutic options.

Figure 8.


A


B


C
Enteric neuron #
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15000
10000
5000
0
6
7
8
9
10
11
12
Intestinal length (mm)

Figure 8: Pan-intestinal imaging permits quantification of all neurons within the enteric nervous system.

8a) After wholemount IHC versus HuC/D and optically clearing with RIMS, tiled z-stacks were acquired of the entire adult zebrafish intestine, from foregut to hindgut, using a Zeiss Z1 lightsheet microscope. The tiled images were stitched into a single montage using Arevis software and further analysis performed on Imaris software. On the left is an example of an adult foregut with dense HuC/D staining pattern. On the right, the 3-dimensional image is rotated to obtain an intraluminal view of the intestine.

8b) Another adult zebrafish intestine that was pan-imaged as above, but then underwent automated cell counting using Imaris software (marked by white dots). This program's "Spots" feature allows thresholds to be set regarding cell size and fluorescent intensity, thus reducing false positive and false negative cell counts. This intestine revealed a total of 8,978 enteric neurons.

8c) Applying this assay to N=12 adult male zebrafish, we found that enteric neuron number is positively correlated to intestinal length. The data in this graph represent a linear regression analysis, with an R^2 of 0.62 and a slope that was significantly non-zero ($p=0.0021$). The dashed lines represent the 95% confidence interval of the linear regression.

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