“Progenitor Populations Within the Vertebrate Enteric Nervous System”

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I, Sarah McCallum, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
Abstract

Tissue maintenance and repair depends on specialised cell types with the capacity to replenish cellular compartments lost by normal turnover or disease. Within the enteric nervous system (ENS), enteric glial cells (EGCs) play roles in: supporting enteric neurons, modulating inflammation, enhancing epithelial barrier fitness and, under certain experimental conditions, regenerating the ENS. Nevertheless, the cellular and molecular basis of the regenerative capacity and the extent of regeneration of the ENS is unknown. We use zebrafish, a model organism particularly amenable to genetic, developmental and functional studies, to study the organisation and function of EGCs, including their potential role in ENS maintenance and regeneration. Since limited studies have examined EGCs in zebrafish, we first combined transgenic reporter analysis with immunostaining to characterise the EGC population in zebrafish. Our experiments led to the identification of a population of EGCs within the ENS that are closely associated with enteric neurons and express the neural crest marker Sox10, but surprisingly lack canonical peripheral glial markers. However, these cells express the Notch effector bHLH transcription factor Her4.1, and have characteristic ultrastructural features of EGCs. Since, Her4.1 is an established marker of radial glia in the zebrafish CNS, a known progenitor cell type we used EdU chase experiments and mathematical modelling to reveal the proliferative and neurogenic potential of Her4.1 cells in the adult zebrafish ENS. Moreover, we employed Notch inhibitors to show that Notch signalling promotes EGC quiescence and maintenance of the non-neuronal cell fate. Finally, using transgenic lines we identified expression of HES5, the mammalian orthologue of Her4.1, within a subpopulation of Type I mammalian EGCs, identifying the first subtype marker of mammalian EGCs. This work provides the first characterisation of zebrafish EGCs, and identifies a novel marker of EGCs in both the zebrafish and mouse ENS with putative stem cell properties.
Impact Statement

Stem cells are vital for tissue homeostasis. Understanding the cellular properties and functions of stem cells has clinical impact for elucidating their roles in regeneration during homeostasis, after pathology, and as a source of cells for transplantation studies. The enteric nervous system (ENS) is an attractive source of autologous neural stem cells, since they can be easily collected via routine biopsies. Moreover, functional enteric neural progenitor cells (ENPCs) can be collected from the guts of aged patients, suggesting that ENPCs can act as a source of stem cells throughout life. Nevertheless, despite knowing that functional neural stem cells capable of generating neurons and glia, exist within the glial population of the ENS, we have no molecular markers of these cells. In addition, it is unclear if there are restricted neuronal and glial progenitors or whether multipotent progenitors exist.

The work described herein identifies a stem cell population within the zebrafish ENS, which express the transcription factor Her4.1. We show that these Her4.1+ cells are capable of generating both themselves and new neurons during homeostasis. In addition, we show that Notch signalling maintains these stem cells in a quiescent state and that Notch signalling is important in maintaining the non-neuronal cell fate. Further, within mammals we have identified a population of enteric glial cells (EGCs) that express the mammalian orthologue of Her4.1, HES5. Identification of stem cell characteristics within the Her4.1+ zebrafish population suggests that the mammalian HES5 expressing EGCs may have similar functional characteristics, and warrants further analysis of their potential progenitor properties. Moreover, identification of a genetic marker (her4.1+) that is associated with stem cell properties within the ENS suggests potential clinical implications for the Her4.1+ cells in stem cell transplant studies. Multiple studies have shown that ENPCs are capable of acting as material for stem cell transplants to treat ENS pathologies such as Hirschsprung disease or chronic pseudo-obstruction. However, despite promising results, these studies have had limitations such as a limited spread of the transplanted material and altered differentiation potential of transplanted cells. It has been suggested that these limitations are partially due to the use of heterogeneous starting material. However, if a pure stem cell population was used, such as Her4.1+ cells, this may overcome some of these problems. Moreover, readily accessible
ENPCs have been shown to be more successful when transplanted to treat central nervous system (CNS) pathologies than CNS-derived progenitors. Therefore, the Her4.1+ cells identified are a potential resource for treatment of CNS pathologies.

Further elucidation of the roles of specific aspects of Notch signalling and its downstream mediators, in controlling the proliferative potential of the Her4.1+ population may also be instructive for future therapies. Moreover, understanding the mechanisms mediating the proliferative potential of Her4.1+ cells in homeostasis and disease will give insight into how and why pathology occurs.
Acknowledgement

My family often ask me “how long does it take to get a PhD”, I can now (almost) answer: it takes several new friends, lots of old ones, trips all over the world, three weddings, many more engagements, three babies, lots of laughs, occasionally some tears, too many beers, learning MCB in as many languages as you can, a supply of cake and coffee, a visit from the queen, and all those that supported me along the way.

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## Abbreviations

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<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix loop helix</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CLEM</td>
<td>Correlative light and electron microscopy</td>
</tr>
<tr>
<td>dpf</td>
<td>days post fertilisation</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>EdU</td>
<td>5-Ethynyl-2’-deoxyuridine</td>
</tr>
<tr>
<td>EGCs</td>
<td>enteric glial cells</td>
</tr>
<tr>
<td>eNCCs</td>
<td>enteric derived neural crest cells</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>Hes</td>
<td>Hairy enhancer of split</td>
</tr>
<tr>
<td>hpf</td>
<td>hours post fertilisation</td>
</tr>
<tr>
<td>HSCR</td>
<td>Hirschsprung disease</td>
</tr>
<tr>
<td>mo</td>
<td>Month Old</td>
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<tr>
<td>NC</td>
<td>Neural crest</td>
</tr>
<tr>
<td>NEXT</td>
<td>Notch external truncated domain</td>
</tr>
<tr>
<td>NICD</td>
<td>Notch intracellular domain</td>
</tr>
<tr>
<td>NSC</td>
<td>Neural stem cell</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>RGCs</td>
<td>Radial glial cells</td>
</tr>
<tr>
<td>SCP</td>
<td>Schwann cell precursors</td>
</tr>
<tr>
<td>Su(H)</td>
<td>suppressor of hairless</td>
</tr>
<tr>
<td>SEZ</td>
<td>Subependymal Zone</td>
</tr>
<tr>
<td>SGZ</td>
<td>Subgranular Zone</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
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Chapter 1. Introduction

1.1 The Enteric Nervous System

The nervous system is composed of central and peripheral sub-divisions. The enteric nervous system (ENS) is the largest subdivision of the peripheral nervous system (PNS). Due to its complexity, the ENS has more similarities to the central nervous system (CNS), than the other subdivisions of the PNS, the sympathetic and parasympathetic branches of the autonomic nervous system and sensory ganglia (Furness, 2006).

1.1.1 Organisation

The ENS is composed of neurons and glia. In the mammalian gut, the majority of neurons and glia are found in two interconnected gangliated plexi, the outer myenteric (Auerbach’s) and the inner submucosal (Meissner’s) plexus (Figure 1.1A and C). The myenteric plexus is situated between the circular and longitudinal muscle layers and is found throughout the gastrointestinal (GI) tract. Neurons from the myenteric plexus primarily innervate and interact with the muscular layers, although primary afferent neurons that contact the mucosa have also been described in the myenteric ganglia. The submucosal plexus, on the other hand, is situated between the circular smooth muscle and the mucosa, and is only found within the small and large intestines. Submucosal neurons largely project into the mucosa. Despite large variations in ganglia size, the ganglia in the myenteric plexus are generally larger in comparison to those in the submucosal plexus. Moreover, there are further variations between gut regions, such as ganglia being generally larger in the colon (Furness, 2006). The cell bodies of enteric neurons are restricted to the ganglia, but enteric glial cell (EGCs) and neuronal processes are found throughout the wall of the gut including the mucosa (Figure 1.1A and C) (Gulbransen and Sharkey, 2012, Kabouridis et al., 2015, Ruhl, 2005). Many different types of enteric neurons have been described which are distributed in a “salt and pepper” fashion within the ganglia with an apparently stochastic mixture of neuronal subtypes (Furness, 2006).
Figure 1.1. Organisation of the ENS. (A) The mammalian ENS is organised into two plexi comprised of neurons (yellow) and glia (green): the myenteric plexus is located between the longitudinal smooth muscle and the circular smooth muscle, and the submucosal plexus, is located between the circular smooth muscle and the mucosa. Glial cells are also found within the mucosa. (B) The zebrafish ENS is located within the myenteric plexus between longitudinal smooth muscle and circular smooth muscle. Enteric neurons (yellow) are found as individual cells within the plexus. (C) Image of the mouse myenteric plexus showing ganglia containing neurons (green, arrows) and glia (red, arrowheads), glial cells are also found outside the ganglia. (D) Image from the zebrafish myenteric plexus showing neurons (green, arrows) as individual cells or in small groups. (A) was modified from Furness (2006) with permission from Blackwell Publishing.

In teleosts, such as zebrafish (*Danio rerio*), the ENS has a simpler organisation, in that it is comprised only of the myenteric plexus (Figure 1.1B and D). Despite neurons having similar subtype diversity observed in the ENS of the mouse (Chapter 1.1.1.1), they are found as individual cells or small groups and are not in organised into ganglia (Figure 1.1D). However, in contrast to the mammalian ENS, there are few regional differences in the organisation of the zebrafish ENS (Wallace
et al., 2005), which may reflect the lack of anatomically distinct subdivisions of the zebrafish intestine (Wang et al., 2010, Wallace and Pack, 2003). Currently, there have been only a few reports on zebrafish EGCs, and therefore their identity, cellular and morphological properties and anatomical positioning relative to enteric neurons is currently unknown.

1.1.1.1 Enteric Neurons

Enteric neurons are found in large numbers throughout the gut. In vertebrates, enteric neuron numbers are equivalent to the number of neurons found in the entire spinal cord (Furness, 2006). Adding to the complexity, subsets of enteric neurons express all the neurochemical markers found in the CNS. Due to the large numbers of neurons and their neurochemical diversity, the ENS is often referred to as “the second brain” (Furness, 2006). The first classification of mammalian enteric neurons was performed by Dogeil in 1899, which classified neurons based on the axonal and dendritic arborisation (Figure 1.2). Dogeil Type I neurons have a single axon with multiple dendrites. In contrast, Dogeil Type II neurons have multiple axons originating from the cell body (or from a single process) and lack dendrites. Finally, Dogeil Type III neurons have multipolar dendrites which are confined within their resident ganglia. Since this initial characterisation, neuronal classifications within the ENS have been expanded to include five additional morphologically defined subtypes. Dogeil Type IV neurons have asymmetrically arising dendrites with scarce branching, Dogeil Type V neurons have clustered dendrites that normally project aborally, and Dogeil Type VI neurons have dendrites arising from the proximal axon. In addition, Filamentous neurons have multiple short tapering processes and a single long process. The final morphological subtype is known as “small neurons”, which as their name suggests, are significantly smaller in size than the other neurons described, and have a simple axonal and dendritic arbour (Furness, 2006, Brehmer et al., 1999). Currently, axonal markers (such as AcTu: Acetylated tubulin and NF: Neurofilament) can be used to distinguish, to some extent between morphological subtypes and projection targets.

Enteric neurons are also subdivided on the basis of function, which relates to the localisation of their neuronal projections. Thus, motor neurons target the muscle layers and control peristalsis, interneurons target other enteric neurons, and sensory neurons detect mechanical stimulation or metabolites in the muscle and mucosal
layers. Secretomotor enteric neurons, which project to the mucosal epithelia and vasodilator neurons that project to blood vessels, are considered to be a special categories of motor neurons (Brehmer et al., 1999, Furness, 2006). The ENS is the only part of the PNS that contains motor neurons, sensory neurons and interneurons, a feature that adds to the complexity of this network and further justifies its name as the “second brain”.

Figure 1.2. Subtypes of enteric neurons.
Morphologies of Dogiel type I neurons (1, 3, 4, 5, 6, 7, 8, 9, 12), Dogiel type II neurons (2, 11), Dogiel type III neurons (10), Dogiel type IV neuron (14) are shown. Specifically, neurons shown are classified according to their position, projections, connections and functions: (1) ascending (orally projecting) interneuron; (2) Myenteric intrinsic primary afferent neuron; (3) intestinoefugal neuron (projects to sympathetic ganglia); (4) excitatory longitudinal muscle motor neuron; (5) inhibitory longitudinal muscle motor neuron; (6) excitatory circular muscle motor neuron; (7) inhibitory circular muscle motor neuron; (8) descending (anally projecting) interneuron (local reflex); (9) descending interneuron (secretomotor reflex); (10) descending interneuron (migrating myoelectric complex); (11) submucosal intrinsic primary afferent neuron; (12) non-cholinergic secretomotor/vasodilator neuron; (13) cholinergic secretomotor/vasodilator neuron; (14) cholinergic secretomotor (non-vasodilator) neuron. LM: longitudinal muscle, MP: myenteric
Further classification of enteric neurons by neurochemical coding has revealed an even greater variety of neuronal subtypes, of which there are believed to be more than 20 in both mammals and zebrafish (Furness, 2006, Shepherd and Eisen, 2011). Using these methods, enteric neurons can be classified by: 1) expression of enzymes involved in neurotransmitter synthesis or processing (ChAT: Choline acetyl-transferase, NOS: nitric oxide synthase, TH: tyrosine hydroxylase); 2) the presence of neuropeptides (CGRP: calcitonin gene-related peptide, NKA: neurokinin A, NPY: neuropeptide Y, PACAP: pituitary adenylate cyclase-activating polypeptide, SOM: somatostatin, TK: tachykinin, VIP: vasoactive intestinal peptide); 3) neurotransmitters (5-HT: 5-hydroxy tryptamine a.k.a. serotonin, Ach: acetylcholine, NO: nitric oxide), 4) calcium binding proteins (calbindin and calretinin) (Shepherd and Eisen, 2011, Uyttebroek et al., 2010, Qu et al., 2008, Furness, 2000). The main excitatory neurotransmitters within the ENS are Ach and TK whereas there are many inhibitory neurotransmitters such as NO, VIP and ATP (Furness, 2006). Neurochemical marker expression can be widespread, or can be restricted to only a minority of neurons. Within the mouse, there are two largely non-overlapping subtypes of enteric neurons that express either calretinin and nNOS (Qu et al., 2008, Lasrado et al., 2017), and similarly, in zebrafish, ChAT and nNOS expression do not overlap (Uyttebroek et al., 2010). The two distinct major subtypes of enteric neurons differ in marker expression, between mouse and zebrafish, however it is unknown whether these differences signify different functions or characteristics of the major ENS neuron populations in mouse and zebrafish. Recent single-cell bioinformatics analysis of the murine ENS has suggested there may be up to 9 transcriptionally distinct populations of enteric neurons within the mammalian ENS, and indicates a molecular basis for understanding subtype diversity (Zeisel et al., 2018).

Finally, neurons can be subdivided based on their electrophysiological properties. In adulthood, two electrophysiological subtypes exist: AH (after hyperpolarization) neurons, which are primarily composed of Dogeil Type II neurons; and S (synaptic) neurons, which are largely Dogeil Type I neurons. The AH subtype of neurons have a slow hyperpolarisation after a broad action potential. In contrast
S neurons have a sharp action potential profile in response to fast excitatory postsynaptic potentials (Nurgali, 2009, Nurgali et al., 2004).

1.1.1.2 Enteric Glia and Non-Neuronal Cells

Enteric glia outnumber neurons by 2-4 times in the adult ENS in all the mammalian species studied (Ruhl, 2005, Gabella, 1981, Furness, 2006) and have traditionally thought to be similar to astrocytes (Gabella, 1981, Jessen and Mirsky, 1983, Gulbransen and Sharkey, 2012). Characterization of EGCs has described four subtypes based on their morphology and location within the intestine (Figure 1.3). Type I EGCs are found in the submucosal and myenteric ganglia, closely associated with the enteric neuron cell bodies and have short fibrous processes that remain within the ganglia where the cell resides (Figure 1.3A). Type I EGCs are believed to be similar to astrocytes of the CNS (Chapter 1.2.1). Type II EGCs are observed within the inter-ganglionic strands that connect neighbouring ganglia. The Type II EGCs have elongated processes which are closely associated with neuronal fibres within the tracts (Figure 1.3B), but do not myelinate these fibres (Hanani and Reichenbach, 1994, Boesmans et al., 2014). Type III EGCs are found in the extra-ganglionic spaces, between the ganglia and the interganglionic strands, and in the mucosal layers. Morphologically, Type III EGCs have four main processes which branch further and contact a wide variety of cell types such as neurons, the vasculature and epithelial cells (Figure 1.3C) (Boesmans et al., 2014, Kabouridis et al., 2015, Bohorquez et al., 2015). Finally, Type IV EGCs are bipolar cells that are found within the smooth muscle layers (Figure 1.3D) (Boesmans et al., 2014).
Figure 1.3. *Enteric glial subtypes are defined exclusively by their morphologies and locations.* (A) Type I EGCs are found in the ganglia and have a star-shaped morphology. (B) Type II EGCs are found in the interconnective strands and have elongated processes. (C) Type III EGCs are found in the extra ganglionic space and stereotypically have four main processes. (D) Bipolar Type IV EGCs are found in the muscle layers. Modified from Boesmans (2014) with permission from Wiley Periodicals.

At the ultrastructural level, EGCs within the ganglia have stereotypic features. EGC nuclei have multiple lobes/crenations and take up with large majority of the soma. Glial processes often contact neuronal cell bodies, nevertheless the majority of the neuron remains exposed to the extracellular matrix. Despite detailed ultrastructural characterisation, no subtype specification has been found at the ultrastructural level (Gabella, 1981).

The majority of known glial cell markers (SOX10, S100β, BFABP, SOX2, proteolipid-1 (PLP1) and glutamate synthase (GS)) are expressed by all EGCs, and
these markers cannot be used to distinguish between EGC subtypes (Boesmans et al., 2014, Heanue and Pachnis, 2011, Jessen and Mirsky, 1983, Rao et al., 2015, Young et al., 2003). Interestingly, the majority of PLP1+ EGCs do not colocalise with GFAP, particularly in the colon (Rao et al., 2015). In addition, GFAP expression within adult EGCs fluctuates during normal homeostatic conditions (Boesmans et al., 2014), although the functional consequences of this are unknown. Transcriptionally, EGCs have been shown to be molecularly distinct from other glial cells of the CNS and PNS, however EGCs show clear similarities to Schwann cells and oligodendrocytes (Rao et al., 2015, Zeisel et al., 2018). Moreover, single-cell sequencing of the murine myenteric plexus has recently described seven transcriptionally distinct subsets of EGCs, one of which is proliferative, suggesting that EGCs are likely to have further heterogeneity within the previously described morphological subtypes (Zeisel et al., 2018). GFAP expression has also been described within the ENS of zebrafish (Doodnath et al., 2010, Hagstrom and Olsson, 2010, Kelsh and Eisen, 2000) but no expression of S100, which is widely expressed in mammalian EGCs, has been reported within the teleost ENS (Germana et al., 2008).

Unlike enteric neurons, which give rise to distinct action potentials, EGCs exhibit large passive currents (Hanani et al., 2000). EGCs exhibit calcium (Ca\(^{2+}\)) responses to neuroactive molecules such as ATP and ADP\(\beta\), via P2Y\(_1\) and P2Y\(_4\) receptors, and to neurotransmitters such as 5-HT and ACh (Boesmans et al., 2013b, Grubisic and Parpura, 2017, Gomes et al., 2009, Kimball and Mulholland, 1996). In addition, neuronal action potentials can stimulate calcium responses within EGCs (Gulbransen et al., 2010, Gulbransen and Sharkey, 2009, Boesmans et al., 2013a). Interestingly, Type I and Type II EGCs show increased Ca\(^{2+}\) responses to ATP when compared to Type III EGCs, suggesting functional heterogeneity within the EGC population (Boesmans et al., 2014). Moreover, EGCs have gap junctions, mediated by connexin 43, which allow for ATP and Ca\(^{2+}\) responses to spread between large numbers of adjacent EGCs (Zhang et al., 2003, Hanani and Reichenbach, 1994, McClain et al., 2014, Fung et al., 2017). Such exchange is believed to be important in gliotransmission, a process essential for glial-glial communication, which has been implicated in modulation of neuronal circuitry (Halassa et al., 2007).
1.1.2 Development

In all vertebrates, the ENS develops from neural crest (NC) cells that migrate into the gut. Once in the gut, they are known as enteric neural crest derived cells (eNCCs), which proliferate, migrate to fully colonise the gut, and differentiated to generate the neurons and glial cells of the ENS (Furness, 2006). Key aspects of ENS development will be discussed below.

1.1.2.1 Migration of NC cells into the developing gut

In vertebrates, the majority of the ENS is derived from vagal NC cells which arise from somites 1-7 (Figure 1.4A), and invade the fore-gut during embryogenesis. In mice, NC cells enter the fore-gut at around embryonic day 9.5 (E9.5), and by E14.5 eNCCs have colonised the entire length of the gastrointestinal tract (Young et al., 1999, Kapur et al., 1992, Le Douarin and Teillet, 1973). NC cells migrate in a rostrocaudal manner, in chains of associated NC cells, a pattern that is essential for effective directional migration (Young et al., 2003, Young et al., 2014). Individual chains migrate at variable speeds, leading to alterations in the individual cell that occupies the leading edge. Interestingly, the cells at the leading edge have more variable migratory behaviours, often leap-frogging one another, whereas, the follower eNCCs tend to migrate in the same path as the leaders (Young et al., 2014, Young et al., 2003). Recently, it has been shown that developing eNCCs, that have largely finished colonisation, function to supply enteric neurons and EGCs to a local gut region within the myenteric plexus, before seeding the submucosal and mucosal layers (Lasrado et al., 2017).

Trans-mesenteric migration has also been described as necessary, and sufficient for hind-gut colonisation. Trans-mesenteric migration describes the process of vagal eNCCs migrating from the juxtaposed mid-gut into the hind-gut, through the mesentery, which occurs between E10.5-E11.5 (Figure 1.4A). From E10.5-E11.5, the eNCCs dissociate from chains in the mid-gut and migrate as solitary cells across the mesentery. The solitary eNCCs invade the hind-gut mesenchyme between E11.5-E12.5, where they reform as chains of cells with one another, or with eNCCs that had migrated through the caecum. Once the chains are reformed, the eNCCs continue to migrate and colonise the hind-gut (Nishiyama et al., 2012).
The second source of the mammalian and avian ENS arises from the sacral NC, around E14, after the vagal eNCCs have entered the hind-gut (Figure 1.4B). Sacral eNCCs contribute to enteric neurons and glia specifically within the hind-gut (Kapur, 2000, Le Douarin and Teillet, 1973, Serbedzija et al., 1991). However, the sacral eNCC population is not required for hind-gut colonisation (Nishiyama et al., 2012).

Within mammals, a third NC cell source has been described to contribute to ENS colonisation, which originates from the truncal-crest. Truncal eNCCs enter the fore-gut around E9.5 and contribute to a small proportion of the ENS within the fore-gut (Kapur, 2000, Newgreen and Young, 2002).

Finally, in the mammalian gut, Schwann cell precursor (SCP) cells associated with extrinsic nerves of the gut migrate into the small intestine (E14.5), and large intestine (E16.5) and contribute to ENS development. Within the small intestine, SCPs are only found within the submucosal plexus and generate around 5% of the submucosal neurons found in adulthood. In contrast, SPCs have a much greater contribution to the large intestine, where they contribute about 20% of the postnatally generated neurons in both the myenteric and submucosal plexi (Uesaka et al., 2016, Espinosa-Medina et al., 2017). This diverse range of colonisation methods may allow for some compensation between colonisation sources, and therefore cooperatively ensure complete colonisation of the rapidly expanding intestine.
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Figure 1.4. Development of the zebrafish and mouse ENS. (A) The majority of the mammalian ENS is derived from vagal NCCs (red), whereas the sacral NCCs (blue) contribute to the hindgut. The inset in (A) shows the process of transmesenteric colonisation of the hind gut. (B) Zebrafish vagal NCCs (red) colonise the gut and generate the entire ENS. Adapted from Heanue (2016) with permission from Wiley Inc.

In zebrafish, the ENS is thought to derive purely from vagal NC cells that enter the gut at approximately 32 hours post fertilisation (32hpf) and completely colonise the gut by 3 days post fertilisation (dpf) (Figure 1.4A) (Kelsh and Raible, 2002, Shepherd and Eisen, 2011). Unlike other vertebrates, there is no evidence of a sacral NC cells contribution to the ENS (Shepherd and Eisen, 2011, Heanue et al., 2016b). The vagal NC cells migrate into the developing gut in two streams, found on the left and right sides of the gut (Elworthy et al., 2005, Heanue et al., 2016b, Olden et al., 2008), which subsequently cross circumferentially to complete colonisation (Olden et al., 2008, Shepherd et al., 2004, Uribe and Bronner, 2015, Kelsh and Raible, 2002). However, little is known about the behaviour of individual cells within the steams. In addition, the contribution of SCPs and transmesenteric migration has yet to be characterised in the zebrafish ENS.

1.1.2.2 Key genetic and signalling pathways involved in ENS Development

The key genetic pathways involved in the development of the ENS are largely conserved between human, mouse and zebrafish. In fact, zebrafish contains ~70%
of all human disease genes (Heanue et al., 2016b), whereas mouse contains ~90% of all human disease genes (Schriml et al., 2003).

1.1.2.2.1 SOX10

One of the earliest NC cell markers expressed during ENS development is the transcription factor SRY (sex determining region Y)-box 10 (Sox10), which is initially expressed when the NC cells delaminate from the neural tube, and is maintained in eNCCs and EGCs, but is downregulated in enteric neurons (Kim et al., 2003, Charrier and Pilon, 2017, Laranjeira et al., 2011). Mutations in Sox10 result in lethality, due to widespread depletion of NC-derived cell types, including the ENS. Specifically, Sox10 null homozygous animals are characterised by total intestinal aganglionosis due to loss of eNCCs (Kapur et al., 1992, Paratore et al., 2001, Dutton et al., 2001), while in heterozygous animals show, partial ENS colonisation due to, defective migration and adhesion of eNCCs (Watanabe et al., 2017). Sox10 interacts with many important genes and signalling pathways in ENS development, such as EDNRB (endothelin receptor type B) and EDN3 (endothelin 3). Thus, combined Sox10;Ednrb and Sox10;Edn3 heterozygous mutants have more severe aganglionosis than either of the single mutants. Therefore, Ednrb, Edn3 and Sox10 function cooperatively to regulate NC cell progenitor migration into the gut (Stanchina et al., 2006).

Sox10 also has important roles in maintaining the multipotency of NC cells and appropriate SOX10 levels are essential for normal ENS development. Overexpression of SOX10 prevents differentiation of the neuronal lineage (Kim et al., 2003, Bondurand et al., 2001) and results in persistence of glial or non-neuronal cell fates. Conversely, when levels of SOX10 are insufficient, eNCCs, differentiate prematurely into neurons, leading to depletion of the eNCC pool (Kapur et al., 1992, Paratore et al., 2001). Sox10 is required for induction of pro-neurogenic genes such as Ascl1 (Kim et al., 2003, Delfino-Machin et al., 2017), but the mechanisms whereby SOX10 regulates the transition between undifferentiated and neuronal states of ENS progenitors remain unknown.

While deletion of sox10 in zebrafish results in loss of NC cells and results in intestinal aganglionosis (Dutton et al., 2001, Kelsh and Eisen, 2000) the effects on Sox10 dosage within the zebrafish has yet to be described. The mammalian data
suggests that SOX10 has important roles in maintaining the balance between neuronal differentiation and maintaining non-neuronal cell fate. In adulthood, Sox10 expression is maintained within adult EGCs, however, little is known about its function.

1.1.2.2.2 FOXD3

Another early NC cell marker is the transcriptional repressor FoxD3, a member of the forkhead family of proteins. FoxD3 has similar onset and expression to Sox10, in that it is expressed in early NC cells and later becomes restricted to EGCs (Mundell and Labosky, 2011, Mundell et al., 2012, Lister et al., 2006, Teng et al., 2008). Deletion of FoxD3 from the NC cell lineage at early developmental stages, results in widespread NC disruption (Mundell and Labosky, 2011, Teng et al., 2008, Lister et al., 2006), similar to the phenotypes observed with Sox10 mutant animals (Kapur et al., 1992, Paratore et al., 2001, Dutton et al., 2001). Within the ENS, of FoxD3 knockout mutants, the vagal NC cells colonising the gut are severely depleted, as uncommitted NC cells are lost to mesenchymal cell fates resulting in intestinal aganglionosis (Mundell and Labosky, 2011, Teng et al., 2008, Lister et al., 2006). This suggests that at early stages, FOXD3 is important in fate specification of the early NC cells towards the neuronal lineage. Interestingly, deletion of Foxd3 after the vagal NC cells have entered the gut disrupts gliogenesis and premature neuronal differentiation is observed (Mundell et al., 2012). These results suggest that at later stages, FoxD3 is a key factor that maintains glial cells fate and prevents neuronal differentiation (Mundell et al., 2012).

1.1.2.2.3 RET and GDNF

One of the most well-studied genes involved in ENS development is the Ret receptor tyrosine kinase (Pachnis et al., 1993, Marcos-Gutierrez et al., 1997). Ret is expressed at around E9-9.5 in NC cells, immediately before entering the gut. However, during eNCC colonisation, Ret expression gradually becomes restricted to enteric neurons (Pachnis et al., 1993, Young et al., 1998, Young et al., 1999, Young et al., 2003, Marcos-Gutierrez et al., 1997, Heanue and Pachnis, 2008). Activation of RET occurs upon dimerization of the RET receptor following binding to its ligand GDNF (glial derived neurotrophic factor) and its co-receptor GFRα1 (GDNF family
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receptor alpha 1). Dimerization activates a signalling pathway responsible for eNCC survival, proliferation, migration and differentiation (Natarajan et al., 2002). In the majority of vertebrates, RET has two isoforms, RET9 and RET51, which arise due to differential splicing. RET51 is sufficient to support colonisation of the gut by NC cells and ENS development, but RET9 appears to be dispensable (Heanue and Pachnis, 2008, de Graaff et al., 2001). Interestingly, primates have an additional splicing isoform, RET43, but its role in ENS development is unclear (Carter et al., 2001, Ivanchuk et al., 1998). RET mutations result in intestinal aganglionosis and account for approximately 50% of familial cases of Hirschsprung disease (HSCR), (see 1.1.4.1). Prior to NC cell entry into the gut, GDNF is expressed in the developing stomach mesenchyme while RET expressing NC cells migrate towards the GDNF expression in the developing gut. However, once eNCCs are in the intestine, a GDNF gradient is formed, rostrocaudally, with the highest GDNF expression in the caecum mesenchyme. It appears therefore that, eNCCs migrate toward this site as they advance along the length of the gut (Natarajan et al., 2002, Young et al., 2001). If there is insufficient RET signalling, due to mutations in Ret, GDNF or GFR1α, NC cells fail to migrate and proliferate, and eventually undergo apoptosis (Heuckeroth et al., 1998, Srinivasan et al., 2005, Uesaka and Enomoto, 2010). These results suggest that RET signalling is important for eNCC function. However, recent analysis of Ret hypomorph mutants suggests that once eNCCs have entered the gut, ret may also plays a role in the efficiency of eNCC migration (Heanue et al., 2016a). In addition, after gut colonisation, RET inhibits proliferation and drives neurogenesis (Lasrado et al., 2017). These data suggest that RET may have different functions during the early colonisation of the gut and during later neuronal differentiation.

1.1.2.2.4 ASCL1

A late-acting molecule of interest is Ascl1 (also known as MASH1), which is a basic Helix-loop-helix transcription (bHLH) factor with key roles in neurogenesis (Urban et al., 2016, Kageyama et al., 2018). ASCL1 is widely expressed in eNCCs during early stages, after the onset of Phox2b and Ret expression (Durbec et al., 1996, Blaugrund et al., 1996, Pattyn et al., 1999). SOX10 is also required for induction of Ascl1 expression (Kim et al., 2003). After induction, ASCL1 expression
is maintained throughout eNCC colonisation (Memic et al., 2016, Blaugrund et al., 1996, Guillemot et al., 1993, Lo et al., 1991). Ascl1 knockout mice exhibit delayed ENS neurogenesis and mild reduction in gliogenesis within the posterior gut. However, despite the early onset of Ascl1, loss of function experiments do not indicate a role of ASCL1 in eNCCs colonisation of the gut (Memic et al., 2016, Guillemot et al., 1993).

Ascl1 plays important roles in enteric neurogenesis (Guillemot et al., 1993, Blaugrund et al., 1996, Kim et al., 2003, Memic et al., 2016), and was originally thought to have a role in the differentiation of serotonergic neurons (Blaugrund et al., 1996). However, the development of genetic tools that can restore the neurogenic potential of Ascl1 mutants demonstrated that ASCL1 has roles in the specification of late born neurons of the stomach and small intestine such as those expressing Calbindin, VIP and TH (Memic et al., 2016). Since no effects of Ascl1 deletion were observed in the large intestine, it is possible that there is compensation from the sacral NC or SCP, which may not express ASCL1 at high levels and therefore are not affected by ASCL1 deletion.

1.1.2.2.5 NOTCH

**NOTCH** signalling (Chapter 1.3.1) has been implicated in regulating many processes within the nervous system, from controlling progenitor potential (Chapter 1.3.2) to neuronal circuitry (Chapter 1.3.3). Specifically, within eNCCs, NOTCH receptors (Notch 1 and Notch2), and NOTCH ligands (Delta-like 1, 3 and 4 (Dll1, Dll3 Dll4)) have been shown to be expressed within the developing ENS (Okamura and Saga, 2008). Similarly, within the adult ENS, Jagged1 is expressed within the majority of neurons, whereas Notch1 has limited expression within the somas of Calretinin or nNOS expressing neurons (Sander et al., 2003). Similarly, a variety of downstream NOTCH effectors have also been observed within the ENS (Okamura and Saga, 2008, Ngan et al., 2011, Theocharatos et al., 2013, Charrier and Pilon, 2017).

Within the ENS, NOTCH signalling has been implicated in eNCC progenitor function. Thus, disruption of NOTCH signalling leads to premature differentiation of eNCCs into neurons and loss of Sox10 expressing eNCCs (Okamura and Saga, 2008, Taylor et al., 2007). Similar observations have been described *in-vitro*; where
inhibition of NOTCH signalling in enteric neurospheres results in a reduced expression of downstream NOTCH target genes such as *Hairy enhancer of split 1 and 5 (Hes1 and Hes5)*, leading to premature neuronal differentiation and reduced proliferation rates of ENS progenitors (Theocharatos et al., 2013).

Interestingly, Sonic Hedgehog (SHH) signalling has been implicated in mediating at least some aspects of NOTCH signalling within the ENS. Disruption of SHH signalling in eNCCs leads to upregulation of *Dll1* and activation of the NOTCH signalling pathway. This activation of NOTCH signalling leads to premature gliogenesis and results in aganglionosis in the developing ENS (Ngan et al., 2011, Jia et al., 2012, Kubu et al., 2002, Morrison et al., 2000). In support of a model in which NOTCH signalling plays an active role within the ENS, mutations in *NOTCH1, JAGGED2* and *DLL3* are associated with Hirschsprung disease (HSCR) (Ngan et al., 2011, Jia et al., 2012). Interestingly, while NOTCH signalling has been well described during ENS development, with important roles in maintaining glial cell fates, the role of NOTCH signalling in the adult remains largely unexplored.

### 1.1.2.3 Differentiation of NC cells into enteric neurons and glia

Differentiation of enteric neurons and EGCs occurs while the eNCCs are still colonising the gut (Furness, 2006). The eNCCs colonising the gastrointestinal tract are initially multipotent, however they become progressively restricted along gliogenic or neurogenic lineages. Neuronal differentiation is associated with expression of *Tubb3, Elavl4, Phox2b* and *Ret*, whereas gliogenic differentiation and a progenitor state are associated with expression of *Erbb3, Sox10, PLP1* and *FABP7* (Lasrado et al., 2017).

Neurogenesis begins in the mammalian gut around E10.5 and occurs in a rostrocaudal manner, reflected by the expression of the pro-neurogenic gene *Phox2b*, which directs multipotent eNCCs into neurogenic progenitors (Young et al., 2003). Neurogenic restriction is followed by upregulation of pan-neuronal markers such as HuC/D, and PGP9.5, beginning at E12.5 (Young et al., 1999, Young et al., 2003, Baetge and Gershon, 1989). Since neuronal precursors maintain the ability to form multiple neuronal subtypes until the last few cell divisions (Lasrado et al., 2017), this suggests that neuronal restricted progenitors retain some multipotency.
Birthdating and neurohistochemical studies have shown that different neuronal subtypes appear in overlapping waves in both mammals and zebrafish (Table 1-1). In mammals, early born neurons include serotonergic (5-HT) and cholinergic (Ach) neuronal subtypes, whereas late neuronal subtypes include NF, Calcitonin, ENK, NPY, VIP, CGRP, TH, NOS and Calretinin neurons (Pham et al., 1991, Bergner et al., 2014). Interestingly, recent studies have also suggested that some neurochemical markers may only be transiently expressed during development, particularly in the case of NOS neurons (Bergner et al., 2014). In zebrafish enteric neurogenesis begins at approximately 48hpf (Shepherd and Eisen, 2011, Heanue et al., 2016b), when the first nitrergic neurons are identified (Holmqvist et al., 2004, Holmberg et al., 2006), which is followed by NKA, PACAP, VIP, NOS, and Calbindin neurons, and later with 5-HT and CGRP neurons (Olden et al., 2008, Olsson et al., 2008, Olsson, 2011, Olsson, 2009). The different time course of neuronal subtype appearance between zebrafish and mouse may reflect different developmental pathways or requirements. Alternatively, these differences may be related to technical factors, since the onset of marker expression and birth-date of a neuronal subtype are not necessarily correlated. Therefore, the appearance of neuronal subtypes described in the zebrafish may misrepresent the actual neuronal birthdate, since current descriptions are based on marker expression. Further examination of neuronal subtype appearance using birth dating in zebrafish is required to determine similarities, or differences, to the mammalian system.

<table>
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<th>Zebrafish Earliest Detection</th>
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<td>PACAP</td>
<td>ND</td>
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<td>NKA</td>
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*latest time analysed 13dpf
ND = Not Determined

Little is known about the developmental pathways important for neuronal subtype differentiation. However, recent transcriptomic analysis has highlighted many transcription factors and signalling pathways that may be important in the process of neuronal subtype specification. This study has implicated Sox6 in dopaminergic neuronal development within the stomach. However, no role for Sox6 was observed in the small or large intestine (Memic et al., 2016). This suggests that neuronal subtypes may be influenced by the local environment and genetic cues. While neurogenesis continues into early postnatal stages, it is rarely observed after
P10 (Laranjeira et al., 2011, Joseph et al., 2011, Belkind-Gerson et al., 2015, Bergner et al., 2014, Pham et al., 1991). Recently, it has been suggested that neurons are generated throughout adult life and that 80% of the neurons within a ganglia are turned over within a two-week period (Kulkarni et al., 2017), though these results have not been independently confirmed (personal communications see Appendix Chapter 8.1), and remain controversial.

Unlike the differentiation of enteric neurons, EGC differentiation is less well understood. A major stumbling block has been that expression of NC cell markers such as Sox10 and Foxd3 are maintained in EGCs (Mundell and Labosky, 2011, Mundell et al., 2012, Kim et al., 2003), preventing definitive distinction between progenitors and EGCs. Nevertheless, within the myenteric plexus, marker expression analysis has shown that BFABP is the first “mature” glial marker to be expressed, at E11.5, a time frame similar to the onset of neurogenesis. Mature glial markers such as S100β are not detected until E14.5, and are only found in small number of EGCs (Young et al., 2003), whereas GFAP is not expressed until E16.5 (Rothman et al., 1986). While EGC differentiation begins at mid-gestation stages, mucosal EGCs develop postnatally, in response to the colonisation of the gut by microbiota, and do not reach adult numbers until after weaning (Kabouridis et al., 2015). Unlike neurons, there is a homeostatic turnover of EGCs throughout adult life (Kabouridis et al., 2015, Joseph et al., 2011). Within the zebrafish, EGCs have not been extensively described or studied (Doodnath et al., 2010, Hagstrom and Olsson, 2010, Kelsh and Eisen, 2000), leaving the potential to use this elegant developmental and genetic system to further explore EGC biology.

1.1.3 The Function of the ENS

The ENS controls many basic gut functions, largely independently from the CNS. The diverse functions controlled by the ENS include gut peristalsis, local blood flow, epithelial cell secretion, absorption and regulation of immune responses (Furness, 2006). Both enteric glia and neurons have been implicated in many aspects of gut function.
1.1.3.1 Neuronal Functions within the ENS

One of the important functions mediated by enteric neurons is peristalsis, the regulated contractions of the smooth muscle cell layers of the gut wall, which are important for the mixing and movement of luminal contents along the length of the gut (Heanue et al., 2016a, Kunze and Furness, 1999). Enteric neurons respond to mechanical or chemical stimulation from within the lumen, and initiate coordinated contractions that result in propulsive peristalsis (Kunze and Furness, 1999, Lundgren, 2002). Interestingly, lineage related enteric neurons are found within a local region and are functionally connected (Lasrado et al., 2017), a feature that may mediate the coordinated local responses to luminal stimuli. In pathological conditions, such as HSCR, where segments of the gastrointestinal tract are devoid of enteric neurons, there is an absence of peristalsis (Heanue and Pachnis, 2007, Heanue et al., 2016b, Becker et al., 2018), highlighting the essential roles of the ENS for intestinal motility. Animal models of HSCR in mouse and zebrafish similarly exhibit loss of motility (Heanue et al., 2016a, Heanue and Pachnis, 2007). In addition, when neuronal circuitry is disrupted in certain mouse mutants, motility defects are also observed (Sasselli et al., 2013).

Enteric neurons have other important roles within the gut, such as modulating local blood supply in the muscle layers and the mucosa (Patton et al., 2005, Vanner and Macnaughton, 2004), and controlling epithelial cell absorption and secretion (Kunze and Furness, 1999, Lundgren, 2002). In addition, enteric neurons modulate intestinal epithelial cells barrier function (Neunlist et al., 2014). For example, neuronally derived NPY increases epithelial barrier permeability (Chandrasekharan et al., 2013), whereas neuronal ACh and VIP decrease epithelial barrier permeability (Neunlist et al., 2003). Disruption of enteric neuronal signalling can disrupt gastrointestinal homeostasis, and lead to gut pathology due to infiltration of toxins from the intestinal lumen.

Enteric neurons are also thought to mediate signalling between the gut microbiota, immune system and the CNS. It is now established that the gut microbiota influence behaviour and stress responses of the CNS (Sudo et al., 2004, Neufeld et al., 2011) and it is thought that the ENS interacting with the endocrine and immune system, mediate such interactions along the microbiota-gut-brain axis (Cryan and Dinan, 2012). Interestingly, enteric neurons are able to differentiate
between different subclasses of bacteria within the lumen and modulate their response specifically (Burgueno et al., 2016). Reciprocally, the ability of neurons to regulate peristalsis has been shown to influence microbiota composition. Mutants that lack enteric neurons, and consequently have peristaltic defects, exhibit increased abundance of pro-inflammatory strains of microbiota, which results in increased levels of inflammation. This inflammatory phenotype can be reversed upon restoring ENS function (Rolig et al., 2017).

The interactions between enteric neurons and macrophages residing within the outer muscular layers (muscularis externa) are an important component of the ENS-immune system interactions, and occur in a bi-directional manner. Enteric neurons stimulated by macrophage-derived BMP produce CSF1 (colony stimulating factor 1), which is important in controlling the number of macrophage within the muscularis externa (Muller et al., 2014). Conversely, muscularis externa macrophages have been shown to be important in establishing the correct neuronal circuitry, since their depletion, or loss of BMP2 production, leads to reduced colonic motility (Muller et al., 2014, Chalazonitis et al., 2008, Chalazonitis et al., 2004, De Schepper et al., 2018). Similarly, loss of macrophages results in enhanced enteric neuron death, alters peristalsis, and results in vascular leakage/haemorrhage and increased epithelial barrier permeability (De Schepper et al., 2018). During ageing, macrophages become more inflammatory, which results in apoptosis and neuronal loss and leads to reduced intestinal transit times (Becker et al., 2018). In addition, enteric neurons release many immunomodulatory molecules, such as proinflammatory prostaglandin D2, tumour necrosis factor-α (TNFα), interleukin6 and 8 (IL-6 and IL-8) that can influence inflammatory responses of the gut (Le Loupp et al., 2015, Burgueno et al., 2016). This has been demonstrated in mice, where animals with an experimentally induced increase in neuron numbers exhibit increased sensitivity to inflammation (Margolis et al., 2011). Taken together, these data indicate that enteric neurons and the immune system establish a dynamic and bidirectional interaction, with further input from environmental factors, all of which are required for normal gut function and homeostasis.
1.1.3.2 Glial functions within the ENS

Glial cells of the ENS were traditionally thought to function primarily in neuronal support. Such a role is suggested by the extensive gliofillaments in EGCs, which can be revealed by GFAP immunoreactivity, and by the fact that EGCs form sheaths around enteric neurons within the ganglia. The gliofillament sheath is thought to protect enteric neurons from damage caused by mechanical stress associated with intestinal peristalsis (Gabella, 1981, Hanani and Reichenbach, 1994). Consistent with this idea, mechanical stimulation of single EGCs results in intracellular calcium waves that can be propagated to neighbouring EGCs (Zhang et al., 2003), indicating that enteric glia are responsive to mechanical stimuli. EGCs may be able to modulate molecules such as GFAP in response to mechanical stimuli to increase the protection provided to neurons.

In addition to their structural roles, EGCs also have developmental roles and provide functional support to neurons. For example, during development EGCs modulate neuronal maturation via purinergic signalling (Le Berre-Scoul et al., 2017). In adulthood, reductions in EGCs purinergic signalling decreases coordinated peristaltic contractions, suggesting that EGCs continue to modulate enteric neuronal circuitry throughout life (Gulbransen and Sharkey, 2009, McClain et al., 2014, McClain et al., 2015). EGCs have also been shown to modulate neuronal synapses by altering potassium ion (K⁺) levels (Hanani et al., 2000), and to clear potentially toxic neurotransmitters such as glutamate and GABA, to prevent damage to enteric neurons (Fletcher et al., 2002, Galligan et al., 2000). In addition, recent work indicates an intriguing sex-dependant requirement of EGCs in neuronal support and function in mice, highlighted by the female-specific intestinal dysmotility observed following ablation of EGCs in adult animals (Rao et al., 2017).

Enteric glia may also be involved in the regulation of intestinal epithelial barrier function. Thus, loss of EGCs results in severe epithelial barrier disruption and vascular haemorrhage (Bush et al., 1998, Savidge et al., 2007), and subsequent work identified some of the glial-derived mediators that are important for barrier fitness such as S-nitrosoglutathione (Savidge et al., 2007). However, these observations could not be reproduced by subsequent studies, carried out by a different group (Rao et al., 2017). This discrepancy may be accounted for by the ectopic expression of the transgenic drivers used in the earlier study and the
unintended loss of additional cell types (Rao et al., 2017). Nevertheless, EGCs have close associations and interactions with epithelial cells (Bohorquez et al., 2015, Kabouridis et al., 2015, Savidge et al., 2007), consistent with a role in modulating epithelial cell secretions. In addition, in adulthood, EGCs have been shown to contribute to epithelial barrier fitness by upregulating epithelial tight junctions (Savidge et al., 2007). Moreover, EGCs modulate the development and maturation of intestinal epithelial cells through GDNF, which further contributes to the maintenance of the epithelial barrier by reducing the epithelial barrier permeability (Meir et al., 2015).

Enteric glia are also found in close association with the vascular system of the gut (Boesmans et al., 2014, Kabouridis et al., 2015, Hanani and Reichenbach, 1994). In the CNS, astrocytic association with blood vessels is essential for the establishment of the blood brain barrier (BBB). Therefore, the close association of EGCs to the vasculature of the intestine may suggest the presence of an equivalent gut-vasculature barrier (GVB) (Bush et al., 1998, Savidge et al., 2007, Spadoni et al., 2015). Similar to the BBB, EGCs extend end-feet that ensheathe local capillaries (Gabella, 1981, Gabella, 1972, Hanani and Reichenbach, 1994, Boesmans et al., 2014, Gershon and Bursztajn, 1978, Spadoni et al., 2015), thus establishing a close association that allows EGCs to modulate endothelial permeability (Gershon 1978, Corner 2001). Alternatively, the close association of EGCs and the vasculature could prevent pathogenic molecules within the microbiome of the intestinal lumen from entering the vasculature, and the rest of the body. Therefore, EGCs could be an important component of the putative GVB that prevents microbiota and/or their metabolites from entering host tissues (Spadoni et al., 2015). In support of this, human EGCs are capable of differentiating between probiotic and pathogenic bacteria, which specifically induce S100β over-expression and NO release from enteric glia (Turco et al., 2014).7dpf miss

Similar to neurons, EGCs also interact with the immune system. EGCs express immune system related molecules such as MHC class II (major histocompatibility complex class II) and have been shown to have phagocytic properties, suggesting they can act as antigen presenting cells within the ENS (Turco et al., 2014, Esposito et al., 2016, Geboes et al., 1992). Additionally, pro-inflammatory cytokines and inflammation can induce gliosis-like responses in EGCs, such as upregulation of GFAP and S100β (Cirillo et al., 2011, Esposito et al., 2007,
Stoffels et al., 2014, von Boyen et al., 2004), therefore EGCs are responsive to immuno-active molecules. Since EGCs are both responsive to immune signals and capable of interacting with the immune system, this suggests a model in which EGCs play an active role in mediating local immune responses.

Finally, recent studies have suggested that EGCs can act as a stem cell pool within the ENS. It is unknown whether all EGC subtypes have progenitor potential, or whether there are distinct lineage restricted progenitors that give rise to either neurons or glial cells or both. The majority of studies suggest that EGC proliferation is a low frequency event during homeostasis (Belkind-Gerson et al., 2017, Belkind-Gerson et al., 2015, Joseph et al., 2011, Laranjeira et al., 2011, Kabouridis et al., 2015) and that the progeny derived from proliferating EGCs are glia, with neurons generated only rarely. Our recent work has demonstrated that in the mouse EGCs respond to signals from the microbiota and that, newly generated EGCs migrate into the mucosa to replace those that are lost during homeostasis (Kabouridis et al., 2015). However, EGCs can proliferate and generate new neurons after injury (Belkind-Gerson et al., 2017, Laranjeira et al., 2011, Joseph et al., 2011, Kabouridis et al., 2015). These studies suggest that, depending on the environmental stimuli EGCs are able to modulate their proliferative and differentiation potential. Although the mechanisms mediating EGC proliferation and neurogenesis remain largely unknown, serotonergic stimulation, via the 5HT4 receptor, has been shown to increase neurogenesis in-vitro (Belkind-Gerson et al., 2015, Liu et al., 2009) and may represent a pathway mediating the neurogenic response during injury. Recent (and highly controversial studies), have also suggested that a Sox10-Nestin+ non-neuronal population within enteric ganglia is highly proliferative and neurogenic. This study speculates that 80% of the neurons within ganglia are replaced every week (Kulkarni et al., 2017), a rate of cell turnover that vastly exceeds those predicted from previous studies (Belkind-Gerson et al., 2015, Joseph et al., 2011, Laranjeira et al., 2011). While the rate of turnover may be disputed, all studies agree that there is a progenitor population within EGCs/non-neuronal lineage of the ENS that is capable of giving rise mainly to glia (during homeostasis) and possibly to neurons (following injury conditions).

In summary, EGCs have a diverse range of functions within the ENS from neuronal support to mediating immune responses. Understanding how the diverse EGC functions relates to the known morphological and positional EGC subtypes is
an unexplored aspect of ENS biology. Functional characterisation of EGCs is essential to enhance our understanding of ENS function and pathology.

1.1.4 Pathologies of the ENS

A wide range of gastrointestinal pathologies can affect the ENS. These pathologies can be split into three main subgroups: developmental, acquired and neurodegenerative pathologies, which will be discussed below. In addition, we will discuss potential treatments for ENS pathologies, with a particular focus on stem cell therapy.

1.1.4.1 Developmental ENS Pathologies

The most well studied developmental ENS pathology is Hirschsprung disease (HSCR), which occurs in 1:5000 live births, and is characterised by the presence of aganglionic distal bowel segments. HSCR disease is normally detected within the first 48 hours after birth, with affected children presenting with megacolon, vomiting and inability to pass the meconium and stools (Spouge and Baird, 1985). HSCR disease results from a failure of NC cells to fully colonise the gut, due to defects in NC cell migration, survival and proliferation (Heanue and Pachnis, 2007). Current treatment of HSCR disease involves removal of the aganglionic portion of the bowel and surgical resection of the remaining bowel and anastomosis with the anus (Hackam et al., 1998). While surgery is life-saving and mortality rates are low, patients present with many gastrointestinal complications following this treatment, such as enterocolitis and chronic constipation. These complications may be accounted for by the fact that the transition zone, the area between the aganglionic and ganglionic regions, has neuroanatomically abnormal ganglia, being either sparse or having a reduction in neuron numbers or subtypes (Thapar, 2009, Kapur and Kennedy, 2012). Preservation of the transition zone may, therefore, contribute to gastrointestinal dysfunction following surgery. In light of this, alternative treatments using stem cell therapies are being developed and will be discussed in detail below (Chapter 1.1.4.4).

HSCR disease can be split into two forms: familial, which occurs within families in a non-mendelian manner, and sporadic, which appears in individuals with no family history of HSCR disease (Amiel et al., 2008, Brooks et al., 2005). HSCR
disease is a multifactorial disease and can be caused by mutations in many genes, including those important for NC cell functions such as RET, GDNRB, GDNF, and Sox10 (Heanue and Pachnis, 2007). The penetrance and severity of aganglionosis also varies between individuals, indicating that the genetic basis of HSCR is complex (Brooks et al., 2005, Heanue et al., 2016a, Amiel and Lyonnet, 2001). Moreover, there is a 4:1 male bias is observed in HSCR disease presentation (Emison et al., 2005). HSCR disease also often occurs alongside many other developmental diseases, particularly those with chromosomal abnormalities, for example, trisomy 21 (Downs syndrome), which accounts for 10% of all HSCR cases (Amiel and Lyonnet, 2001, Spouge and Baird, 1985, Goldberg, 1984). Together these examples highlight the complex genetic nature of HSCR, and much research is now focused on understanding this complexity, and identifying new HSCR disease genes.

One of the most well studied genes associated with HSCR disease is RET, a receptor tyrosine kinase, which has roles in eNCC proliferation, differentiation and migration (discussed Chapter 1.1.2.2.3). Mutations in RET occur in 50% of familial HSCR disease cases and 10-35% of sporadic cases (Amiel et al., 2008). RET coding mutations are rare, and HSCR is most commonly associated with non-coding mutations that influence the level of expression of RET. One particular RET enhancer mutation is suggested to be involved in most forms of HSCR, yet this mutation is common within the population as a whole, underscoring the fact that HSCR is a multifactorial disease (Amiel et al., 2008). Many studies are currently focusing on identification of modifier loci which are associated with RET mutations and result in disease phenotype (Heanue et al., 2016a, Emison et al., 2005). Work on zebrafish has recently identified, a modifier locus map kinase10 (mapk10) and demonstrate that Mapk10 mutations increase the incidence and severity of phenotypes in ret heterozygotes mutants (Heanue et al., 2016a). Identification of additional modifier loci will help to unravel the complex genetics of HSCR and improve our understanding of ENS development.

While HSCR disease can be easily diagnosed, due to the complete absence of enteric ganglia within specific gut segments (usually colon), other developmental diseases may occur as a consequence of more subtle ENS defects. Mouse models of chronic constipation have been shown to arise due to altered organisation of neuronal circuitry (Sasselli et al., 2013). Similarly, mice lacking specific NOS neurons present with disrupted peristalsis within the colon, which is consistent with the fact
that reduction in the NOS expressing enteric neurons is thought to be the cause of a variety of enteric neuropathies such as oesophageal achalasia, gastroparesis and colonic dysfunction (McCann et al., 2017). Constipation and dysmotility in human patients, which may affect the entire length of the gut, may require complex treatments, including possible stem cell therapies (disused below Chapter 1.1.4.4).

1.1.4.2 Acquired ENS pathologies

Acquired ENS pathologies occur in adulthood and normally result from inflammation. The impact of inflammatory disease on the ENS is less well understood, however exciting new research has started to uncover the relationship between the ENS and immune system (Obata and Pachnis, 2016, Veiga-Fernandes and Pachnis, 2017). The most common acquired ENS pathologies are irritable bowel diseases (IBDs), such as Crohn's disease and ulcerative colitis. IBDs are characterised by recurrent inflammatory episodes in localised regions of the gut, and often originate from an inflammatory insult (Spiller, 2003). The ENS undergoes significant changes during inflammation: enteric neurons become hyper-excitabile (Lindon et al., 2003), neuronal synapses become more plastic or are remodelled (Lomax et al., 2005, Krauter et al., 2007, Mawe, 2015), and there is a decrease in descending purinergic inhibition (Roberts et al., 2012, Roberts et al., 2014). Together these changes result in a loss of neurons and neuronal signalling, ultimately resulting in dysmotility within the intestine (Mawe, 2015, Lindon et al., 2003). In addition, due to the altered connectivity, ENS dysfunction can also be observed in non-inflamed regions and can continue long after the initial inflammation has subsided (Hons et al., 2009). Interestingly, recent evidence suggests that neuronal density can influence the level of pathological inflammation observed in IBDs. Specifically, low density of neurons confers a protective, anti-inflammatory effect, whereas a high density of neurons is pro-inflammatory (Margolis et al., 2011). This may explain why the individual responses to inflammation are highly variable and suggests that regional differences in neuronal density may impact on the inflammatory phenotype.

In response to inflammation the majority of EGCs undergo gliosis. Specifically, EGCs upregulate GFAP in response to pro-inflammatory cytokines (von Boyen et al., 2004), a response associated with the formation of glial scars in the CNS (Sofroniew, 2009, Adams and Gallo, 2018). Inflammation-induced glial scars
may interfere with restoration of neuronal circuitry, accounting for continuing ENS dysfunction. In addition, EGCs release increased amounts of S100β in response to inflammation (Cirillo et al., 2011, Esposito et al., 2007, Cirillo et al., 2009). In the CNS S100β is neuro-protective at the nanomolar scale, whereas on the micromolar scale observed in inflammation, it has pro-inflammatory effects (Michetti et al., 2018, Van Eldik and Wainwright, 2003). In addition, increased S100β expression has been observed in neuropathies such as Alzheimer’s disease and Down’s syndrome (Griffin et al., 1989, Van Eldik and Wainwright, 2003), further suggesting that increased S100β levels can contribute to neurodegeneration. Together, these data suggest that EGCs undergoing gliosis during inflammation may lead to further pathology of the ENS. Although EGCs can increase their proliferative potential and generate new neurons (Belkind-Gerson et al., 2017, Joseph et al., 2011, Laranjeira et al., 2011, Belkind-Gerson et al., 2015) this process is slow, and evidently cannot compensate for the loss of neurons and neuronal circuitry observed during inflammation. Understanding the mechanisms by which EGCs enter a proliferative and regenerative state, instead of gliosis, may instruct future treatments.

### 1.1.4.3 Neurodegenerative ENS pathology

Recent studies have suggested that neurodegenerative diseases associated with the CNS, such as Alzheimer’s disease (AD) and Parkinson’s disease (PD), present in the ENS before clinical signs appear and/or diagnosis is possible (Braak et al., 2006, Chalazonitis and Rao, 2018, Shannon and Vanden Berghe, 2018, Semar et al., 2013). Moreover, due to the connectivity between the CNS and ENS, known as the gut-brain axis, it is believed that the ENS could potentially spread pathogenic molecules such as β-amyloid (βA) plaques, or prions, to the CNS via the vagus nerve (Holmqvist et al., 2014, Liddle, 2018, Pan-Montojo et al., 2010, Liu et al., 2017). Moreover, it is suggested that this communication is bidirectional, and therefore CNS pathologies could be spread to the ENS, via the vagus nerve, and cause ENS pathology (Noorian et al., 2012). The pathogenic potential of vagal mediated gut-brain communication is supported by the fact that people with a truncal vagotomy have a reduced risk of developing PD (Liu et al., 2017, Svensson et al., 2015).
Due to the high co-morbidity of intestinal motility deficits and PD, the effects of PD on the ENS are the best characterised of all the neurodegenerative enteric neuropathies. PD-associated enteric neuropathologies are most commonly observed within the submucosa (Beach et al., 2016). In addition, although there is not a loss of enteric neurons, there is an associated downregulation of VIP expression in submucosal neurons (Giancola et al., 2017). In AD, pathogenesis has also been observed within the submucosal plexus (Joachim et al., 1989), however, this observation has not been supported by other studies (Shankle et al., 1993). The discrepancies between these studies may be due to the limited samples and studies of AD patients, due to the less common co-occurrence of gastrointestinal dysfunctions in these patients. Understanding the role that the ENS plays in neurodegenerative disease has the potential to improve our understanding of CNS pathology and gut functions.

1.1.4.4 Stem-cell therapy as a treatment for ENS pathology

A potential treatment for both developmental and acquired ENS pathologies is the use of stem cells to replace the enteric neurons and glia lost and/or damaged in these conditions. Many different cell sources are currently being investigated for use in stem cell transplants, such as enteric neural stem cells (ENSC), including NC cells and EGCs, iPSCs, or CNS derived neural stem cells (Burns et al., 2016). Interestingly, stem cells derived from the ENS show better engraftment, proliferation and neuronal differentiation than those originating from the CNS (Findlay et al., 2014, Fattahi et al., 2016), suggesting there may be some pre-programming or epigenetic differences between CNS and ENS progenitors. Moreover, the most clinically relevant stem cell populations are those that are host derived and easily accessible, such as EGCs or iPSCs (Burns et al., 2016), making ENSCs an attractive population for stem cell therapies. Once a stem cell population has been selected, and isolation and culture conditions optimised, the stem cell population must be able to survive, migrate, proliferate within the recipient gut. Moreover, the stem cell derived progeny must be able to integrate into functional networks within the gut for successful restoration of gut function. To date, model systems have been used to explore stem cell potential (Burns et al., 2016), and will be discussed below.
The majority of cell transplantation studies have been carried out using embryonic or early postnatal ENSCs. All studies have shown that transplanted ENSCs are able to proliferate and differentiate into both neurons and glia within the host gut (Cooper et al., 2016, Cooper et al., 2017, Dettmann et al., 2014, Findlay et al., 2014, Heanue and Pachnis, 2011, Hotta et al., 2016, Hotta et al., 2013, McCann et al., 2017, Nishikawa et al., 2015, Cheng et al., 2017, Binder et al., 2015, Stamp et al., 2017, Belkind-Gerson et al., 2013). Additionally, many studies have shown that the progeny of the transplanted cells integrate within the existing endogenous host neural circuitry (Binder et al., 2015, Cooper et al., 2016, Cooper et al., 2017, Hotta et al., 2013, McCann et al., 2017), and transplanted cells are assuming appropriate positions and neuronal connections to enable functional participation with endogenous circuitry (Cheng et al., 2017, Cooper et al., 2016, Cooper et al., 2017, Hotta et al., 2013, McCann et al., 2017). Despite this, cells transplanted into HRSC disease models failed to show successful incorporation into the host circuitry, due to poor survival or migration (Cooper et al., 2016, Hotta et al., 2013, Nishikawa et al., 2015). In contrast, in functional disease models where a particular neural subtype is missing, transplanted cells successfully incorporate and can restore motility defects (McCann et al., 2017). This suggests that mutations that have a secondary effect on the extracellular environment (Ret, Ednrb) may also adversely affect the success of transplantation (Burns et al., 2016).

More recently, human stem cells or iPSC, have been used as a source of stem cells in transplantation studies. Similar to transplantation studies carried out with ENSC, iPSCs are able to proliferate and differentiate into neurons and glial cells (Schlieve et al., 2017, Fattahi et al., 2016, Workman et al., 2017, Hetz et al., 2014, Li et al., 2018), and can even migrate large distances within the host gut (Fattahi et al., 2016). Additionally, iPSCs are able to integrate into existing circuitry (Schlieve et al., 2017, Hetz et al., 2014) and form functional connections within the intestine (Hetz et al., 2014, Fattahi et al., 2016, Schlieve et al., 2017, Workman et al., 2017). Moreover, transplantation of iPSCs into aganglionic guts had apparently better success rates in comparison to ENSCs, and have been shown to mediate functional improvements in gut motility (Fattahi et al., 2016, Hetz et al., 2014).

Despite these promising studies, we still lack an understanding of many of the underlying biological events necessary to devise a robust clinical application (Burns et al., 2016). For example, while transplanted cells can undergo neuronal
differentiation, there are often altered proportions of specific neuronal subtypes derived from these progenitors (Workman et al., 2017, Cooper et al., 2016). An additional limitation of current transplantation studies is the heterogeneity of the starting population, which contains both stem cells and differentiated cell types. Restriction of stem cell character has been shown to reduce the heterogeneity of the starting population and may increase the success of transplantation (Cooper et al., 2016, Heanue and Pachnis, 2011).

Ideally, clinical use of stem cells therapies would utilise autologous stem cells in order to avoid rejection of transplanted cells (Burns et al., 2016). Recently there has been some success in establishing autologous cells as a potential treatment (Cheng et al., 2017, Metzger et al., 2009). Importantly, stem cells can be collected from the submucosal plexus, or mucosa by endoscopy (Cheng et al., 2017, Metzger et al., 2009, Belkind-Gerson et al., 2013). In addition, human ENSC obtained from patients up to 84 years old can generate both neurons and glia, suggesting that patient-derived ENSCs are a viable source for transplantation studies for treating both developmental diseases and those that occur in adulthood (Metzger et al., 2009). Despite this, it is possible ENSCs acquired from older animals, or patients may be unable to differentiate into the full neurogenic spectrum (Stamp et al., 2017, Kruger et al., 2002). ENSCs isolated from normal and HSCR human patients have the same neurogenic and proliferative potential. However, ENSCs from individual patients showed high degrees of variability in proliferation and differentiation potentials, which may reflect the disease state of the patient, or variability in the source material (Cheng et al., 2017, Hotta et al., 2016). While transplanted cells were able to migrate, proliferate and differentiate within healthy host tissue, it remains to be tested whether ENSCs derived from HSCR patients would show the same potential when transplanted into a HSCR model gut, where the environment may be altered (Burns et al., 2016, Cooper et al., 2016, Hotta et al., 2016, Hotta et al., 2013, Nishikawa et al., 2015). Finally, a long term study of transplanted ENSCs has shown that there is no tumour formation or spread of transplanted cells to other organs, which suggests that stem cell therapy is relatively safe for treatment of enteric neuropathies. The fact that transplanted ENSCs have long term survival, makes the cell transplantation model a promising therapeutic strategy (Cooper et al., 2016).

ENSCs may also represent a viable source of stem cells for treatment of pathologies outside of the ENS. Due to the common marker expression and neuronal
subtypes between the ENS and CNS (Furness 2005), ENSCs have potential to generate the required neuronal subtypes when used in transplantation studies in CNS pathologies. Use of ENSCs has the additional advantage of the cells being easier to obtain that equivalent populations of CNS stem cells, since endoscopy biopsies are a routine procedure and do not carry risks associated with isolation of cells from the CNS, with the exception of stem cells isolated from olfactory biopsies (Barnett, 2004, Lindsay et al., 2010). ENSC can be locally or systemically delivered via intravenous injections, to the injured brain, with systemic delivery having greater therapeutic potential since this strategy prevents any damage to the CNS that might occur during local delivery. Once in the brain, ENSCs proliferate and differentiate into neurons and glia, and modulate the local microenvironment to promote endogenous neurogenesis (Belkind-Gerson et al., 2016). Similarly, in the injured spinal cord, transplanted ENSCs are able to proliferate and differentiate, and newly differentiated neurons have been shown to bridge the gap of the injury site (Jevans et al., 2018). Moreover, in neurodegenerative diseases such as AD and PD, astrocytes are required to regulate neuroinflammation and degeneration (Verkhratsky et al., 2014). Transplantations of EGCs into the brains of an AD rat model showed an arrest of disease progression and improved cognitive function. Moreover, transplanted EGCs prevented the spread of neuroinflammation and neurodegeneration and released the neurotrophic factors necessary to encourage endogenous neuronal regeneration (Esposito et al., 2016). Similarly, in a model of PD, when ENSCs were transplanted into diseased brains, there was a recovery of motor skills and partial recovery of dopaminergic neurons (Parra-Cid et al., 2014). These studies suggest that not only could ENSC stem cell therapies be useful not only in enteric pathologies, but also to treat pathology’s in the CNS.

1.2 Glial Cells of the Central Nervous System

Glia, or glial-like cells, are found in a broad range of species, from Caenorhabditis elegans to humans (Hartline, 2011, Freeman and Rowitch, 2013). Glial cells were originally thought to function as structural scaffolding to support the multitude of neurons and their anatomical and functional connections (Zuchero and Barres, 2015). However, in the past decade, diverse roles of glial cells have been uncovered and their study has become an exciting and complex area of research.
Subtypes of glial cells are now known to have specialised roles to serve a wide range of functions within the nervous system. Within the CNS of mammals, four populations of glial cells exist: astrocytes, radial glial cells (RGCs), oligodendrocytes and microglia. Astrocytes are the most numerous glial cell type within the CNS (Bayraktar et al., 2014), and have roles in trophic support of neurons, maintenance of the BBB and modulating neuronal connectivity (Molofsky and Deneen, 2015). RGCs are a progenitor population that gives rise to all the neurons and glial cells within the CNS (Rakic, 2003, Than-Trong and Bally-Cuif, 2015, Alunni and Bally-Cuif, 2016, Taverna et al., 2014). Oligodendrocytes ensheath axons with insulating myelin, allowing for fast neuronal transmission (Bear et al., 2001). Finally, microglia are the resident CNS macrophages and have additional roles in synaptic pruning (Eyo and Dailey, 2013, Vainchtein et al., 2018).

Since the ENS lacks myelination, and no cell population equivalent to microglia has been described within the ENS, we provide here relevant background on astrocytes and RGCs, cell types whose function can be informative with regards to our own work.

### 1.2.1 Astrocytes

Astrocytes are the most numerous cell type in the CNS (Bayraktar et al., 2014) and are found throughout all regions of the CNS (Molofsky and Deneen, 2015). Astrocytes are highly branched with star-like morphology, allowing them to interact with a variety of cell types such as endothelial cells, neurons and microglia (Poskanzer and Molofsky, 2018, Bayraktar et al., 2014). Similar to EGCs, astrocytes express many of the same molecules as the RGC progenitors they are derived from (Figure 1.5) (Than-Trong and Bally-Cuif, 2015, Molofsky and Deneen, 2015) (RGCs are discussed in Chapter 1.2.2). Despite sharing similar marker expression with RGCs, astrocytes have a distinct gene expression profile, and can be broadly split into two subtypes based on location and marker expression. Fibrous astrocytes are found in the grey matter and express high levels of GFAP. In contrast, protoplasmic astrocytes reside within the white matter, and have little or no GFAP expression (Miller and Raff, 1984).
Chapter 1 Introduction

**Figure 1.5. Marker expression in CNS glial cells.** Marker expression is largely shared between glial cells and progenitors of the CNS. Markers in black are used in mouse, but their expression in the zebrafish CNS has yet to be tested. Modified from Than-Thong (2015).

### 1.2.1.1 Astrocyte development

Within the CNS, astrocytes develop after neurogenesis is largely completed (Rowitch and Kriegstein, 2010, Gallo and Deneen, 2014, Kizil et al., 2012b). Differentiation along the astrocytic pathway is largely dependent on *nuclear factor 1A (NF1A)* and *Sox9* (Deneen et al., 2006, Stolt et al., 2003, Kang et al., 2013). All astrocyte progenitors are capable of generating both fibrous and protoplasmic astrocytes (Tsai et al., 2012). Moreover, astrocytes develop in a patterned manner,
similar to the neurons of the CNS, and astrocytes derived from the same progenitors are found in spatially distinct domains within the CNS (Tsai et al., 2012, Zeisel et al., 2018, Bayraktar et al., 2018). Recent single cell transcriptomic analysis has revealed there are up to seven transcriptomically distinct subtypes of astrocyte within the brain, which are spatially distinct. The fact that their position is associated with local differences in neuronal glutamate and glycine signalling (Zeisel et al., 2018, John Lin et al., 2017) suggests that astrocytic character may be initially dependent upon lineage and then further refined by the local environment (Farmer et al., 2016, Lanjakornsiripan et al., 2018). Consistent with this idea, within the cerebellum, Purkinje neurons supply SHH to Bergmann glia to maintain their distinct astrocytic subtype identity (Farmer et al., 2016). The spatial restriction of astrocytes subtypes within local brain regions, is believed to help maintain local cues derived from astrocytes that are required for neuronal identity, axon guidance and formation of neural circuitry (Farmer et al., 2016, Molofsky et al., 2014, Tsai et al., 2012, Zeisel et al., 2018) However, there is currently conflicting evidence as to whether a similar spatial restriction of astrocytes exists within the spinal cord (Tsai et al., 2012, Zeisel et al., 2018, Hochstim et al., 2008). Within the postnatal brain, astrocytes undergo further development, which is associated with the upregulation of gap junctions, synapse inducing proteins, secreted molecules, neurotransmitter recycling components and transmembrane receptors (Zhang et al., 2016). Therefore, mature neuronal signalling could further refine local astrocyte character. Interestingly, reports have shown that methylation states within mature astrocytes are similar to those observed in foetal astrocytes. This transcriptional fluidity may explain astrocytic heterogeneity and the ability of astrocytes to adapt to the local environment (Lister et al., 2013).

### 1.2.1.2 Astrocytic involvement in BBB formation and maintenance

Astrocytes have important roles in the formation and maintenance of the BBB, along with endothelial cells and pericytes (Attwell et al., 2010). The close contact between astrocytes and both the vascular system and neurons allows astrocytes to provide trophic support to neurons through the uptake of nutrients (McAllister et al., 2001). All astrocytes express Aquaporin 4 (AQP4) on their end-feet (Zeisel et al., 2018), which enables interaction with the vasculature of the BBB. The close proximity
of astrocytic end-feet to the blood vessels of the brain serves to modulate local blood flow, and therefore oxygen availability, thus affecting neuron activity (Metea and Newman, 2006, Gordon et al., 2008, Rouach et al., 2008).

Among their other functions within the BBB, astrocytes have important roles in modulating the movement of immune cells from the circulatory system into the CNS tissue (Sofroniew, 2015). Therefore, astrocytes play a significant role in maintaining a sterile environment for neurons (Attwell et al., 2010, Engelhardt and Coisne, 2011). Leucocytes that leave the vascular system do not directly enter the brain, but instead are ensheathed by astrocytic processes and basement membranes surrounding the blood vessels (Brosnan and Raine, 2013, Owens et al., 2008, Engelhardt and Coisne, 2011). Depending on the stimulus, astrocytes can modify their response to act either as pro-inflammatory mediators, by release of molecules promoting leucocyte migration and BBB disruption, or anti-inflammatory mediators, by restricting leucocyte migration and promoting BBB tightening and/or repair (Sofroniew, 2015).

1.2.1.3 Astrocytic roles in neuronal support

Astrocytes play important roles in supporting CNS neurons. Aside from the traditionally recognised function of structural support, astrocytes also provide trophic support to neurons (Molofsky and Deneen, 2015, Poskanzer and Molofsky, 2018, Kanski et al., 2014). Local depletion of adult astrocytes results in misdistribution of neurons, loss of neuropil, and altered synaptic connections, exclusively in the regions where the astrocytes are depleted (Molofsky et al., 2014, Tsai et al., 2012). However, this reduction in astrocytic numbers does not affect inflammation, gliosis or the BBB (Tsai et al., 2012). This suggests that sufficient regional astrocytic numbers are required for proper neuronal support. Additionally, the regional specification of astrocytic subtypes suggests that astrocytes vary their neuronal support functions based on the local population of neurons (Molofsky et al., 2014, Zeisel et al., 2018, John Lin et al., 2017).

1.2.1.4 Roles of astrocytes in neuronal circuitry

Evidence of direct interactions between astrocytes and neurons was initially described in-vitro, using fluorescent calcium indicators. Both spontaneous neuronal
activity and neurotransmitter release were shown to induce calcium transients within astrocytes (Charles et al., 1991, Cornell-Bell et al., 1990). Interestingly, astrocytic calcium transients occur in discrete microdomains that are mediated by specific neurotransmitters (Grosche et al., 1999, Di Castro et al., 2011). Moreover, there is increasing evidence for complex processing of neuronal signals in individual astrocytes, since astrocytes are able to integrate signals from multiple neural inputs (Di Castro et al., 2011, Perea and Araque, 2005, Zhang et al., 2016). Therefore, astrocytes constantly survey local neuronal activity and can modulate their responses appropriately.

Close association of astrocytes and neurons, and their ability to detect neuronal signalling, allows them to function within the neuronal circuitry. Aside from detecting neural signals, astrocytes are also able to modulate neuronal signalling via a calcium dependant release of gliotransmitters, which are neuroactive molecules such as Glutamate, ATP, D-Serine or TNFα (Perea et al., 2014), and modulation of neuroactive molecules within the extracellular environment (Poskanzer and Molofsky, 2018). Most astrocytic calcium release is mediated by G-protein coupled receptors, and therefore it occurs at a much slower timescale than synaptic neurotransmission, and may play an important role in synaptic plasticity (Serrano et al., 2006, Pascual et al., 2005). For example, astrocytic glutamate has been shown to modulate neuronal excitation and may provide non-synaptic neuronal synchronisation to hippocampal neurons (Serrano et al., 2006, Verbich et al., 2012).

Around 40% of neuronal synapses are believed to be tripartite synapses, in which there is communication between a pre- and post- synaptic neuron and an astrocyte (Perea et al., 2009). Interestingly, astrocytic ensheathment of synapses and neuronal processes is dynamic and occurs over both long and short timescales, in a stimulus dependant manner (Gordon et al., 2009a, Panatier and Oliet, 2006). Region specific molecules secreted from astrocytes are important in establishment of neuronal axon initiation segments and resulting synaptogenesis (Molofsky et al., 2014). Moreover, synapse associated astrocytes can mediate neuronal circuit refinement in the postnatal CNS (Vainchtein et al., 2018, Stevens et al., 2007). For example, astrocyte-derived interleukin-33 targets synapses for engulfment by microglia (Vainchtein et al., 2018). Additionally, astrocytes themselves can upregulate molecules important in phagocytic engulfment of excitatory and inhibitory synapses, and therefore contribute to synaptic refinement within the brain (Chung et
al., 2013). These data highlight the dynamic roles astrocytes play in neuronal signalling and circuitry.

1.2.1.5 Evolution of the astrocytic glial subtype

Glial cells vary greatly across animal phyla, in their cell types and functions. Higher organisms, including vertebrates, generally have greater glial cell-type diversity, which is thought to correlate with more complex neuronal functions and circuitry (Freeman and Rowitch, 2013, Hartline, 2011). Nevertheless, many astrocytic functions are conserved across species, although the glial subclass providing a specific function may vary. For example, in *Drosophila melanogaster* (*D. melanogaster*), astrocytic CNS glial cells play important roles in modulating neuronal signalling (Ma et al., 2016, Jennings et al., 2017, Muthukumar et al., 2014), similar to what is observed in the mammalian CNS (Molofsky et al., 2014). In contrast, zebrafish do not have protoplasmic astrocytes and instead have radial glial cells (RGCs). However, zebrafish RGCs take-up many astrocytic functions such as supporting the BBB (Grupp et al., 2010). Moreover, in adult stages, zebrafish RGCs become highly branched and morphologically resemble astrocytes of the mammalian CNS (Poskanzer and Molofsky, 2018), suggesting they may have similar roles in neuronal support and signalling. Therefore, despite different developmental and morphological appearances of glial cells across species, functional characteristics of glial cells are similar (Hartline, 2011).

All glial subpopulations arise from RGCs, however the genetic pathways mediating glial cell differentiation also appear to be distinct between species. In *D. melanogaster*, astrocyte development is dependent on *glial cell missing* (*gcm*), however the mammalian orthologue of this gene is not expressed in the CNS (Jones et al., 1995, Kim et al., 1998). Instead, the genetic pathway for astrocyte cell differentiation in the mammalian CNS employs a distinct mechanism, and astrocyte differentiation is dependent on *NF1A* and *Sox9* (Deneen et al., 2006, Stolt et al., 2003, Kang et al., 2013).

1.2.2 Radial Glia Cells (RGCs)

RGCs are the resident stem cell population within the developing and adult CNS, however they are not the only neural stem cell population within the brain (Falk
and Gotz, 2017, Rakic, 2003). As their name suggests, RGCs have their cell bodies located at the ventricular surface, with a radially projecting process that extends to the basal surface (Gotz and Huttner, 2005, Rakic, 2003, Taverna et al., 2014). As mentioned previously, RGCs express a large majority known of glial markers (Figure 1.5), however they also express many genes and markers reflecting their unique progenitor function, such as components of the NOTCH pathway and Nestin (Than-Trong and Bally-Cuif, 2015, Molofsky and Deneen, 2015). However, it is believed that the RGC population is heterogeneous, since at least two subpopulations can be distinguished based on their morphologies (Harris et al., 2018, Gebara et al., 2016). Below we will discuss the functions of RGCs during development, adulthood and injury. The roles of NOTCH signalling that are critical for RGC function will be disused in more detail later (Chapter 1.3.2).

1.2.2.1 RGCs in development

During CNS development, RGCs are the first cells to be generated from by neuroependymal cells (Gotz and Huttner, 2005). In the embryonic brain, RGCs are the most numerous cell type and are found in all brain areas (Rakic, 2003). During development, RGCs function as progenitors that give rise to all neurons and glia that make up the CNS (Furlan et al., 2017, Antony et al., 2004, Noctor et al., 2001, Miyata et al., 2001). RGCs initially divide symmetrically to expand the cortical area, which is followed by a neurogenic phase where RGCs undergo asymmetric divisions generating one neuron, or intermediate progenitor, and a RGC (Noctor et al., 2002, Noctor et al., 2007a, Noctor et al., 2007b). At early stages of mammalian development (until E15.5), RGCs can directly differentiate into a neuron without the need for an intermediate progenitor (Telley et al., 2016), resulting in an early depletion of the stem cell pool.

RGCs generate the neurons of the CNS in an inside-out manner, before generating glial cells. During development RGCs progenitor potential becomes increasingly restricted (Ming and Song, 2011, Zhao et al., 2008). The gradual suppression of high mobility complex (Hmga1 and Hmga2) by HES5, a downstream target of NOTCH signalling, has been shown to be a key factor in restricting RGC competency during development (Bansod et al., 2017, Kishi et al., 2012). The declining expression of Hmga1/2 is associated with the switch from generating
neuronal subtypes associated with deep cortical layers to the generation of neuronal subtypes found in superficial cortical layers. At later stages Hmga1/2 controls the switch from neurogenesis to astrogeneisis (Kishi et al., 2012). Interestingly, recent evidence suggests that while mammalian RGCs are first generated in early embryogenesis, a specific subset of adult RGCs are generated in embryogenesis, but remain quiescent until required in adulthood (Falk and Gotz, 2017, Furutachi et al., 2015). The early birth of adult RGCs may prevent them from becoming restricted in their progenitor potential. In addition, their entry into quiescence prevents depletion of the stem cell pool during development, therefore allowing neurons to be generated throughout life.

1.2.2.2 RGCs in adulthood

In adulthood, RGCs reduce their proliferative rate and generally have more restricted progenitor potential. However, in neuro-regenerative animals, such as the zebrafish, the neurogenic and proliferative domains containing RGCs are more abundant relative to their mammalian counterparts (Alunni and Bally-Cuif, 2016). In mammals, the neurogenic zones are largely confined to the subventricular zone (SVZ) and subgranular zone (SGZ) of the dentate gyrus. The SGZ generates new neurons in the dentate gyrus of the hippocampus, which plays important roles in learning and memory (Goncalves et al., 2016). On the other hand, neurogenesis in the SVZ generates new interneurons in the olfactory bulb and has been implicated in olfaction-mediated behaviours (Lazarini and Lledo, 2011). The SVZ is also thought to contribute to the regeneration of neurons generated within the cortex following injury (Palma-Tortosa et al., 2017). In contrast, in zebrafish there are around 16 distinct neurogenic domains (Alunni and Bally-Cuif, 2016, Grandel and Brand, 2013). The majority of these domains are able to generate new neurons during homeostasis. Despite the abundance of neurogenic domains in zebrafish, the most well studied region is the adult pallium (part of the telencephalon), which is believed to be similar to the mammalian SGZ.

In both zebrafish and mammals, RGCs act as a source of new neurons and glial cells throughout life, however the proliferative potential varies throughout the nervous system, depending on the area (Barbosa et al., 2015, Alunni and Bally-Cuif, 2016). The variability of proliferative and neurogenic potentials is largely dependent
on signals from the local niche, which includes many signalling pathways important during embryogenesis (Lie et al., 2005, Moreno-Estelles et al., 2012, Peretto et al., 2004, Basak and Taylor, 2007). Even within the same brain region, proliferative rates of RGCs vary, suggesting that RGCs can respond to local cues (Piatti et al., 2011). The neurogenic niche is composed of many cell types including: astrocytes, ependymal cells, endothelial cells, neurons, intermediate progenitors, microglia, neighbouring RGCs and the cerebral spinal fluid (Song et al., 2003, Urban and Guillemot, 2014). The niche is both permissive and instructive for RGC proliferative and neurogenic potential, in that if RGCs from the SGZ are transplanted into the SEZ, they produce SEZ specific neurons (Merkle et al., 2007, Gage, 2000). One notable difference between adult RGCs and embryonic RGCs is that unlike the stereotyped neurogenesis observed during development, adult RGCs respond to environmental cues. Enriched environments, or exercise, can increase the proliferative rate of adult RGCs (So et al., 2017, Ashokan et al., 2018, van Praag et al., 1999), highlighting the fact that adult neurogenesis is not a passive process, but actively adapts to the environment.

In zebrafish, newly generated neurons do not migrate from the area where they were born, but remain directly below the RGCs that generated them (Barbosa et al., 2015, Rothenaigner et al., 2011). Similarly, in the SGZ of the mammalian CNS, newborn neurons remain within the dentate gyrus (Altman and Bayer, 1990). The local position of newly generated neurons may act to control further proliferation events, by contributing to the local niche. In contrast, newborn neurons generated from the mammalian SVZ migrate extensively to reach their target area (Menezes et al., 2002). How this migration affects the local niche remains to be determined.

In adulthood, the majority of RGCs are quiescent. Upon receipt of appropriate signals, asymmetric proliferation is triggered, but then the RGCs re-enter quiescence (Urban et al., 2016). The maintenance of a quiescent state is essential to retain the progenitor pool. If cells are prevented from returning to quiescence after activation, the RGC pool is depleted (Urban et al., 2016). However, in both mouse and zebrafish, the adult RGCs have a limited proliferative potential, which leads to a gradual exhaustion of the RGC pool and ultimately to an age-dependant decline in neurogenesis. However, the time scale of RGC depletion varies in a species- and potentially area-dependant manner (Edelmann et al., 2013, Barbosa et al., 2015, Lugert et al., 2010, Seib et al., 2013). Interestingly, quiescent RGCs in an aged brain
can be reactivated to achieve proliferative rates that are typical of younger animals by exposure to systemic factors, such as GDF11, from young animals (Katsimpardi et al., 2014). Understanding how ageing affects quiescence will be instructive for understanding how aging affects the CNS. The return to quiescence is extremely important, since the majority of adult RGCs undergo symmetric divisions and generate two neurons, or neuronal progenitors (Obernier et al., 2018), leading to depletion of the stem cell pool with ageing. Similarly, adult RGCs can also directly differentiate into neurons (Barbosa et al., 2015, Edelmann et al., 2013). Together these behaviours result in a gradual depletion of the RGC pool.

1.2.2.3 RGC roles during injury

After CNS injury, both mammalian and zebrafish RGCs increase their proliferative and neurogenic behaviour. However, the relative success of RGC-mediated repair varies between zebrafish and mammals. Neuronal repair following traumatic injury to the mammalian CNS is largely unsuccessful, due to slow and/or low level neurogenic potential. Additionally, the formation of a glial scar is inhibitory to neurogenesis and circuit restoration (Jones and Bouvier, 2014, Yiu and He, 2006). Similarly, in mammalian models of neurodegeneration, local inflammation results in neuronal death, brain wasting and a decrease in proliferative potential (Tincer et al., 2016, LaFerla and Green, 2012). In contrast, there is little scaring present in the zebrafish CNS following injury, and neurogenesis is sufficient to regenerate the damaged area (Kroehne et al., 2011, Baumgart et al., 2012). In zebrafish, although toxic mediators of neurodegeneration result in neuroinflammation and death of neurons, the RGC population increases its proliferation rates and generates new neurons that can integrate into the existing circuitry (Bhattarai et al., 2016, Bhattarai et al., 2017). Therefore, understanding how neurogenesis is induced during injury conditions in the zebrafish brain might give insight into possible treatments for human neurodegenerative diseases.

Within the zebrafish brain, there are distinct mechanisms mediating homeostatic and regenerative behaviours. This was highlighted in a study that showed that oestrogen is capable of reducing proliferation in homeostatic conditions, but has no effect on proliferation during injury (Diotel et al., 2013). During injury, it is possible that the mechanism promoting proliferation in the zebrafish brain is
mediated via the microglia of the innate immune system of the brain. In traumatic brain injury, the immune system increases the proliferative and neurogenic potential of zebrafish RGCs through Leukotriene-C4 and increased expression of cxcr5 (Kizil et al., 2012a, Kyritsis et al., 2012). Injury induced expression of the quiescence-inducing molecule ld1 prevents over-activation of RGCs during injury and returns RGCs to quiescence (Rodriguez Viales et al., 2015). Similarly, Interleukin-4, which is expressed as a result of neurodegeneration, stimulates proliferation and neurogenesis of RGCs (Bhattarai et al., 2016). Interestingly, the regenerative response of RGCs during injury is dependent upon the insult, and is likely to involve divergent pathways that mediate neurogenesis. In support of this model, regeneration in zebrafish traumatic brain injury is dependent on gata3 expression in RGCs. However, during neurodegenerative disease, gata3 does not influence the proliferative or neurogenic potential of RGCs (Bhattarai et al., 2016, Kyritsis et al., 2012). This suggests that within the zebrafish brain, RGCs are able to modulate their proliferative response to the local environment, resulting in targeted regenerative programmes during injury. Understanding the molecular and signalling responses during discrete injury events may identify candidate therapeutic targets for mammalian CNS regeneration.

1.3 Roles of Notch Signalling

1.3.1 The NOTCH Receptor

NOTCH is a type 1 transmembrane protein (characterised by a single transmembrane span and the N-terminus in the extracellular space) with a stereotypic structure. All NOTCH receptors have an extracellular domain that has 29-36 epidermal growth factor (EGF) tandem repeats, a negative regulatory region (NRR) and a heterodimerisation domain. Within the extracellular domain, EGF-repeats 11-12 mediate trans-interaction with the NOTCH ligands (Kopan and Ilagan, 2009, Cordle et al., 2008, Komatsu et al., 2008), whereas EGF-repeats 24-29 mediate cis-inhibitory interaction, when the receptor and ligand are on the same cell (Kopan and Ilagan, 2009, Cordle et al., 2008, Komatsu et al., 2008). The specificity and ligand binding efficiency of NOTCH receptors is dependent on the presence of calcium ions within the EGF domain (Yamamoto et al., 2014, Raya et al., 2004). In addition, post-translational sugar modification of the extracellular EGF domain, by
enzymes such as POFUT-1, play additional roles in modulating receptor specificity. For example, it has been suggested that these modifications lead to differential recognition of the two subclasses of NOTCH ligands, DELTA or SERRATE (JAGGED) (Bruckner et al., 2000, Munro and Freeman, 2000). Finally, the NNR domain prevents promiscuous NOTCH activation (Weng et al., 2004, Gordon et al., 2007, Gordon et al., 2009b).

The NOTCH intracellular domain (NICD) (Figure 1.6A) is composed of a RBPJκ associated molecule (RAM) domain, several nuclear localization sequences, seven ankyrin repeats (ANK domain) and a conserved region that harbours degradation signals (degrons) at the C-terminus (Kopan and Ilagan, 2009, Cordle et al., 2008, Komatsu et al., 2008) that largely determines the half-life of the NICD (Kopan and Ilagan, 2009, Cordle et al., 2008, Komatsu et al., 2008).
**Figure 1.6. NOTCH receptor structure and canonical NOTCH signalling.** (A) Schematic structure of the NOTCH receptor, and position of cleavage sites. The majority of the extracellular domain is made up of EGF repeats. EGFs 11-12 are essential for trans-activating ligand binding, whereas EGF repeats 24-29 are essential for cis-inhibition. The NEXT domain represents the intermediate NOTCH receptor after cleavage at S1 and S2. The NICD is released after S3 cleavage, the RAM domain and CLS are important in DNA binding, whereas the PEST domains is required for signal termination. (B) Schematic of canonical NOTCH signalling. (1) Post translation NOTCH modifications in the receiver cell (2) NOTCH is trafficked to the membrane. (3) NOTCH is expressed on the cell surface. (4) NOTCH ligands are translated and transported to the membrane in the sender cell. (5) NOTCH and ligands interact via trans-activation. (6) Endocytosis occurs in the sender cell, exposing S1 and S2. (7) Cleavage of S1 and S2 by enzymes such as ADAM to generate NEXT (8) NEXT is cleaved at S3 by γ-secretase. (9)
Cleavage of NEXT/NICD can also occur on the endosomal membrane. (10) NICD translocated to the nucleus. (11) NICD interacts with DNA binding proteins such as CLS and MAM. See main text for abbreviations. Adapted from Yamamoto (2015) with permission from Springer Nature.

1.3.2 Canonical NOTCH Signalling

Canonical NOTCH signalling is dependent on cell-cell contact. Trans-activation occurs when a NOTCH ligand is expressed on one cell (sender cell) and interacts with the NOTCH receptor on its neighbouring cell (receiver cell) (Kopan and Ilagan, 2009, Yamamoto et al., 2014). Upon binding of a NOTCH ligand, (Table 1-2), the sender cell begins endocytosis of the NOTCH-ligand complex, thus exposing the first cleavage site (S1). The extracellular component of NOTCH is cleaved by ADAM enzymes at S1, just before the transmembrane domain, followed by further cleavage at site 2 (S2) (Weinmaster and Fischer, 2011). Once the ectodomain has been removed, the NOTCH receptor forms a membrane intermediate known as the NOTCH external truncation domain (NEXT), which is the substrate for γ-secretase (Jorissen et al., 2010). Subsequently, γ-secretase progressively cleaves the transmembrane portion of NEXT. Only after cleavage of the transmembrane domain by γ-secretase can the NICD translocate to the nucleus (Huenniger et al., 2010). Once in the nucleus, the NICD interacts with DNA binding proteins CLS (RBPJκ in vertebrates and Su(H) in Drosophila) via the RAM domain (Johnson and Barrick, 2012). Subsequently, the ANK domain of the NICD associates with CSL to recruit a coactivator Mastermind (MAML1-3) (Tanigaki and Honjo, 2010). These coactivators further recruit MED8, a mediator of the transcriptional activation complex, which results in upregulation of downstream NOTCH target genes (Morel et al., 2001, Furriols and Bray, 2001). The downstream activation of the NICD-CLS complex is terminated by phosphorylation, of the NICD (Fryer et al., 2004). Finally, after phosphorylation the NICD is degraded via the ubiquitin ligase pathway to prevent further DNA binding (Oberg et al., 2001, Wu et al., 2001).
NOTCH signalling can be modulated through multiple mechanisms, to facilitate specific and targeted signalling. One principal mechanism for such signalling, is through *cis*-inhibition, which blocks the appropriate conformational changes in the NOTCH receptor necessary to expose the S2 cleavage site within the NOTCH receptor, thus preventing downstream NOTCH signalling (del Alamo et al., 2011). Therefore, ligands expressed on the same cell as the receptor must compete for receptor binding with trans-activating ligands. This ultimately prevents, or limits, the amount of trans-activation that can occur within a cell, and serves to modulate the downstream NOTCH response (Becam et al., 2010, Sprinzak et al., 2010). In addition, there are multiple NOTCH receptors and ligands in both zebrafish and mammals (Table 1-2). Differential expression leads to various combinations of NOTCH ligands and receptors being present in different tissue environments, and leads to differential modulations of downstream target genes. For example, recent data shows that cells can distinguish between DELTA-LIKE 1 (DLL1) and DELTA-LIKE 4 (DLL4) signalling though the NOTCH1 by virtue of their distinct binding dynamics to NOTCH1 receptor. DLL1 binding modulates the frequency of NICD release, leading to downstream expression of *Hes1*, whereas, DLL4 binding has sustained binding to the NOTCH1 receptor to modulate the amplitude of NICD

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Table 1-2. Notch ligands and receptors found in mammals and zebrafish.
molecules within the cell. The increased amplitude of NICD signalling results in preferential expression Hey1 and HeyL (Nandagopal et al., 2018). Furthermore, the short half-life of the NICD, and its downstream targets, allows dynamic NOTCH signalling to be responsive to environmental changes (Kageyama et al., 2018, Ilagan et al., 2011).

NOTCH signalling is a key pathway involved in many developmental processes, however we shall focus on its roles in the nervous system.

1.3.3 Roles of NOTCH in Neural Stem Cells

Although, NOTCH signalling plays a critical role within NSCs and is essential for cell maintenance and proliferative potential, it is not required for NSC development (Hitoshi et al., 2002). In NSCs, NICD-CLS signalling induces the transcription of the Hairy and enhancer of split (Hes) genes, Hes1 and Hes5. Hes genes inhibit neurogenesis by repressing transcription of basic helix-loop-helix (bHLH) transcription factors, such as Ascl1, and Neurogenin (Takke et al., 1999, Chen et al., 1997, Kageyama et al., 2007a, Kageyama et al., 2007b). In addition, HES proteins also form heterodimers with target bHLH activators, such as ASCL1, to prevent these activators from binding to DNA and initiating neurogenic programs (Chen et al., 1997, Kageyama et al., 2007b).

Since HES proteins negatively regulate their own expression, and both Hes mRNA and proteins are highly unstable, Hes expression oscillates, with a periodicity of around 2 hours (Hirata et al., 2002, Fongang and Kudlicki, 2016). Optogenetic studies have suggested that oscillation of bHLH genes, such as Hes5 and Ascl1, are essential to maintain progenitor and proliferation potential. Low expression or absence of bHLH genes results in extremely low levels of proliferation. In contrast, once levels of bHLH gene expression becomes stable at high levels, fate restriction and differentiation occur (Imayoshi et al., 2010, Imayoshi et al., 2013). Similarly, if the periodicity of bHLH oscillations are altered, the proliferation and maintenance of the stem cells pool is defective and the stem cell pool becomes depleted (Shimojo et al., 2016). Slight changes in NOTCH ligand expression levels could be sufficient to result in a switch from oscillatory to stable bHLH expression, resulting in loss of progenitor potential and onset of differentiation. However, it is unknown how gene expression is altered in response to oscillatory bHLH behaviours. It is speculated
that oscillations of *Hes* genes and *Ascl1* may prime NSCs for neurogenesis, without initiating neural differentiation (Imayoshi et al., 2013, Shimojo et al., 2008).

Upregulation of pro-neural genes, such as *Ascl1*, promotes the expression of NOTCH ligands within the cell. Due to the requirement for cell-cell contact in NOTCH signalling, upregulation of ligands in turn leads to active NOTCH signalling in neighbouring cells. This process is known as lateral inhibition and prevents all NSCs from differentiating or proliferating at the same time (Kageyama et al., 2015, Kageyama et al., 2008b). Lateral inhibition may, therefore, have a role in influencing cell fate decisions of RGC progeny, by maintaining cells in a progenitor state until later stages. Since neuronal birth date is instructive for neuronal fate and subtype, the reserved progenitors can go on to form the different neuronal subtypes typical of later neurogenic stages. In support of this model, in the absence of *Hes* genes, only early-born neurons and astrocytes are formed, suggesting that *Hes* genes are important in maintaining some cells in a NSC state to enable late born neuronal subtypes to be generated (Hatakeyama et al., 2004).

### 1.3.4 Roles of Notch in Gliogenesis

Later in development, NOTCH signalling promotes gliogenesis rather than, directing stem cell fate (Kishi et al., 2012, Kageyama et al., 2008a, Kanski et al., 2014). NOTCH signalling also promotes a RGC fate switch from neurogenic to gliogenic programs. HES5 mediated downregulation of *hmga1/2* expression results in the switch from late neurogenesis to astrogenesis (Bansod et al., 2017, Kishi et al., 2012). One mechanism that may determine the timing of this switch in cell fate is the methylation state of promoter regions of genes associated with astrocytic fate. After the bulk of neurogenesis is complete, the NICD upregulates the transcription factor *NF1A*, which in turn binds to the promoter regions of various astrocytic genes, resulting in their subsequent upregulation (Namihira et al., 2009). In addition, NOTCH dependant upregulation of *Sox9* further promotes astrogenesis, through SOX9 dependant upregulation of *NF1A* (Stolt et al., 2003, Kang et al., 2013). However, *NF1A* also inhibits NOTCH, acting as an autoregulatory feedback mechanism to prevent exhaustion of the RGC pool and overproduction of astrocytes (Piper et al., 2010). Additional mechanisms, such as HES mediated upregulation of *STAT3*, further promotes astrocytic differentiation (kamakura S 2004). Therefore,
NOTCH signalling promotes astrogenesis using a variety of mechanisms. It has been proposed that the continued responsiveness of astrocytes to NOTCH signalling underlies their proliferative behaviour during injury conditions (Magnusson et al., 2014).

NOTCH signalling plays multiple roles within the ENS (Chapter 1.1.2.2.5), in particular NOTCH signalling has been implicated in maintaining the enteric glial/non-neuronal cell fate. NOTCH signalling at late embryonic stages is essential to upregulate Sox10, a key gene associated with non-neuronal/EGC cell fate (Kim et al., 2003). Moreover, loss of NOTCH signalling results in premature neuronal differentiation (Ngan et al., 2011, Okamura and Saga, 2008, Taylor et al., 2007), further suggesting a critical role in NOTCH in maintaining the non-neuronal cell fate. However, these studies have largely focused on early stages of ENS development, and the contribution of NOTCH signalling to mature gliogenesis has yet to be determined.

1.3.5 NOTCH Signalling in Neuronal Circuitry

Within the CNS, NOTCH signalling influences neuronal morphology and circuitry. NOTCH has been implicated in regulating microtubule dynamics in neurons (Ferrari-Toninel 2008), a key process in establishing neuronal morphology and axonal outgrowth. NOTCH1 expression within neurons is also associated with the development of a complex dendritic arbour (Redmond et al., 2000, Breunig et al., 2007). Interestingly, it is likely that there is a critical period where NOTCH signalling can influence dendritic branching. Although immature neurons are highly sensitive to NOTCH signalling, there is little change in the dendritic tree of mature neurons when NOTCH signalling is perturbed (Zhao et al., 2008). Nevertheless, within mature neurons, NOTCH plays a role in modulating spine formation (Dahlhaus et al., 2008), suggesting that NOTCH is required for neuronal plasticity. In support of this idea, NOTCH mutants have defects in synaptic plasticity that results in deficits in spatial learning and memory (Costa et al., 2003, Presente et al., 2004, Wang et al., 2004), and these effects can be reversed upon restoration of NOTCH signalling (Ge et al., 2004). A role in mature neurons is also indicated by the fact that there is an increase in NOTCH ligands and receptors at the synapse, in response to synaptic activity.
(Alberi et al., 2011, Lieber et al., 2011), suggesting that NOTCH signalling in neurons is highly dynamic.

While the roles of NOTCH signalling in neuronal circuitry remains to be determined, the presence of NOTCH1 and JAGGED1 on adult neurons suggests such mechanisms could also take place within the ENS.

In conclusion, NOTCH signalling is critical for the development and homeostasis of the nervous system it maintains the stem cell population, directs glial cell differentiation and modulates neuronal circuitry.

1.4 Thesis Aims

Recent studies have demonstrated that in the mammalian ENS EGCs represent a topologically and morphologically heterogeneous cell population with similarities to astrocytes and other peripheral glia (Boesmans et al., 2014). In addition to their roles as “professional” glial cells, it has been suggested that under normal conditions, at least a subset of mammalian EGCs undergo low rate proliferation and replenish glial cells lost to physiological turnover (Kabouridis et al., 2015). Moreover, a number of reports have suggested recently that under certain conditions, EGCs can acquire neurogenic potential but the generation of new enteric neurons in the adult ENS in-vivo is remarkably inefficient (Laranjeira et al., 2011, Joseph et al., 2011). Controversially, a recent study has provided evidence for extensive neuron turnover and unprecedented levels of neurogenesis in the ENS of adult mice (Kulkarni et al., 2017). The aim of this thesis is to address outstanding questions regarding the properties and potential of EGCs in the vertebrate ENS. We reasoned that a better understanding of the non-neuronal population in the ENS of teleosts (zebrafish), an organism with a generally high regenerative potential of the nervous system, would provide valuable insight into the dynamics of enteric glia within vertebrates, including mammals.

The specific aims of this thesis are:

1. Characterisation of the enteric glial population in larval and adult zebrafish
2. Description of the cellular dynamics of zebrafish enteric glia during development and adulthood
3. Description of the neurogenic potential of zebrafish enteric glia
Chapter 2. Materials & Methods

2.1 Reagents and Equipment Used

Common solutions are listed in Table 2-1. Those marked with an asterisks were made by in-house facilities at the Francis Crick institute. Common chemicals and equipment used for experiments are listed in Table 2-2 and Table 2-3. In all cases, where appropriate, the catalogue number and supplier has been provided. A list of suppliers can be found in Table 2-4.

<table>
<thead>
<tr>
<th>Name</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Hydroxy tamoxifen (4-OHT)</td>
<td>Immersion: 5μM in DMSO</td>
</tr>
<tr>
<td></td>
<td>Injection: 50μM in 9:1 ethanol:corn oil</td>
</tr>
<tr>
<td>BrdU</td>
<td>Injection: 10mM BrdU in PBS</td>
</tr>
<tr>
<td>E3 embryo media</td>
<td>5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl, 0.33 mM MgSO4 in dH2O (pH=7.4)</td>
</tr>
<tr>
<td>2'-Deoxy-5-ethyluridine (EdU)</td>
<td>Immersion: 1.6M EdU in DMSO</td>
</tr>
<tr>
<td></td>
<td>Injection: 10mM EdU in PBS</td>
</tr>
<tr>
<td>Embedding Solution (cryosections)</td>
<td>15% sucrose 7.5 gelatin in PBS</td>
</tr>
<tr>
<td>Danios solution</td>
<td>58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5 mM HEPES in 1x PBS (pH=7.6)</td>
</tr>
<tr>
<td>Lidocane*</td>
<td>0.53 Lidocaine hydrochloride monohydrate in 1L RO water</td>
</tr>
<tr>
<td>Loading Buffer for DNA</td>
<td>30% (v/v) glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF in MilliQ H2O</td>
</tr>
<tr>
<td>LY11575</td>
<td>2.5M LY11575 in DMSO</td>
</tr>
<tr>
<td>Lysis Buffer</td>
<td>100mM Tris HCl (pH=8), 5mM EDTA, 0.2% SDS, 200mM NaCl, 0.1mg/ml Proteinase K</td>
</tr>
<tr>
<td>PB (Phosphate Buffer)*</td>
<td>Sodium phosphate dibasic 20.2g, Sodium phosphate monobasic 3.4g in dH2O (pH=7.4)</td>
</tr>
<tr>
<td>PBS (Phosphate Buffered Saline)*</td>
<td>137mM NaCl, 3mM KCl, 8mM Na2HPO4, 1.5mM KH2PO4 in dH2O (pH=7.4)</td>
</tr>
<tr>
<td>PBT (PBS with Triton-X 100)</td>
<td>0.1%, 0.3% or 0.5% Triton-X 100 in PBS</td>
</tr>
<tr>
<td>PFA (paraformaldehyde)</td>
<td>4% PFA in PBS (pH=7.2)</td>
</tr>
<tr>
<td>1-phenyl 2-thiourea (PTU)</td>
<td>0.003% 1-phenyl-2-thiourea in 10% Hank's saline</td>
</tr>
<tr>
<td>Sodium citrate buffer</td>
<td>10mM in dH2O (pH=6)</td>
</tr>
<tr>
<td>Sodium Tetraborate</td>
<td>0.1M in dH2O (pH=8.5)</td>
</tr>
<tr>
<td>Sucrose</td>
<td>30g in 100ml PBS (30%)</td>
</tr>
<tr>
<td>3-aminobenzoic acid ethyl ester (MSS-222, or Tricane)</td>
<td>400mg MS222 in 97.9ml RO water, and 2.1ml Tris 9 (Ph=7.5)</td>
</tr>
<tr>
<td>Zebrafish Water</td>
<td>0.2g Ocean salts in 20L of dH2O</td>
</tr>
</tbody>
</table>

* made in house at the Francis Crick Institute
Table 2-2. List of chemicals used

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Source (Catalogue number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>Roche (11 388 991 001)</td>
</tr>
<tr>
<td>BrdU (5-Bromo-2’-deoxyuridine)</td>
<td>Sigma (B5002)</td>
</tr>
<tr>
<td>EdU (5-ethynyl-2’-deoxyuridine)</td>
<td>Thermo Fisher (A10044)</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>Sigma (L5647)</td>
</tr>
<tr>
<td>PTU (1-phenyl-2-thiourea)</td>
<td>Sigma (P7629)</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>Sigma (71405)</td>
</tr>
<tr>
<td>Sodium tetraborate</td>
<td>Sigma (221732)</td>
</tr>
<tr>
<td>Tricane (MS222)</td>
<td>Sigma (A-5040)</td>
</tr>
<tr>
<td>LY411545</td>
<td>Cambridge Bioscience (SM25)</td>
</tr>
</tbody>
</table>
### Table 2-3. List of equipment

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Centrifuge</strong></td>
<td></td>
</tr>
<tr>
<td>Heraeus PICO 17 microcentrifuge</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>Centrifuge 55702 R</td>
<td>Eppendorf</td>
</tr>
<tr>
<td><strong>Cryostat</strong></td>
<td></td>
</tr>
<tr>
<td>Microm HM 560 cryostat</td>
<td>Carl Zeiss</td>
</tr>
<tr>
<td><strong>Fridges and Freezers</strong></td>
<td></td>
</tr>
<tr>
<td>Innova U725 -86°C freezer</td>
<td>Scientific</td>
</tr>
<tr>
<td>Whirlpool fridge</td>
<td>Whirlpool</td>
</tr>
<tr>
<td>Labcold -20°C freezer</td>
<td>Labcold</td>
</tr>
<tr>
<td><strong>Incubators, ovens and shakers</strong></td>
<td></td>
</tr>
<tr>
<td>Thermo Hybaid shake n stack hybridisation oven</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>Gyro-rocker SSL3</td>
<td>Stuart</td>
</tr>
<tr>
<td>Block heater SBH2200D</td>
<td>Stuart</td>
</tr>
<tr>
<td>Memmit incubator</td>
<td>GbMH and Co.</td>
</tr>
<tr>
<td>Labdancer</td>
<td>VWR International Ltd</td>
</tr>
<tr>
<td><strong>Microscopes and Cameras</strong></td>
<td></td>
</tr>
<tr>
<td>Leica MZ6 stereoscope</td>
<td>Leica</td>
</tr>
<tr>
<td>Axiophot</td>
<td></td>
</tr>
<tr>
<td>GFPscope</td>
<td></td>
</tr>
<tr>
<td>Leica DM6000 confocal microscope fitted with multiphoton spectra physics (SP5) Mai Tai Ti:Sapphire Deep Sea Laser system</td>
<td>Leica</td>
</tr>
<tr>
<td>Zieiss 880-inverted</td>
<td>Zieiss</td>
</tr>
<tr>
<td><strong>PCR machines</strong></td>
<td></td>
</tr>
<tr>
<td>DNA Engine eDyad Thermal Cycler Chassis</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>Electrophoresis</td>
<td></td>
</tr>
<tr>
<td>MultiSub horizontal electrophoresis units with consort E884 power supply</td>
<td></td>
</tr>
<tr>
<td><strong>Safety Cabinets</strong></td>
<td></td>
</tr>
<tr>
<td>Walker fume hood cabinet</td>
<td>cabinets</td>
</tr>
<tr>
<td><strong>Scales</strong></td>
<td></td>
</tr>
<tr>
<td>XB620M balance</td>
<td>Precisa</td>
</tr>
<tr>
<td>M-Power presision balance</td>
<td>Sartorius</td>
</tr>
<tr>
<td><strong>Water Purifier</strong></td>
<td></td>
</tr>
<tr>
<td>Milli-Q ultrapure water</td>
<td>Millipore</td>
</tr>
</tbody>
</table>
Table 2-4. List of Companies

<table>
<thead>
<tr>
<th>Company</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abcam</td>
<td>Cambridge, UK</td>
</tr>
<tr>
<td>Adobe Systems</td>
<td>San Jose, CA, USA</td>
</tr>
<tr>
<td>BD Biosciences</td>
<td>Oxford, UK</td>
</tr>
<tr>
<td>Bio-rad Laboratories</td>
<td>Hemel Hempstead, UK</td>
</tr>
<tr>
<td>Cambridge Bioscience</td>
<td>Cambridge, UK</td>
</tr>
<tr>
<td>Covance Berkeley</td>
<td>CA, USA</td>
</tr>
<tr>
<td>Dako, UK, Ltd.</td>
<td>Cambridge, UK</td>
</tr>
<tr>
<td>Dow Corning</td>
<td>Seneffe, Belgium</td>
</tr>
<tr>
<td>Eppendorf</td>
<td>UK, Ltd. Cambridge, UK</td>
</tr>
<tr>
<td>GraphPad Software</td>
<td>Inc. San Diego, CA, USA</td>
</tr>
<tr>
<td>Immune Systems, Ltd.</td>
<td>Devon, UK</td>
</tr>
<tr>
<td>Jackson ImmunoResearch Laboratories, Inc.</td>
<td>West Grove, PA, USA</td>
</tr>
<tr>
<td>Leica Microsystems, UK, Ltd.</td>
<td>Milton Keynes, UK</td>
</tr>
<tr>
<td>Life Technologies (Invitrogen)</td>
<td>Paisley, UK</td>
</tr>
<tr>
<td>Millipore Billerica,</td>
<td>MA, USA</td>
</tr>
<tr>
<td>Molecular Probes, Inc.</td>
<td>Eugene, OR, USA</td>
</tr>
<tr>
<td>New Brunswick Scientific (Eppendorf)</td>
<td>Cambridge, UK</td>
</tr>
<tr>
<td>New England Biolabs, UK, Ltd.</td>
<td>Hitchin, UK</td>
</tr>
<tr>
<td>Promega Corporation</td>
<td>Southampton, UK</td>
</tr>
<tr>
<td>Santa Cruz Biotechnology, Inc.</td>
<td>Santa Cruz, CA, USA</td>
</tr>
<tr>
<td>Sigma-Aldrich Company, Ltd.</td>
<td>Dorset, UK</td>
</tr>
<tr>
<td>Source BioScience LifeSciences</td>
<td>Nottingham, UK</td>
</tr>
<tr>
<td>Stuart</td>
<td>Staffordshire, UK</td>
</tr>
<tr>
<td>Thermo Scientific</td>
<td>Loughborough, UK</td>
</tr>
<tr>
<td>Ultra-violet products, Ltd.</td>
<td>Cambridge, UK</td>
</tr>
<tr>
<td>Vector Laboratories, Ltd.</td>
<td>Peterborough, UK</td>
</tr>
<tr>
<td>VWR International</td>
<td>Lutterworth, UK</td>
</tr>
<tr>
<td>Walker Safety Cabinets, Ltd.</td>
<td>Glossop, UK</td>
</tr>
<tr>
<td>Whirlpool, UK</td>
<td>Croydon, UK</td>
</tr>
<tr>
<td>ZM systems</td>
<td>Hampshire, UK</td>
</tr>
</tbody>
</table>

2.2 Animal Husbandry

Zebrafish and mice were kept in accordance to the British Home Office Scientific Procedures Act 1986 and approved by local ethics committee. All procedures were carried out by trained personal licence holders.
2.2.1 Zebrafish

Zebrafish embryos were obtained by natural spawning (Westerfield, 1993). Embryos and larvae were staged according to their morphological features (Westerfield, 1993) as hours or days post fertilisation (hpf and dpf, respectively). Zebrafish are considered embryos before feeding age (5dpf) and larvae afterwards. Animals are considered adults when sexual maturity is reached (~3 months old (mo)). Embryos and larvae were raised in E3 media or zebrafish water (Table 2-1) in an 28.5°C illuminated incubator on 14hour:10hour light:dark cycle. For live imaging, and embryonic analysis, 0.15M 1-phenyl 2-thiourea (PTU) was added to the zebrafish water at 24hpf to block differentiation of pigmented melanocytes, which interfere with imaging, and maintained until the zebrafish were culled. From 5-7dpf, zebrafish were fed daily with ZM-000 (ZM systems). Larvae, and adults, analysed after 5dpf were fasted the evening before being culled to prevent luminal content from disrupting subsequent analysis. Zebrafish were culled by overdose of MS222 (S1K). Zebrafish embryos and larvae are kept at a stocking density of 50 zebrafish per 20mls. At 5dpf, larvae are moved onto a Tecniplast system and maintained under standard husbandry conditions, during adulthood zebrafish are maintained at a density of 17 adult zebrafish per 3 litres.

The zebrafish transgenic lines were used in the heterozygous state and have been previously described: \( Tg(gfap:GFP) \) (Bernardos and Raymond, 2006), \( Tg(ret_hu2846) \) (Knight et al., 2011), \( Tg(nestin:GFP) \) (Lam et al., 2009) \( Tg(zFoxD3:GFP) \) (Gilmour et al., 2002), \( Tg(-4.7sox10:GFP) \) (Dutton et al., 2008) \( Tg(her4.1:EGFP) \) (Yeo et al., 2007), \( Tg(-4725sox10:cre)ba74 \) (Rodrigues et al., 2012), \( Tg(\beta actin-\text{LoxP-STOP-LoxP-hmgb1-mCherry}) \) (Wang et al., 2011), \( Tg(SAGFF234A) \) (Heanue et al., 2016a, Kawakami et al., 2010), \( Tg(SAGFF217B) \) (Kawakami et al., 2010), \( Tg(UAS:mCherry) \) (Qiling Xu, Francis Crick Institute), \( Tg(UAS:GFP) \) (Kawakami et al., 2010); \( Tg(\text{her4.1ERT2creERT2}) \) (Boniface et al., 2009), \( Tg(\text{EF1a:loxp-gfp-pA-loxp-dsRed-pA}) \) (Sinha et al., 2010).

2.2.1.1 Genotyping

The majority of transgenic zebrafish larvae were genotyped based on fluorescent reporter expression, using a fluorescent dissecting microscope (Table 2-5). Most fluorescent reporters (i.e. GFP) were directly conjugated to a promoter
region of interest, resulting in GFP expression whenever the gene of interest was expressed.

Visualising expression of the \( Tg(-4725sox10:cre)ba74 \) expression required crossing it with a Cre-reporter line \( Tg(\beta\text{actin-LoxP}-STOP-LoxP-hmgb1-mCherry) \), or \( Tg(EF1\alpha:loxp-gfp-pA-loxp-dsRed-pA) \). In resulting double transgenic lines (\( Tg(-4725sox10:cre; \beta\text{actin-LoxP}-STOP-LoxP-hmgb1-mCherry) \), \( Tg(-4725sox10:cre;EF1\alpha:loxp-gfp-pA-loxp-dsRed-pA) \), in which bacterial Cre recombinase expression is under the control of a \( \text{sox10} \) promoter region. Cre recombinase specifically recognises LoxP sites, and when Cre binds to the LoxP sites it excises and recombines the DNA, removing the stop codon (\( Tg(\beta\text{actin-LoxP}-STOP-LoxP-hmgb1-mCherry) \)), or GFP transgene (\( Tg(EF1\alpha:loxp-gfp-pA-loxp-dsRed-pA) \)).

Genotyping of transgenics using the binary Gal4-UAS system required crossing tissue specific Gal4 drivers (\( Tg(\text{SAGFF234A}) \) and \( Tg(\text{SAGFF217B}) \)) to a UAS reporter (\( Tg(\text{UAS:GFP}) \) or \( Tg(\text{UAS:mCherry}) \)). The tissue specific Gal4 binds specifically to the UAS region on the reporter line and induces downstream fluorophore expression. Double transgenic zebrafish (\( Tg(\text{SAGFF234A;}\text{UAS:GFP}) \), \( Tg(\text{SAGFF234A;}\text{UAS:mCherry}) \) and \( Tg(\text{SAGFF217B;}\text{UAS:mCherry}) \)) have tissue specific fluorophore expression.

**Table 2-5. Genotyping by fluorescence**

<table>
<thead>
<tr>
<th>Transgenic line</th>
<th>Expected Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>( Tg(EF1\alpha:loxp-gfp-pA-loxp-dsRed-pA) )</td>
<td>GFP is expressed in every cell</td>
</tr>
<tr>
<td>( Tg(\text{gFoxD3:GFP}) )</td>
<td>GFP is observed in the NCCs and in the pineal gland</td>
</tr>
<tr>
<td>( Tg(gfp:GFP) )</td>
<td>GFP is highly expressed within the CNS</td>
</tr>
<tr>
<td>( Tg(\text{her4.1:EGFP}) )</td>
<td>GFP is highly expressed within the CNS</td>
</tr>
<tr>
<td>( Tg(\text{her4.1ERT2creERT2;}\beta\text{actin-LoxP}-STOP-LoxP-hmgb1-mCherry) )</td>
<td>After tamoxifen induction Cherry is highly expressed in the CNS</td>
</tr>
<tr>
<td>( Tg(\text{neslin:GFP}) )</td>
<td>GFP is highly expressed within the CNS</td>
</tr>
<tr>
<td>( Tg(\text{SAGFF234A;}\text{UASGFP}) )</td>
<td>GFP/mCherry is highly expressed within the CNS and heart</td>
</tr>
<tr>
<td>( Tg(\text{SAGFF234A;}\text{UASmCherry}) )</td>
<td>mCherry is expressed within a subset of neurons in the CNS</td>
</tr>
<tr>
<td>( Tg(\text{sox10:GFP}) )</td>
<td>GFP is highly expressed within NCCs. Obvious expression in the jaw</td>
</tr>
<tr>
<td>( Tg(-4725sox10:cre)ba74;\beta\text{actin-LoxP}-STOP-LoxP-hmgb1-mCherry) )</td>
<td>Cherry is observed in the neural crest cells lineage</td>
</tr>
<tr>
<td>( Tg(-4725sox10:cre)ba74;EF1\alpha:loxp-gfp-pA-loxp-dsRed-pA) )</td>
<td>GFP is expressed in every cell. Cherry is observed in the neural crest cells lineage</td>
</tr>
</tbody>
</table>
For genotyping that required a PCR based approach, tissue biopsies were collected as follows. Adult transgenic zebrafish were given analgesic (2.1 mM lidocaine), then anesthetised in 0.15 mg/ml MS222 before removal of the caudal part of the tail fin with surgical scissors. Zebrafish were allowed to recover in individual tanks, with analgesic lidocaine provided for 24 hours after fin-clipping. Tail pieces from transgenic larvae were removed from fixed samples after immunohistochemistry was performed. The process of DNA extraction and subsequent PCR is described below (Chapter 2.3).

2.2.1.2 Immersion

The γ-secretase inhibitor LY411575 and thymidine analogue 2’-Deoxy-5-ethynyluridine (EdU), were provided by immersion to adult zebrafish, to study the effects of Notch signalling and proliferation, respectively. All solutions were prepared as described previously (Table 2-1). 3L tanks were maintained off the system, and filled with required solutions: 10 µM LY411575, 0.04% DMSO, and/or 1 mM EdU in system water. For Notch inhibition experiments, control zebrafish were incubated with the equivalent concentration of DMSO (0.04%) in system water. Adult zebrafish were maintained off the system at a stocking density of 4 zebrafish/litre, while off the system, and at least one piece of bio-media was provided for every two zebrafish, to improve water quality (Mocho, 2016, Mocho et al., 2017). Zebrafish were fed only once a day with paramecium and water was changed every 48-72 hours, to prevent the build-up of waste products, which adversely affect animal health. Zebrafish in LY411575, and the equivalent controls, were additionally supplied with analgesic (2.1 mM lidocaine) to limit suffering due to the global effects of γ-secretase inhibition. Environmental enrichment was provided by a picture of stones placed underneath the tank.

Immersion of Tg(her4.1ERT2creERT2; β-actin-LoxP-STOP-LoxP-hmgb1-mCherry) larval zebrafish occurred between 1-2 dpf. 4-hydroxytamoxifen (4-OHT) (stock solutions were prepared as previously described in Table 2-1), was diluted to 10 mM 4-OHT in E3 embryo media (Table 2-1). Larval zebrafish were stocked at 50 larvae per 20mls of E3 embryo media. After incubation, zebrafish were transferred to a new container and washed quickly 5 times in E3 embryo media. Subsequently, the embryo media was changed daily before putting larvae on the system at 5 dpf.
48 hours after 4-OHT incubation, embryos were genotyped based on their fluorescence pattern (Table 2-5), and afterwards mCherry expressing embryos were grown to adulthood as normal.

2.2.1.3 Injections

Adult zebrafish were provided with analgesic (2.1mM lidocaine) for at least 20 minutes before intra-coleomic injections. Zebrafish were anaesthetised in 0.15mg/ml MS222 until their ocular movements stopped. Adult zebrafish were removed from the anaesthetic and placed on a sponge, which was pre-wetted with system water. Using an insulin syringe, zebrafish were injected with 10µl of 50µM 4-OHT, 10mM EdU or 10mM BrdU into the intra-coelomic cavity. The injection site is along the midline approximately “1 pelvic fin-length” rostral to the base of the pelvic fins. Zebrafish were allowed to recover in a tank with 2.1mM lidocaine in system water for a minimum of 24 hours after injection. After injections, zebrafish were maintained off the system, and water was changed every 48-72 hours. Zebrafish were maintained at a density of 4 zebrafish/litre and supplied with at least 1 piece of bio-media for every 2 zebrafish. Environmental enrichment was provided by a picture of stones placed underneath the tank.

2.2.2 Mouse

Hes5:EGFP transgenic mouse lines have been described previously (Basak and Taylor, 2007), and were used in the heterozygote state. For embryonic studies, timed mating were set up between Hes5:EGFP males and C57Bl6/J females. Midday on the day of the vaginal plug was considered as embryonic day 0.5 (E0.5). Mice were raised in ambient temperature of 22-24°C on a 12hr:12hr light:dark cycle. Standard laboratory chow and drinking water were provided ad-libitum. Adult mice were culled by cervical dislocation, whereas embryonic mice were culled by decapitation.

2.2.2.1 Genotyping

Genotyping of Hes5:EGFP adult mice was carried out by PCR (Chapter 2.3.2) with DNA extracted from ear biopsies (Chapter 2.3.1). At embryonic stages, Hes5:EGFP transgenic mice were genotyped using a dissecting GFP scope to
identify transgene expression based on their visible fluorescent pattern, which includes strong EGFP expression in the forebrain. A piece of tail was also collected for PCR genotyping if required.

2.2.3 Tissue Manipulation

2.2.3.1 Dissection

2.2.3.1.1 Zebrafish

Adult zebrafish guts were dissected, in ice cold PBS, and included the intestinal bulb and the full length of the intestine, including the anal pore. Adult zebrafish brains were also dissected and collected as control samples. Individual guts and brains were fixed in 4% PFA in 2ml tubes (Chapter 2.2.3.2).

2.2.3.1.2 Mouse

Guts from adult mice were collected to include the full length from the stomach to the colon, with adipose tissue removed. The intestine was divided into its constituent parts, duodenum, jejunum, ileum and colon. Small segments, of approximately 2cm, were cut from their constituent subdivisions. The tissue segments were kept in PBS on ice while individual pieces were threaded over a 1ml pastette, which was held in a clamp stand. A small incision was made into the myenteric layer, along the mesenteric border, with a surgical scalpel blade. The serosa, longitudinal muscle and myenteric plexus were then gently rolled off from the underlying tissue using a PBS soaked cotton swab. To generate flat preparations, the longitudinal muscle-myenteric plexus (LMMP) preparations were stretched out and pinned on Sylgard plates (Down Corning, Sylgard 184), using insect pins, before fixation. The underlying submucosal and mucosa left on the pastette was removed and cut along its length to generate a flat preparation, before being stretched and pinned onto Sylgard plates, mucosal side facing up and then fixed in 4% PFA.

At the relevant time points, pregnant mice were culled by cervical dislocation and embryonic mice were dissected from the uterine horns in ice cold PBS. Embryos were removed from the uterus and culled by decapitation. Transgenic embryos were genotyped on the basis of their fluorescence patterns and guts were dissected from transgenic embryos. The intestine was collected to include the full length from the
stomach to the anus and all mesentery was removed. Intestines were pinned onto silgard plates to straighten the gut, and then fixed in 4% PFA.

### 2.2.3.2 Fixation

Adult tissue and larval zebrafish were fixed in 4% PFA overnight (O/N) at 4°C, shaking on a rocker. After fixation, samples were washed 3 times with 0.1% TritonX-100 in PBS (PBT) (Table 2-1) for 5 minutes. After washing, samples were used immediately or preserved at 4°C for future use in 0.1% PBT with Sodium Azide (0.01%) to avoid any bacterial or fungal contamination. For Sox10 antibody (GTX128374) staining, zebrafish tissue underwent a secondary fixation step in 100% methanol at -20°C O/N.

For correlated light and electron microscopy (CLEM), whole zebrafish guts were fixed in 4% PFA with 0.1% Gluteraldehyde O/N at 4°C. Tissue was washed with PB 3 times for 5 minutes and stored in 2% PFA in PB.

Murine embryos and adult LMMP preparations were fixed in 4% PFA for 4 hours at 4°C. After fixation, samples were washed for 5 minutes, three times in PBS. Tissue was preserved at 4°C, in PBS and 0.01% Sodium Azide.

### 2.2.3.3 Sectioning

Adult zebrafish guts and brains were sectioned for antibody staining (Chapter 2.5). Fixed gut tissue was cut into thirds at the points of curvature of the tissue. To generate blocks that contained three parallel gut pieces (corresponding to the intestinal bulb/fore-gut, mid-gut and hind-gut) for immunohistochemistry or in three separate blocks for CLEM analysis.

### 2.2.3.4 Vibratome

For immunohistochemistry, tissue was embedded in 3% agarose-PBS. For CLEM, tissue was embedded in 3% agarose-PB. Once set, the block was trimmed glued to the stage of the vibratome. The bath was filled with PBS, or PB, and tissue was cut to 60µm or 150 µm, for immunohistochemistry and CLEM, respectively. Samples were collected using a paintbrush into a 7ml bijou tube for immunohistochemistry, or in sequence, in 96well plates, for CLEM. Tissue sections were used immediately for immunohistochemistry or stored in 0.1% PBT, with 0.01%
sodium azide at 4°C for long term storage. For CLEM analysis, tissue was kept in 2% PFA in PB at 4°C for storage.

2.2.3.5 Cryostat

Fixed tissue was immersed in 30% sucrose in PBS at 4°C O/N for cryoprotection. Subsequently, the tissue was embedded in 7.5% sucrose, 30% gelatin. Once set, embedded samples were trimmed and snap frozen in isopentane at -60°C. At this point, embedded tissues could be stored at -80°C until sectioning. Using a Microm HM 560 CryoStar cryostat (Thermo Scientific), sections were cut to 14µm and collected serially onto Superfrost Plus™ slides (J1800AMNT, Thermo Scientific). Sections were air-dried for 1-2 hours before being processed for immunohistochemistry, or kept at -80°C in a dehydrated slide box.

2.3 Molecular Biology

2.3.1 Genomic DNA extraction

For adult mice and zebrafish, genomic DNA was extracted from ear or fin biopsies, respectively. Tissue was digested at 55°C O/N in tail lysis buffer with 0.1mg/ml Proteinase K (Roche, 03 115 879 001). The following day, isopropanol was added (1:1) to the lysate. To precipitate, the DNA samples were centrifuged at 13000rpm for 10 minutes, at room temperature (RT). The supernatant was discarded and the DNA pellet air dried, after which 50µl of MilliQ water was added. The DNA solution was then used immediately for PCR or kept at -20°C for long term storage.

For larval zebrafish tissue, 50µl of TE was added to the tail biopsy and heated at 95°C for 10 minutes. The sample was allowed to cool to RT before 5µl of proteinase K was added, and then tissue was digested at 55°C O/N. Afterwards, PK was deactivated by heating the sample to 95°C for 10 minutes. Larva DNA preparations need no further processing and were used immediately for subsequent PCRs.

2.3.2 Polymerase Chain Reaction (PCR)

PCR was used to genotype transgenic mice and zebrafish, by amplifying a region of genomic DNA. PCR reagents were supplied by Invitrogen (Taq polymerase,
10x Buffer, MgCl₂, Promega (dNTPs), and Sigma (primers). Each reaction contained a mixture of Taq polymerase, 10x reaction buffer, MgCl₂, dNTPs, primers and genomic DNA. When genotyping Hes5:EGFP animals premixed REDTaq® ReadyMix™ was used (Sigma, R2523). The appropriate volumes for each reaction, and cycling parameters, are summarised in Table 2-6 and Table 2-7, respectively. Primers were supplied as lyophilised powder and were suspended at 100µM in MilliQ water, and stored at -20°C.

Table 2-6. PCR Master mixes

<table>
<thead>
<tr>
<th>Reagent (Stock concentration)</th>
<th>Hes5:GFP</th>
<th>ERT2creERT2</th>
<th>NLSCherry</th>
<th>ref hu2846</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction Buffer (10x)</td>
<td>n/a</td>
<td>2.5</td>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td>MgCl₂ (50mM)</td>
<td>n/a</td>
<td>0.75</td>
<td>1</td>
<td>2.5</td>
</tr>
<tr>
<td>dNTP (10mM)</td>
<td>n/a</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>Primer1</td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>Primer2</td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>Taq DNA Pol (5u/µL)</td>
<td>12.5*</td>
<td>0.3</td>
<td>0.3</td>
<td>0.6</td>
</tr>
<tr>
<td>MilliQ water</td>
<td>9.25</td>
<td>17.95</td>
<td>17.45</td>
<td>8.6</td>
</tr>
<tr>
<td>belaine</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>10</td>
</tr>
<tr>
<td>DNA</td>
<td>2.5</td>
<td>1</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Reaction Volume</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>50</td>
</tr>
</tbody>
</table>

* RedTaq used
2.3.3 Restriction-Enzyme Digestion

To genotype *Tg(ret<sup>hu2846</sup>)*, mutants, heterozygotes and wild types, further digestion of the PCR product is needed. Restriction digests were carried out using 15µl of PCR product, 2.5µl of 10x NE Buffer 4, 6.5µl of MilliQ water, and 1 µl of Hpy1881 (NEB) enzyme. The digest was incubated O/N at 37°C before being visualised using gel electrophoresis, as described in Heanue et al. (2016a).

2.3.4 Gel electrophoresis

To measure the size of the DNA fragment and determine the genotype of the animal, the PCR/restriction-enzyme digestion products were visualised using Horizontal MultiSub gel electrophoresis apparatus. Agarose gels were cast at 1.5%
for single-band PCR products and 3% for PCR restriction-enzyme digestions in 1X TAE buffer, with the addition of 1:10000 GelRed™ (41003, Biotium) to enable visualisation of the DNA products. DNA products were mixed with loading buffer (Table 2-1) in a ratio of 9:1 prior to loading. Samples underwent electrophoresis in 1x TAE buffer at 70-100V. Visualisation was carried out using ultraviolet trans-illuminator at 302 (UVP) in a BioDoc-IT lightproof cabinet, and captured using a CCD camera. The size of the PCR/enzyme digestion products were determined by comparison to a 1 Kb Plus DNA ladder (10787018, Thermo Scientific).

2.4 Immunohistochemistry

Immunohistochemistry on zebrafish adults (whole-mount tissue and sections) and larvae was performed after fixation, essentially as previously described (Uyttebroek et al., 2010). Specifically, larvae or adult guts were washed three times with 0.1% PBT for 5 minutes, before a quick wash (1 minute) with deionised water. Tissue was then permeabilized in acetone at -20°C for 10 minutes, and washed again for 1 minute with deionised water. This was followed by three washes in 0.1% PBT for 5 minutes. Tissue was incubated in blocking solution (5% sheep serum, 1% DMSO, 1% Bovine Serum Albumin and 0.2% Sodium Azide in 0.5% PBT) for at least one hour. Primary antibodies (Table 2-8) were diluted in blocking solution and incubated O/N at RT, on a rocker. The following day, the tissue received three 30 minute washes in 0.1% PBT before transferring to secondary antibodies (Table 2-8) diluted in blocking solution and left O/N at RT. Tissue was washed again, at least three times in 0.1% PBT for 30 minutes before mounting.
### Table 2.8. Antibodies and relevant dilutions

<table>
<thead>
<tr>
<th>Target</th>
<th>Host</th>
<th>Dilution</th>
<th>Catalog no</th>
<th>Target</th>
<th>Host</th>
<th>Fluorophore</th>
<th>Dilution</th>
<th>Catalog no</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFABP</td>
<td>Rabbit</td>
<td>1:500</td>
<td>sc-546</td>
<td>Chicken</td>
<td>Donkey</td>
<td>488</td>
<td>1:500</td>
<td>703-545-155</td>
</tr>
<tr>
<td>BrdU</td>
<td>Rat</td>
<td>1:200</td>
<td>ab6326</td>
<td>Goat</td>
<td>Donkey</td>
<td>568</td>
<td>1:500</td>
<td>ab175474</td>
</tr>
<tr>
<td>Cherry</td>
<td>Goat</td>
<td>1:500</td>
<td>ABIN1440057</td>
<td>Goat</td>
<td>Donkey</td>
<td>488</td>
<td>1:500</td>
<td>ab150129</td>
</tr>
<tr>
<td>GFP</td>
<td>Chicken</td>
<td>1:500</td>
<td>ab13970</td>
<td>Goat</td>
<td>Donkey</td>
<td>647</td>
<td>1:500</td>
<td>ab150131</td>
</tr>
<tr>
<td>HuC/D</td>
<td>Mouse IgG2b</td>
<td>1:200</td>
<td>A-21271</td>
<td>Mouse</td>
<td>Donkey</td>
<td>488</td>
<td>1:500</td>
<td>ab150105</td>
</tr>
<tr>
<td>MCM5</td>
<td>Rabbit</td>
<td>1:200</td>
<td>Provided by Soojin Ryu</td>
<td>Mouse</td>
<td>Donkey</td>
<td>568</td>
<td>1:500</td>
<td>A10037</td>
</tr>
<tr>
<td>mGFAP</td>
<td>Rabbit</td>
<td>1:500</td>
<td>G-9269</td>
<td>Mouse</td>
<td>Donkey</td>
<td>647</td>
<td>1:500</td>
<td>A-31571</td>
</tr>
<tr>
<td>PCNA</td>
<td>Rabbit</td>
<td>1:200</td>
<td>P8825</td>
<td>Mouse</td>
<td>Goat</td>
<td>405</td>
<td>1:500</td>
<td>ab175660</td>
</tr>
<tr>
<td>S100b</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>z-0311</td>
<td>Rabbit</td>
<td>Donkey</td>
<td>488</td>
<td>1:500</td>
<td>ab150073</td>
</tr>
<tr>
<td>Sox2</td>
<td>Goat</td>
<td>1:200</td>
<td>GT15098</td>
<td>Rabbit</td>
<td>Donkey</td>
<td>568</td>
<td>1:500</td>
<td>ab175470</td>
</tr>
<tr>
<td>Sox10</td>
<td>Goat</td>
<td>1:200</td>
<td>sc 17342</td>
<td>Rabbit</td>
<td>Donkey</td>
<td>647</td>
<td>1:500</td>
<td>ab150075</td>
</tr>
<tr>
<td>Sox10</td>
<td>Rabbit</td>
<td>1:200</td>
<td>GTX128374</td>
<td>Rat</td>
<td>Donkey</td>
<td>594</td>
<td>1:500</td>
<td>ab150156</td>
</tr>
<tr>
<td>zrf-1 (gfa)</td>
<td>Mouse IgG1</td>
<td>1:500</td>
<td>ab154474</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Whole-mount embryonic mouse guts, LMMP and mucosal preparations were prepared as previously described (Chapter 2.2.3.1.2). Tissue was permeabilized by three 15 minute washes in 0.3% PBT, before blocking solution (10% Donkey serum in 1% PBT) was applied for at least 2 hours. Primary antibodies (Table 2-8) were diluted in an incubation solution (10% Donkey Serum and 0.3% PBT) and incubated O/N at 4°C. After three 15 minutes washes, secondary antibodies (Table 2-8) diluted in incubation solution were applied for 3-4 hours at RT. Finally, At least three 15 minute washes with 0.3% PBT were carried out before mounting.

Both zebrafish and mouse tissue was mounted with Vectasheild with, or without, DAPI (H1200 and H1000, Vector Laboratories) depending on the combinations of antibodies used.

### 2.4.1 Modifications to Immunohistochemistry Protocol

#### 2.4.1.1 Antigen retrieval

Sections were used for immunohistochemical markers that required antigen retrieval (PCNA, MCM5). Immunohistochemistry for the other antibodies used was carried out, as described above, before antigen retrieval. Floating vibratome sections were incubated in 10mM sodium citrate buffer (Table 2-1), for 30 minutes at 50°C. Alternatively, slides containing cryosections were microwaved in coplin jars containing sodium citrate buffer for 2 minutes at 100% power, followed by 7 minutes at 30% power. In both cases, after antigen retrieval, the tissue was allowed to cool for 20mins, before half the sodium citrate buffer was removed and replaced with MilliQ water. After a further 10 minutes, the solution was removed and immunohistochemistry was performed as described above.

#### 2.4.1.2 DNA hydrolysis

For visualisation of BrdU, floating tissue sections were processed for the other antibodies required before DNA was denatured, to allow for BrdU detection. Sections were added to pre-warmed 1M HCl for 30 minutes at 37°C. HCl was removed and the tissue sections were neutralised with 0.1M sodium tetraborate (PH=8.5) for 10 minutes at RT. The sample was then washed (3x 15 minutes) in 0.1%PBT before proceeding with immunohistochemistry against BrdU as described above.
2.4.2 Developing EdU

After immunohistochemistry was performed, EdU was developed following the manufactures protocol. EdU was detected using the Click-It Alexa Fluor 594 or 647 kits (Invitrogen, C10339, or C10419), which is based on the “click chemistry” reaction between EdU and azide-fluorophore. Whole-mount adult zebrafish guts were incubated in EdU developing solution (75µl of 1x solution F, 645µl of reaction buffer, 30µl of 100mM copper sulphate (CuSO4) and 1.8µl of the appropriate Alexa Fluor dye) for 45 minutes at RT. Samples were then washed at least three times for 30mins with 0.1%PBT and mounted as previously described.

2.5 Imaging

2.5.1 Live Imaging

Moulds were made using 1% agarose in 0.5% Danios solution (Table 2-1) in the lid of a 60mm dish as previously described (Heanue et al., 2016a, Megason, 2010). Zebrafish larvae were anesthetised with 0.15mg/ml Tricane and placed into warmed 0.6% low-melt agarose (LMA) in 0.5% Danios solution with 0.15M PTU and transferred into the mould. Once the zebrafish embryos were positioned, the LMA was allowed to set, before being overlaid with zebrafish water containing 0.15mg/ml Tricane and 0.15M PTU, which was replaced at least every 24 hours. Larvae were imaged using a Leica CM6000 confocal microscope with a Mai Tai Ti:Sapphire Deep Sea Laser System run by the Leica Application Suite Advanced Fluorescence (LAS AF) software. Images were acquired with a 20X dipping objective, and standard excitation and emission filters were used to visualise EGFP and mCherry expression. For each individual larva, 33 z-stacks (z-thickness= 2.014µm) were collected at a frame rate of 602ms. Throughout the imaging process, the temperature and humidity were maintained at 29°C. For figures and movies, z-ranges were selected to encompass both eNCC migratory streams.

2.5.2 Fixed imaging

Imaging of fixed whole-mount samples and sections were captured using a Leica CM6000 confocal microscope run by the Leica Application Suite Advanced Fluorescence (LAS AF) software with a standard 405, Argon, 561 and 633nm laser
set. Standard excitation and emission filters were used to visualise DAPI, Alexa Fluor 488, Alexa Fluor 568, Alexa Flour 594 and Alexa Fluor 647. Each fluorescent image is a projection of z-stacks (1.5µm thick), using “Fiji” software stacks were selected to show the muscle layer or mucosa (adult zebrafish guts), or to encompass the entire sample (tissue sections, larval zebrafish, adult and embryonic mouse tissues). Images were taken with 20x dry or 40x oil objectives, using a zoom of 1.7.

2.5.3 Super-Resolution Imaging

Super-resolution imaging was used to acquire images for subsequent correlated light and electron microscopy (CLEM) analysis. Sections were mounted in PB and imaged using an inverted Zeiss 880 confocal microscope with Airyscan and with 405, Argon, 561 and 633nm laser set, run using Zeiss Zen software. An overview was generated using standard emission and excitation filters for EGFP and mCherry. The z-stack acquired covered the entire section and images were acquired every 3µm. Afterwards, 2-3 regions of interest (ROIs) were identified per section that contained at least one EGFP cell of interest. The Airyscan was aligned for EGFP and mCherry using an area outside our ROIs where both fluorophores were visible, since photo-bleaching occurs during alignment. After Airyscan alignment, the ROI was captured with pixel size, z-depth and zoom (>1.8) defined by Nyquist’s theorem. Overview images were taken using a 20x dry lens, whereas Airyscan images were taken with a 63x glycerol immersion lens.

2.5.4 Combined Light and Electron Microscopy (CLEM)

The electron microscopy (EM) and subsequent data processing was performed by Christopher Peddie (the Francis Crick institute) with a protocol similar to previously published work (Russell et al., 2016). Briefly, after super-resolution imaging, sections were stained using high contrast inducing agents and embedded in resin. The sample was then cut using serial blockface scanning electron microscopy. Using features such as the vasculature, epithelial layer and cell morphology, cells of interest that had been identified in the light microscopy ROIs were located at the EM level.
2.6 Mathematical Modelling

Modelling analysis was carried out by Carmen Pinn (AztraZenica, Cambridge) and was based on cell-counting data acquired using the CellCounter plugin on Fiji. Since the zebrafish ENS is confined to the myenteric plexus, and hence the zebrafish ENS resides within a two dimensional plane, only X and Y coordinates were used for subsequent analysis. Each image covered a 450µm × 450µm area.

2.6.1 Mote-Carlo Modelling

Using XY coordinates from our experimental data, the density of various cell types around a cell of interest can be calculated within circumferential areas of increasing radius (20-500µm). The experimental data is compared to a simulated model that represents a null-hypothesis, where cells are distributed homogeneously. Densities for the simulated null-hypothesis were obtained by Mote-Carlo simulations and were based experimental data. Briefly, for each image, the experimentally recorded cells were rearranged randomly, to assume uniform distribution, and densities were calculated. This process was repeated 50 times for each experimental image, to obtain 90% confidence intervals. The simulated and experimental densities could then be compared. Significance was reached when the experimental values were outside the 90% confidence interval of the simulated data.

2.6.2 Differentiation vs Proliferation Model

Carmen Pinn developed a model based the birth-death stochastic process model (Kendall, 1948). This model was adapted to describe proliferation and differentiation and assumed that GFP+ EdU+ cell are the progenitors for HuC/D+ EdU+ cells. Furthermore, the model assumes that only one proliferation or differentiation event can occur at a time and that differentiation into HuC/D+ EdU+ cells is an irreversible process. Based on our Monte-Carlo analysis, clusters of GFP+ EdU+ and/or HuC/D+ EdU+ were considered clonally related if cells were located within 60µm of any cell within that clone. Clones were converted into matrices, and the model predicted the probability of these clusters changing over time. Finally, proliferation (λ) and differentiation rates (δ) could be calculated from the probabilities. Proliferation was considered to be significantly lower than differentiation when λ-δ<0.
2.7 Image Processing and Analysis

2.7.1 Counting

Cells were manually counted using the CellCounter plugin in Fiji. Cells were counted if a DAPI+ nuclei were readily visualised within a cell expressing the marker examined. For larval zebrafish, cells along the entire intestine were counted. For 7dpf larval zebrafish, approximately 150 HuC/D+ neurons were counted per individual. In adult zebrafish, cells were counted from three images per gut segment being analysed (intestinal bulb/fore-gut, mid-gut and hind-gut). In adult zebrafish samples, between 1500-2000 HuC/D+ neurons were counted for each animal. In mammalian LMMP samples, two 2x2 tiles were counted for each subdivision of the gut. Approximately 1800 glial cells (Sox10+ or S100β+) were counted per adult mouse.

2.7.2 Live Imaging

Cells were manually tracked using MtrackJ (Fiji), using original Liff files. Resulting MtrackJ data was used to calculate cell migration speed, distance and proliferation. A reference point for each embryo was taken as the point the most anterior spinal nerve, that was visible in the FOV, touched the gut. The reference point was tracked throughout the movies to correct for any growth, or movement, of the animal during the imaging process.

2.7.3 Statistics

In all cases, at least three replicates were performed in an independent manner. In animals in which Notch was inhibited, experimental and control zebrafish were from the same clutch and tank. Data are expressed as mean ± SEM unless otherwise specified. Statistical analysis was carried out using Graphpad Prism 7 (GraphPad Software Inc.) software, using the appropriate statistical test mentioned in the text. For comparisons of two data sets, paired and unpaired t-tests were used. One-way and two-way ANOVAs were used when comparison between multiple data-sets were required, followed by a multiple comparison test. Data was considered significant when P values were less than 0.05 (*), 0.01(**), and 0.001 (***) Graphs were generated in Graphpad Prism 7 (GraphPad Software Inc.).
2.7.4 Figures

Adobe Photoshop CS5 was used to compose final figures and adjust image brightness and contrast. For images requiring direct comparison the same adjustments were applied to all images. All larval images are shown with rostral gut to the left, and adult tissues are from the mid-gut regions with rostral to the top, unless otherwise stated. Copies of the results figures can be found on the DVD, in the pocket attached to the back of this thesis.

2.8 Nomenclature

Gene and protein naming for zebrafish and mouse have been used in accordance with ZFIN (ZFIN and Dunn, 2018) and MGI (MGI, 2018) guidelines, respectively. When referring to both zebrafish and mouse genes or proteins, the mouse conventions have been used.
Chapter 3. Characterisation of Zebrafish Enteric Glial Cells

3.1 Introduction

Mammalian EGCs are thought to outnumber enteric neurons by a factor of 2-4 (Furness, 2006, Ruhl, 2005). Work in the mouse has identified four types of enteric glia based on morphological criteria and anatomical location within the gut wall (Boesmans et al., 2014). However, despite the morphological and positional variation of subtypes, the large majority of EGCs express the canonical peripheral glia markers (GFAP, S100β, SOX10, BFABP, PLP1) (Boesmans et al., 2014, Rao et al., 2015). No histochemical markers that mark a specific EGC subtype have been identified. Another current feature of EGC marker expression is that we are unable to distinguish terminally differentiated EGCs from their precursors because of the expression of genes such as Sox10 and FoxD3, are expressed in early NC cells and are maintained in the mature EGCs (Boesmans et al., 2014, Mundell and Labosky, 2011, Mundell et al., 2012). The maintenance of expression may suggest that EGCs in adult animals can function as progenitors, or that progenitor genes, such as Sox10 and FoxD3, are necessary to prevent neuronal differentiation. We argue that the relatively simple ENS of the zebrafish may provide insight into the functional and neurochemical characterisation of EGCs.

Many studies of the zebrafish CNS have generated a variety of tools suitable for the zebrafish glial cell biology field. Despite this, there is very little work on the glial cells of the zebrafish ENS. Some reports have suggested that GFAP is expressed in the zebrafish gut (Bernardos and Raymond, 2006, Doodnath et al., 2010, Hagstrom and Olsson, 2010, Kelsh and Eisen, 2000), however these studies analysed limited developmental stages and used a small number of markers. In contrast, it has been reported that there is no expression of S100 in the zebrafish ENS, despite a broad distribution of this marker throughout the zebrafish CNS (Germana et al., 2004, Germana et al., 2008). These latter studies suggest potential differences between mammalian and zebrafish EGCs and raises questions about the onset and dynamics of glial marker expression in the zebrafish ENS. Moreover, it is unknown whether the relatively simple zebrafish ENS exhibits the same glial subtype
Chapter 3 Results

diversity. Our aim was to characterise the non-neuronal component of the ENS in zebrafish and to provide a clear description of zebrafish EGCs.

3.2 Results

3.2.1 Examination of Enteric Glial Cell Markers within the Zebrafish ENS

To begin an extensive characterisation of zebrafish EGC marker expression, we performed immunohistochemistry and exploited the available glial specific transgenic lines against markers known to be expressed in mammalian EGCs. For this analysis, we focused on two developmental stages of zebrafish: a 7dpf larval stage and adults, older than 3 months old (>3mo). At 7dpf, zebrafish larvae are of feeding age, and have a functional ENS with organised peristalsis (Holmberg et al., 2003, Heanue et al., 2016a), while at >3mo zebrafish are considered adult animals capable of breeding (Parichy et al., 2009, Westerfield, 1993). We focused on these two later stages, rather than earlier embryonic stages, since neurons are functional from 7dpf to adulthood (>3 mo) they presumably require the support of EGCs at these stages, therefore, EGCs should also be present (Ruhl, 2005, Gulbransen and Sharkey, 2012). In addition, characterisation of EGCs at adult stages allows us to exclude the possibility that our analysis of 7dpf animals misses the time window of EGC differentiation. Moreover, using available transgenic lines, in which fluorescent reporters, such as GFP, are under the control of a glial specific promoter should allow us to detect the glial marker of interest. Since GFP is a stable protein capable of accumulating at high levels in the cytoplasm, this should allow us to detect markers of interest even if they are expressed at low levels. In addition, use of GFP reporters also avoids detection issues cause by the marker of interest having specific subcellular localisation. Below, we describe the expression in the zebrafish ENS of a battery of glial markers known to label mammalian glia, in combination with immunostaining for the neuronal marker HuC/D, which acts as a positive control for antibody staining and labels the neuronal elements of the ENS.

3.2.1.1 Brain fatty acid binding protein (Bfabi)

BFABP is one of the first differentiated glial markers observed during development within mammalian EGCs (Young et al., 2003) and is widely expressed by the glial cells of the CNS (Than-Trong and Bally-Cuif, 2015, Giachino et al., 2014).
Therefore, we proposed that Bfabp is likely to be expressed by zebrafish EGCs. We performed immunohistochemistry against Bfabp and HuC/D on whole-mount 7dpf larvae (N=20), enabling the CNS to serve as an internal positive control. Surprisingly, we were unable to detect any Bfabp+ cells within the ENS of 7dpf larvae (Figure 3.1A), despite the fact that enteric neurons were readily detected by HuC/D and that we observed the expected glial Bfabp staining within the CNS (Figure 3.1B). To determine whether Bfabp expression might arise later, we examined its expression in the adult zebrafish ENS (N=9), by performing immunohistochemistry with Bfabp and HuC/D on whole-mount adult gut preparations. While we detected HuC/D+ neurons throughout the adult gut, we failed to identify Bfabp staining within the ENS (Figure 3.1E), despite observing the expected Bfabp staining pattern within the brain (Figure 3.1F). Therefore, we conclude that Bfabp is not expressed within the zebrafish ENS.

### 3.2.1.2 S100β

Within the CNS, the majority of glial cells express the Ca$^{2+}$ binding protein S100β (Than-Trong and Bally-Cuif, 2015, Germana et al., 2008). Likewise, within the mammalian ENS, the majority of EGCs are labelled with S100β from late embryogenesis until adulthood (Young et al., 2003, Boesmans et al., 2014). In contrast, it has been reported that S100 is not expressed within the zebrafish ENS (Germana et al., 2008), an apparent inconsistency between mouse and zebrafish. In an attempt to resolve this discrepancy, we analysed the expression of S100β in the zebrafish ENS at both 7dpf larval (N=30) and adult stages (N=5), by performing immunohistochemistry against S100β and HuC/D. While we clearly observed HuC/D+ neurons throughout the gut, we were unable to visualise any S100β marker expression within the ENS, in both stages examined (Figure 3.1C and G). As expected, S100β+ cells were readily detected in radial glial cell in control brain areas (Figure 3.1D and H). Therefore, in agreement with previous work (Germana et al., 2008), our data suggests that S100β is not expressed within the zebrafish ENS.
### Chapter 3 Results

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<th>Spinal Cord</th>
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### Adult

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Figure 3.1. Absence of Blabp, S100β, and Nestin expression within the zebrafish gut at larval (7dpf) and adult (>3mo) stages.

(A) Blabp (green) does not label EGCs in the 7dpf intestine, a time when HuC/D+ neurons (red) are readily detected. (B) Blabp is observed in the expected pattern in the CNS, spinal cord shown (arrows). (C) At 7dpf, S100β (green) is not expressed in the intestine, although HuC/D+ neurons are detected (red). (D) Within the CNS of 7dpf larvae, S100β expression is observed as expected (spinal cord shown, arrows) (E) In adulthood, Blabp is not detected in the adult gut (green), in contrast to readily identifiable HuC/D+ identifying enteric neurons (red). (F) Blabp is expressed in expected patterns throughout the adult brain, pallium region shown (arrows). (G) S100β was not expressed in the adult intestine (green), in contrast to clearly detected HuC/D+ (red) neurons. (H) In the adult CNS, pallium region shown, S100β shows the expected radial glial staining (arrows). (I) In adulthood, no GFP+ cells (green) are observed within the ENS of the transgenic line Tg(-3.6nestin:GFP), while HuC/D+ neurons are readily detected (red). (J) GFP+ cells (green) are readily detected within the radial glial cells of the adult brain of Tg(-3.6nestin:GFP) zebrafish, pallium region shown. All larval images are shown with rostral to the left, whereas all adult gut images are from the mid-gut region with rostral to the top. The gut in larval animals is highlighted with dotted outlines. Within the CNS, representative images from the spinal cord are shown for larval stages and from the pallium for adult stages.

3.2.1.3 Nestin

Next, we characterised the expression of the Tg(-3.9nestin:GFP) transgenic line in the zebrafish ENS. In this transgenic line, GFP is under the control of a -3.9kb domain of the nestin promoter (Lam et al., 2009). NESTIN has been shown to be expressed within the RGC population of the CNS in both zebrafish (Johnson et al., 2016, Lam et al., 2009) and mouse (Belkind-Gerson et al., 2013, Bonaguidi et al., 2011). Moreover, it has been reported recently that NESTIN is expressed in a subpopulation of the non-neuronal cells of the murine ENS (Kulkarni et al., 2017, Belkind-Gerson et al., 2013). Therefore, it is conceivable that Nestin is also expressed by zebrafish EGCs. However, there was no detectable GFP signal in the adult zebrafish ENS (Figure 3.1I), although we were able to detect GFP+ radial glial cells in the CNS (N=4, Figure 3.1J). Our data indicates that unlike the mouse (Belkind-Gerson 2013, Kulkarni 2017), Nestin is not expressed in the zebrafish ENS.
3.2.1.4 Glial fibrillary acidic protein (GFAP)

Within the CNS, GFAP is widely expressed by many glial subtypes, including astrocytes and radial glia (Molofsky et al., 2012, Than-Trong and Bally-Cuif, 2015, Bernardos and Raymond, 2006). In addition, GFAP is expressed in EGCs of various model organisms, including mouse (Boesmans et al., 2014, Jessen and Mirsky, 1980) and chick (Balaskas and Gabella, 1998). GFAP expression has also been described in the zebrafish ENS (Doodnath et al., 2010, Hagstrom and Olsson, 2010, Bernardos and Raymond, 2006, Kelsh and Eisen, 2000). To independently examine the expression of GFAP in the zebrafish ENS, we used two commercially available antibodies raised against zebrafish Gfap (zrf-1) (Kroehne et al., 2011, Briona and Dorsky, 2014) and mammalian GFAP (mGFAP) (Boesmans et al., 2014), and we employed a widely used Tg(gfap:GFP) transgenic line (Alunni et al., 2005, Lam et al., 2009, Grupp et al., 2010). EGFP expression, in the Tg(gfap:GFP) transgenic line, is driven by a 7.4kb fragment of the zebrafish gfap regulatory element that includes its promoter and part of the first intron and exon (Bernardos and Raymond, 2006). Previous studies using this transgenic line have described GFP expression in the zebrafish ENS (Bernardos and Raymond, 2006, Doodnath et al., 2010). We have employed each of these tools to examine Gfap expression in the zebrafish ENS.

Our initial analysis was performed at 7dpf, in which the antibody against zebrafish Gfap (zrf-1) labelled cells in the expected pattern with the CNS (Figure 3.2B). Within the gut, zrf-1 labelled cellular processes along the circumferential axis within the zebrafish intestine (Figure 3.2A). However, this staining was not associated with HuC/D+ enteric neurons, as would be expected of EGCs. Furthermore, in ret mutant zebrafish (ret^{hu2846/nu2846}), in which the gut fails to be colonised by eNCCs and enteric neurons are absent (Heanue et al., 2016a), the zrf-1 staining within the gut remained similar to that of wild-type animals (Figure 3.2C-D). Since studies in mouse show that all ENS lineages are derived from the same Ret-dependant progenitors (Durbec et al., 1996, Taraviras et al., 1999), and NC cells in zebrafish ret mutants fail to colonise the ENS (Heanue and Pachnis, 2008, Heanue et al., 2016a), the continued expression of zrf-1 in ret mutants suggests that the zrf-1 staining does not identify EGCs and is independent of the ENS lineage. Consistent with this idea, the zrf-1 signal is reminiscent of muscle specific staining patterns (Wallace et al., 2005). Thus, we concluded that although the zrf-1 antibody
staining appeared specific to glial cells of the CNS, within the gut this antibody is likely to label smooth muscle cells only. For this reason, the zrf-1 antibody was not investigated further.

Figure 3.2. Zebrafish Gfap (zrf-1) staining only labels cells outside the ENS. (A) zrf-1 (green, single colour in A) labels circumferential fibres (open arrowheads) within the 7dpf ENS and is not associated with HuC/D+ enteric neurons (red, merge A'). Outside of the gut, zrf-1+ glial fibers are visualised (arrowheads), however these fibres do not enter the intestine. (B) zrf-1 (green) labels the radial glial cells within the spinal cord, as previously described (arrowhead). (C-D) Analysis of zrf-1 expression in wild type and ret+hu2846/hu2846 mutants. (C) At 7dpf, the wild-type (ret+/+), gut is fully colonised by eNCCs labelled using the transgenic line Tg(SAGFF234A;UAS:GFP) (green), in which the zrf-1 staining (red, single colour C’) is visualised in a circumferential pattern in the gut (open arrowhead), and zrf-1+ fibres are visualised descending from the spinal cord (arrowhead). (D) In 7dpf ret+hu2846/hu2846 mutants, there is no colonisation of the gut by eNCCs, labelled by Tg(SAGFF234A;UAS:GFP) (green), however, the zrf-1 staining (red, single colour D’) is still observed in a circumferential pattern, and the zrf-1+ fibres descending from the spinal cord (arrowheads) remain intact. The few GFP+ cells observed in the intestine are smooth muscle cells that are also labelled with the transgenic at late larval stages (arrows), which are also detected in the ret+ ret+ gut (C, arrows). All larval images are from the mid-gut and are shown with rostral to the left. The gut in larval animals is highlighted with dotted outlines.
Next, we examined the expression Gfap, using the mGFAP antibody. At 7dpf, we observed that mGFAP+ fibres emerge from the spinal cord and extend ventrally, but stop before entering the gut, the gut itself lacks mGFAP+ signal (Figure 3.3A-B). These findings were confirmed by using the Tg(gfap:GFP) transgenic line. In 7dpf Tg(gfap:GFP) larvae, we observed GFP+ fibres, which, similar to those stained with the mGFAP antibody, emerged from the spinal cord towards the gut, but never entered the wall of the intestine (Figure 3.3C-D, N=53). As expected HuC/D+ enteric neurons and GFP+ RGCs were readily detected in the gut and CNS, respectively. This result suggests that at 7dpf there is no gut-intrinsic Gfap+ population of EGCs, and also that there are no external Gfap+ glial fibres entering the gut to support enteric neurons.

We next analysed Gfap expression at adult stages. Using the mGFAP antibody, we observed the expected RGCs staining throughout the brain (Figure 3.3F). In contrast to our results using the mGFAP antibody in 7dpf larvae, (Figure 3.3A-D), we detected fibrous mGFAP staining within the intestine of adult zebrafish (n=26, Figure 3.3E). Interestingly, the mGFAP staining in the adult gut is closely associated with HuC/D+ neurons, suggesting that it corresponds to cellular elements of the ENS. Nevertheless, we were unable to detect any clear mGFAP+ cell bodies within the gut (Figure 3.3E). This could suggest that, due to the subcellular localisation of Gfap we are unable to resolve the EGC cell bodies, or that the mGFAP+ fibres originate from non-ENS cells that are external to the intestine. To explore these possibilities further, we took advantage of the Tg(gfap:GFP) transgenic line, in which stable GFP expression should allow us to visualise the EGC cell bodies of Gfap expressing cells. Although GFP expression was readily detected in the CNS of adult zebrafish (>3mo, N=13, Figure 3.3H), we were unable to detect GFP+ signal in the intestine of these animals (Figure 3.3G, N=12/13), despite abundance an of HuC/D+ neurons. In one intestinal preparation, we detected GFP+ fibres within the proximal intestinal bulb, but we were unable to visualise any cell bodies, suggesting that these fibres originated from cells outside the gut and are not gut-intrinsic EGCs (N=1/13). These observations contrast our immunohistochemical analysis, with the mGFAP antibody (Figure 3.3E) and could be accounted for by a lack of necessary regulatory elements in the Tg(gfap:GFP) line. Alternatively, these inconsistencies could suggest Gfap is indeed not expressed within the zebrafish ENS from internal or external glial cells, and the apparent mGFAP antibody staining we
observe in the gut results from detection of other intermediate filaments within the ENS cells. This would be consistent with the failure to detect Gfap+ cell bodies in the zebrafish gut. Taken together, we concluded that Gfap is not a useful marker for studying the intrinsic EGC population of the ENS.

Figure 3.3. Gfap expression is not detected in the 7dpf zebrafish gut, however Gfap immunoreactivity is observed within the adult intestine using the mGFAP antibody. (A) The mammalian GFAP antibody (mGFAP, green) did not label any cells in the 7dpf gut, although
mGFAP fibres were detected descending toward, but not entering, the gut (arrowheads) and HuC/D+ neurons (red) were detected within the gut. (B) We readily detect the expected mGFAP pattern within the spinal cord of 7dpf larvae. (C) Using 7dpf Tg(gfap:GFP) larvae, we do not detect GFP+ cells within the ENS (green), although GFP+ fibres are seen descending from the spinal cord, but do not enter the gut (arrowheads). At this time, HuC/D+ neurons (red) are visualised throughout the gut. (D) We detect the expected GFP+ staining (green) within the spinal cord using Tg(gfap:GFP). (E) In the adult gut, mGFAP labels fibres (green, open arrow) closely associated with HuC/D+ neurons (red). However, no cell bodies are labelled with mGFAP. (F) Throughout the adult brain, mGFAP+ RGCs are visualised, pallium region shown. (G) Within the adult gut of Tg(gfap:GFP) transgenics, no GFP+ cell bodies (green) were observed, although HuC/D neurons (red) were readily detected. (H) As previously reported, RGCs throughout the brain are labelled with GFP (green). All larval images are shown with rostral to the left, whereas all adult gut images are from the mid-gut region with rostral to the top. The gut in larval animals is highlighted with dotted outlines. Within the CNS, representative images from the spinal cord are shown for larval stages and from the pallium for adult stages.

3.2.1.5 Foxd3

In the absence of obvious expression of “mature” glial markers in the zebrafish ENS, using Bfapb, S100β, Nestin or Gfap, we looked for alternative markers. Therefore we examined the Tg(zFoxD3:GFP) line, in which GFP is under the control of a 14kb segment of the zebrafish foxD3 promoter (Gilmour et al., 2002). FoxD3 expression is of particular interest, as it has been shown to be expressed in the early NC cells which colonise the gut (Dutton et al., 2008, Mundell et al., 2012, Mundell and Labosky, 2011) and FoxD3 expression is maintained in mammalian EGCs (Mundell et al., 2012, Coelho-Aguiar Jde et al., 2015). Therefore, we speculated that FoxD3 is likely to be expressed in the zebrafish ENS. At early embryonic stages, during gut colonisation by NC cells, we were able to see GFP+ cells in the NC cells as previously described (Gilmour et al., 2002). By 7dpf, we observed a small number of cells with very weak GFP+ signal in the gut, which were distinct from HuC/D+ neurons (Figure 3.4A). This weak expression contrasts with strong EGFP expression within the CNS (in areas such as the pineal gland, Figure 3.4B) and in other neural crest lineages, such as the melanocytes surrounding the gut (N=20, Figure 3.4A) Since GFP levels within the gut were so low as to be almost
undetectable, we initially discounted this line as a useful tool to study zebrafish EGCs.
Chapter 3 Results

7dpf

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<th>Pineal Gland</th>
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<td><strong>A’</strong> GFP</td>
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<td>GFP HuC/D</td>
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<td>Jaw</td>
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<td><strong>C’</strong> GFP</td>
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<td>GFP HuC/D</td>
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<td>GFP HuC/D</td>
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Adult (>3mo)

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Figure 3.4. Characterisation of neural crest cell markers within the ENS at 7dpf and adulthood. (A) Within the gut of 7dpf Tg(zFoxd3:GFP) transgenic larvae, we are able to visualise relatively weak GFP expression in non-neuronal cells (green, open arrowheads, single colour A'), in close association with HuC/D+ neurons (red, merge A). In contrast, other FoxD3+ NC cell lineages, such as melanocytes exhibit strong GFP expression (arrowheads). (B) As previously described, GFP expressing cells (green) are visualised within the pineal gland in the CNS of 7dpf Tg(zFoxd3:GFP) transgenic larvae. (C) At 7dpf, immunohistochemistry performed with Sox10 reveals a Sox10 expressing non-neuronal population (green, open arrowheads, single colour C') that is closely associated with HuC/D+ neurons (red, merge C). (D) Sox10+ cells (green) are visualised within the developing jaw (arrowheads) of 7dpf embryos. (E) At 7dpf, the Tg(-4.7sox10:egfp) line (green, single colour E') does not label cells within the zebrafish ENS, although sox10+ expressing melanocytes surrounding the gut express high levels of GFP (arrowheads) and HuC/D+ neurons (red, merge E) are present within the ENS. (F) GFP expression (green) is observed in other sox10 expressing NC cell lineages, such as the developing jaw and pectoral fin (arrowheads). (G) In adulthood, the Sox10 antibody labels cells within the ENS (green, arrowheads, single colour G') that are distinct from, but closely associated with HuC/D neurons (red, merge G). The inset in (G) highlights the fact that the Sox10 antibody (green, arrowheads) labels nuclei (grey), but also weakly labels non-neuronal fibres (open arrowheads). (H) In the adult brain, the Sox10 antibody (green) labels oligodendrocytes, as previously reported (midbrain region shown). (I) Within the adult Tg(-4.7sox10:egfp) gut, GFP expressing cells or processes (green, single colour I') are not detected within the ENS, or associated with HuC/D+ neurons (red, merge I). (J) Within the adult brain, sox10 expressing cells, such as oligodendrocytes, are labelled with GFP throughout the brain (green, arrowheads, midbrain shown). All larval images are from the mid-gut with rostral to the left, whereas all adult gut images are from the mid-gut region with rostral to the top. The gut in larval animals is highlighted with dotted outlines. Representative control regions for immunohistochemistry and transgenic lines are shown.

3.2.1.6 Sox10

Another marker of interest is the transcription factor Sox10, which is expressed widely in multiple NC cell lineages (Haldin and LaBonne, 2010). In mice, SOX10 is one of the earliest markers observed in eNCCs, and is maintained in the entire population of EGCs throughout life (Young et al., 2003, Elworthy et al., 2005, Bondurand et al., 2006, Sasselli et al., 2012, Boesmans et al., 2014). In zebrafish, Sox10 is expressed in all NC cells, including the early population of ENS precursors.
that invade the gut (Carney et al., 2006, Dutton et al., 2008, Rodrigues et al., 2012), but it is unknown whether Sox10 expression is maintained at later stages and potentially within the population of EGCs. To examine this issue, we initially used the Tg(-4.7sox10:GFP) line, in which GFP expression is under the control of a 4.7kb fragment of the zebrafish Sox10 promoter (Dutton et al., 2008). During early embryonic development, while NC cells are invading the gut, we detected expression of Tg(-4.7sox10:GFP) in the migrating NC cells, as previously reported (Carney et al., 2006, Dutton et al., 2008). However, from around 3dpf, the GFP signal was progressively lost and by 7dpf GFP was no longer detectable within the gut (N=30, Figure 3.4E, Appendix Chapter 8.2 Figure 8.1A). This is unlikely to be related to technical or sensitivity issues, since at this stage (7dpf) GFP expression was maintained in other NC cell lineages such as the developing jaw (cranial NC cells) (Figure 3.4F) and melanocytes (Figure 3.4E), as well as other Sox10 expressing populations such as oligodendrocytes (data not shown). To determine whether the loss of GFP signal in the ENS of zebrafish is a specific feature of the Tg(-4.7sox10:GFP) transgene employed, we immunostained 7dpf larvae with a Sox10 specific antibody, raised against zebrafish Sox10, which has been used to detect a wide range of Sox10 expressing cells (Asad et al., 2016, Williams et al., 2018, Kroehne et al., 2017). Using this antibody, we were able to detect nuclear Sox10 staining in the zebrafish ENS of 7dpf larvae (N=25)(Figure 3.4C). These Sox10+ cells were closely associated, but non-overlapping, with HuC/D+ neurons (Figure 3.4C). These experiments suggest, that the non-neuronal component of the zebrafish ENS can be identified by Sox10 at 7dpf, which is composed of EGCs and/or progenitors.

To test whether Sox10 expression was maintained into adulthood, we first examined the gut of Tg(-4.7sox10:GFP) transgenic animals at adult stages. In ~1 year old (1yo) animals (N=4), we failed to detect any GFP+ signal within the gut (Figure 3.4I), despite the presence of GFP+ oligodendrocytes within the adult brain (Figure 3.4J). In contrast to these results, immunostaining with the Sox10 antibody revealed Sox10+ nuclei within the adult ENS. The Sox10+ cells within the ENS are distinct but closely associated with HuC/D neurons (Figure 3.4G). This result is consistent with observations using this antibody at 7dpf, and together suggests that Sox10 can identify the non-neuronal component of the ENS throughout life (Figure 3.4C and G). Surprisingly, Sox10 immunostaining in the adult ENS also reveals a
relatively weak staining within fibres (Figure 3.4G), despite Sox10+ signal being exclusively nuclear in the CNS (Figure 3.4H). The altered cellular distribution of Sox10 immunostaining may suggest different roles or functions of this transcription factor in the adult gut (Chaoui et al., 2015). The reason for the discrepancy between our immunohistochemical and transgenic data, at both 7dpf and adulthood, remains unclear but may reflect a lack of regulatory elements in $Tg(-4.7sox10:GFP)$ transgene that are required for faithful expression of EGFP within the ENS. Consistent with this idea, previous work has shown the presence of several ENS specific regulatory elements that reside exist outside the 4.7kb element used to generate the $Tg(-4.7sox10:GFP)$ transgenic line (Carney et al., 2006, Antonellis et al., 2008). Nevertheless, we propose that the zebrafish non-neuronal compartment of the ENS can be identified by expression of Sox10.

In summary, our analysis so far indicates that the non-neuronal compartment of the zebrafish ENS lacks peripheral glial markers (BFABP, S100β, Nestin, Gfap, and potentially FoxD3), that are normally expressed by mammalian EGCs. However, the non-neuronal cellular compartment can be identified by expression of the transcription factor Sox10. Due to the maintained expression of NC cell markers, the non-neuronal compartment of the zebrafish may exhibit more progenitor-like characteristics. Such a population within the zebrafish ENS has not been previously described.

3.2.2 Lineage Tracing to Identify the Origins of the Non-Neuronal Population of the Zebrafish Enteric Nervous System

The neuronal and glial lineages of the ENS arise from the same pool of Sox10-expressing NC cell progenitors in multiple vertebrate models, and they differentiate within a similar time frame (Uesaka et al., 2016). The unusual properties of the non-neuronal compartment of the zebrafish ENS, in terms of glial marker expression, raises the possibility that they have an alternative non-NC cell origin. To explore this possibility, we carried out genetic lineage tracing of early NC cells in zebrafish using a combination of transgenic drivers and reporters.

The initial lineage tracing tool used was the Gal4 driver $Tg(SAGFF234A)$ transgene, which drives Gal4 expression in the eNCCs that migrate into the gut and is maintained in the ENS at later stages (Kawakami et al., 2010, Heanue et al.,
When combined with the \textit{Tg(UAS:GFP)}, in which the GFP reporter is under the control of the Gal4 recognition sequence, GFP is expressed within the ENS (Heanue et al., 2016a, Kawakami et al., 2010). Previous studies have shown that the majority of neurons are labelled with the \textit{Tg(SAGFF234A;UAS:GFP)} transgenic line (Heanue et al., 2016a), therefore we carried out a more in-depth analysis of the ENS lineage labelled with this transgenic. At 7dpf, \textit{Tg(SAGFF234A;UAS:GFP)} embryos were immunostained for GFP and HuC/D. Given our expectation that the neuronal and non-neuronal component of the zebrafish ENS arise from eNCCs, and that both populations would be present at 7dpf, we expect that this line will also label the majority of the non-neuronal compartment of the ENS. In light of this, we visualised that 87.78 ± 0.93% of all the HuC/D+ neurons were labelled with the GFP+ reporter (Figure 3.5D), therefore if the non-neuronal component is derived from the same eNCC progenitors we would expect the same efficiency of labelling. We observe that the vast majority of GFP+ cells (93.8 ± 1.00%) are HuC/D+ neurons whereas only 6.24 ± 1.00% were non-neuronal (N=9, Figure 3.5C and E). This suggests that the majority of cells within the zebrafish ENS at 7dpf are neurons. Unfortunately, we were unable to examine later developmental stages using the \textit{Tg(SAGFF234A;UAS:GFP)} transgenics since smooth muscle cells within the gut of these animals start to express GFP from around 5dpf. Nevertheless, our analysis suggests that the non-neuronal cells (EGCs and/or progenitors) of the zebrafish ENS share a common origin with enteric neurons, but constitute a smaller proportion of the ENS relative to their mammalian counterparts.
Chapter 3 Results

$Tg(-4.7s\text{ox}10:cre; B\text{actin-LoxP-STOP-LoxP-hmgb1-mCherry})$

A

Cherry HuC/D

A'

Cherry

A''

HuC/D

B

Cherry HuC/D

B'

Cherry

B''

HuC/D

$Tg(SAGFF234A; U\text{AS}:GFP})$

C

GFP HuC/D

C'

GFP

C''

HuC/D

D

% Lineage+HuC/D+/Total HuC/D+

E

Lineage+Marker+/Lineage+
Figure 3.5. Lineage tracing reveals that there is a small non-neuronal lineage component of the zebrafish ENS. (A-B) Tg(-4.7sox10cre; βactin-LoxP-STOP-LoxP-hmgb1-mCherry) labels the ENS lineage. (A) At 7dpf, the sox10 lineage (red, single colour A’) is primarily composed of HuC/D+ neurons (arrows, cyan, single colour A’). (B) Within the adult ENS, the majority of sox10-lineage cells (red, single colour B’) are also HuC/D+ neurons (arrows, cyan, single colour B’'). (C) Using the Tg(SAGFF234A;UAS:GFP) line at 7dpf, the ENS lineage is labelled in GFP (Green, single colour C’). Immunostaining with HuC/D (cyan, single colour C’’) reveals that the majority of the ENS lineage is neuronal (arrow). (D) Quantification of the percentage of neurons labelled by lineage tools highlights the variability in labelling with Tg(βactin-LoxP-STOP-LoxP-hmgb1-mCherry) reporter at 7dpf that is not seen using the other reporters, which consistently label the majority of the neuronal populations. (E) Quantification of neuronal (dark grey) and non-neuronal (light grey) populations within the ENS lineage, using any of the different transgenic lineage reporters shows only a small non-neuronal population. For clarity transgenic lines in (E and F) were abbreviated to 234:GFP= Tg(SAGFF234A;UAS:GFP), sox10cre;bactin= Tg(-4.7sox10cre; βactin-LoxP-STOP-LoxP-hmgb1-mCherry) and sox10cre;EF1α= Tg(-4.7sox10cre;EF1α;loxp-gfp-pA-loxp-dsRed-pA). All larval images are from the mid-gut and shown with rostral to the left whereas all adult gut images are from the mid-gut region with rostral to the left. The gut in larval animals is highlighted with dotted outlines. Data is given as mean ± SEM.

To independently verify these results, we used an alternative, transgenic line Tg(-4.7sox10:cre), in which Cre recombinase is expressed under the control of a 4.7kb fragment of the sox10 promoter element (Rodrigues et al., 2012). When this transgene is combined with the Cre-dependent reporter Tg(βactin-LoxP-STOP-LoxP-hmgb1-mCherry) (Wang et al., 2011), sox10 expressing cells, and their progeny are indelibly labelled with the nuclear localised Cherry reporter. Immunostaining of 7dpf Tg(-4.7sox10:cre;βactin-LoxP-STOP-LoxP-hmgb1-mCherry) double transgenic zebrafish for Cherry and HuC/D expression revealed that 84.79 ± 2.13% of the Cherry+ ENS lineage were HuC/D+ neurons, whereas only 15.21 ±2.13% were non-neuronal (N=13, Figure 3.5A and 3.5E). We noted that the percentage of enteric neurons labelled with Cherry in 7dpf Tg(-4.7sox10:cre;βactin-LoxP-STOP-LoxP-hmgb1-mCherry) animals varied from 15-78% (mean = 47.06 ± 5.527%, Figure 3.5D). This variability in neuronal labelling may reflect delayed onset, or inefficient Cre expression and/or reporter recombination. Consistent with this latter interpretation, when the Tg(-4.7sox10:cre) is crossed with the alternative Tg(EF1α;loxp-gfp-pA-loxp-dsRed-pA) reporter, we find 40-99% of the HuC/D+
neurons were labelled with this reporter (mean= 61.64 ± 7.83%, Figure 3.5D). Nevertheless, we observe that the majority of the sox10-derived ENS cells are HuC/D+ neurons (86.78 ± 2.42, Figure 3.5A andE). These experiments support our lineage analysis with Tg(SAGFF234A;UAS:GFP) animals, and suggest that the non-neuronal compartment in the ENS of 7dpf larvae represents relatively small fraction of the ENS.

To determine whether there was expansion of the non-neuronal cell population at later developmental stages, we examined adult (>3mo) Tg(-4.7sox10:cre;βactin-LoxP-STOP-LoxP-hmgb1-mCherry) double transgenic zebrafish. We observed that, similar to findings at 7dpf, the majority of the ENS lineage were HuC/D+ neurons (65.49 ± 1.39%), with non-neuronal HuC/D- cells representing 34.41 ± 4.88% (N=8, Figure 3.5B and E). In adult Tg(-4.7sox10:cre;βactin-LoxP-STOP-LoxP-hmgb1-mCherry) animals, the fraction of neurons labelled with Cherry is on average higher than observed at 7dpf (range of 11-97%, mean=71.86 ± 5.77%, Figure 3.5D). The relatively small labelling index of this transgenic tool is a limitation that may be explained by the lack of sox10 regulatory elements in the Tg(-4.7sox10:cre) transgene (Antonellis et al., 2008). Nevertheless, using this tool further suggests that the majority of the ENS lineage is neuronal. Interestingly, the non-neuronal population has increased from 7dpf to adulthood, an expansion of this population may be explained by potential roles in supporting more complex neuronal networks. However, we still never observed the large numbers of non-neuronal cells that we would expect from the number of EGCs seen within the mammalian ENS, where EGCs outnumber neuronal cells by at least two fold (Ruhl, 2005).

In summary, our lineage analysis indicates that the NC cell lineage in zebrafish gives rise to two ENS cell populations: enteric neurons and non-neuronal cells (progenitors and/or EGCs). Surprisingly, unlike the mammalian ENS where EGCs (non-neuronal cells) outnumber enteric neurons, in the zebrafish ENS the non-neuronal cells represent a relatively small fraction of the total ENS cell population. Finally, the increase of the non-neuronal ENS cell compartment in the adult zebrafish (34.41 ± 1.21%) relative to 7dpf animals (15.21 ± 2.13%) suggests a role of this cell population supporting the presumably more complex neuronal circuits of the adult gut.
3.3 Conclusion

Enteric glia in the mouse have been well characterised in terms of morphology, location and marker expression. Using this information, we performed screens with antibodies and transgenic reporters to characterise the putative zebrafish EGCs. Unlike mammals, in which all non-neuronal ENS cells in the adult ENS are thought to represent EGCs, the non-neuronal compartment of the zebrafish ENS lacks expression of canonical glial markers such as BFABP and S100β. Also, and in contrast to previous observations (Doodnath et al., 2010, Hagstrom and Olsson, 2010, Kelsh and Eisen, 2000, Bernardos and Raymond, 2006), our own analysis indicates that the zebrafish ENS does not express Gfap, a characteristic marker of peripheral glia and mammalian EGCs. We show instead, that the zrf-1 antibody raised against zebrafish Gfap, labels smooth muscle and that an antibody specific for mammalian Gfap labelled only cellular processes within the gut but not cell bodies, suggesting that the identified Gfap fibres come from a gut-extrinsic origin. Furthermore, there were no cells bodies or fibres labelled in the gut of Tg(gfap:GFP) transgenic line at any stage analysed. We hypothesise that the mGFAP signal detected represents glial fibres that enter the gut at late stages of development, after peristalsis is under neuronal control and the ENS is fully functional, and that Gfap is not expressed within the ENS itself.

Despite the absence of peripheral glial markers, we were able to visualise Sox10+ cells in the larval and adult ENS. Sox10 is a NC cell marker which is maintained throughout mammalian gliogenesis. Within the zebrafish ENS, after 3dpf Sox10+ non-neuronal cells were visualised using the zebrafish Sox10 antibody but not the Tg(-4.7sox10:GFP) transgene. This difference is likely to be due to the Tg(-4.7sox10:GFP) transgene being under the control of only the first 4.7kb of the sox10 promoter (Dutton et al., 2008) and therefore lacking regulatory elements necessary to exhibit the complete sox10 expression pattern within the ENS. Consistent with this idea, regulatory elements outside of the 4.7kb region used are capable of driving EGFP expression in the zebrafish ENS (Rodrigues et al., 2012, Antonellis et al., 2008, Dutton et al., 2008). In light of the maintained expression of Sox10 within the zebrafish ENS, it would be interesting to perform a more detailed analysis of other NC cell markers such as foxD3, for which our previous examination of the Tg(zFoxd3:GFP) transgenic line showed weak expression of EGFP within non-
neuronal ENS cells. The relatively low levels of expression of this transgene lead us initially to discount this tool as a useful marker of the non-neuronal lineage. However, in light of our results with Sox10, it will be of interest to revisit FoxD3 as a putative non-neuronal marker of the ENS. It may be that analysis of FoxD3 in the adult gut will reveal expression in the non-neuronal component of the ENS. The existing Tg(zFoxd3:GFP) transgenic line and FoxD3 antibodies could be employed for this analysis.

Finally, we used two independent lineage tracing tools, the Tg(SAGFF234A;UAS:GFP) and Tg(-4.7sox10:cre;βactin-LoxP-STOP-LoxP-hmgb1-mCherry), to assess the population of non-neuronal cells within the zebrafish ENS. To our surprise, and in contrast to the mammalian ENS, we observed that the majority of the zebrafish ENS was composed of neurons and only a minority of the cells were non-neuronal (progenitors and/or EGCS). We postulated that the apparently small percentage of non-neuronal cells could be due to inefficient labelling of the NC cell/progenitors that give rise to the ENS, and/or biased labelling of neuron-committed eNCCs. We can discount the inefficient labelling possibility, since in the vast majority of the animals studied we observed that nearly all the ENS neurons were labelled with our lineage markers. In addition, in the mouse, equivalent tools as the Sox10::Cre, do not show any bias towards specific subpopulation of NC cells and ENS progenitors (Laranjeira et al., 2011, Lasrado et al., 2017). Since all the key developmental genes are conserved between mouse and zebrafish, we do not anticipate that zebrafish sox10-derivved eNCCs would have a neuronal bias. Moreover, two independent lines reveal a similar and relatively small non-neuronal population and a larger neuronal population. Since, other glial lineages such as Schwann cells, are labelled with the Tg(-4.7sox10:cre;βactin-loxp-stop-loxp-NLSCherry) reporter system in the expected numbers, we find no evidence to suggest that this reporter shows a disproportionate bias towards neuro-committed progenitors. Therefore, we can argue that non-neuronal cells of the zebrafish ENS represent a relatively small proportion of the ENS.

In conclusion, we have identified a small non-neuronal population within the zebrafish ENS that are likely to represent putative EGCs. These non-neuronal cells do not express the majority of expected glial markers such as BFABP, GFAP, S100β and Nestin. However, this population expresses Sox10. The lack of mature marker expression together with the expression of the NC marker Sox10, suggests that
instead of a “typical” glial population the non-neuronal population of the zebrafish ENS may be more progenitor-like. Perhaps this is similar to the situation in the zebrafish telencephalon, an area of the CNS most similar to the mammalian cortex, where there are no protoplasmic astrocytes and the astrocytic functions are performed by the radial glia progenitor cells (Grupp et al., 2010). Given the fact that RGCs are the progenitor cells of the CNS, we hypothesise that EGCs of the zebrafish ENS share properties with this CNS glia progenitor population.
Chapter 4. Identification of Her4.1 as a Marker of Enteric Glial Cells

4.1 Introduction

Mammalian EGCs are traditionally thought to be most similar to astrocytes (Gabella, 1981, Jessen and Mirsky, 1983, Gulbransen and Sharkey, 2012). However, the zebrafish CNS lacks protoplasmic astrocytes, and instead have radial glial cells (RGCs) that take up astrocytic functions (Grupp et al., 2010). We identified that the non-neuronal cells in the zebrafish ENS lack “astrocytic” glial markers such as GFAP, BFABP and S100β, however they maintained the NC cell marker Sox10. In light of this, we hypothesised that since NC cell markers are maintained within the non-neuronal population in the zebrafish ENS, this population may have more progenitor-like characteristics.

Zebrafish RGCs express many common glial markers, but in addition express progenitor markers such as nestin and her4.1. We had previously discounted nestin as a useful marker for zebrafish EGC (Chapter 3.2.1.3) therefore, we were particularly interested in the expression of her4.1 within zebrafish EGCs. Her4.1, the zebrafish orthologue of mammalian HES5, is a basic helix-loop-helix (bHLH) transcription factor upregulated by Notch signalling and downregulated by Neurogenin-1 (Takke et al., 1999). In mammals, Hes5 has also been implicated in maintaining astrocytic cell fates (Kageyama et al., 2015, Bansod et al., 2017). Moreover, within the mammalian ENS, NOTCH signalling, mediated by HES5, has been implicated in ENS development (Theocharatos et al., 2013, Liu and Ngan, 2014, Okamura and Saga, 2008, Memic et al., 2016, Charrier and Pilon, 2017).

We hypothesised that similar to the CNS, the zebrafish ENS may lack an astrocyte-like EGCs population, and instead have EGCs with enriched RGC-like progenitor characteristics. We therefore explored the expression of the RGC marker her4.1 in the zebrafish intestine at larval stages and in adulthood.
4.2 Results

4.2.1 Characterisation of Tg(her4.1:EGFP) in the zebrafish intestine.

4.2.1.1 Immunohistochemical analysis of the Tg(her4.1:EGFP) transgenic

We postulated that her4.1 would be a potential marker of zebrafish EGCs due to its expression in RGCs of the CNS (Yeo et al., 2007) and the previous evidence that NOTCH signalling is important in the developing ENS and gliogenesis (Okamura and Saga, 2008, Theocharatos et al., 2013, Ngan et al., 2011, Kanski et al., 2014). Therefore, we analysed the well-established her4.1 reporter line Tg(her4.1:EGFP), which expresses EGFP under the control of a 3.4kb regulatory element of the her4.1 promoter. This element contains 5 of the Notch mediated Su(H) binding sites required for Notch-mediated transcriptional activation, and therefore should recapitulate the spatiotemporal dynamics of her4.1 signalling in response to Notch signalling (Yeo et al., 2007). Examining the Tg(her4.1:EGFP) transgenic for EGFP expression in conjunction with immunostaining for GFP and HuC/D allowed us to assess the association with neurons within the ENS. As expected, at 7dpf Tg(her4.1:EGFP) larvae exhibited strong EGFP expression in the CNS (Yeo et al., 2007). Interestingly, at this stage we also detected a relatively small number of individual GFP+ cells within the gut, which were closely associated with HuC/D+ neurons (Figure 4.1A-B). Despite their close proximity to neurons, Her4.1+ cells were generally negative for HuC/D+ signal (Figure 4.1A-B), suggesting, that similar to the CNS, her4.1 expression within the gut belongs to the non-neuronal compartment of the zebrafish ENS. Occasionally, however, we observed cells with a perinuclear HuC/D+ staining that were weakly GFP+ (Figure 4.1B). We suggest that GFP+ cells represent progenitors, and that the HuC/D+GFP+ cells identified in the gut of 7dpf zebrafish larvae represent neurons generated recently from Her4.1+ progenitors that still retain some of the EGFP signal. In support of this idea, a similar phenomenon has been described in the developing mammalian gut, where HuC/D staining has a characteristic perinuclear location in newborn neurons (Young et al., 2003). Taken together, our experiments suggest that her4.1 is expressed in a non-neuronal population within the ENS of zebrafish larvae, and henceforth cells labelled with by the Tg(her4.1:EGFP) transgene will be referred to as Her4.1+ cells.
Figure 4.1. Characterisation of *Tg(her4.1:EGFP)* expression in the intestine at larval and adult stages. (A-B) In 7dpf *Tg(her4.1:EGFP)* larvae, GFP⁺ cells (green, arrowheads) are closely associated with, but distinct from, HuC/D⁺ neurons (red). (B) Occasionally, weakly expressing EGFP cells (green) are co-localised with a perinuclear HuC/D staining (red) (C-D) In adult *Tg(her4.1:EGFP)* whole-mount gut preparations, EGFP expressing cells (green, arrowheads) are closely associated with, yet distinct from, HuC/D⁺ neuronal cell bodies (red) and AcTu⁺ processes (red). (E-H) The morphology and position of adult *Tg(her4.1:EGFP)* expressing cells (green) resemble mammalian enteric glial cells, for example: GFP⁺ cell processes are wrapped around HuC/D⁺ neuronal cell bodies (red) (E), GFP⁺ cells bodies (green) have elongated fibres that are associated with AcTu⁺ neuronal fibres (red) (F), GFP⁺ cells (green) are seen close to the intestinal epithelia (DAPI, grey) (G), and bipolar GFP⁺ cells (green) are found in the muscle layers (H). (I) GFP⁺ cells (green, single colour I") also colocalise with Sox10⁺ (red, arrowhead, single colour I") and do not overlap with HuC/D⁺ neurons (blue, single colour I"') within the adult gut. All larval images are from the mid-gut with rostral to the left, whereas all adult gut images are from the mid-gut region with rostral to the top. The gut in larval animals is highlighted with dotted outlines.

Next we examined whether Her4.1⁺ expressing cells were also found in the adult zebrafish ENS. For this analysis, we immunostained for GFP and HuC/D in whole-mount gut and brain preparations (the latter serving as a positive control) from >3mo
Tg(her4.1:EGFP) transgenics. In certain preparations immunostaining for GFP and HuC/D were combined with Sox10 or acetylated tubulin (AcTu), to reveal the non-neuronal cells or neuronal processes, respectively. As expected, a large number of GFP+ cells were present within the RGCs found throughout the brain, in a distribution pattern similar to that described previously (Appendix Chapter 8.3, Figure 8.2) (Kroehne et al., 2011, Galant et al., 2016, Lupperger et al., 2018). Interestingly, GFP+ cells were also detected in the outer smooth muscle layers of the gut in close association HuC/D+ neuron cell bodies (Figure 4.1C) and acetylated tubulin+ (AcTu+) neuronal processes (Figure 4.1D). Unlike in the larval stages, we almost never saw an overlap of EGFP expressing cells with HuC/D+ neurons, suggesting that the generation of enteric neurons from EGFP expressing cells in adult animals (if it indeed occurs) is less pronounced relative to larvae. Alternatively, these observations could indicate that neuronal differentiation occurs at a much slower rate and that EGFP is degraded before the onset of HuC/D expression. Finally, combined immunostaining for GFP and Sox10 demonstrated that all GFP+ cells within the outer smooth muscle layers co-expressed Sox10 (Figure 4.1I), suggesting that they belong to the NC cell-derived non-neuronal lineage of the zebrafish ENS. Together our data suggests that Her4.1 marks a non-neuronal ENS population at larval and adult stages.

4.2.1.2 Morphological characterisation of Her4.1+ cells

The cytoplasmic distribution of the EGFP expressed by the Tg(her4.1:EGFP) transgene, allowed us to examine in detail the morphology of Her4.1+ cells in the adult intestine. Intriguingly, the morphology of these cells is similar to that of EGCs present in the mammalian ENS. In general, GFP+ cells were closely associated to HuC/D+ neurons and their processes wrapped around the neuron cell bodies (Figure 4.1E), similar to the Type I mammalian EGCs found within enteric ganglia (Boesmans et al., 2014, Hanani and Reichenbach, 1994). We also observed GFP+ cell bodies that were associated with neuronal processes (Figure 4.1F). Often, GFP+ cellular processes were elongated and followed the neuronal fibres (Figure 4.1F), in a manner analogous to mammalian Type II EGCs present in the interganglionic connectives (Boesmans et al., 2014, Hanani and Reichenbach, 1994). Although most of the cells labelled with the Tg(her4.1:EGFP) transgene in the gut are found
next to neuron bodies or fibres, we also detected GFP+ cells close to the mucosal layers, such as close to the mucosal epithelia (Figure 4.1G), similar to mammalian Type III EGCs (Boesmans et al., 2014). Finally, we detected GFP+ bipolar cells within the muscle layers (Figure 4.1H), which resemble Type IV mammalian EGCs (Boesmans et al., 2014). This suggests that the non-neuronal cells of the ENS are likely to be distributed in all gut layers and to be morphologically heterogeneous, similar to mammalian EGCs. Together, these observations suggest that the non-neuronal cells of the zebrafish gut labelled by her4.1 correspond to mammalian EGCs, on the basis of gene expression (sox10), morphology and locations within the gut layers.

4.2.1.3 Ultrastructural characterisation of Her4.1+ cells

Since we unable to detect any expression of “mature” glial markers within the zebrafish gut, we wished to confirm the glial characteristics of Tg(her4.1:EGFP) expressing cells by comparing their ultrastructural features to those of mammalian EGCs that have been reported previously (Gabella, 1981). Given the relative sparsity of EGFP expressing cells within the gut of Tg(her4.1:EGFP) transgenics, we performed a correlative light and electron microscopy (CLEM) study, in collaboration with Christopher Peddie (EM facility at the Francis Crick Institute). CLEM allows the identification of regions of interest (ROIs) that contain fluorescently labelled cells of interest (by confocal laser microscopy) and subsequent analysis of their ultrastructural characterisation by electron microscopy (EM) (Russell et al., 2016, Brama et al., 2015). To ensure accurate alignment of the light microscopy images with the EM data, we crossed the Tg(her4.1:EGFP) zebrafish with the Tg(SAGFF217B:UAS:mCherry) line, which labels a subset of enteric neurons with the mCherry reporter (T. Heanue unpublished data, Kawakami et al., 2010). The resulting Tg(her4.1:EGFP;SAGFF217B;UAS:mCherry) triple transgenic zebrafish allowed us to simultaneously visualise the Her4.1+ non-neuronal population and a subpopulation of enteric neurons. CLEM analysis, carried out by Christopher Peddie, revealed that Tg(her4.1:EGFP) cells, located within the adult zebrafish gut, have remarkably similar ultrastructural morphologies to mammalian EGCs (Figure 4.2). Specifically, zebrafish enteric Tg(her4.1:EGFP) expressing cells have large multi-lobed nuclei that take up the majority of the soma (Figure 4.2A+C), a characteristic
feature of mammalian EGCs (Gabella, 1981). Furthermore, the radially projecting processes of EGFP expressing cells compartmentalised axonal bundles (Figure 4.2A and C) and established several contacts with the soma of enteric neurons (Figure 4.2A-B). However, EGFP expressing processes never completely surround neurons or neuronal processes, leaving them exposed to the basal lamina (Figure 4.2A-C), these features have also been described for mammalian EGCs (Gabella, 1981). Despite these similarities to mammalian EGCs, we were unable to detect any glio-filaments within the Tg(her4.1:EGFP) expressing cells of the zebrafish intestine. This observation is in stark contrast to the mammalian EGCs, which have abundant glio-filaments throughout their processes. The lack of glio-filaments within the Her4.1+ cells is also constant with the lack of Gfap staining visualised within the zebrafish ENS (Eliasson et al., 1999, Middeldorp and Hol, 2011, see also Chapter 3.2). Glio-filaments are thought to provide mechanical support within the ENS, since the expansive glio-filaments within EGC fibres form a sheath around the ganglia and prevent damage to cells within the ganglia during gut contraction (Gabella, 1981). Therefore, the lack of Gfap and glio-filaments may perhaps reflect that zebrafish do not need the extra mechanical support provided by glio-filaments since zebrafish lack ENS ganglia. Nevertheless, the striking similarities between the ultrastructural characteristics of the Her4.1+ cells with mammalian EGCs at the ultrastructural levels suggests that the Her4.1+ non-neuronal cell population of the zebrafish ENS may have glial functions in supporting neurons.

In conclusion, due to the close association of Her4.1+ cells to enteric neurons, their similarity to mammalian EGCs at both morphological and ultrastructural levels, and their expression of Sox10 suggest that Her4.1+ cells are the non-neuronal EGC population of the mature zebrafish ENS and are likely to share properties with mammalian EGGs.
Figure 4.2. Correlative Light and Electron Microscopy (CLEM), performed in collaboration with Christopher Peddie, reveals that *Tg(her4.1:EGFP)* cells have characteristic ultrastructural features of glial cells. (A) Super-resolution image of a *Tg(her4.1:EGFP)* labelled cell (green) with three radial projecting processes (arrowheads). (A’) Electron microscopy (EM)
image of the ROI shown in (A). The EGFP expressing cell (green) has a lobed nuclei (open arrowheads) and radially projecting EGFP expressing processes that segment unlabelled axon bundles (arrowheads). The EGFP expressing processes contact an unlabelled neuronal soma (arrow). (B) Super-resolution image of a Tg(her4.1:EGFP) labelled process (green, arrowheads) wrapping around a neuron cell body (red, open arrow) labelled with Tg(SAGFF217B;UAS:mCherry). (B') EM image of the ROI in (B), the EGFP expressing process (green, arrow) is shown to partially surround (arrows) the mCherry expressing neuron cell body (red, open arrow). (C) Super-resolution image of a Tg(her4.1:EGFP) labelled cell (green) in close contact with a Tg(SAGFF217B;UAS:mCherry) labelled neuron (red). The EGFP expressing processes are in close proximity to the neuronal process (arrowheads). (C') EM image of the ROI in (C), with the EGFP expressing cell (green, open arrowhead) and neuron (red, open arrow). The EGFP expressing processes compartmentalise the unlabelled neuronal axons (arrowheads) and has contacts with the neuron cell body (arrows). All images are orientated with the intestinal lumen to the top. Unlabelled enteric neurons were identified by large soma, spherical nuclei, and expansive cytoplasm, unlabelled neuronal axons were characterised by the presence of synaptic vesicles, and pre-/post- synaptic densities as previously described by Gabella (1981), Gabella (1972).

4.2.2 The Tg(her4.1:EGFP) Expressing Cell Population is Part of the ENS Lineage

Our experiments have indicated that Her4.1 is expressed in a non-neuronal population of cells within the zebrafish gut that are morphologically similar to mammalian EGCs. In the mammalian ENS, eNCCs that invade the fore-gut during embryogenesis give rise to both neurons and glial cells (Uesaka et al., 2016). Our previous analysis using the Tg(SAGFF234A) and Tg(-4.7sox10cre) lineage markers demonstrated that neurons and a non-neuronal population derive from eNCCs (Chapter 3.2.2). To test whether the Her4.1+ cell population in the zebrafish derives from NC cells, we generated triple transgenic Tg(her4.1:EGFP;SAGFF234A;UAS:mCherry) zebrafish larvae, which were immunostained for GFP, HuC/D and the reporter Cherry, at various developmental stages (Figure 4.3A-C). This analysis identified a relatively small number of GFP+ cells that expressed relatively low EGFP levels (Figure 4.3B-C) relative to the CNS. Interestingly, all the GFP+ cells were also positive for Cherry+, this result confirms that Tg(her4.1:EGFP) expressing cells belong to the eNCC lineage. The first
GFP*Cherry* cells were detected at 60hpf (Fig 4.3B), a stage at where the majority of the gut has been colonised by eNCCs (Uyttebroek et al., 2010, Shepherd and Eisen, 2011) and neurogenesis has been initiated. Therefore, appearance of the Tg(her4.1:EGFP) cell population is coincident with neurogenesis (Olden et al., 2008, Shepherd and Eisen, 2011). Since Her4.1 is downstream of Notch signalling, this may suggest that neurons are providing the Notch ligands to induce Her4.1 expression.

To further examine the origin of Her4.1+ cells, we introduced the Tg(her4.1:EGFP) transgene into the Tg(-4.7sox10cre;βactin-LoxP-STOP-LoxP-hmgb1-mCherry) background, which marks all NC cell lineages, including the ENS (Figure 4.3F) (Rodrigues et al., 2012). We observed that virtually all EGFP expressing cells were also mCherry+ (Figure 4.3D-E), confirming that the Her4.1+ population is an intrinsic NC-derived cell population of the zebrafish ENS. The majority of the HuC/D-Cherry+ non-neuronal cell population of the triple transgenic animals expressed EGFP (64.65 ±12.28%, Figure 4.3G). However, a small fraction of non-neuronal cells were EGFP- (34.06 ±12.79%, Figure 4.3G), raising the possibly of an EGFP-HuC/D-sox10-derived cell lineage in the adult ENS. Alternatively, the level of Her4.1 within these cells is below the limit of detection, due to the oscillatory nature of her4.1 (Pasini et al., 2004, Kageyama et al., 2008a), or low level her4.1 expression that is insufficient to drive EGFP (Yeo et al., 2007).

In conclusion, we have confirmed that the Her4.1 expressing cell population in the zebrafish gut are derived from NC cells and represents the majority of non-neuronal ENS cells. The glial-like morphologies and ultrastructural features support the idea that that at least a subset, if not all, of the Her4.1+ cells are equivalent to the mammalian EGCs.
Figure 4.3. Lineage analysis reveals that Tg(her4.1:EGFP) drives expression in the eNCC population that gives rise to the ENS. (A-C) Analysis of Tg(her4.1:EGFP) expressing population (green) relative to the Tg(SAGFF234A;UAS:mCherry) expressing eNCC population (red) that colonises the gut. (A) No EGFP expression (green) is detected in migrating eNCC cells
(red) in the 54hpf gut, although differentiated ENS neurons are present, as revealed by HuC/D (blue) staining. (B) At 60hpf, weakly expressing EGFP expressing cells are detected (green, arrowhead) within the eNCC cell streams colonising the gut (red) near HuC/D+ neurons (blue). Insets in B show single z-stacks of the GFP+ cells within the Cherry+ eNCC cell lineage. (C) At 4dpf, GFP+ cells (green, arrowheads, single colour C’) were observed next to HuC/D expressing neurons (blue). (D-E) Analysis of Tg(her4.1:EGFP;4.7sox10cre;βactin-LoxP-STOP-LoxP-hmgb1-mCherry) adult zebrafish reveals EGFP expressing cells (green, arrowheads, single colour E) are within the sox10-derived lineage (red, single colour E’) of the adult zebrafish. (D) In a whole-mount gut preparation, EGFP expressing cells (green) represent the majority of the non-neuronal cells of the ENS lineage (red, arrowhead). (E) High magnification views reveal that the majority of the sox10-derived ENS lineage (red, single colour E’) is neuronal (magenta, arrow). Within the non-neuronal population, EGFP expressing cells (green, single colour E) represent the majority of non-neuronal cells (yellow, arrowhead). However, there is a small population of GFP HuC/D cells within the sox10-derived lineage (red, open arrowhead). (F) Quantification of the different cell populations observed within the sox10-derived lineage. (G) Quantification of the different cell types observed within the sox10-derived non-neuronal population of the ENS. All larval images are shown with rostral to the left, whereas all adult gut images are from the mid-gut region with rostral to the top. The gut in larval animals is highlighted with dotted outlines.

4.2.3 Development of Her4.1 Expressing Cell Population within the Zebrafish ENS.

Since detection of Her4.1 expressing cells in fixed embryo preparations was limited (due to small numbers of cells present and relatively low levels of EGFP expression, see Chapter 4.2.2), we reasoned that live imaging would provide a more detailed and accurate analysis of the developmental profile of the Her4.1+ cell population. We therefore, performed live imaging on Tg(her4.1:EGFP;SAGFF234A;UAS:mCherry) embryos to track individual EGFP+ cells relative to the population of eNCCs that colonise the gut. Zebrafish embryos were mounted in low melt agarose moulds and imaged from 56hpf for 40.33 hours, with images taken every 10 minutes (mins). The field of view (FOV) was chosen so that at the beginning of the recording (time 00:00, hours: minutes), the migratory wavefront of mCherry expressing cells were visible at the rostral side of the FOV (Figure 4.4A). The most anterior EGFP+ spinal nerve that was visible in the FOV was used as a reference point and all distances were relative to the reference point.
As previously described (Heanue et al., 2016a), the mCherry expressing wavefront moved in a rostrocaudal manner and colonized the entire FOV in 984 ± 33.73 mins. Similar to what we observed in our static lineage analysis (Chapter 4.2.2), the EGFP+ cells were always found within the mCherry expressing eNCC populations and expressed both EGFP and mCherry, confirming their origin from the eNCC population that gives rise to the ENS (N=18, Figure 4.4B-F). Furthermore, in all cases, the EGFP expressing cells appeared behind the wavefront of mCherry expressing eNCCs that colonise the gut (N=18 Figure 4.4A-C). Specifically, the first EGFP expressing cells to appear within the FOV were found approximately 90.76 ± 34.44 µm behind the mCherry expressing wavefront (n=4, N=4, Figure 4.4B). Together these data support our static analysis, to demonstrate that the Her4.1+ population originates from NC-derived eNCCs. Moreover, this analysis suggests that Her4.1+ cells appear relatively late during development and behind the migratory wavefront.

EGFP+ cells appear within the mCherry expressing eNCC population in two stereotypic ways: by entering the FOV or by de-novo EGFP expression in mCherry expressing populations within the FOV. EGFP expressing cells that appear by de-novo EGFP expression are found at significantly later time points (1185 ± 82.78 mins, n=47, N=4, Figure 4.4G) than the EGFP expressing cells which enter the FOV (908.4± 107.5mins n=32, N=4, P=0.042, Unpaired T-Test, Figure 4.4G), suggesting a rostrocaudal wave of EGFP induction. To further investigate the rostrocaudal appearance of Her4.1+ cells, we recorded the position that the EGFP expressing cell appeared along the length of the gut against the time of appearance. We excluded any EGFP expressing cells that had entered the FOV from this analysis, because this would bias the analysis to more rostral areas of the FOV where EGFP expressing cells are first observed. This analysis revealed that EGFP expressing cells appear in a rostrocaudal manner (R²=0.155, P=0.0004, Figure 4.4H).

Next, we examined how many Her4.1+ cells were present within the FOV at different developmental stages. As we previously described, there are very few EGFP expressing cells at early time points. However, the total numbers of EGFP expressing cells significantly increased until approximately 1000 mins (approximately 72hpf) when the population numbers became more stable (R²=0.796, P<0.0001, n=132, N=4, Figure 4.4I). The population of EGFP expressing cells remains constant for the rest of the recording (1410 mins, ending when the embryos
are approximately 96hpf). Therefore, the rate at which new EGFP expressing cells appear is presumably equivalent to any potential loss of EGFP expressing cells, or loss of EGFP expression.

Subsequently, we examined the migratory behaviour of Her4.1\(^+\) cells relative to the mCherry expressing wavefront, by tracking the migration of individual cells (Figure 4.4J and K). Similar to what has been previously described (Heanue et al., 2016a), we observed that the mCherry expressing wavefront migrated with a constant velocity of 0.312 ± 0.0163 µm/min (Figure 4.4K). Interestingly, the EGFP expressing cells, within the mCherry expressing population, migrated significantly slower than the wavefront at approximately 0.043 ± 0.011 µm/min (P<0.0001, One-way ANOVA, Tukey’s test, Figure 4.4K). We were unable to observe any significant difference in the velocities between EGFP expressing cells entering the FOV (0.0427 ± 0.017 µm/min) or those inducing EGFP de-novo (0.0438 ± 0.0151 µm/min, P>0.999, One-way ANOVA, Tukey’s test, Figure 4.4K). Taken together, our live imaging experiments suggest that Her4.1\(^+\) cells have limited migratory potential.
Figure 4.4. Live imaging analysis reveals that Tg(her4.1:EGFP) cells are within the eNCC population have limited migratory potential and exhibit proliferative behaviours. (A-F) Still images from time-lapse recordings of Tg(her4.1:EGFP;SAFGFF234A;UAS:mCherry) zebrafish, starting at 56hpf, reveal that EGFP expressing cells (grey, arrowhead, single colour A’-F’) appear behind the mCherry expressing wavefront (red arrowhead, merge A’-F’), and are capable of dividing (grey, arrow, D). (G) Quantification of the time of appearance of EGFP expressing cells that enter the FOV or by de-novo EGFP expression. (H) Quantification of the rostrocaudal position of EGFP+ cell appearance against its time of appearance. (I) The average number of EGFP+ cells observed over time. (J) Tracking average cell movement of EGFP expressing cells (green) over time in comparison to the mCherry expressing wavefront (red). (K) Quantification of the velocities of cell migration for the mCherry expressing wavefront (dark grey), all EGFP expressing cells (light grey), cells that express EGFP de-novo (vertical stripes) and EGFP expressing cells that enter the FOV (diagonal stripes). (L) Schematic of lineage events observed in the live imaging movies. (M) Quantification of timings of Type A, B and C lineage events occur. (N) Analysis of lineage events in EGFP expressing cells that have entered the FOV and express de-novo EGFP. Type A events (blue), Type B events (red) and Type C events (green). (O) Ratios of timing of EGFP loss after a Type A lineage event between sister cells. (P) Duration between the time of appearance and division in de-novo EGFP+ expressing cells and EGFP+ cells born from division. Time denoted as hh:mm. ns= not significant, *=P<0.05, **P<0.01 ***P<0.001. (G, H, L, N, O, P, Q) Data are given as mean ±SEM. (I and K) data are given as mean ± SD.

4.2.3.1 Proliferative Potential of Her4.1+ Cells Within the Developing Gut

The use of live imaging has provided a unique opportunity to assess the proliferative capacity of Her4.1+ cells during development, and to assess the lineage relationship of their progeny. By tracking individual EGFP expressing cells, we observed that 38.61 ± 4.56% (n=53/132, N=4) of the cells underwent cell division during the recording session. All cell divisions observed were symmetrical giving rise to two EGFP expressing daughter cells (Figure 4.4L). Moreover, in 76.63 ±11.44% (n=26/53, N=4) of the cases, both first generation (1’) daughter cells lost EGFP+ expression at some point during the recording (Type A, Figure 4.4L). In 10.28 ± 7.08% (n=9/53, N=4) of the cases, one of the 1’ daughters lost EGFP expressing expression, while the other cell divided again symmetrically to generate two second-generation (2’) EGFP expressing daughter cells (Type B, Figure 4.4L). In most cases, the 2’ EGFP expressing daughters lost EGFP expression, or the recording ended.
However, in three cases we observed that one 2’ EGFP expressing daughter maintained EGFP expression for some time, while the other 2’ EGFP expressing daughter divided again symmetrically to generate a third-generation (3’) of EGFP expressing daughters. Finally, on rare occasions (4.77 ± 2.76%, n=2/53, N=4) we observed a Type C event (Figure 4.4L), in which after the first symmetric division both 1’ EGFP expressing daughters divided again symmetrically to generate two sets of 2’ EGFP expressing daughters. However, in these cases we were unable to track the 2’ EGFP expressing daughters further, since the recordings ended before EGFP expression was lost, or the 2’ EGFP+ daughters divided again.

We then asked when these lineage events occur over time, by examining the time point of the initial symmetric division. We found that the Type A events occurred at significantly later time points (1443 ± 105.7 mins, n=26, N=4) than Type B events (581.3 ± 95.42 mins, n=8, N=4, P=0.0012, one-way ANOVA, Dunns test, Figure 4.4M). In addition, the Type A events appeared clustered in time, occurring in three waves. Finally, we saw no significant difference between the time of Type C events (1240 ± 270mins, n=2, N=4) and Type A or Type B events (P>0.999 and P=0.56 respectively, one-way ANOVA, Dunns test, Figure 4.4M). However, since there were only two Type C events, we lack appropriate numbers to truly assess the statistical significance.

Next, we examined the proliferative behaviours of the EGFP expressing cells that entered the FOV and those inducing EGFP expression de-novo. We observed that the first group was significantly more likely to undergo a division event (43.18 ± 17.87) than de-novo EGFP expressing cells (29.84 ±15.96%, P=0.0013, Two-way ANOVA, Sidack test, Figure 4.4N). When we further analysed the types of lineage events that occurred (Figure 4.4N), we saw no significant difference between Type A, B or C events when comparing the two groups of EGFP expressing cells (P=0.935, P=0.208 and P0.999, respectively, Two-way ANOVA, Sidack test, Figure 4.4N). Despite this, it is of note that the majority of Type B events were observed in EGFP expressing cells that entered the FOV (n=7/9, N=4), while only a few examples were observed in de-novo EGFP expressing cells (n=2/9, N=4). The lack of statistical significance here may be due to the small sample size, and this would likely become significant with more data.

During our analysis, we observed that EGFP expressing sisters derived from a Type A event often lose EGFP expression at similar time-points. To investigate this
further, we compared the length of time the sister cells maintained EGFP expression. Since the ratio of EGFP duration is close to 1 (1.095 ± 0.063, Figure 4.4O), we suggest that the sister cells born from Type A events undergo a synchronised loss of EGFP expression.

Finally, we hypothesised that by recording the time EGFP expressing cells take to divide, we can predict the cell cycle length during development. We found that de novo EGFP expressing cells divide after approximately 325± 77.46 mins, approximately 5.5 hours (n=22, N=4, Figure 4.4P). In contrast, the time taken for EGFP+ cells born from division, to divide again is significantly slower at 670 ± 66.5 mins, approximately 11 hours (n=16, N=4, P=0.0024, Mann-Whitney test, Figure 4.4P). These data suggest that the time taken for an initial cell division is shorter than any subsequent cell divisions.

4.3 Conclusion

We hypothesised that since mammalian EGCs are thought to be similar to astrocytes of the cortex (Gulbransen and Sharkey, 2012) and since zebrafish lack an equivalent astrocytic population (Grupp et al., 2010), the zebrafish ENS may also lack canonical enteric glia and instead have a more progenitor-like population of non-neuronal cells. In support of this idea, our studies show that Her4.1, a commonly used RGC marker in zebrafish, is expressed in the non-neuronal Sox10 expressing population of the ENS. Further analysis in the adult ENS has allowed us to gain insight into the morphology of adult Her4.1+ cells. The morphological similarities between mammalian EGCs and zebrafish Her4.1+ cells are striking, especially given the difference in ENS organisation, such as the lack of ganglia in the zebrafish ENS. Moreover, our CLEM analysis further confirmed that the Her4.1+ cells share many ultrastructural characteristics with mammalian EGCs such as multi-lobed nuclei, compartmentalisation of axon bundles, and close, but incomplete, association with neuronal somas. However, Her4.1 expressing cells in the zebrafish ENS lack gliofilaments, a characteristic and abundant component of mammalian EGCs. This absence of gliofilaments is consistent with the fact that the Her4.1 expressing population also lack Gfap expression (Chapter 3.2.1), and underscores the differences between the zebrafish and mouse ENS. It is possible that due the relatively simple organisation of the zebrafish ENS (i.e. lack of ganglia), there is less
need for glio-filaments for neuronal structural support (Gabella, 1981). Taken together, these data suggest that Her4.1 is a marker of non-neuronal cells within the zebrafish ENS. Due to the strikingly similar topology (location within smooth muscle layers of the gut and close association with enteric neurons and neuronal bundles) and morphologies of Her4.1 expressing cells relative to mammalian EGCs, we propose that Her4.1 expressing cells are likely to have enteric glial functions. For example, the close association of Her4.1+ cells and neurons indicates that these cells are likely to have functions in neuronal support. From mammalian work, we propose the roles for zebrafish EGCs could include modulating gut inflammation (Gulbransen and Sharkey, 2012, Sharkey and Savidge, 2014) and intestinal motility (Rao et al., 2017, McClain et al., 2014). However, direct examination will be necessary to establish if these functions are carried out by Her4.1 expressing zebrafish EGCs.

We also investigated whether the Her4.1+ population was derived from the group of NC cells that colonise the gut. From static image analysis, we found that sparse weakly labelled Her4.1+ cells are part of the migratory NC cell streams and first arise at around 60hpf. This result confirms that the Her4.1+ population is part of the ENS lineage. This relatively late appearance of Her4.1+ cells can be accounted for by induction via Notch ligands originating from enteric neurons that differentiate at relatively late stages of ENS ontogenesis and are not present when the first eNCCs are invading the fore-gut and establishing themselves within the gut wall. However, it has been reported that in some areas of the PNS and CNS, the Tg(her4.1:EGFP) transgenic line does not induce high levels of EGFP expression and therefore, prolonged her4.1 expression is required for EGFP to be visualised (Yeo et al., 2007). If this is true in the ENS, it is possible that we are missing the true onset of her4.1 expression in early development. Nevertheless, in adulthood we showed that the majority of the non-neuronal cells in the ENS are Her4.1+ cells. This further confirms that Her4.1+ cells are part of the NC cell-derived intestinal cell lineages and suggests the Her4.1+ cells are the glial-like component of the adult ENS. It is intriguing that there is a small non-neuronal population that lacks Tg(her4.1:EGFP) expression. This could be due to the oscillatory nature of her4.1 limiting our detection, or alternatively, the Her4.1+ population could represent another non-neuronal population within the ENS.

The NC cell origin of Her4.1+ population within the gut was also confirmed by live imaging of zebrafish embryos. Interestingly the Her4.1+ cells were found behind
the wavefront of migrating eNCCs, and arose in a rostrocaudal manner that mimics the rostrocaudal wave of neuronal differentiation. This further suggests a possible mechanism for the induction of Her4.1 expression: that neurons are providing the Notch ligands needed to induce Her4.1 expression. Moreover, our imaging analysis suggest that the number of EGFP expressing cells in the imaging window (of Tg(her4.1:EGFP) transgenic embryos) increases until a time where enteric neurons should have colonised the entire window (Olden et al., 2008), indicating a further link between neuronal differentiation and Her4.1 expression. Our recordings stop at approximately 96hpf, at which point there is a large expansion of neurons (Uyttebroek et al., 2010). It would be interesting to determine whether there is a further increase in the Her4.1+ population after 96hpf, which would lead to a new homeostatic level.

Another interesting behaviour of the Her4.1+ population is that they have a limited migratory potential relative to the migratory stream of NC cells that colonise the gut. Given the largely sedentary character of Her4.1+ expressing cells, we argue that they are in an excellent position to form the resident glial or progenitor-like population of the ENS that provide continuous support to enteric neurons or generate progeny to fill in emerging gaps of the ENS as a result of normal gut growth or local injury. However, if the limited migratory behaviour is unique to the Her4.1+ population or is a feature of all ENS cells behind the eNCC wavefront has yet to be determined.

Within the CNS, the Her4.1+ population has a significant progenitor function (Kizil et al., 2012b, Than-Trong and Bally-Cuif, 2015). Likewise, we observe that a large proportion of the enteric Her4.1+ cells are capable of dividing during development. All the Her4.1+ cell divisions examined were symmetric, mimicking what is observed in the murine ENS (Lasrado et al., 2017). This may suggest that conserved mechanisms mediate cell division within the ENS. Additionally, we observed three lineage event patterns during zebrafish ENS development. We hypothesised that the majority of lineage events observed were symmetric neurogenic divisions, since EGFP is lost from both daughter cells. Within the CNS, loss of RGC reporter expression is coincident with neuronal differentiation (Barbosa et al., 2015, Rothenaigner et al., 2011). Moreover, our data suggests that symmetric neurogenic divisions occur in waves reminiscent of the waves of neuronal differentiation within the ENS (Olden et al., 2008, Uyttebroek et al., 2010, Pham et al., 1991, Bergner et al., 2014, Young et al., 2003). It is possible that loss of EGFP signal associated with
Her4.1+ cell progeny could result from cell death within the developing ENS, however there is very little evidence of programmed cell death within the vertebrate ENS (Konstantinidou et al., 2016, Young et al., 2003). Therefore, it is unlikely that cell death explains the loss of EGFP expression observed. To a lesser extent we find that some Her4.1+ lineage events are characterised by prolonged expression of Her4.1 in one daughter, while the other daughter proliferates. Intriguingly, such asymmetric behaviour is mostly observed at early time points and within migratory Her4.1+ cells, suggesting that they are important in seeding the gut with sufficient ENS cell population while it is being colonised by eNCCs. The final lineage event we observed in the Her4.1+ population is characterised by the maintenance of proliferative potential in both daughters. We believe that this form of division is a symmetric progenitor division. Symmetrical progenitor division events rarely occurred, and may be important in expanding the Her4.1+ population, to maintain the ENS in the correct density, especially at particular periods of rapid gut growth. Intriguingly, within the pallium of the CNS, similar types of division have been observed (Barbosa et al., 2015, Rothenaigner et al., 2011, Alunni et al., 2013). Moreover, the most common type of RGC division within the pallium is a neuronal division (Alunni et al., 2013, Rothenaigner et al., 2011, Barbosa et al., 2015), which mimics what we observed in the developing ENS.

Finally, based on our live-imaging data we found that Her4.1+ cells which divide de-novo have a significantly faster cell cycle that those Her4.1+ that undergo a second division. We theorise that differential Notch signalling may impact cell cycle lengths or cellular behaviours, such as division characteristics. This behaviour is also reminiscent of RGCs within the CNS, which enter a resting state following proliferation, but retain the ability to divide at a later point, thus leading to a longer cell cycle (Barbosa et al., 2015, Rothenaigner et al., 2011, Urban et al., 2016).

In conclusion, we have identified Her4.1 as a marker of the non-neuronal population within the zebrafish ENS. In adulthood, Her4.1 expression is maintained and Her4.1 expressing cells form the majority of the non-neuronal ENS population. Due to the striking resemblance of Her4.1+ cells to mammalian EGCs at both light and ultrastructural levels we speculate that the Her4.1+ population within the zebrafish gut has some traditional glial functions. In addition, we have shown that Her4.1+ cells arise within the eNCC population that colonises the gut and are therefore a true component of the zebrafish ENS. During ENS development, the
Her4.1+ cells are heterogeneous and have limited migratory potential during development. Moreover, since Her4.1+ cells are able to divide in a variety of ways, they have a progenitor function, and potentially act as stem cells during ENS development. Finally, since Her4.1 is a marker of RGCs, and because Her4.1 expression is maintained in the adult ENS in the absence of mature glial markers, we speculate that Her4.1+ cells are the EGCs of the zebrafish ENS and could possess a progenitor function into adulthood.
Chapter 5. Examining Her4.1+ Cell Progenitor Potential Within the Zebrafish ENS

5.1 Introduction

We previously identified Her4.1 as a marker for zebrafish EGCs, however it was unclear what roles the Her4.1+ population may have within the zebrafish ENS. From our embryonic analysis, we observed that the Her4.1+ cells can proliferate during development. Moreover, the Her4.1+ cells in the zebrafish CNS represent RGCs, which act as neural progenitors throughout life. Therefore, we wanted to test whether the Her4.1+ cells within the adult ENS are also maintained their progenitor potential.

Within the mammalian ENS, EGCs have been shown to have proliferative potential. Despite discrepancies regarding the scale of proliferation and neurogenesis (Belkind-Gerson et al., 2017, Laranjeira et al., 2011, Joseph et al., 2011, Kabouridis et al., 2015, Kulkarni et al., 2017), all the data suggests that the mammalian EGCs have some proliferative potential. However, it is unclear whether all EGCs have this potential or only a subset, and it is unclear whether there are restricted neurogenic and gliogenic progenitors. Therefore, given the fact that zebrafish exhibit a high turnover of cells in many lineages including the CNS, we reasoned that zebrafish would be an ideal experimental model in which to explore the proliferate potential of EGCs.

NOTCH signalling plays a critical role in the dynamics of RGC proliferation. Inhibition of NOTCH, using γ-secretase inhibitors, which prevent downstream NOTCH signalling, releases RGCs from quiescence and can lead to exhaustion of the stem cell pool (Alunni et al., 2013, Basak and Taylor, 2007). Within the developing ENS, NOTCH signalling, via Hes5, has also been implicated in the function of eNCCs (Charrier and Pilon, 2017, Ngan et al., 2011, Okamura and Saga, 2008, Theocharatos et al., 2013). Therefore, it is feasible that HES5/Her4.1 are important mediators in regulating the proliferative potential of EGCs.

Given the known proliferative potential within adult Her4.1+ RGCs, the known proliferative capacity of adult mammalian EGCs, and our results showing Her4.1+
cells undergo cell divisions during embryogenesis, we hypothesised that adult zebrafish EGCs expressing Her4.1 have proliferative potential.

5.2 Results

5.2.1 Proliferation of Her4.1+ Cells in the Gut of the Adult Zebrafish During Homeostasis

5.2.1.1 Short-term proliferation analysis

To assess the basal rate of proliferation during adult ENS homeostasis, we used antibodies against PCNA and MCM5, two proteins expressed specifically during the cell cycle. PCNA is expressed from late $G_1$ to $G_2$, with peak expression at S phase. Expression of MCM5 largely mimics that of PCNA, but it has a slightly earlier onset in $G_1$ and is less variable. Consequently, MCM5 is thought to be a more reliable marker for detection of proliferating cells (Gonzalez et al., 2005, Dray et al., 2015, Stoeber et al., 2001). Cryosections from the guts of adult $Tg(her4.1:EGFP)$ animals were stained for GFP, HuC/D and MCM5 or PCNA. As expected, MCM5+ and PCNA+ cells were readily detected in the highly proliferative gut epithelium (Figure 5.1A and C) and among the RGCs throughout the brain (Figure 5.1B and D). In contrast, no PCNA+ and only one MCM5+ cell was identified among ~200 EGFP expressing cells analysed (Figure 5.1A), suggesting that the majority of Her4.1+ expressing ENS cells are quiescent. Since, in our hands MCM5 and PCNA antibodies only work on gut sections, and each section includes only a small number of ENS cells, we were able to examine only a relatively small number of Her4.1+ cells.

Another technique used for cell cycle analysis is incorporation of thymidine analogues, such as BrdU or EdU (Yu et al., 2009, Larison and Bremiller, 1990). Thymidine analogues are incorporated into the DNA during S phase, and are maintained in the DNA of the daughter cells, but are increasingly diluted with each subsequent division. Adult zebrafish were injected once with 10µl of BrdU (10mM) or EdU (10mM), into the, intracoelemic space, and culled 3 days later. Guts and brains from these animals were immunostained for GFP and BrdU or EdU in floating sections or whole-mount preparations, respectively. Although, strong BrdU or EdU staining was observed in the epithelial layer of the gut (Figure 5.1E) and the progeny
of RGCs in the brain (Figure 5.1F), we were unable to detect any BrdU+ or EdU+ cells within the EGFP+ cell population of the gut, despite being able to visualise EdU+ cells in whole-mount preparations. Taken together, these data suggest that the majority of her4.1+ cells in the gut of adult zebrafish are quiescent.
### Chapter 5. Results

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**Tg(her4.1:EGFP)**
Figure 5.1. Immunohistochemical analysis of cell cycle markers in adult *Tg(her4.1:EGFP)* transgenics. (A-B) Immunohistochemistry for MCM5 (red, single colour A) in adult *Tg(her4.1:EGFP)* gut (green,) and brain (green) sections, revealed only one GFP+MCM5+ cell within the ENS (arrowhead, A), and the expected high numbers of MCM5+ cells in the intestinal epithelia (open arrowheads, A) and telencephalon (arrow, B). (C-D) Immunohistochemistry for PCNA (red) in adult *Tg(her4.1:EGFP)* gut (green) and brain (green) sections did not identify any GFP+PCNA+ cells in the ENS, although proliferative (PCNA+) cells are detected within the intestinal epithelia (open arrowheads, C) and within the telencephalon (GFP+PCNA+) (arrows, D). (E-F) Following a single injection of 10mM BrdU and 3 day chase, we did not visualise any BrdU+ cells (red, single colour E) within the ENS of *Tg(her4.1:EGFP)* (green), however we did observe BrdU+ cells within the intestinal epithelia (open arrowheads, E) and within the telencephalon (arrows B). All gut sections are from the mid-gut, images are shown with the lumen to the top. The brain sections are from the telencephalon.

5.2.1.2 Long-term proliferation assays in young adults (3-4 months old)

To enhance the sensitivity of our proliferative assay, we modified the EdU labelling protocol to allow for continuous exposure of tissues to EdU over a time period that we estimated would allow for the completion of at least one cell cycle. We therefore maintained 3-4 mo *Tg(her4.1:EGFP)* zebrafish for 3-days in swimming water containing 1mM of EdU (Figure 5.2A). At the end of the 3-day labelling period (pulse), whole-mount gut preparations were immunostained for GFP and HuC/D and subsequently processed for EdU. We observed that in the gut, 7.91 ± 1.18% of EGFP expressing cells were co-labelled with EdU+ (arrowheads Figure 5.2B-D and J) indicating that Her4.1+ cells of the adult zebrafish ENS proliferate under physiological husbandry conditions. Next, we analysed if the rate of proliferation of Her4.1+ cells varied across the length of the gut. We observed no significant difference in the fraction of proliferating EGFP expressing cells (GFP+EdU+/GFP+ cells) along the length of the gut (8.44 ± 2.13 in the fore-gut, 6.453 ± 1.88 in the mid-gut, 7.872± 1.51 in the hind-gut, P=0.284, ANOVA, Figure 5.2K), suggesting that Her4.1+ cells have similar proliferative potential across different gut segments.

The majority of GFP+EdU+ cells were found in doublets, or in rare cases, in small clusters (60.84 ± 3.61, Figure 5.2B-D and M), suggesting that most of the proliferating Her4.1+ have undergone at least one symmetrical division during the 3-
day exposure to EdU. Intriguingly, we observed that in females, EGFP expressing cells are more proliferative (10.15 ± 0.83 %), relative to their male counterparts (5.75 ± 0.53%, P<0.0001, Unpaired T-Test, Figure 5.2L), suggesting sex dependant differences in the ENS of zebrafish during homeostasis.
Figure 5.2. Incubating adult Tg(her4,1:EGFP) zebrafish in 1mM EdU for three days reveals EGFP expressing cells undergoing proliferation. (A) Schematic representation of the experimental design: adult Tg(her4,1:EGFP) zebrafish are incubated in 1mM EdU continuously for 3 days before guts and brains were collected. (B–E) Representative images of the gut (B–D) and pallium (E) of 3-4mo zebrafish immunostained for DAPI (grey), GFP (green), EdU (red), HuC/D (blue). (B–D) In 3-4mo adult zebrafish, the majority of GFP+ cells (green) that take up EdU (red) are found in doublets. Within the doublets, we observe that the level of EGFP expression is
either high in both cells (arrowheads, B and B'), high in one EGFP expressing cell (arrowhead, C and C') and low in the other EGFP expressing cell (open arrowhead, D), or low in both EGFP expressing cells (open arrowhead, D). (E) GFP+EdU+ cells are readily observed in the brain. (F-I) Representative images of the gut (F-H) and brain (I) of 6-7mo zebrafish immunostained for DAPI (grey), GFP (green), EdU (red), HuC/D (blue). (F-H) At 6-7mo the majority of GFP+EDU+ cells are found as individuals in the ENS (arrowheads, F-G), whereas doublets are only occasionally observed (arrowheads, H). (I) GFP+EdU+ cells are readily observed in the brain. (J) Quantification of the GFP+EDU+ population at 3-4mo (dark grey) and 6-7mo (light grey) highlights the fact that the EGFP expressing population is more proliferative at 3-4mo than 6-7mo. (K) Quantification of GFP+EDU+ cells in the fore-, mid- and hind-gut reveals that the proliferation rate is equivalent in all areas and declines from 3-4mo (dark grey) to 6-7mo (light grey). (L) Quantification of sex-dependent differences in the proliferative potential of EGFP expressing cells reveals that females exhibit a higher proliferative rate than males, although proliferative potential decreases equivalently in both males and females with age. (M) Quantification of GFP+EDU+ cell clusters, at 3-4mo to 6-7mo, highlights that the majority of cells at 3-4mo are found in doublets or in small groups, whereas at 6-7mo the GFP+EdU+ cells are mainly found as individual cells. ns= not significant, *=P=0.05, **P<0.01 ***P<0.001. All adult gut images are from the mid-gut region with rostral to the top. Brain images are from the pallium.

5.2.1.3 Analysis of proliferative potential within the Her4.1− non-neuronal cells of young adults (3-4 months old)

We have previously demonstrated that although the Her4.1 population comprises the majority of the non-neuronal lineage in the adult zebrafish ENS, there is a small non-neuronal population that is Her4.1− (Chapter 4.2.2). To assess whether the Her4.1− non-neuronal population within the sox10-derived ENS lineage were also proliferative, we repeated our 3-day EdU incorporation assay on 3-4mo adult Tg(her4.1:EGFP;−4.7sox10cre;βactin-LoxP-STOP-LoxP-hmgb1-mCherry) zebrafish generated as previously described (Chapter 4.2.2). Although the vast majority of non-neuronal ENS cells that incorporated EdU within the Cherry+ sox10-derived lineage were also GFP+ (GFP+HuC/D-Cherry+EdU+/Cherry+ = 0.69 ±0.13%, Figure 5.4B and D), there was a very small number of GFP+HuC/D-Cherry+EdU+ cells (0.059 ±0.03%, Figure 5.4B and D). However, there is no significant difference between the percentage of the her4.1− non-neuronal cells (Cherry+GFP+HuC/D) that had incorporated EdU (0.059 ±0.03%) and the HuC/D+ neurons which had incorporated
EdU (0.009 ± 0.01%, P>0.999, one-way ANOVA, Dunn’s test, Figure 5.4B and D). Therefore, the Her4.1 expressing cell population represents the main proliferative cell type within the adult zebrafish ENS.

In summary, we demonstrate that although the majority of the Her4.1* expressing population in the young adult ENS are quiescent, a fraction are able to undergo symmetrical cell divisions during homeostasis.

### 5.2.1.4 Her4.1* cell proliferation in adults (6-7 months old)

Ageing is known to have an effect on the proliferative potential in many stem cell populations, including that of the RGCs found in the zebrafish CNS (Barbosa et al., 2015). Therefore we examined the proliferation of Her4.1+ cells in older (6-7mo) Tg(her4.1:EGFP) zebrafish using our 3-day EdU incorporation assay. We observed that 2.14 ± 0.84% of the EGFP expressing cells in the gut were labelled by EdU (Figure 5.2F-H and J), significantly fewer in comparison to 3-4mo animals (7.91 ± 1.18%, Figure 5.2J, P=0.0007, Two-way ANOVA, Sidak’s test). The reduction in proliferation of GFP+EdU+ cells was observed in all gut regions (fore-gut, P=0.0041, mid-gut P=0.02, hind-gut P=0.001, Two-way ANOVA, Sidak’s test, Figure 5.2K). No significant difference was detected between the proliferative potential of GFP+EdU+ cells in the fore-gut (3.22 ± 1.10), mid-gut (1.95± 0.75) or hind-gut (1.45 ± 0.79) in 6-7mo zebrafish (P=0.078, One-way ANOVA, Figure 5.2K).

Moreover, we recorded a significant reduction in the number of GFP+EdU+ doublets observed in 6-7mo zebrafish (28.94 ± 13.26%), in comparison to 3-4mo zebrafish (60.84 ± 3.61, P= 0.0086, Two-way ANOVA, Sidak’s test, Figure 5.2M), and we never observed clusters of more than two GFP+EdU+ cells in older animals. This result suggests, once again, that at 6-7mo the Her4.1+ population is less proliferative or has a longer cell cycle. In addition, preliminary experiments indicated no significant difference between the GFP+EdU+ population of male (0.65 ± 0.123%) and female zebrafish (2.89 ± 2.205%, P= 0.133, Mann-Whitney test, Figure 5.2L). Nevertheless, we observed a significant decrease in the percentage of GFP+EdU+ cells labelled in both female (P=0.034, Two-way ANOVA, Sidak’s test) and male (P=0.0019, Two-way ANOVA, Sidak’s test) zebrafish from 3-4mo to 6-7mo (Figure 5.2L), suggesting that the age dependant decline in Her4.1 cell proliferative potential is not sex-specific. Therefore, our experiments indicate that Her4.1+ cells in adults
5.2.2 Generation of New Neurons during Homeostasis from the Her4.1+ Population in the Adult ENS

5.2.2.1 Neurogenesis in the ENS of young adults (3-4 months old)

Next, we examined the fate of the progeny of Her4.1+ cells in adult zebrafish. We first labelled proliferative Her4.1+ cells using an EdU incorporation protocol and used immunohistochemistry with specific markers to determine the identity of their progeny. Groups of 3-4mo Tg(her4.1:EGFP) zebrafish were incubated in EdU for 3 days (pulse) then the EdU was removed and zebrafish were left for a 0, 4, or 11 day chase period, hereafter referred to as t0, t4 and t11, respectively (Figure 5.3A). Whole-mount gut preparations, were then immunostained for GFP and HuC/D, followed by EdU detection.

The percentage of GFP+EdU+ cells relative to the total GFP+ cell population at different time points were as follows t0= 7.91 ± 2.88%; t4= 3.37 ± 2.23%; t11= 3.8 ± 2.72%. Based on this analysis, there was a statistically significant decrease in the GFP+EdU+ population over the chase period (P=0.0239, ANOVA, Figure 5.3B-D and H). Specifically, this indicates that the percentage of GFP+EdU+/GFP+ cells at t4 (Figure 5.3C and H, P=0.0416, ANOVA, Holm-Sidak’s Test) and t11 (P=0.0464, ANOVA, Holm-Sidak’s test, Figure 5.3D and H) are significantly lower relative to t0 (Figure 5.3H). However, there was no further significant decrease in the percentage of GFP+EdU+/GFP+ cells from t4 to t11 (P=0.8005, ANOVA, Holm-Sidak’s test, Figure 5.3H).

The decrease in the percentage of GFP+EdU+ cells could result from differentiation of the population of GFP+ cells into neurons. To examine this possibility, we determined whether EdU was incorporated into enteric neurons during the chase period. While very few HuC/D+EdU+ cells were found at t0, we observed a significant increase in the percentage of HuC/D+EdU+/HuC/D+ cells during the chase period: t0=0.068 ± 0.128%; t4=0.611 ±0.28%; t11=0.729 ± 0.433% (Figure 5.3B-D and K, P=0.0041, One-way ANOVA). Specifically, statistical analysis indicated that the t11 stage is significantly higher relative to t0 (P=0.0125, One-way ANOVA, Dunn’s test, Figure 5.3K). Conversely, no significant difference was observed
between t4 and t0 (P=0.0908, One-way ANOVA, Dunn’s test, Figure 5.3K) or t11 (P>0.999, One-way ANOVA, Dunn’s test, Figure 5.3K). Interestingly, this increase in HuC/D+/EdU+ neurons correlates with the loss of GFP+/EdU+ cells, suggesting that Her4.1+ cells are giving rise to neurons after cell division.
Figure 5.3. Adult neurogenesis in Tg(her4,1:EGFP) zebrafish is revealed after pulse-chase experiments. (A) Schematic of experimental design: 3-4mo or 6-7mo Tg(her4,1:EGFP) zebrafish are exposed to 3 days of 1mM EdU. Then after a 0 day (t0), 4 day (t4) or 11 day (t11) chase period, guts are collected for analysis. (B) In 3-4mo animals, at t0, we observe that the majority of GFP+ EdU+ (yellow) cells are found in doublets. (C) After 4 days chase, we begin to observe HuC/D+ neurons (blue, single colour C’) that have incorporated EdU (red, arrows), which are found close to GFP+EdU+ cells (arrowhead). (D) At t11, we observe an increased number of HuC/D+ EdU+ cells (arrows) and...
GFP*EdU* cells (arrowhead). (E-G) EdU pulse-chase experiments were repeated in 6-7mo animals. (E) At t0, we observe that the majority of GFP* cells (green) that have incorporated EdU (red) are found individually (arrowhead). (F) At t4, increased numbers of GFP*EdU* cells (arrowheads) are observed in doublets. (G) By t11, we observed HuC/D* neurons (blue) that had incorporated EdU* (arrows, red). (H and K) Quantification of GFP*EdU* cells at 3-4 mo (H) and 6-7 mo (K) reveals a significant decrease in double positive cells over the chase period. (I and L) Quantification of HuC/D*EdU* cells at 3-4 mo (I) and 6-7 mo (L) reveals a significant increase in these double positive cells over the chase period. (J) Comparison of the GFP*EdU* population between 3-4 mo (dark grey) and 6-7 mo (light grey) reveals that while there is initial increased rate of proliferation at t0, the percentage of GFP*EdU* becomes equivalent at t4 and t11. (M) Comparing the population of HuC/D*EdU* cells between 3-4 mo (dark grey) and 6-7 mo (light grey) reveals that no HuC/D*EdU* cells are found at t0 at either age, however by t4, 3-4 mo zebrafish have significantly more HuC/D*EdU* neurons than 6-7 mo zebrafish. By t11, this difference is no longer significant. ns= not significant, *=P=0.05, **P<0.01 ***P<0.001. All adult gut images are from the mid-gut region with rostral to the top.

5.2.2.2 Neurogenesis in the adult ENS (6-7 months old)

A similar pulse-chase analysis was carried out in older Tg(her4.1:EGFP) zebrafish (6-7mo), to assess the neurogenic potential in older animals. At 6-7mo, the percentage of GFP*EdU*/GFP* remained constant over all chase periods (t0= 2.14 ± 0.84%; t4= 1.56 ± 0.474; t11= 3.72 ± 1.08) and no significant changes in the proportion of GFP*EdU*/GFP* cells could be observed (P=0.393, ANOVA, Figure 5.3E-G and I). Therefore, the GFP*EdU* population appears to be a more stable population in older 6-7 mo zebrafish. Next, we assessed the neurogenic capacity of the Her4.1* cells in 6-7 mo zebrafish. Similar to our observations at 3-4 mo, at 6-7 mo we observed a significant increase in the percentage of HuC/D*EdU*/HuC/D* cells over the chase period as follows: t0= 0.004 ± 0.067%; t4= 0.11 ± 0.157%; t11=0.389 ± 0.358% (P=0.026, One-way ANOVA, Figure 5.3E-G and I). Specifically, statistical analysis indicated that the percentage of HuC/D*EdU*/HuC/D* cells is significantly higher at t11 than t0 (P=0.033, ANOVA, Dunn’s test, Figure 5.4L). However, no significant difference in the percentage of HuC/D*EdU*/HuC/D* cells could be observed between t4 and t0 (P=0.999, ANOVA, Dunn’s Test, Figure 5.3L) or t11 (P=0.229, ANOVA, Dunn’s Test, Figure 5.3L). These data suggest that in older animals (6-7 mo) there is still active neurogenesis in the ENS.
Subsequently, we compared the proliferative and neurogenic potential between 3-4mo and 6-7mo zebrafish. As previously mentioned, we observed a significant increase in proliferative $\text{GFP}^+\text{EdU}^+/\text{GFP}^+$ cells at t0 between 3-4mo ($7.91\pm 2.88\%$) and 6-7mo ($2.14\pm 0.84\%$) zebrafish ($P=0.0007$, Two-way ANOVA, Sidak’s test, Figure 5.3J). However, there was no significant difference in the percentage of $\text{GFP}^+\text{EdU}^+/\text{GFP}^+$ cells between 3-4mo and 6-7mo zebrafish at t4 ($P=0.497$, Two-way ANOVA, Sidak’s test, Figure 5.3J), suggesting that the $\text{GFP}^+\text{EdU}^+$ cells have reached a similar homeostatic or quiescent level. These cells may represent cells that have returned to quiescence after undergoing proliferation. Next, we assessed the neurogenic potential between 3-4mo and 6-7mo zebrafish. We observed that there was no significant difference in the percentage of $\text{HuC/D}^+\text{EdU}^+/\text{HuC/D}^+$ cells at t0 ($P=0.9988$, Two-way ANOVA, Sidak’s test, Figure 5.3M) and t11 ($P=0.3057$, Two-way ANOVA, Sidak’s test, Figure 5.3M) when comparing 3-4mo and 6-7mo zebrafish. However, at t4 there was a significant increase in the percentage of $\text{HuC/D}^+\text{EdU}^+/\text{HuC/D}^+$ cells at 3-4mo relative to 6-7mo ($P=0.596$, Two-way ANOVA, Sidak’s test, Figure 5.3M). While this difference could reflect the reduced numbers of Her4.1+ cells labelled with EdU at t0 in 6-7mo animals, it could also suggest that the differentiation of neurons is slower in older zebrafish.

In summary, our findings suggest that the enteric Her4.1+ population is neurogenic throughout the adult life of the zebrafish. However, by 6-7mo the neurogenic potential is reduced, which may reflect a more homeostatic level of proliferation.

5.2.2.3 Lineage relationship of Her4.1+ cells and other non-neuronal cells of the ENS.

Our studies suggest that the Her4.1 population gives rise to new neurons during adult homeostasis. However, we wanted determine whether Her4.1+ cells can also give rise to the Her4.1- non-neuronal population. To examine this, we applied our EdU labelling protocol to adult $\text{Tg(her4.1.EGFP};^{-4.7}\text{sox10cre; βactin-LoxP-STOP-LoxP-hmgb1-mCherry)}$ zebrafish and analysed incorporation of EdU into the Her4.1- non-neuronal cells after chase (Figure 5.4A). We observed that the fraction of $\text{GFP}\cdot\text{Cherry}^+\text{HuC/D}^+\text{EdU}^+/\text{Cherry}^+$ cells at t4 ($0.612\pm 0.140\%$) was significantly
higher than those observed at t0 (0.059 ± 0.003%, P=0.0151, P=0.001, Mann-Whitney Test, Figure 5.4C and D), which suggests that Her4.1+ cells give rise to the Her4.1− non-neuronal cells. Moreover, the increase in the GFP+Cherry+HuC/D+ EdU+/Cherry+ non-neuronal cells (0.611 ± 0.134%) is significantly greater than the increase in Cherry+HuC/D+EdU+/Cherry+ neurons (0.1609 ± 0.045%) at t4 chase (P=0.0146, Two-way ANOVA, Sidak’s test, Figure 5.4C and D). Since HuC/D is a mature neuronal marker and EGFP has a limited perdurance (Dray et al., 2015), it is possible that GFP+HuC/D− cells correspond to cells transitioning between a Her4+ state and a HuC/D+ state. However, the relationship of the Her4.1− non-neuronal population and newborn neurons remains unknown.

Figure 5.4. Analysis of Tg(her4.1:EGFP;−4.7sox10cre;βactin-LoxP-STOP-LoxP-hmgb1-mCherry) zebrafish reveals that Her4.1+ cells are the main proliferative population within the ENS. (A) Schematic of experimental design: 3-4mo Tg(her4.1:EGFP;−4.7sox10cre;βactin-LoxP-STOP-LoxP-hmgb1-mCherry) zebrafish are exposed to 3days of 1mM EdU. Then after a 0 day (t0) or 4 day (t4) chase period guts are collected for analysis. While the Tg(-
4.7sox10cre;βactin-LoxP-STOP-LoxP-hmgb1-mCherry) line labels the majority of the ENS, it is not completely efficient, therefore some HuC/D+ neurons are not mCherry+. (B) At 0d chase, the majority ENS cells that have taken up EdU+ (blue) are EGFP+ cells (green) that are within the Cherry lineage (red, arrowheads). (C) After 4 days chase, we observe an increase in the Cherry-GFP-EdU+ cells (open arrowheads). All adult gut images are from the mid-gut region with rostral to the top. (D) Quantification of ENS populations (mCherry-GFP-EdU+ (green), mCherry-GFP-HuC/D-EdU+ (red) and mCherry-HuC/D-EdU+ (blue)) that have taken up EdU at 0d chase and after 4 days chase.

### 5.2.2.4 Clustering of newborn cells in the ENS of young adults (3-4 months old).

Analysis of the adult Tg(her4.1:EGFP) zebrafish exposed to EdU for 3 days revealed that GFP*EdU+ cells appeared clustered next to other GFP*EdU+ and/or HuC/D*EdU+ cells (hereafter referred to as ENS*EDU+ cells) at all chase time-points. To determine whether this apparent clustering was genuine, we used Monte-Carlo simulations to further explore the relative distribution of GFP*EdU+ cells and GFP*ENS+ cells (in collaboration with Carmen Pinn). The density of the different cell types around a cell of interest was calculated over a range of increasing radial areas (Figure 5.5A). These observed (experimental) densities were compared to a simulated model that represents the null hypothesis, according to which cells are distributed uniformly independently of their identity, across the range of areas. To generate the null hypothesis, the number of recorded cells for a given analysis is fed through a Monte Carlo simulation to generate uniformly distributed cells, from which simulated densities can be calculated (see methods, Chapter 2.6.1). Therefore, for each given analysis, the density of cells (shown below as N cells/0.001mm²) calculated in the null hypothesis varies, reflecting the number of cells recorded in the corresponding experimental data. 90% confidence intervals are calculated for each radius and time point in the relevant figures. This simulated data was then compared to our observed experimental data. If our experimental densities are higher at areas close to the cell type of interest, this would indicate a specific clustering suggestive of a lineage relationship.

Initially, we examined the relationship of different cell types around GFP*EdU+, cells in 3-4mo zebrafish, to assess possible clustering. (Figure 5.5B-D).
Our simulated null hypothesis predicts that GFP\(^+\)EdU\(^+\) cells around other GFP\(^+\)EdU\(^+\) cells would have a density of 0.023 cells at all areas, throughout the chase period (Figure 5.5B-D). Interestingly, at areas with a radius of 20-60\(\mu\)m our experimental data recorded significantly higher GFP\(^+\)EdU\(^+\) densities (0.05-0.2 cells Figure 5.5B-D), indicating that proliferative GFP\(^+\) cells are not distributed homogeneously. Moreover, since significance was only found at areas with a radius 20-60\(\mu\)m, this suggests that cells are clustered closely together within several cell diameters. This analysis indicates a close association between GFP\(^+\)EdU\(^+\) cells, consistent with their origin from a common progenitor.

We next examined the relationship of newborn neurons (HuC/D\(^+\)EdU\(^+\)), to GFP\(^+\)EdU\(^+\). At 0 days chase, only very few EdU labelled neurons were present and analysis could not be reliably performed. However, at 4 and 11 days chase, we observed that HuC/D\(^+\)EdU\(^+\) cells have significantly higher experimental densities (0.02-0.05 cells/0.001mm\(^2\)) around GFP\(^+\)EdU\(^+\) cells than that predicted by the null hypothesis (0.018 cells), at areas with a radius of 20-50\(\mu\)m (Figure 5.5C' and 5.5D'). Therefore, HuC/D\(^+\)EdU\(^+\) cells are not distributed homogeneously with respect to GFP\(^+\)EdU\(^+\) cells and are instead clustered around these cells within an area with a radius of 20-50\(\mu\)m, which corresponds to approximately 2-5 cell diameters. Again this indicates a potential lineage relationship between GFP\(^+\)EdU\(^+\) cells and EdU labelled neurons. In contrast, we found no significant differences between our simulated null hypothesis and experimental observations when we examined the association of GFP\(^+\)EdU\(^+\) cells to non-proliferative ENS cells: HuC/D\(^+\)EdU\(^-\) (1.02 cells vs 1.08 cells) or GFP\(^+\)EdU\(^-\) (0.36 cells vs 0.36 cells) cells or cells which had incorporated EdU within the muscle layer, ENS-EdU\(^+\), (0.18 cells vs 0.18 cells) at any area or time examined (Appendix Chapter 8.6, Figure 8.4). Together these data show, that in the ENS of 3-4mo zebrafish, ENS cells that have incorporated EdU are specifically clustered near other GFP\(^+\)EdU\(^+\) cells and HuC/D\(^+\)EdU\(^+\) cells (Figure 5.5B-D), suggesting that Her4.1\(^+\) cells give rise to other Her4.1\(^+\) cells and to neurons.

An alternative method to explore the relationship between Her4.1\(^+\) cells and neurons is to anchor the analysis to newborn neurons, HuC/D\(^+\)EdU\(^+\). Because only very few HuC/D\(^+\)EdU\(^+\) cells were visualised at 0 days chase (n=3), our analysis was restricted to 4 and 11 days chase. At 3-4mo, GFP\(^+\)EdU\(^+\) were found at significantly higher densities (0.04-0.13 cells) around newborn neurons, than expected from our simulated null hypothesis (0.018 cells) at areas with a radius between 20-30\(\mu\)m.
(Figure 5.5H and I). These data suggest that EGFP+EDU+ cells are not distributed randomly around the HuC/D+EdU+ population. Similarly, we found that newborn neurons were found at higher densities (0.011-0.045 cells) relative to other newborn neurons than predicted from our simulated null hypothesis (0.01 cells, Figure 5.5H’ and I’). This non-homogeneous distribution is observed at areas with a radius of 20-30 µm, indicating that clustering of newly born neurons occurs within a few cell diameters. Newborn neurons show no evidence of clustering with other cell populations within the gut wall, since the experimental and simulated null hypotheses are not significantly different: GFP+EdU- (0.38 cells vs 0.34 cells, Appendix Chapter 8.6, Figure 8.4), ENS+EdU+ (0.17 cells vs 0.16 cells, Appendix Chapter 8.6, Figure 8.4), and HuC/D+EdU- (1.07 cells vs 1.08 cells, Appendix Chapter 8.6, Figure 8.4) In summary, these data show that newly born neurons are clustered with other EdU+ENS+ cells and, therefore, it is likely, that these cells are lineage related.
Chapter 5. Results

A. **HuC/D** • **GFP** • **EdU**

A'.

**3mo**

B. **GFP**<sup>+</sup>**EdU**<sup>+</sup> Cells

E. **GFP**<sup>+</sup>**EdU**<sup>+</sup> Cells

**6mo**

C. 0d Chase

C'. 0d Chase

D. 4d Chase

D'. 4d Chase

F. **HuC/D**<sup>+</sup>**EdU**<sup>+</sup> Cells

F'. **HuC/D**<sup>+</sup>**EdU**<sup>+</sup> Cells

G. 11d Chase

G'. 11d Chase

H. **GFP**<sup>+</sup>**EdU**<sup>+</sup> Cells

J. **HuC/D**<sup>+</sup>**EdU**<sup>+</sup> Cells

I. **HuC/D**<sup>+</sup>**EdU**<sup>+</sup> Cells
Figure 5.5. Mathematical modelling reveals that the EdU$^+$ cells within the ENS are significantly closer to each other than expected from the simulated null hypothesis. (A) Schematic model of experimental design, where the density of the cells is calculated from increasingly large areas around a cell of interest. (A') High magnification of box in (A) highlights an area with a radius of 40µm in yellow. (B-G) At all time-points analysed, modelling reveals that the experimental densities (red) of GFP$^+$EdU$^+$ cells (B, C, D, E, F, G) and HuC/D$^+$EdU$^+$ cells (C', D', F', G') are significant higher around GFP$^+$EdU$^+$ cells than expected from simulated data (blue) at 3-4mo (A-D) and 6-7mo (E-G). (H-K) At all time-points analysed, modelling reveals that the experimental densities (red) of GFP$^+$EdU$^+$ cells (H, I, J, K) and HuC/D$^+$EdU$^+$ cells (H', I', J', K') are significantly higher around HuC/D$^+$EdU$^+$ cells than expected from simulated data (blue) at 3-4mo (H-I) and 6-7mo (J-K). Areas with a significant radius are denoted with an asterisk, $P=0.05$, error bars represent 90% confidence intervals.

5.2.2.5 Clustering of newborn cells in the adult ENS (6-7 months old).

Our analysis provides evidence for a proliferating Her4.1$^+$ population that gives rise to ENS neurons in 3-4mo zebrafish. To test whether this population is maintained in older fish (6-7mo), we repeated the Monte-Carlo simulation with the data generated from 6-7mo animals. We found that GFP$^+$EDU$^+$ cells were at significantly higher densities (0.02-1.9 cells) around GFP$^+$EDU$^+$ cells than predicted by our simulated null hypothesis (0.006 cells), at areas with a radius of 20-100µm (Figure 5.5E-G). This clustering is indicative of a lineage relationship. Similarly, at 4 and 11days chase, the experimental densities (0.01-0.07 cells) of HuC/D$^+$EdU$^+$ cells around GFP$^+$EdU$^+$ cells were significantly higher than the simulated null hypothesis (0.005 cells), at areas with a radius of 20-60µm (Figure 5.6F'-G'). Therefore, newly born neurons exhibit clustering around GFP$^+$EdU$^+$ cells. Conversely, experimental densities from GFP$^+$EdU$^+$ (0.22 cells), ENS$^+$EdU$^+$ (0.16 cells), and HuC/D$^+$EdU$^+$ cells (0.76 cells) did not show any significant difference to the simulated null hypotheses (0.21, 0.2, 0.81 respectively), at any areas or time points examined, and therefore show no evidence of clustering (Appendix Chapter 8.6, Figure 8.4). Taken together, these data suggest that in older animals, GFP$^+$EdU$^+$ cells are clustered relative to other GFP$^+$EdU$^+$ cells and newborn neurons, and are likely to be derived from the same Her4.1$^+$ progenitor.
Finally, as a complimentary strategy, the potential relationship between Her4.1+ cells and newly born neurons in 6-7 mo zebrafish was explored by focusing the analysis on HuC/D*EDU+. This analysis shows that the experimental densities of EGFP*EDU+ cells around newborn neurons (0.004-0.1 cells) were significantly higher than our simulated null hypothesis (0.003 cells, Figure 5.5J-K), at a radius of 20-80 µm. Therefore, GFP*EDU+ cells are clustered around HuC/D*EDU+ cells. Likewise, we found the experimental densities of HuC/D*EDU+ cells, around other newly born neurons were significantly higher (0.01-0.2 cells) than the simulated null hypothesis (0.002 cells), in areas which had a radius of 20-80 µm (Figure 5.5J-‘K’). Hence, newborn neurons are clustered together. Conversely, we do not find any evidence of clustering between newborn neurons with other cell types within the gut wall, since our experimental densities and simulated densities are not significantly different: GFP*EdU- cells (0.18 cells vs 0.17 cells, Figure 8.4, Appendix Chapter 8.6), ENS*EdU+ cells (0.15 cells vs 0.14 cells, Appendix chapter 8.6, Figure 8.4), and HuC/D*EdU- cells (0.92 cells vs 0.88 cells, Appendix Chapter 8.6, Figure 8.4). Consequently, at 6-7mo we find that newborn neurons are clustered together, and with GFP*EdU+ cells. This finding suggests that in older zebrafish HuC/D*EdU+ cells have a lineage relationship with ENS*EdU+ cells. This is consistent with a model in which proliferating Her4.1+ cells give rise to enteric neurons.

Intriguingly, significantly higher densities of ENS*EdU+ cells are found at greater distances from the HuC/D*EdU+ cell of interest at 6-7mo than at 3-4mo (80µm vs 30µm). While this increased significance could be due to noise in the system, from the relatively small clusters found in older zebrafish (6-7mo) it could also suggest changes in cell dynamics or tissue environment with increasing age. Finally, at both 3-4mo and 6-7mo, clusters centred around non-neuronal, GFP*EdU+, cells span larger areas (<60µm and <100µm) when compared with those centred around neuronal clusters (HuC/D*EdU+, <30µm and <80µm). Since, neurons are known to be non-migratory (Hao et al., 2009), this may explain in the smaller areas of clusters containing HuC/D*EdU+ cells.

In conclusion, Monte-Carlo analysis of cell densities provides clear evidence that proliferative Her4.1+ cells are clustered with ENS*EdU+ cells (from being proliferative, or arising from a proliferative cell). Complimentary analysis shows that newborn neurons are clustered with proliferative Her4.1+ cells and other newborn neurons. This evidence strengthens our current model that Her4.1+ cells of the ENS
function as progenitor cells capable of giving rise to more Her4.1+ cells and to neurons.

5.2.2.6 Proliferation and differentiation rates in Her4.1+ cells

The Monte-Carlo simulation model suggests that the Her4.1+ population gives rise to neurons in the adult ENS during homeostasis. However, it is unclear whether neurons were the only progeny of the Her4.1+ population and what were the dynamics of proliferation and differentiation were. Therefore, Carmen used a stochastic birth vs differentiation model, to distinguish rates of differentiation and proliferation within the adult ENS (Figure 5.6A). Because we see clusters of GFP+EdU+ and HuC/D+EdU+ cells, we assessed the probability that the composition of the clusters would change over time. At 3-4 mo we observed that the differentiation rate, δ, which is calculated by assessing the probability that a GFP+EdU+ cell within a cluster will differentiate into a HuC/D+EdU+ cell, is faster than the generation of a new GFP+EdU+ cell, λ, calculated by assessing the probability that a GFP+EdU+ cell within a cluster will divide and generate a new GFP+EdU+ cell (Figure 5.6B and D, λ - δ = -0.02). Through quantifying the probability of a cell state change over time, we can predict that every 29 days a GFP+EdU+ cell will divide and generate itself, whereas a HuC/D+EdU+ neuron will differentiate every 20 days. In contrast, by 6-7 mo, the probability of a GFP+EdU+ cell differentiating to generate a HuC/D+EdU+ neuron, δ, or dividing to generate a GFP+EdU+ cell, λ, are now equal (Figure 5.6C and E, λ - δ = -0.0081). Therefore, we can calculate that GFP+EdU+ cells in the 6-7 mo zebrafish ENS, proliferate to generate a GFP+EdU+ cell every 30 days and differentiate into a HuC/D+EdU+ cell every 30 days. Since, proliferation and differentiation are equal, this suggests the ENS at 6-7 mo has reached a homeostatic state (Figure 5.6D). The decline in GFP+EdU+ cell proliferative potential observed at 6mo, is in agreement with our EdU labelling analysis where we observe a reduction in Her4.1+ cell proliferative capacity within the ENS at 6-7 mo.

Since differentiation exceeds proliferation in 3-4 mo, zebrafish this would suggest that the Her4.1+ population would be depleted and the number of neurons increased by 6-7 mo. We found that, indeed, there is a significant decrease in the percentage of GFP+ cells from 3-4 mo (28.81 ± 2.48%) to 6-7 mo (20.42 ± 2.00%) within the ENS (Figure 5.6F, P=0.0147, Two-way ANOVA, Sidak’s test). In addition,
there is a significant increase in the HuC/D positive neuronal population from 3-4mo (71.19 ± 2.48%) to 6-7mo (79.59 ± 2.00%, Figure 5.6F, P=0.0147, Two-way ANOVA, Sidak’s test). In contrast we see no significant difference in the EdU population which is labelled within the muscle layer between 3-4mo (14.15 ± 1.87%) and 6-7mo (12.06 ± 1.31%) zebrafish (Figure 5.6F P=0.8559, Two-way ANOVA, Sidak’s test), suggesting that there is a specific shift in the cellular populations of the ENS, and not merely a general change in all cell populations due to ageing.
Figure 5.6. Modelling of proliferation and neuronal differentiation reveals that the Her4.1+ population generates either Her4.1+ cells or neurons. (A) Schematic of mathematical modelling highlighting the possible steps of proliferation, $\lambda$, generating a GFP+EdU+ cell, or differentiation, $\delta$, generating a HuC/D+EdU+ cell from a single GFP+EdU+ progenitor over time. (B) Graph of proliferation-differentiation, $\lambda-\delta$, at 3-4mo highlights that differentiation occurs at a greater rate than proliferation. (C) Graph of proliferation-differentiation, $\lambda-\delta$, at 6-7mo highlights that differentiation and proliferation are now equal. (D-E) Probabilities of the composition of GFP+EdU+ and HuC/D+GFP+ clusters changing over time, at 3-4 mo (C) and 6-7 mo (F). Quantification of changes in the ENS populations over time reveals that the GFP+ population decreases and the HuC/D+ population increases from 3-4mo to 6-7mo, while no significant difference is observed in the EdU+ population within the muscle layers. ns= not significant, *=P=0.05.

5.2.2.7 Embryonic lineage analysis confirms Her4.1+ cells have progenitor and neurogenic potential

To independently assess the capacity of Her4.1+ cells to proliferate and generate new neurons, we performed lineage analysis with the Tg(her4.1ERT2creERT2;\beta actin-LoxP-STOP-LoxP-hmgb1-mCherry) line. Initially we carried out lineage analysis at embryonic stages by incubating zebrafish embryos with 10mM 4-OHT for 24 hours between 1-2dpf and subsequently analysed the zebrafish at adult stages (Figure 5.7A). At adult stages, we occasionally observe small clusters of mCherry expressing cells within the ENS (Figure 5.7B), suggesting they arose from a common progenitor. Interestingly, within the mCherry expressing clusters, the majority of cells are neurons (Figure 5.7B-D), confirming that Her4.1 expressing cells are able to generate new neurons. The fact that clusters are observed indicates that the progeny derived from Her4.1+ progenitors have a restricted spatial migration during development. Moreover, this observation suggests that within a cluster, all the cells originated from a common progenitor.

Unfortunately, when inducing Cre expression with 4-OHT incubation or intracolemic injection in adult animals, although we always achieved the expected Cre recombination within the brain, we were unable to detect reporter recombination in the adult gut. We propose that low her4.1 levels within the gut, relative to the CNS, results in insufficient Cre recombinase induction and failure to recombine the
Therefore, due to current technical limitations, the neurogenic potential of Her4.1+ cells cannot be assessed directly.

![Schematic diagram](image)

**Figure 5.7.** Analysis of Tg(her4.1ERT2creERT2;βactin-LoxP-STOP-LoxP-hmgb1-mCherry) confirms that neurons are derived from larval Her4.1+ progenitors. (A) Schematic of experimental design: Tg(her4.1ERT2creERT2;βactin-LoxP-STOP-LoxP-hmgb1-mCherry) embryos were incubated with 10mM 4-OHT in E3 media for 24 hours from 1dpf to 2dpf to lineage label cells expressing her4.1 at this early time window. Animals were analysed at adult stages (>3mo) (B) Low magnification image of a whole-mount gut preparation showing local restriction of a Cherry+ (red) clone. (C) Inset of boxed area in (B) reveals that the majority of Cherry+ cells are HuC/D+ neurons (green). Adult gut images are from the hind-gut region with rostral to the top (D) Quantification of the percentage of Cherry cells that are HuC/D+ neurons or Cherry only, highlighting that the majority of labelled cells are neuronal.
5.2.3 Effects of Notch Inhibition on the Zebrafish ENS

5.2.3.1 Her4.1 is Downstream of Notch Signalling Within the Zebrafish ENS

Her4.1 is a target of Notch signalling that has been implicated, within the CNS, in the regulation of RGCs quiescence (Alunni et al., 2013). We therefore examined whether the proliferative potential of ENS Her4.1+ cells was also regulated by Notch signalling. For this analysis, we inhibited Notch signalling using the γ-secretase inhibitor, LY411575, which prevents cleavage of the NICD and therefore prevents any downstream Notch signalling. Tg(her4.1:EGFP) embryos were incubated with 100µM LY411575 for 24 hours from 3dpf to 4dpf, after which we performed immunohistochemistry for GFP and HuC/D. We observed loss of GFP signal within the gut in LY411575 treated embryos (Figure 5.9B and L) relative to DMSO controls (Figure 5.8A and L). This confirms that Notch signalling is required for Her4.1 expression and shows that Her4.1 is downstream of Notch signalling. Surprisingly, treatment with LY411575 did not change the number of HuC/D+ neurons (Figure 5.8L). We did, however, observe an apparent increase in neuronal density, which might reflect a change in ENS organisation, and possibly in neuronal numbers themselves (Figure 5.8A and L). It is worth noting that 3dpf embryos treated with LY411575 show growth defects and developmental delay. Despite the fact the gut is already colonised by eNCCs, such delay could influence the timing of ENS neuronal differentiation and thus confound our analysis. In this case performing the analysis at alternate time-points might circumvent this problem.

We also assessed the contribution of Notch signalling on Tg(her4.1:EGFP) expression at adult stages. Incubation of Tg(her4.1:EGFP) adults with 10µM LY411575 for 7 days decreased the GFP signal in the ENS (Figure 5.8D), when compared with DMSO treated controls (Figure 5.8C). Therefore, Her4.1 is downstream of Notch signalling at embryonic and adult stages, and active Notch signalling in the adult ENS is required for Tg(her4.1:EGFP) transgene expression throughout life.
Figure 5.8. Notch inhibition with the γ-secretase inhibitor LY411575 in *Tg(her4.1:EGFP)* larvae and adults reduces EGFP expression levels. (A-B) 3dpf *Tg(her4.1:EGFP)* larvae are treated with DMSO (A) or 100µM LY411575 (B) for 24hpf, and analysed at 4dpf for EGFP expression (green) and HuC/D (red). (A) In DMSO controls, GFP+ cells (green, arrowheads) are clearly visible within the gut, and are associated with HuC/D+ neurons (red). (B) When larvae are treated with 100µM LY411575, no GFP+ cells (green) are visible within the gut despite still observing HuC/D+ neurons. (C-D) Adult (>3mo) *Tg(her4.1:EGFP)* transgenics are treated with DMSO (C) or 10µM LY411575 (D) for 7dpf and analysed for EGFP expression (green) and HuC/D (red). (C) DMSO controls show abundant GFP+ cells (green, arrowheads) situated amongst HuC/D+ neurons (red). (D) LY411575 treated animals show a substantial reduction in GFP+ cells (green), despite HuC/D neurons (red) being found throughout the gut. (E) Quantification of the composition of the ENS in DMSO (blue) and LY411575 conditions (red) in 4dpf animals. All larval images are shown with rostral to the left, whereas all adult gut images are from the mid-gut region with rostral to the top. The gut in larval animals is highlighted with dotted outlines. All adult gut images are from the mid-gut region, with rostral to the top.

5.2.3.2 Notch Inhibition Enhances Non-Neuronal Cell Proliferation

Throughout our previous experiments, we have used the *Tg(her4.1:EGFP)* transgenic line as a tool to identify the novel non-neuronal population of the ENS (Her4.1+ cells). However, since Notch inhibition leads to loss of EGFP expression within these cells, we require an alternate method to visualise our cells of interest. For this reason, we have performed subsequent Notch inhibition experiments using adult *Tg(-4.7sox10cre;βactin-LoxP-STOP-LoxP-hmgb1-mCherry)* zebrafish, of which the Her4.1+ population makes up the majority of the non-neuronal lineage (Chapter 4.2.2). Adult fish were incubated for 7 days with 10µM of the γ-secretase inhibitor LY411575, or DMSO, and with 1mM EdU for the final 3 days (Figure 5.9A). We stained whole-mount gut preparations, from 3-4mo or 6-7mo adult zebrafish for HuC/D, Cherry and EdU to assess the effect of Notch inhibition on the ENS. In both 3-4mo and 6-7mo, LY411575 treated zebrafish showed a significant increase in the non-neuronal population (Cherry+HuC/D- cells) of the ENS that had incorporated EdU. Specifically, at 3-4mo we observed an increase in the Cherry+HuC/D-EdU+ cells, which comprise only 0.085 ± 0.085% of the non-neuronal cells in the DMSO control, but make up to 14.84± 4.10% of the non-neuronal cells in LY41155 treated zebrafish (P=0.0114, Unpaired T-Test, Figure 5.9B-D and H). Similarly, at 6-7mo, LY411575 treatment led to an increase in EdU incorporation in non-neuronal cells.
(Cherry⁺HuC/D⁺) cells: 7.814 ± 0.94% as compared to 1.05 ± 0.38% in DMSO controls (P=0.0005, Unpaired T-Test, Figure 5.9E-G and I). These results suggest that Notch normally maintains the non-neuronal population in a quiescent state, and upon release from Notch signalling, non-neuronal cells undergo proliferation. Moreover, since we see very little proliferation in the DMSO control condition, this suggests that Notch inhibition recruits non-neuronal cells into the cell cycle.

Interestingly, Notch inhibition also resulted in an increase in the percentage of HuC/D⁺EdU⁺/HuC/D⁺ in the LY411575 treated animals relative to DMSO controls. At 3-4mo, we observed there were essentially no HuC/D⁺EdU⁺ neurons in the DMSO control condition at the end of EdU incubation (0.059 ± 0.046%, Figure 5.9E and J). However, after inhibition of Notch signalling, there was an increase in the HuC/D⁺EdU⁺ population (0.533 ± 0.21%, P=0.069, Unpaired T-test, Figure 5.9D and J). At 6-7mo, we were also unable to observe HuC/D⁺EdU⁺ cells in DMSO control conditions after EdU incubation (0.098 ± 0.013%, Figure 5.9E and K). However, after Notch inhibition with LY411575, we observed a significant increase in the HuC/D⁺EdU⁺ population (1.06 ± 0.175, P= 0.0015, Unpaired T-test, Figure 5.9G and K). Therefore, we hypothesised that neuronal differentiation is also enhanced after Notch inhibition.

In conclusion, we have confirmed that the Her4.1 expression in the non-neuronal cells of the ENS is regulated and maintained by active Notch signalling. Moreover, inhibition of Notch signalling shows that Notch signalling is required to maintain the non-neuronal population of the ENS in quiescence. In addition, active Notch signalling prevents neuronal differentiation within the non-neuronal population, thus promoting maintenance of the progenitor fate.
Figure 5.9. Inhibition of Notch signalling with the γ-secretase inhibitor LY411575 releases the non-neuronal ENS population from quiescence. (A) Schematic of experimental design to assess the effect of Notch inhibition on proliferation rate: adult Tg(-4.7sox10cre;βactin-LoxP-STOP-LoxP-hmgb1-mCherry) zebrafish are immersed in 10μM LY411575 or 0.04%DMSO for 7 days. EdU was added for the final 3 days to label cells undergoing proliferation. While the Tg(-4.7sox10cre;βactin-LoxP-STOP-LoxP-hmgb1-mCherry) line labels the majority of the ENS, it is
not completely efficient. Therefore, some HuC/D+ neurons are not Cherry+. (B-D) 3-4mo Tg(-4.7sox10cre;βactin-LoxP-STOP-LoxP-hmgb1-mCherry) were treated with DMSO (B) or 10µm LY411575 (C-D). (B) In DMSO controls, there are few non-neuronal cells (Cherry+HuC/D- (red)) and HuC/D+ neurons (green) that have incorporated EdU (blue, arrowheads). (C-D) In LY411575 conditions, however, there is an increase in the Cherry+HuC/D+EdU+ cells (arrowheads, C) and HuC/D+EdU+ cells (arrows, D). (E-G) 6-7mo Tg(-4.7sox10cre;βactin-LoxP-STOP-LoxP-hmgb1-mCherry) animals were treated with DMSO (E) or 10µm LY411575 (F-G). (E) Very few non-neuronal cells Cherry+HuC/D- (red)) and HuC/D+ neurons (green) had incorporated EdU (blue, arrowheads). (F-G) In LY411575 conditions, there is an increase in the Cherry+HuC/D+EdU+ cells (arrowheads, F) and HuC/D+EdU+ (arrows, G). (H-I) Quantification of the increase in the non-neuronal population (H) and HuC/D+ neurons (I) that have incorporated EdU in DMSO (blue) and 10µm LY411575 treated (red) zebrafish at 3-4 mo. (J-K) Quantification of the increase in the non-neuronal population (J) and HuC/D+ neurons (K) that had incorporated EdU in DMSO (blue) and 10µm LY411575 treated (red) zebrafish at 6-7 mo. ns= not significant, *=P<0.05, **P<0.01 ***P<0.001. All adult gut images are from the mid-gut region with rostral to the top.

5.3 Conclusion.

Notch signalling and its downstream mediators, including Her4.1, have been implicated in the maintenance of progenitor characteristics of neural stem cells and RGCs in vertebrates. Consistent with this view, we found that expression of the Notch target Her4.1 in the non-neuronal population of the zebrafish ENS was also associated with progenitor behaviour. Specifically, we observed that approximately 8% of Her4.1 expressing cells of the 3-4mo zebrafish incorporate the nucleotide analogue EdU following a continuous 3 day exposure EdU. This indicates that the rate of proliferation of Her4.1+ cells in the zebrafish ENS is considerably lower relative to that observed for RGCs in the telencephalon, where approximately 8-10% of the RGCs are proliferating at any one time, and proliferation in these cells is detected by short EdU exposure (Than-Trong and Bally-Cuif, 2015, Adolf et al., 2006, Marz et al., 2011). Moreover, whether all Her4.1+ cells have the ability to proliferate, or whether this property is restricted to a specific subpopulation, remains to be determined directly. In the ENS of adult mammals most studies have identified only a very small number of EGCs that express cell cycle markers or incorporate BrdU following a short pulse (Laranjeira et al., 2011, Joseph et al., 2011, Belkind-Gerson
et al., 2017). In fact, identification of cycling EGCs in adult mice requires continuous exposure to BrdU for several weeks (Joseph et al., 2011), further supporting the idea that proliferation of non-neuronal cells in the ENS of vertebrates during homeostatic conditions is generally low. This low rate of proliferation is not unique to the ENS, and has been observed in other peripheral glial cells such as Schwann cells, which are largely quiescent during homeostasis (Stierli et al., 2018). Investigations into the molecular pathways, and signalling molecules required to maintain EGCs in a quiescent state will raise the possibility of having molecular control over EGC activation.

Although at 3-4mo zebrafish have reached breeding age, they can be still considered to be young adults. Therefore, we analysed the proliferation of Her4.1+ ENS cells in older adults, at 6-7mo, and observed a significant reduction in proliferative potential (from ~8% to ~2% of GFP+ cells), suggesting that ageing alters the dynamics and equilibrium of teleost ENS cell populations. Further investigation is required to determine whether this results from a reduction in proliferative potential of all Her4.1+ cells or the loss of a dedicated ENS progenitor population within the Her4.1 expressing population. While the effects of ageing on the proliferative potential of enteric glia has yet to be studied in mammals, loss of enteric neurons and glia has been associated with ageing (Phillips et al., 2004; Saffrey, 2013; Becker et al., 2018). In addition, decrease in intestinal motility in aged mice, has been associated with a reduction in glial cell communication (McClain et al., 2014). The decline in the Her4.1+ cell proliferative potential observed in the ENS of older zebrafish mimics the reduced proliferation of RGCs in the zebrafish CNS (Barbosa et al., 2015; Edelmann et al., 2013). This data could suggest that there is a depletion of the neural stem cell pool with age, however currently there is conflicting evidence as to whether there is a loss of stem cells or simply an increase in their quiescence (Edelmann et al., 2013; Barbosa et al., 2015).

In our zebrafish experiments, the majority of Her4.1+EdU+ cells observed in the adult ENS after 3 days of EdU incubation formed doublets or small groups, suggesting that these cells proliferate by symmetrical cell division. Similarly, in the adult mammalian ENS after 6 weeks of BrdU labelling, the majority of EGCs that had taken up the EdU label were found in doublets within the ganglia (Joseph et al., 2011). These observations echo the symmetrical cell divisions observed of SOX10+ cells in the mouse ENS (which includes ENS progenitors and EGCs) observed during
development (Lasrado et al., 2017). Moreover, the presence of doublets suggests that, like in mammals, the progeny of proliferative EGCs remain within the local vicinity and may act to replenish cells lost during normal homeostasis (Kabouridis et al., 2015, Lasrado et al., 2017). We suggest that the main role of cell divisions observed in the non-neuronal cell population of the vertebrate ENS is to either add more glial cells to a growing gut/ENS or replenish glial cells lost to physiological turnover or injury/disease, a view which is consistent with the reduced proliferation of Her4.1+ cells in older zebrafish (Barbosa et al., 2015, Edelmann et al., 2013). It will be interesting to determine the effect of gut injury/inflammation on the proliferation of non-neuronal ENS cells in the gut of “young” and “older” zebrafish.

Our zebrafish experiments also revealed that Her4.1+ cells are more proliferative in female zebrafish than in males. In mammals, sex differences have been noted in the roles of EGCs, in that loss of EGCs in females leads to motility defects that have not been observed in males (Rao et al., 2017). Since females are more susceptible to glial loss, it is possible that females have an increased progenitor potential to prevent glial loss.

Active neurogenesis within the adult ENS remains a controversial issue within the field. The vast majority of studies suggest that there is essentially no neurogenesis under homeostatic conditions (Joseph et al., 2011), and minimal neurogenesis is only observed after injury (Laranjeira et al., 2011) or inflammation (Belkind-Gerson et al., 2017). However, a recent controversial study has suggested that there is high rate of non-neuronal cell proliferation and neuronal turnover in the mammalian ENS (Kulkarni et al., 2017) though this remains to be independently validated. Since the majority of this work has been performed in mammals, a model organism with low neurogenic potential, our work in zebrafish has given us a unique opportunity to address the neurogenic potential within the ENS, in a model organism that has a characteristically higher regenerative and neurogenic potential (Alunni and Bally-Cuif, 2016, Chapouton et al., 2007, Grandel and Brand, 2013). We performed EdU pulse-chase experiments to directly test the proliferative capacity and neurogenic potential within the adult zebrafish ENS. In both 3-4mo and 6-7mo animals, we observed HuC/D+EdU+ neurons after 4 days of chase. Since neurons are post-mitotic, our results suggest HuC/D+EdU+ cells are newborn neurons, and therefore there is active neurogenesis during homeostasis within the adult zebrafish ENS. Moreover, the decrease in Her4.1+EdU+ cells mimics the increase in
HuC/D\textsuperscript{+}EdU\textsuperscript{+} cells, in the chase period, suggesting that Her4.1\textsuperscript{+} cells are giving rise to the newly born neurons. Alternatively, the decrease in the GFP\textsuperscript{+}EdU\textsuperscript{+} population could be due to rapid proliferation of Her4.1\textsuperscript{+} cells, which would lead to a dilution of EdU and loss of signal. However, since we were previously unable to detect a proliferative Her4.1\textsuperscript{+} population using cell cycle antibodies (Chapter 5.2.1), our model suggested Her4.1 cells are a largely quiescent and slow cycling population. Therefore, Her4.1\textsuperscript{+} cells would not be expected to proliferate fast enough to dilute the EdU label. In addition, the highly proliferative epithelial cells in the lamina propria retain the EdU label up to 11 days of chase (Appendix Chapter 8.5, Figure 8.3), indicating that EdU labelling is unlikely to be lost through rapid proliferation. Therefore, while our EdU-chase experiments are suggestive of a lineage relationship between Her4.1\textsuperscript{+} cells and enteric neurons, they do not allow us to determine the origin of newborn neurons directly. To address this question, we collaborated with Carmen Pinn, to perform mathematical modelling, which showed that cells of the ENS that had taken up EdU (Her4.1\textsuperscript{+}EdU\textsuperscript{+} and HuC/D\textsuperscript{+}EdU\textsuperscript{+}) were significantly closer to one another than expected if the cells were distributed randomly. These data suggest a clustering of newborn lineage-related ENS cells that are generated from a common Her4.1\textsuperscript{+} progenitor. To further explore the fate of lineage-related cells, we performed modelling to estimate the probability of a Her4.1\textsuperscript{+} cell undergoing division or differentiation. We observed that Her4.1 cells are biased toward neuronal differentiation at 3-4 mo. However, by 6-7 mo, the probability of neuronal differentiation is equal to division. Since, neuronal differentiation either matches or slightly exceeds proliferation, this suggests that the only cell type that Her4.1\textsuperscript{+} cells generate are new Her4.1\textsuperscript{+} cells and neurons. Together these data suggest the Her4.1\textsuperscript{+} cells represent a stem cell population within the ENS, which only generate new Her4.1 expressing cells or neurons, and that neurogenesis within the ENS occurs at a low rate throughout adult zebrafish homeostasis. Despite the fact that zebrafish are far more neurogenic than mice, we observed that in the adult zebrafish ENS a new neuron was generated, on average, every 20-30 days. This low level of neurogenesis contrasts with recent work in mouse suggesting that 80% of neurons within a ganglia are newly generated every two weeks (Kulkarni et al., 2017) but is consistent with the bulk of previous mouse work that suggests a low level of ENS neurogenesis, relative to that of the CNS (Laranjeira et al., 2011, Joseph et al., 2011, Belkind-Gerson et al., 2017). The discrepancies between previous studies, our work
and Kulkarni et al could be due to a multitude of factors such as: different transgenic lines or genetic backgrounds used, the age of the animals or even influences from the external environment. Understanding the molecular mechanisms underlying EGC proliferation and neurogenesis could provide insight into these inconsistencies.

Finally, we investigated the role of Notch signalling on the proliferative potential of Her4.1+ cells within the zebrafish ENS since, within the CNS, Notch signalling maintains RGCs in a quiescent state. Inhibition of Notch signalling in embryonic and adult Tg(her4.1:EGFP) transgenics lead to loss of EGFP expression within the ENS. This demonstrates that in the ENS her4.1 expression (and consequently EGFP expression) is downstream of Notch signalling, consistent with published work in the CNS (Yeo et al., 2007). To enable the non-neuronal lineage to be tracked after Notch inhibition, we used the Tg(-4.7sox10cre:bactin-LoxP-STOP-LoxP-hmgb1-mCherry) transgenic line. We observed that Notch inhibition significantly increased the number of non-neuronal cells that enter the cell cycle, relative to control conditions, indicating that Notch inhibition recruits non-neuronal cells into the cell cycle. Therefore, like the CNS, it suggests that the non-neuronal population within the ENS, which is largely Her4.1+, is maintained in a quiescent state by active Notch signalling (Alunni et al., 2013, Chapouton et al., 2010). Moreover, since Notch inhibition recruits non-proliferative non-neuronal cells into the cell cycle, this suggests that the majority of the non-neuronal cells have the capacity to proliferate. Interestingly, we also observed an increase in the number of newborn neurons that had incorporated EdU, after Notch inhibition. Notch signalling is important in inhibiting the neuronal fate (Bansod et al., 2017, Ganz et al., 2010, Chapouton et al., 2010), and Notch inhibition releases a block on pro-neurogenic genes such as Ascl1 or Neurogenin-1 (Yeo et al., 2007, Kageyama et al., 2015, Kanski et al., 2014), therefore enabling neuronal differentiation. We speculate that within the ENS, Notch signalling not only plays a role in maintaining non-neuronal cells in a quiescent state, but also acts to maintain the non-neuronal cell fate.

In conclusion, we have shown that the Her4.1+ population within the ENS is proliferative during homeostasis. Moreover, the proliferative potential of Her4.1+ cells is decreased with age. Using EdU chase experiments, we showed that the Her4.1 expressing population gives rise to HuC/D+ neurons. Moreover, our modelling indicates that neurons and Her4.1+ cells are the sole progeny of proliferative Her4.1 expressing cells, suggesting that Her4.1+ cells are stem cells within the ENS. Finally,
we demonstrated that Her4.1\(^+\) cells are maintained in a quiescence state through active Notch signalling, and implicate Notch signalling the establishment of non-neuronal cell fate.
Chapter 6. Characterising Hes5 Expression in the Mammalian ENS

6.1 Introduction

Expression of Her4.1 marks zebrafish EGCs (Chapter 4), but these cells lack the majority of the canonical peripheral glia markers and make up a smaller population of the ENS than seen in mammals (Chapter 3). Therefore, we wondered whether the Her4.1+ population was unique to zebrafish, or whether an equivalent population exists in the mammalian gut. Moreover, we wished to examine whether the proliferative properties of Her4.1+ cells in zebrafish ENS could instruct studies of the proliferative potential of mammalian EGCs.

Within the mammalian ENS, eNCCs express Notch1, Dll1 and Dll3, and possibly Notch4, Dll4 and Jag2 (Okamura and Saga, 2008, Charrier and Pilon, 2017). Similarly, NOTCH receptors and ligands have been detected on adult enteric neurons (Sander et al., 2003), suggesting that there is active NOTCH signalling in the mammalian ENS throughout life. However, the role of NOTCH signalling in the mature ENS remains unknown. Given the role of NOTCH in eNCCS and RGCs of the CNS, investigation of NOTCH signalling in the mature ENS may give insight into EGC or enteric progenitor cell biology.

Our studies of zebrafish Her4.1 expressing EGCs indicate a possible role for its mammalian orthologue HES5 in mouse EGCs, which we can now begin to address. Moreover, studies implicating NOTCH signalling within the developing mammalian ENS show that Hes5 is a downstream target of NOTCH (Charrier and Pilon, 2017, Theocharatos et al., 2013). Due to the maintenance of many glial and progenitor markers from developmental stages to adult EGCs, we hypothesise that HES5 is likely to be a marker of mammalian enteric glia. A first step toward addressing these possibilities is examining the expression of HES5 in the ENS of adult mice and during development.
6.2 Results

6.2.1 Hes5:EGFP expression is identified within a subset of EGCs in the Mammalian ENS.

Although our studies highlighted many differences between zebrafish and murine EGCs, we speculated that Her4.1 expression in zebrafish EGCs may also give some insight into gene expression in mammalian enteric glia. To test this idea directly, we examined the expression of HES5, the mammalian orthologue of Her4.1, in the adult murine ENS. We have utilised an a Hes5:EGFP transgenic reporter line, that is analogous to Tg(her4.1:EGFP), in which EGFP is under the control of a 3kb fragment of the Hes5 gene. The fragment used includes 1.6kb of the 5’ flanking region and 1.4kb downstream of the transcriptional start site of the Hes5 gene, including three exons and two introns (Basak and Taylor, 2007). Myenteric plexus preparations were obtained from Hes5:EGFP young adult mice (7-8 week old), and stained with GFP, HuC/D, SOX10, SOX2, GFAP and S100β. EGFP signal was detected in a small subset of SOX10 expressing EGCs (1.22±1.84%). Furthermore, we observed that the EGFP expressing population was restricted to the ganglia, making up 3.66±5.15% of the SOX10+ Type I EGCs. In contrast, other glial markers, such as S100β, make up 98.54±2.63% of the total SOX10+ population, or 97.89±3.89% of the SOX10+ cells within ganglia, respectively (Figure 6.1A and G). Moreover, while other EGC markers, such as SOX10 or S100β, have significantly more cells in the extra-ganglionic spaces (62.30 ±3.189%, P=0.0032, and 61.70 ±2.405%, P=0.0042, respectively, Two-way ANOVA, Sidak’s Test), almost all GFP+ cells are found within the ganglia (94.74 ±4.839%, Fig, P<0.0001, Two-way ANOVA, Sidak’s test, Figure 6.1A and H). To further examine the restriction of GFP+ cells within the ganglia, we stained flat-mounted mucosal preparations of the Hes5:EGFP intestines. We were unable to visualise any GFP+ cells within the mucosa, despite readily observing S100β+ and SOX10+ cells in this location (Figure 6.1C). Since we only observed GFP+ cells within the ganglia, this makes HES5 is the first unique subtype marker of Type 1 EGCs. Moreover, the GFP+ cells are found in small clusters or groups within the ganglia, most commonly with 2-4 GFP+ cells clustered per ganglia (Figure 6.1D and J). Additionally, only 21.21 ±4.47% of ganglia within the myenteric plexus contain EGFP+ cells (Figure 6.1I), which may suggest that these
Hes5:EGFP expressing EGCs are responding to local NOTCH signalling from specific neurons within individual ganglia. Finally, we found that there was no significant difference in the distribution of GFP+ cells along the length of the gut (duodenum = 1.51 ± 1.206, Jujenum= 1.80 ± 0.93, Ileum= 0.90 ± 0.90, colon=0.60 ± 0.52, P=0.867, One-way ANOVA), suggesting that the subtype of EGCs identified with this marker is likely to serve a general EGC function.

To further investigate the marker expression of HES5+ cells within the mammalian ENS, we compared HES5 expression to that of GFAP and SOX2. These two markers are of particular interest, since GFAP has dynamic expression within the ENS (Boesmans et al., 2014), and since SOX2 is associated with RGCs and stemness (Belkind-Gerson et al., 2017, Heanue and Pachnis, 2011). We found that all the GFP+ cells were labelled with GFAP, but constitute a subset of the GFAP population (Figure 6.1O). Similarly, we found that all the GFP+ cells also expressed SOX2, although the GFP+ cells represented a subset of the SOX2 population within the ENS (Figure 6.1F-J). The co-expression of HES5 and SOX2 suggests that the GFP+ cells may represent ENS stem cells.
Chapter 6. Results
Figure 6.1. Adult Tg(Hes5:EGFP) mice show EGFP expression within a subpopulation of the EGCs. Analysis of the Hes5:EGFP transgenic line at adult stages with a variety of glial markers to characterise the Hes5+ population within the ENS. (A-B) Low magnification view of the myenteric plexus highlights GFP+ (green, single colour A and B) cells are a subpopulation of SOX10+ (single colour arrowhead, red, A') and SOX2+ (arrow, red, single colour B') glial cells. No GFP+ cells (green) are co-localised with HuC/D+ neurons (blue, single colour A' and B'). (C) Within the mucosal layer, no GFP+ cells (green, single colour C) are observed, despite S100β+ (blue, single colour C'') and SOX10+ (red, single colour C') cells being present. (D-F) High magnification images show GFP+ cells (green) are only found within the ganglia and are a subset of glial cells expressing S100β+ (blue, D and red E), SOX10+ (arrowhead, blue E, and F), SOX2+ (arrows, blue, E) and GFAP (open arrowheads, red, F). (G) Quantification of GFP+ and S100β+ co-expressing SOX10+ cells over all SOX10+ cells within the ganglia or within the extra-ganglionic region. (H) Quantification of S100β+, SOX10+, and GFP+ cells in the ganglia (dark grey) and outside the ganglia (light grey) shows that unlike other markers, GFP+ cells are found predominantly within the ganglia. (I) Quantification showing the percentage of ganglia with (light grey) and without (dark grey) GFP+ cells within them, highlighting the fact that only a minority of ganglia containing GFP+ cells. (J) Quantification of the number of GFP+ cells within a ganglia. All images shown are from the ileum. All data is given as mean ± SEM. ns= not significant, *=P=0.05, **P<0.01 ***P<0.001.

6.2.2 Hes5:EGFP Cell Arise Late within the eNCC Population Colonising the Mammalian Intestine

Next, we investigated the onset of Hes5 expression during mammalian ENS development. We first detected GFP+ cells, at E12.5 when colonisation of the small intestine by NC cells is largely complete (Sasselli et al., 2012). Moreover, the GFP+ cells are within the SOX10+ population that colonises the gut. At E12.5, the GFP+ population was largely restricted to the fore-gut, and we were only able to clearly visualise GFP+ cells in the duodenum and jejunum (Figure 6.2A-D). However, we did occasionally find GFP+ cells within the ilium of E12.5 guts (Figure 6.2C). At E14.5, we observe an increase in the number GFP+ cells within the SOX10+ eNCC population, with GFP+ cells now found throughout the small intestine, with a few GFP+ cells observed in the colon (Figure 6.2E-H). Finally, by E16.5, a relatively large number GFP+ cells were found throughout the gut, in both the small intestine and colon (Figure 6.2I-L). In all stages analysed, there was a greater density of GFP+
cells within the fore-gut region and the density of Hes5+ cells increased caudally as time progressed, suggesting that the Hes5 population appears in a rostrocaudal manner. Our imaging analysis suggests that the greatest proportion of SOX10+GFP+ cells are observed at E16.5, with an apparently smaller population in the adult ENS, suggesting that Hes5 expression becomes restricted during post-natal life.

![Figure 6.2](image.png)

**Figure 6.2. Development of Hes5:EGFP expressing cells occurs in a rostrocaudal manner after E12.5.** Analysis of the Hes5:EGFP transgenic line at various embryonic stages in regions of the intestine, using a SOX10 antibody to identify eNCCs and glial cells, and a GFP antibody to identify the Hes5:EGFP cells. (A-D) GFP+ cells (arrowheads, green) are observed in the duodenum (A) and jejunum (B) of E12.5 guts, within the SOX10+ (red) population. Very occasionally, GFP- cells are observed within the ilium (C), however, no GFP+ cells are observed within the colon (D). (E-F) By E14.5, substantially more GFP+ cells (arrowheads, green) are observed within the SOX10+ population in the duodenum (E) and jejunum (F) and GFP+ cells are now readily observed in the ileum (G) and colon (H). (I-L) By E16.5, GFP+ cells (arrowheads, green) are readily detected throughout all examined regions in the developing gut (I-L), and substantially more GFP·SOX10+ cells are observed in each gut region. Boxed areas are high...
magnification images of the lower magnifications images shown in the figure. All images are orientated with rostral to the top.

6.3 Conclusion

Here we have identified HES5 as a marker for a subset of mammalian EGCs. Interestingly, HES5 represents the first marker that labels a specific subtype of EGCs. Unlike other glial markers such as SOX10, S100β, GFAP and SOX2, HES5 is restricted to the ganglia, and therefore is the first unique subtype marker of Type 1 EGCs. Characterisation of NOTCH ligands in the adult mammal ENS demonstrated that Jag1 was expressed within the majority of neuronal cell bodies within the ganglia, but was absent from neuronal processes (Sander et al., 2003). Therefore, the Hes5 expression we have observed could be the result of NOTCH signalling within the ENS, responding to Jag1 ligand expressed on neuron cell bodies within the ganglia. In addition, HES5 cells were found in clusters or small groups within ganglia, suggesting that local signalling within ganglia may result in upregulation of Hes5. The presence of HES5 in the mammalian ENS suggests exciting similarities between the Her4.1 and HES5 population within the zebrafish and mouse ENS, respectively. We have previously described the proliferative potential of the Her4.1+ population in the EGCs of the zebrafish ENS (Chapter 5). Interestingly, the Hes5+ population of mammalian EGCs is found in approximately the same percentage as the percentage of EGCs that are proliferative within the adult ENS (Joseph et al., 2011, Laranjeira et al., 2011, Belkind-Gerson et al., 2017). Moreover, the majority of proliferative EGCs are found within the ganglia (Belkind-Gerson et al., 2017, Laranjeira et al., 2011, Joseph et al., 2011), which may suggest that like the zebrafish Her4.1+ population, the HES5+ EGCs described above represent the population within the mammalian ENS that has proliferative potential.

The relatively late onset and rostrocaudal appearance of the HES5+ population during mouse ENS development mimics the later onset and rostrocaudal appearance of Her4.1+ population during zebrafish ENS development (Chapter 4.2.2 and 4.2.3). Subsequently, GFP+ cells appear in a rostrocaudal manner. Interestingly, the expression of NOTCH ligands and receptors within the ENS are upregulated around E11.5 (Okamura and Saga, 2008). Active NOTCH signalling from E11.5
could, therefore, lead to the relatively late appearance of HES5+ cells at E12.5. In addition, it has been proposed that migration of the eNCCs as chains of cells allows for the cell-cell contact required for NOTCH signalling (Okamura and Saga, 2008). However, since we do not detect HES5 expression until E12.5, when NC cell migration is largely completed, it is possible that cell-cell contacts between migrating eNCCs are insufficient to activate NOTCH dependant HES5 expression. The later expression of HES5 may be important in preventing neurogenesis in some cells, to provide a reserve of progenitor cells. In agreement with this, in the absence of NOTCH signalling, Sox10 is not detected at E12.5. This result suggests that starting at E12.5, maintenance of glial fates is regulated by NOTCH signalling (Okamura and Saga, 2008). Similarly, in mutant mice that have defective NOTCH signalling, no phenotypes in proliferation or differentiation potential are observed in the ENS until after E12.5 (Okamura and Saga, 2008, Taylor et al., 2007), which suggests that NOTCH signalling is not active before E12.5. Moreover, no eNCC cell migration phenotypes have been observed in NOTCH signalling mutants. This suggests that eNCC cell colonisation of the ENS is independent of NOTCH signalling and that NOTCH signalling may function later to instruct glial cell fate or maturation, and/or to maintain a progenitor lineage important for expanding local ENS population during the extensive growth of the gut during development.
Chapter 7. Concluding Remarks

This study has provided the first extensive characterisation of zebrafish EGCs and allowed us to update our current model of the organisation of the zebrafish ENS to include EGCs, next to neurons and the mucosa (Figure 7.1). We have shown that in the zebrafish ENS, the enteric glial cell population, labelled by Her4.1 and Sox10, is unlike their mammalian counterparts. Specifically, zebrafish EGCs lack many canonical peripheral glial markers. Moreover, the EGC lineage constitutes a much smaller proportion of the ENS than expected from work in mammals (Ruhl, 2005, Furness, 2006). This finding suggests that there are key differences in cellular, and potentially functional, properties between mammalian and zebrafish EGCs. Investigating these differences further will provide key insights into EGC biology, which could help us understand the mechanisms underlying key aspects of EGC function. Since the zebrafish EGC population contains a large progenitor population we speculate that comparisons between zebrafish and murine EGCs could provide insight into conserved mechanisms critical for progenitor or stem cell behaviours within EGCs.

Figure 7.1. Old and new models of the organisation of the Zebrafish ENS. The old model of the organisation of zebrafish ENS lacked any information of EGCs. Our data has shown an EGC population expressing Her4.1 and Sox10 exists in the ENS. These EGCs are found close to enteric neurons and near the mucosal layers.
One technical approach that may shed light on the EGC characteristics is to perform transcriptomic analysis. Performing RNA sequencing analysis of the zebrafish EGC population would allow us to assess their glial and progenitor character. This analysis could further confirm the lack of conical peripheral glial markers in zebrafish EGCs and identify new genes or pathways that are important in EGC cell function. Moreover, single-cell RNAseq would allow us to determine whether there are subpopulations of EGCs, with specialised functions such as blood or epithelial barrier properties, or the ability to mediate immune interactions.

Enteric glial cells have been widely studied in the mammalian intestine. Yet, despite having diverse functions and cellular interactions with both neuronal and non-neuronal cells, EGC subtypes are only delineated by morphology and position (Boesmans et al., 2014, Gulbransen and Sharkey, 2012, Ruhl, 2005). Our characterisation of Her4.1 expressing EGCs within the zebrafish ENS has led us to the identification of a HES5 expressing subpopulation of EGCs within the murine ENS. HES5⁺ EGCs represent the first unique subtype marker of mammalian Type I EGCs, however their function within the ENS has yet to be examined.

Astrocytes are not present within the zebrafish CNS. However, RGCs, take up astrocytic function in neuronal support and BBB formation (Grupp et al., 2010, Jeong et al., 2008, Fleming et al., 2013), along with their primary role as neural stem cells (Kizil et al., 2012b, Than-Trong and Bally-Cuif, 2015). It is our expectation, therefore, that the zebrafish ENS also lacks cells with astrocytic EGC character. Given the fact that mammalian EGCs are traditionally thought to be most similar to astrocytes, this is a surprising idea. Since, all EGCs of the zebrafish ENS are Her4.1 expressing cells, we postulate that the Her4.1 expressing population represents neural progenitors with some astrocytic functions, in a strong parallel to the RGCs of the zebrafish CinfNS. In support of this, our EM studies suggest that Her4.1 expressing cells may provide some glial function, while also acting as progenitors; the close association of Her4.1⁺ cell processes around neuronal cell bodies is indicative of a role for these cells in supporting neurons. The significance of the fact that canonical glial markers are not expressed within the Her4.1 expressing population is unclear. However, similar discrepancies in glial marker expression are also observed in RGC populations within the zebrafish CNS, in that ventral RGCs lack, or have very weak, expression of the canonical glial markers such as GFAP, Vimeintin and Aro-B (Ganz et al., 2010).
Since the Her4.1 expressing population are stem cells within the zebrafish ENS, with both self-renewal and neurogenic potential, we can postulate that the HES5 expressing EGCs in the mammalian gut also have progenitor characteristics. In support of this idea, work in mammalian EGCs show that the majority of proliferative EGCs are contained within the ganglia (Joseph et al., 2011, Laranjeira et al., 2011, Belkind-Gerson et al., 2017), consistent with the location of the HES5 expressing population. Therefore, we propose that the mouse has a full complement of EGCs in the ENS, including astrocyte-like EGCs, expressing SOX10, S100β, GFAP and SOX2, and progenitor-like EGCs, which have additional expression of HES5 (Figure 7.2). In contrast, zebrafish lack the astrocyte-like subtype of EGCs, however retain the progenitor-like population, labelled by Sox10 and Her4.1 (Figure 7.2). Therefore, we hypothesize that Her4.1/HES5 expressing cells represent a core of EGCs with stem cell function within the ENS. This hypothesis could be assessed directly, by examining the proliferative capacity of the HES5 cells within the mammalian ENS under homeostatic and injury conditions.

Figure 7.2. Schematic representing the different glial populations within the mouse and zebrafish ENS. The mammalian ENS has a full complement of EGCs, which constitute a large proportion of the mammalian ENS. Most mammalian EGCs express SOX10, S100β, GFAP, BFABP and SOX2, while only a small subset also express Hes5. In contrast, zebrafish EGCs comprise a smaller proportion of the ENS than their mammalian counterparts. Moreover,
zebrafish EGCs lack the majority of canonical markers like GFAP and S100β and only expresses Sox10 and Her4.1.

Our model suggests that further assessment of the similarities and differences between zebrafish and mouse EGCs will provide valuable information about glial and progenitor character within the ENS. A transcriptomic comparison between these species will allow identification of key transcriptional programs associated with the astrocyte-like subtype of EGCs, which would be present in mammalian EGCs and absent from zebrafish EGCs. Conversely, such analysis could also reveal the transcriptional programme associated with maintenance of Her4.1 expressing cells in a progenitor state. However, due to the diverse subtypes of mammalian EGCs, single-cell RNAseq may need to be employed to avoid masking important transcriptional differences, involved in glial cell type heterogeneity. Furthermore, transcriptomic comparisons between zebrafish Her4.1+ cells and mammalian HES5+ cells could also provide insight into key stem cell and glial properties conserved within the ENS.

Our work highlights a role for Notch signalling within the adult zebrafish ENS, in maintaining the EGC population in quiescence, and inhibiting neurogenesis. A role for NOTCH signalling within the adult ENS has yet to be explored in mammals. Our studies have only examined the immediate effects of Notch inhibition within the zebrafish ENS, and have yet to explore the long term effects of alterations in Notch signalling on the stem cell pool, and resulting neurons. Additionally, upon Notch inhibition, we observe an apparent increase in newborn neurons, which suggests that Notch has roles in the maintenance of non-neuronal fate within the zebrafish ENS. However, the lack of established glial markers in the zebrafish ENS prevents clear separation of the effects of Notch signalling has on maintenance of non-neuronal fate, by preventing neuronal differentiation, or in actively directing gliogenic differentiation.

Due to the relatively late appearance of Her4.1+/HES5+ cells in zebrafish and mouse, we suggest that NOTCH ligands are likely to be expressed by neurons, within the ENS, which begin differentiation around the same time as Her4.1+/HES5 onset. However, we have yet to identify the ligands and receptors that mediate the Her4.1+/HES5+ expression within ENS, in either species. Within the CNS, differential
expression of NOTCH receptors has been implicated in different aspects of neural stem cell function. For example, quiescence is mediated by Notch3 and NOTCH2, in zebrafish and mouse, respectively (Alunni et al., 2013, Engler et al., 2018). Therefore, a greater understanding of the receptor and ligands involved in NOTCH signalling within the ENS may provide further insight into Her4.1/HES5 cell function within the ENS. Previously mentioned transcriptomic analysis, particularly on the single cell level, would identify the candidate NOTCH ligands and receptors mediating Her4.1/HES5 expression, progenitor characteristics, and downstream NOTCH targets. However, it is possible that the NOTCH receptors important in mediating quiescence and proliferation will differ between mouse and zebrafish, which is the case in the CNS (Engler et al., 2018, Alunni et al., 2013). Nevertheless, the downstream pathways activated during quiescence or proliferation may exhibit a greater level of conservation. Since transcriptomic comparisons between RGCs in zebrafish and mouse highlight the same genetic pathways downstream of NOTCH, are responsible for similar biological processes (Than-Trong et al., 2018).

In mammalian ENS development, NOTCH signalling is an important regulator of the proliferative and differentiation potentials of eNCCs. However, these effects are largely observed only after the majority of colonisation is complete, suggesting a late role of NOTCH signalling within the ENS (Ngan et al., 2011, Okamura and Saga, 2008, Taylor et al., 2007). This idea is in agreement with our developmental studies in both zebrafish and mouse, which show that Her4.1/HES5 expressing cells are first identifiable only after the majority of colonisation is complete. After colonisation, Her4.1/HES5 numbers increase, suggesting these cells have potential roles in later phases of ENS development, such as maintaining the correct density of the ENS within local gut regions during growth of the gastrointestinal tract. In support of this idea, our zebrafish data suggests that Her4.1+ expressing cells are largely non-migratory and undergo proliferation during development. Moreover, progeny derived from Her4.1 cells during development are spatially restricted in adulthood. This is similar to what is seen in the developing mammalian ENS, where the progeny derived from individual progenitors are constrained to local gut regions (Lasrado et al., 2017). Together these data suggest a later role for NOTCH signalling during vertebrate ENS development. This model suggests that Her4.1+/HES5+ progenitor cells, responsive to NOTCH signalling, colonise local gut regions as the
gastrointestinal tract grows, and have a lesser role in the initial colonisation of the gut by eNCCs.

In mammals, NOTCH signalling has also been implicated in regulating the differentiation of both EGCs and enteric neurons (Ngan et al., 2011, Okamura and Saga, 2008, Theocharatos et al., 2013, Taylor et al., 2007). In support of this idea, transcriptional studies in mouse have implicated downstream NOTCH targets, such as Heyl, in mediating progenitor/gliogenic fates during mammalian ENS development (Lasrado et al., 2017). Additionally, NOTCH signalling has been implicated in the maintenance of Sox10 expression after colonisation (Okamura and Saga, 2008). Although the mechanism has yet to be described, we can speculate that signalling via Her4.1+/HES5+ may be involved, since the expression of Her4.1/HES5 is coincident with the onset of the NOTCH dependant maintenance of Sox10. Interestingly, Sox10 expression is important for maintenance of progenitor multipotency (Kim et al., 2003, Bondurand et al., 2006), but is not necessarily involved in maintaining proliferative potential; NC cells that lack Sox10 are still capable of proliferating, however they become restricted to pre-neuronal states, resulting in depletion of the stem cell pool (Bondurand et al., 2006, Kim et al., 2003, Paratore et al., 2002). Therefore, it is possible that after colonisation, NOTCH has dual roles, firstly in maintaining Sox10 expression to retain multipotency, and secondly to regulate Her4.1/HES5 expression and thereby maintain progenitor characteristics (Figure 7.3). In addition, this may suggest that in the adult mammalian ENS, all EGCs have the potential to form neurons, however extra factors are required for EGC proliferation. We speculate that HES5 has a role in maintaining proliferative potential in adult EGCs. Therefore, expression of HES5 within the mammalian EGCs may reflect a functional state of the EGCs, that of a progenitor pool.
Figure 7.3. Schematic placing our findings on the emergence HES5/Her4.1 during gut colonisation by eNCCs and their subsequent differentiation in the context of previous work. HES5/Her4.1 cells appear late in eNCCs gut colonisation, in a NOTCH dependant manner. Glial progenitors (GP) and a subset of glia (G) maintain HES5/Her4.1 expression. In contrast
HES5/Her4.1 expression decreases in neural progenitors (NP) and is eventually lost in mature neurons (N). Information from Kim (2003), Okamura (2008), Charrier (2017).

We have shown the Her4.1 expressing cells have stem cell properties within the zebrafish ENS during homeostasis. However, it is still unknown whether these cells would respond, by a similar or alternative mechanism following injury. After an injury, such as chemical injury or inflammation, EGCs of the mammalian gut undergo proliferation and neurogenesis (Joseph et al., 2011, Laranjeira et al., 2011, Belkind-Gerson et al., 2017). Similarly, following injury in the CNS, of both mouse and zebrafish, RGCs (and/or astrocytes in the case of the mammalian CNS) undergo proliferation and subsequent neurogenesis (Kroehne et al., 2011, Alunni and Bally-Cuif, 2016, Torper et al., 2013, Magnusson et al., 2014, Urban and Guillemot, 2014).

Therefore, we propose that after injury, the Her4.1 expressing population would proliferate in to response to the damage caused. We propose, that the molecules and signalling pathways that regulate injury repair are likely to differ from the homeostatic condition, since in the zebrafish CNS there are distinct mechanisms regulating proliferation of RGCs during homeostasis versus repair (Kyritsis et al., 2012, Bhattacharai et al., 2016). Whether the Her4.1 expressing cells play a role in the neuronal rewiring process, similar to glial mediated circuit remodelling that is observed in the CNS, is a compelling question (Vainchtein et al., 2018, Zhan et al., 2014, Eroglu and Barres, 2010).

Our identification of the stem cell potential within the Her4.1 population, and the possible conserved function within the mammalian HES5 population, suggests that these cells may be useful in future cell transplantation studies (Burns et al., 2016, McCann and Thapar, 2018). Recently, many studies have highlighted the potential to use stem cell transplantations to treat gut pathologies. Transplanted EGCs/iPSC are able to differentiate and proliferate within the intestine, and have even been shown to migrate away from the transplantation site (Cooper et al., 2016, Cooper et al., 2017, Findlay et al., 2014, Heanue and Pachnis, 2011, Hotta et al., 2013, McCann et al., 2017, Stamp et al., 2017). A limitation in the majority of transplantation studies is the relatively heterogeneous starting cell populations, comprising of both differentiated glial and progenitor cells. The heterogeneity of the starting stem cell population may limit the success of the transplantation, by limiting
the migratory potential (Burns et al., 2016) or neuronal subtypes derivatives (Cooper et al., 2016, Hotta et al., 2013, Stamp et al., 2017). Use of cells with stem cell character, such as Her4.1/HES5 expressing cells, or cells driven towards this state, may enhance the success of stem-cell transplants in treating ENS related gastrointestinal pathology.

In conclusion, we have shown that zebrafish EGCs do not express the traditional peripheral glial markers. Instead these EGCs have stem cell properties, like RGCs of the CNS, such as expression of the bHLH transcription factor Her4.1, low proliferation rate and neurogenic potential during homeostasis. In addition, we have shown that zebrafish EGCs are derived from the same NC cells that give rise to enteric neurons. Moreover, these cells are late arising in development, suggesting they may represent a more differentiated lineage and/or have distinct roles after colonisation of the ENS. Finally, we identified an equivalent population, HES5 expressing cells, within the mammalian Type I EGCs, which represents the first unique subtype marker for EGCs. Further research into the function of Her4.1/HES5 expressing cells in development, homeostasis, and pathology, will enhance our understanding of EGC biology. Our work gives novel insight into progenitor populations within the vertebrate ENS and in future, these findings may have implications for the treatment of ENS pathologies and the development of stem cell therapies.
Chapter 8. Appendix

8.1 Personal communications p32

Ana Carina Bon Frauches Oliveira and Bethania Garcia-Cassani: – unable to visualise cell death in the ENS
Franze Progatzky and Bethania Garcia-Cassani: Unable to visualise EGC proliferation or neurogenesis to the same extent as seen by Kulkarni et al. 2017.

8.2 Tg(-4.7Sox10:GFP) expression at early stages of eNCC colonisation of the ENS.

![Diagram](image)

Figure 8.1. 60hpf Tg(-4.7Sox10:GFP) embryos show strong GFP expression. (A) GFP expression is visible at early stages (60hpf shown) when eNCCs are colonising the ENS. GFP has some perdurance in the newly born HuC/D+ neurons (red). The larval image is shown with rostral to the left.
8.3 \textit{Tg(Her4.1:GFP)} expression in the adult brain

Figure 8.2. EGFP expression is found in radial glial cells throughout the brain of adult \textit{Tg(her4.1:EGFP)}.  
(A) GFP cells (green) visible on the ventral surface of the brain, in close proximity to HuC/D neurons (red). Brain image shown is from the pallium.

8.4 Live imaging movies for Chapter 4.2.3

Please find movies in the DVD in the pocket attached to the back of this thesis.

Movie 1. Max projection of a time-lapse movie from \textit{Tg(her4.1:EGFP;SAGFF234A;UAS:mCherry)} embryos imaged from 56hpf for 40.33hours, showing EGFP only (grey). EGFP expressing cells (grey) within the ENS are labelled with white arrowheads. Highlighting rostrocaudal appearance of EGFP expressing cells. Images taken every 10 minutes.

Movie 2. Max projection of a time-lapse movie from \textit{Tg(her4.1:EGFP;SAGFF234A;UAS:mCherry)} embryos imaged from 56hpf for 40.33hours, showing mCherry+ wavefront only. mCherry expressing cells migrate in two streams to colonise the FOV. Afterwards the mCherry expressing cells migrate circumferentially to colonise the gut (red arrowhead).

Movie 3. Max projection of a time-lapse movie from \textit{Tg(her4.1:EGFP;SAGFF234A;UAS:mCherry)} embryos imaged from 56hpf for 40.33hours, showing a merge of the mCherry expressing (red) and EGFP expressing
cells (grey). Highlighting that the EGFP cells appear late in development behind the wavefront. Time given is shown as hh:mm.

8.5 EdU is maintained in the intestinal epithelial cells

Figure 8.3. Epithelial cells maintain EdU labelling throughout the chase period. (A) Schematic of experimental design: 3-4mo Tg(her4,1:EGFP) zebrafish are exposed to 3 days of 1mM EdU. Then after 0 days (t0), 4 days (t4) or 11 days (t11) chase period, guts are collected for analysis. (B-D) EdU (red) is readily detected in the epithelial (DAPI, grey) at all chase periods: t0 (B), t4 (C) and t11 (D).
8.6 Controls for Monte-Carlo Modelling

Figure 8.4. Mathematical modelling reveals that the GFP+, HuC/D+ and EdU+ cells within the ENS are homogeneously distributed. (A-C) In 3-4mo Tg(her4.1:GFP) animals, modelling reveals that the experimental densities (red) of GFP+, EdU+ and HuC/D+ cells around GFP+EdU+ cells are not significantly different from simulated data (blue) at t0 (A), t4 (B) and t11 (C). (D-E) The experimental densities (red) of GFP+, EdU+ and HuC/D+ cells around HuC/D+EdU+ cells are also not significantly different from simulated data (blue) at t4 (D) and t11 (E). (F-H) In 6-7mo Tg(her4.1:GFP) animals, modelling reveals that the experimental densities (red) of GFP+, EdU+ and HuC/D+ cells around GFP+EdU+ cells are not significantly different from simulated data (blue) at t0 (F), t4 (G) and t11 (H). (I-J) The experimental densities (red) of GFP+, EdU+ and HuC/D+ cells around HuC/D+EdU+ cells are not significantly different from simulated data (blue) at t4 (I) and t11 (J). Error bars are 90% confidence intervals.
Reference List


