IN VIVO IDENTIFICATION OF NEURAL STEM CELLS IN THE ENTERIC NERVOUS SYSTEM

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"The journey, not the arrival, matters" (T.S. Elliot)

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DECLARATION OF AUTHENTICITY

I, Cátia S. T. Laranjeira declare that all the work presented in this thesis is the result of my own independent investigation. Where information has been derived from other sources, I confirm that this has been indicated and cited accordingly.

This work has not been previously submitted as part of any other degree course or to another university.

Catia Laranjeira

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ABSTRACT

The enteric nervous system (ENS) in vertebrates is derived from neural crest cells which emerge during embryogenesis from the hindbrain and, following stereotypical migratory pathways, colonize the entire gastrointestinal tract. Assembly of enteric ganglia and formation of functional neuronal circuits throughout the gut depends on the highly regulated differentiation of enteric neural crest stem cells (eNCSCs) into a plethora of neuronal subtypes and glia. The identification of eNCSCs and the lineages they generate is fundamental to understand ENS organogenesis. However, the study of the properties of eNCSCs has been hindered by the lack of specific markers and genetic tools to efficiently identify and follow these cells *in vivo*.

Although previous *in vitro* studies have suggested that *Sox10*-expressing cells of the mammalian gut generate both enteric neurons and glia, the differentiation potential of these $Sox10^{+}$ cells *in vivo* is currently unclear. Here, we have developed a genetic marking system which allows us to identify $Sox10^{+}$ cells and follow their fate *in vivo*. Using this system we demonstrate that $Sox10^{+}$ cells of the gut generate both enteric neurons and glia *in vivo*, thus representing multilineage ENS progenitors.

To examine whether the neurogenic potential of $Sox10^{+}$ eNCSCs is temporally regulated over the course of gut organogenesis, we generated additional transgenic mouse lines expressing a tamoxifen-inducible Cre recombinase ($iCreER^{T2}$) under the control of the *Sox10* locus (*Sox10iCreER*^{T2}). Activation of $iCreER^{T2}$ in *Sox10iCreER*^{T2} transgenic mice at specific developmental stages and analysis of enteric ganglia from adult animals showed that the pool of $Sox10^{+}$ cells progressively lose their neurogenic potential.

These findings raise the question of the origin of multilineage ENS progenitors isolated from cultures of post-neurogenic gut. By combining genetic fate mapping in mice, cultures of enteric ganglia and an ENS injury model, we demonstrate that glial cells in the adult ENS retain neurogenic potential which can be activated both *in vitro* and *in vivo*, in response to injury.

The signals that lead $Sox10^{+}$ progenitor cells to become either neurons or glial cells remain unclear. We hypothesized that the receptor tyrosine kinase RET may be part of the molecular fate switch between the two lineages being able to divert differentiation of eNCSCs away from the glial lineage and towards the neuronal fate. Here, we describe a genetic strategy to attain persistent expression of *RET in vivo*, in a temporally and spatially controlled manner. Such a strategy will allow us to assess the role of *RET* in ENS differentiation during development.

Taken together, our data provide a framework for exploring the molecular mechanisms that control enteric neurogenesis *in vivo* and identify glial cells as a potential target for cell replacement therapies in cases associated with congenital absence or acquired loss of enteric neurons.

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ABBREVIATIONS

General Abbreviations:

BAC Benzalkonium chloride

bp base pair

Brd-U 5-bromo-2-deoxyuridine

cDNA complementary DNA

CNS Central Nervous System

cRNA Complementary RNA

DAPI 4,6-diamidino-2-phenylindole dihydrochloride

DNA Deoxyribonucleic acid

dNTPs Deoxyribonucleoside triphosphates

eNCCs Enteric Neural Crest Cells

eNCSCs Enteric Neural Crest Stem Cells

ENS Enteric Nervous System

FMTC Familial medullary thyroid carcinoma

GFP/YFP Green fluorescent protein/Yellow fluorescent protein

HSCR Hirschsprung's disease

Kb Kilobase

MCS Multiple Cloning Site

MEN2A/2B Multiple Endocrine Neoplasia 2A/2B

MS-MP Muscle strips with adherent myenteric plexus

mRNA Messenger RNA

NCCs Neural Crest Cells

NCSCs	Neural Crest Stem cells
NSC	Neural Stem Cells
O/N	overnight
RNA	Ribonucleic acid
PAC	Phage Artificial Chromosome
PCR	Polymerase Chain Reaction
peNCSCs	Pre-enteric Neural Crest Stem Cells
PFGE	Pulse Field Gel Electrophoresis
PNS	Peripheral Nervous System
Rpm	revolution per minute
RTK	Receptor Tyrosine Kinase
4-OHT	4-Hydroxytamoxifen

Units:

cm	centimeter
µm	micrometer
ml	millilitre
µl	microlitre
g	gram
mg	milligram
µg	microgram
ng	nanogram
M	Molar
mM	millimolar
µM	micromolar

V	Volt
U	unit
ºC	degree Celsius

Chapter I

General Introduction

1.1. Enteric Nervous System: structure, organization and function

The autonomic division of the peripheral nervous system (PNS) consists of three sub-divisions: the sympathetic, the parasympathetic and the enteric nervous system (ENS). The ENS is the largest and most complex sub-division of the PNS.

1.1.1. Structure and organization of the ENS

The ENS is composed of a vast number of neurons and glial cells clustered into multiple interconnected ganglia embedded within the gut wall. Enteric ganglia are organized into two major plexi: the myenteric (or Auerbach's) plexus and the submucosal (or Meissner's) plexus (**Fig.1.1**). The myenteric plexus is situated between the longitudinal and circular smooth muscle layers and it runs along the full length of the gastrointestinal tract. The submucosal plexus is closely associated with the connective tissue, between the circular muscle and the innermost mucosal tissue. In small mammals, the submucosal plexus is restricted to the small and large intestines whereas in large mammals, including humans, a few submucosal cell bodies and nerve fibers can be found in the stomach. The size of ENS ganglia is variable between myenteric and submucosal ganglia, between gut regions and, to a lesser extent, between species. Typically, myenteric ganglia are considerably larger than submucosal ganglia in the same region (Furness, 2006).

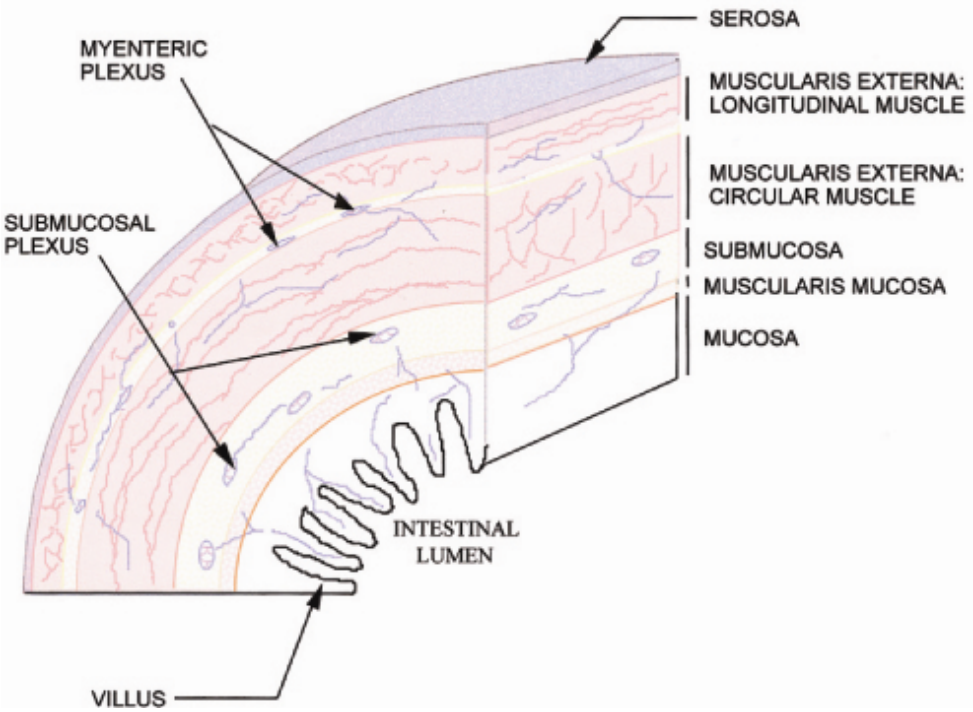

Figure 1.1. ***The enteric nervous system is organized into two ganglionic plexi, the myenteric and the submucosal plexus.*** Schematic representation of a cross-section through the intestinal wall showing its general organization. Ganglia of the myenteric plexus are localized between the longitudinal and circular muscle layers whereas the submucosal plexus resides within the submucosa (modified from Cabarrocas *et al.*, 2003).

1.1.2.Functions of the ENS

Enteric neurons synapse onto other enteric neurons and innervate the muscle, secretory epithelium, and blood vessels of the digestive tract; the biliary system and the pancreas. In accordance, the ENS is involved in regulating fundamental activities of the gut wall such as peristalsis, epithelial secretion, nutrient uptake, blood flow and immune and inflammatory processes (Kunze & Furness, 1999). In small mammals, control of gut motility is mainly carried out by the myenteric plexus whereas submucosal neurons are primarily responsible for the regulation of the secretory, absorptive and vascular functions of the gut. In larger mammals, some submucosal neurons participate directly,

along with the myenteric plexus, in the control of motility reflexes (Timmermans *et al.*, 2001). Because of its critical role in fundamental activities of the gastrointestinal tract, the ENS is absolutely essential for all stages of postnatal life. Hence, mice lacking ENS throughout the gastrointestinal tract (Pattyn *et al.*, 1999; Southard-Smith *et al.*, 1998) or from particular regions, such as the esophagus (Guillemot *et al.*, 1993) or small and large intestines (de Graaff *et al.*, 2001; Schuchardt *et al.*, 1994), usually die within 24 hours after birth.

1.1.3.Characteristics of the ENS

The ENS differs both structurally and functionally from any other component of the PNS (Gabella, 1987; Gershon, 1981). Unlike other autonomic ganglia, enteric ganglia do not contain blood vessels, connective tissue cells or collagen fibrils. Structural support for enteric neurons is not provided by connective tissue or Schwann cells but rather by a unique glial cell population, morphologically and phenotypically more similar to central nervous system (CNS) astrocytes than to support cells of the sympathetic and parasympathetic ganglia (Gershon & Rothman, 1991). The absence of connective tissue and the close packing of neurons and glia give enteric ganglia an appearance similar to the CNS (Gabella, 1981). In terms of cell numbers, the ENS is the largest sub-division of the PNS containing as many neurons as can be found in the spinal cord (Gershon, 1993). Moreover, the ENS is, by far, the most complex PNS component in terms of the number of different subtypes of neurons and their connectivity. Enteric neurons can differ in neurotransmitter expression, morphology, electrophysiology and function (Brookes, 2001; Costa *et al.*, 1996; Furness, 2000). Although clustered into ganglia, neurons do not form identifiable structural units of

morphologically, functionally and biochemically similar neuronal subtypes. In contrast, neurons of different subtypes are grouped in each ganglion forming repeating units of neuronal circuitry along the length of the gastrointestinal tract.

The large number of neurons present in the enteric plexi and their phenotypic diversity is reflected into highly complex reflex circuits, which function largely independent of the CNS. In fact, even though the ENS receives input from the brain and spinal cord nuclei via the sympathetic and parasympathetic branches of the autonomic nervous system (Luckensmeyer & Keast, 1998; Powley, 2000), the intrinsic innervation of the bowel is the only region of the PNS intrinsically capable of mediating reflex activity (Furness *et al.*, 1995). Given its autonomy and the structural and neurochemical resemblance to the CNS, the ENS has been referred to as the "second brain" (Gershon, 1998).

1.2. Enteric Nervous System: origin and development

1.2.1.Embryonic origin of the ENS

As part of the PNS, the ENS is derived from the neural crest, a transient structure that extends along the rostro-caudal axis of developing vertebrate embryos (Le Douarin & Teillet, 1973; Yntema & Hammond, 1954). Neural crest-derived cells (NCCs) comprise a heterogenous population of migratory progenitors that emigrate from the dorsal neural tube during embryogenesis. NCCs, which include neural crest stem cells (NCSCs), follow stereotypical migratory routes and colonise specific embryonic sites where they generate a range of different derivatives, namely neurons and glia of the peripheral sensory and autonomic ganglia, Schwann cells, melanocytes, chromaffin cells of the

adrenal medulla and much of the skeletal and connective tissue of the craniofacial structures (Le Douarin & Dupin, 2003).

The neural crest origin of the ENS was first suggested by Yntema and Hammond (1954). In a series of ablation studies, Yntema and Hammond showed that, in the absence of the neural crest, the ENS fails to develop in a histologically normal gut. These results were later confirmed by Le Douarin and Teillet (1973) using chick-quail interspecies chimaeras. Quail NCCs were grafted into chick embryos prior to the onset of migration and this led to the presence of quail neurons and glia in the gut of the grafted chick embryos supporting the neural crest origin of the ENS (Le Douarin & Teillet, 1973). Cell lineage studies revealed a similar origin for the ENS in mammals and zebrafish (Serbedzija *et al.*, 1991; Shepherd *et al.*, 2001). Even though NCCs are produced from the entire length of the neural axis, only certain tightly defined regions of the neural crest give rise to the ENS, more specifically the vagal and sacral regions **Fig.1.2**).

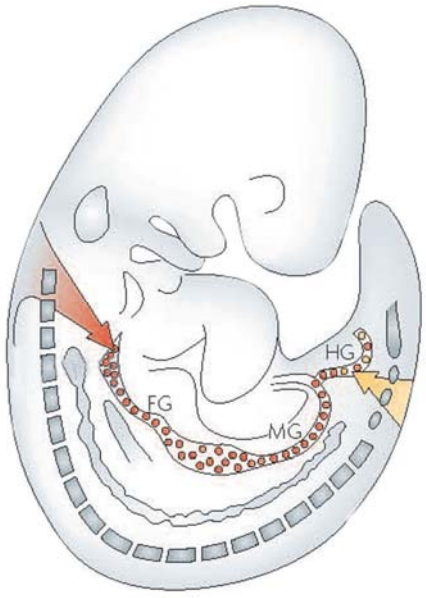

Figure 1.2. ***Enteric neurons and glia arise from neural crest-derived cells.*** At approximately embryonic day (E) 8.5-9 in the mouse, vagal neural crest cells (red arrow) invade the foregut and migrating in a rostrocaudal direction colonise the entire foregut (FG), midgut (MG), caecum and hindgut (HG) giving rise to the majority of the enteric nervous system (red dots). The sacral level neural crest cells (orange arrow) also contribute to the ENS by migrating towards the gut at E13.5 and colonising the colon (orange dots) (modified from (Heanue & Pachnis, 2007).

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Contribution of the vagal neural crest to the ENS

The vagal crest is the only axial level that provides precursors to the entire gastrointestinal tract (Epstein *et al.*, 1994; Yntema & Hammond, 1954). Located at the level of somites 1 to 7, the vagal crest comprises the NCSCs that give rise to the majority of enteric neurons and glia as well as to other structures of the PNS like cardiac ganglia and sensory ganglia of cranial nerves (Durbec *et al.*, 1996; Le Douarin & Teillet, 1973; Newgreen, 1979; Yntema & Hammond, 1954).

Identification of the vagal neural crest as a major source of the ENS throughout the gut was achieved by performing crest ablations in chick embryos at various axial levels (Yntema & Hammond, 1954). More recent studies using chick-quail grafts and fluorescent dyes to label the neural crest confirmed the contribution of the vagal neural axis to the ENS (Le Douarin & Teillet, 1973; Serbedzija *et al.*, 1991).

Colonisation of the embryonic gut by vagal NCCs has been examined using cellular markers and transgenic lines. These studies showed that vagal progenitors of the ENS (called pre-enteric neural crest stem cells-**peNCSCs**) delaminate from the neural tube between embryonic days (E) 8.5-9 in mice and start migrating, first ventrally and then ventromedially, until they enter the foregut by E9.5. Once within the gut, enteric NCCs (**eNCCs**), which include enteric neural crest stem cells (**eNCSCs**), migrate in a rostro-caudal wave to populate the entire gastrointestinal tract (Anderson *et al.*, 2006a; Anderson *et al.*, 2006b; Druckenbrod & Epstein, 2005; Kapur *et al.*, 1992; Pachnis *et al.*, 1993; Young *et al.*, 1998; Young *et al.*, 1999).

Contribution of the sacral neural crest to the ENS

The vagal crest is not the sole source of the ENS. A combination of cell lineage studies using chick-quail grafts, DiI and retrovirus showed that some cells that populate the chick ENS are derived from the sacral level neural crest (Burns & Douarin, 1998; Le Douarin & Teillet, 1973; Pomeranz *et al.*, 1993; Serbedzija *et al.*, 1991). Similarly, studies using transgenic markers revealed an additional minor contribution of sacral-derived NCCs for the mouse ENS (Anderson *et al.*, 2006b; Kapur, 2000). In one of the few species differences this source is absent in zebrafish (Elworthy *et al.*, 2005).

The sacral crest lies caudal to somite 28 in birds and somite 24 in mice and it colonises only the post-umbilical gut (Burns & Douarin, 1998; Le Douarin & Teillet, 1973). The time of arrival of sacral NCCs into the hindgut and the phenotypes of its derivatives was examined in both chick and mouse embryos. Sacral NCCs were observed to migrate ventrally to populate the pelvic mesenchyme and the nerve of Remark (unique to birds) and then pause in their migration outside the hindgut. Upon arrival of the vagal eNCCs, sacral progenitors invade the hindgut (at E7.5 in the birds, E13.5 in mice) contributing to the colonisation of the distal region of the bowel (Anderson *et al.*, 2006b; Burns & Douarin, 1998; Kapur, 2000). Sacral NCCs were found to give rise to neurons and glia but the proportion of ENS cells derived from the sacral crest level is small and declines rostrally (Burns & Douarin, 1998).

1.2.2.Development of the ENS

NCCs that form the ENS emigrate from the neural crest and migrate into the foregut (vagal NCCs) and hindgut (sacral NCCs) during embryogenesis. These cells then migrate along the gut to colonise the entire gastrointestinal tract, which is itself

undergoing growth, morphogenesis and differentiation. A number of studies showed that, from the foregut, vagal eNCCs migrate in a rostral to caudal direction following a specific time course. eNCCs migrate through midgut and approach the ileocaecal junction by E10.5 in the mouse. By E11.5, eNCCs have passed the caecal bulge and by E12.5 they have entered the hindgut. Colonisation is complete around E14.5 when eNCCs reach the end of the hindgut (Anderson *et al.*, 2006a; Druckenbrod & Epstein, 2005; Kapur *et al.*, 1992; Young *et al.*, 1998; Young *et al.*, 1999). At this point, vagal eNCCs mix with cells of sacral origin that are migrating caudorostrally to colonise the hindgut (**Fig.1.3**). During early stages of migration, eNCCs spread through the mesenchyme, but shortly after colonising a region, they form a narrow layer between the longitudinal and circular muscle. In contrast, eNCCs are found in the submucosal plexus only 2-3 days after and it has been suggested that they arise from a secondary centripetal migration from local myenteric cells (Gershon *et al.*, 1980; Jiang *et al.*, 2003; Kapur *et al.*, 1992).

As eNCSCs migrate away from the gut entry points and spread uniformly through the gut, they respond to strong proliferative signals, which increase their numbers dramatically (Gianino *et al.*, 2003). In parallel, subsets of eNCSCs undergo sequential lineage restriction before differentiating into neurons and glia. Thus, the development of the ENS is a complex, asynchronous process that relies on the control of cell migration, proliferation and differentiation of eNCSCs and their progeny in a spatiotemporally controlled manner. Appropriate control of these cellular processes is absolutely essential for the formation of a functional ENS.

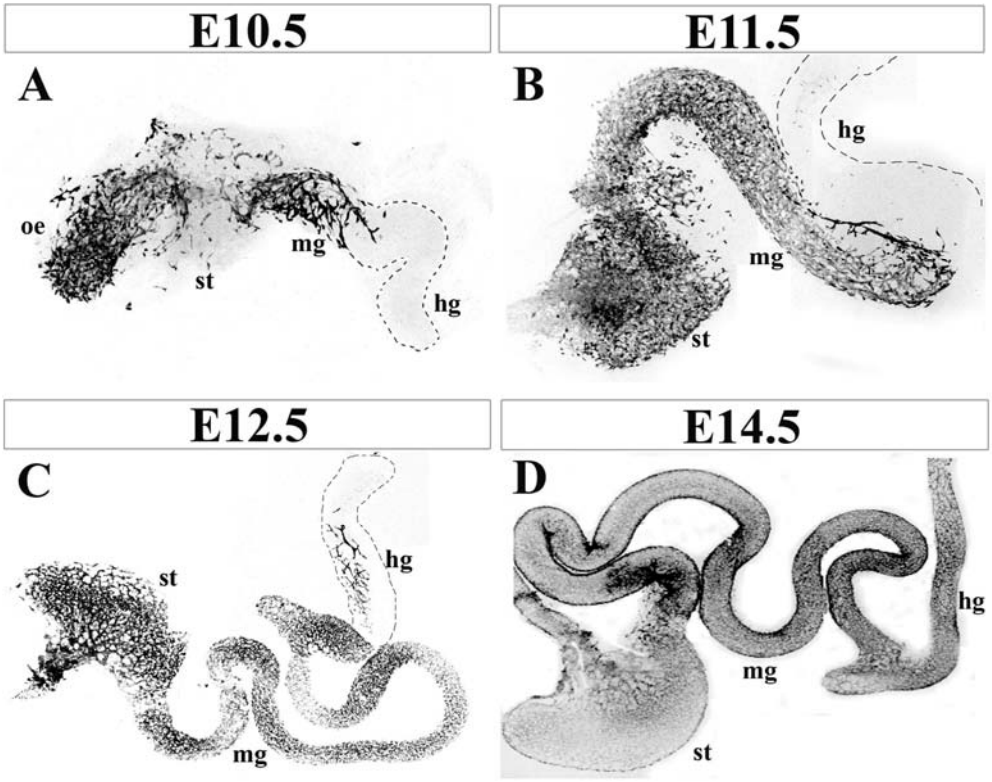


Figure 1.3. ***Temporal and spatial distribution of eNCCs labelled using the bigenic system Wnt1-Cre; R26ReYFP.*** Inverted microscopic confocal images of whole mount gut preparations immunostained for YFP. At E10.5 vagal eNCCs have migrated through the stomach (st) and midgut (mg) and approach the caecal buldge of the intestine (A; x100 magnification). Between E11.5 and E12.5, eNCCs colonise the caecum (B-C; x100 magnification) and, by E14.5, they reach the end of the hindgut (hg) (D; x50 magnification). At that point, vagal eNCCs mix with cells of sacral origin that are migrating caudorostrally to colonise the distal colon.

1.3. Cellular and molecular mechanisms of ENS differentiation

Some of the migrating NCCs are multipotent (Fraser & Bronner-Fraser, 1991) and remain so at the time they reach the foregut mesenchyme (Lo & Anderson, 1995; Rothman *et al.*, 1990; Rothman *et al.*, 1993). Once within the gut, these eNCSCs must generate a vast array of phenotypically diverse neurons and glial cells. In the ENS, unlike some parts of the nervous system, different neuronal subtypes and glia are

generated in the same environment. Furthermore, differentiation of eNCSCs is asynchronous and so multipotent progenitor cells co-exist with fate-restricted cell types (Lo & Anderson, 1995). This raises the question of how and when cell type specification is initiated. A number of studies have suggested that self-renewal of eNCSCs and their orderly differentiation into distinct lineages is dependent on the interplay between extracellular cues and cell-autonomous intracellular signalling (Chalazonitis *et al.*, 1998; Gershon *et al.*, 1993; Paratore *et al.*, 2002; Pisano & Birren, 1999; Taraviras *et al.*, 1999). In the last decades considerable progress has been made in identifying a range of transcription factors, signalling receptors and other molecules implicated in ENS differentiation.

1.3.1.Molecular control of the generation and maintenance of the pool of eNCSCs

To form the mature ENS, eNCSCs must generate correct numbers of neurons and glia in a spatiotemporal controlled manner. This requires a fine-tuned balance between persistence of a pool of eNCSCs and the differentiation of derivatives from this pool. A number of molecules have been implicated in the maintenance of the eNCSCs population by controlling cell survival and renewal. One such molecule is **Sox10**, a transcription factor of the Sox (SRY-box containing) super-family, identified by a DNA-binding domain similar to the high-mobility group (HMG) domain of the mammalian testis-determining factor *Sry* (Koopman *et al.*, 1991). During early stages of development, *Sox10* is specifically expressed in NCCs. Its expression is induced shortly before NCCs exit the neural tube and it continues to be expressed as these cells migrate (Anderson *et al.*, 2006b; Kuhlbrodt *et al.*, 1998; McKeown *et al.*, 2005). Later, when NCCs have reached their final destination and become post-migratory, *Sox10* is strongly

expressed in glial cells, but not the neurons that comprise neural crest derivatives, such as the dorsal root ganglia (DRG), sympathetic chain and ENS (Britsch *et al.*, 2001; Cheng *et al.*, 2000; Cheung & Briscoe, 2003; Kuhlbrodt *et al.*, 1998). Outside the neural crest and its derivatives, *Sox10* is expressed in the otic epithelium and in the central nervous system where it is maintained exclusively in the oligodendrocyte lineage (Cheng *et al.*, 2000; Kuhlbrodt *et al.*, 1998; Stolt *et al.*, 2002).

Consistent with its expression pattern, *Sox10* is required for proper development of various neural crest-derived cell types. Animals homozygous for a spontaneous mutation in the *Sox10* locus, the Dominant megacolon ($Sox10^{Dom}$), show a severe embryonic lethal defect comprising absence of enteric ganglia as well as reduction of sympathetic and parasympathetic ganglia, loss of glia, melanocytes and adrenal chromaffin cells (Kapur, 1999; Southard-Smith *et al.*, 1998). A similar phenotype is exhibited by homozygous mice carrying a targeted *Sox10* null mutation ($Sox10^{LacZ}$; (Britsch *et al.*, 2001). Haploinsufficiency of *Sox10* results in distal enteric aganglionosis, pigmentation defects and cochlear neurosensory deafness (Britsch *et al.*, 2001; Herbarth *et al.*, 1998; Paratore *et al.*, 2002), a phenotype similar to WS4 (Shah-Waardenburg syndrome) observed in human patients carrying *SOX10* mutations (Pingault *et al.*, 1998; Southard-Smith *et al.*, 1998). Studies by Paratore et al. (2001) showed that the cellular basis for these phenotypes is the requirement for Sox10 by NCSCs before lineage segregation. Sox10 is not required for initial NCSCs specification or migration from the neural tube (Britsch *et al.*, 2001; Herbarth *et al.*, 1998; Southard-Smith *et al.*, 1998) but it is essential for the survival of undifferentiated post-migratory NCSCs. In accordance, in the absence of this transcription factor NCSCs undergo apoptotic cell death before

entering the foregut, which leads to the complete elimination of ENS precursors from the entire gastrointestinal tract (Kapur, 1999; Southard-Smith *et al.*, 1998).

A combination of *in vivo* and cell culture studies has suggested that Sox10 might be also implicated in maintaining NCSCs in an undifferentiated state. Paratore et al. (2002) reported that decreased levels of *Sox10* do not impair survival or migration of eNCSCs within the gastrointestinal tract but prevents them from maintaining their progenitor state leading to the acquisition of preneuronal traits (Paratore *et al.*, 2002). In contrast, *Sox10* overexpression in cultured NCSCs inhibits neuronal and glial differentiation (Bondurand *et al.*, 2006; Kim *et al.*, 2003).

Despite its role in the maintenance of the ENS progenitor pool, Sox10 does not appear to be sufficient for conferring progenitor character to eNCSCs as it is also expressed at high levels in the glial lineage of the ENS. This argues that additional factors contribute to the maintenance of the pool of eNCSCs, presumably by interacting with Sox10 either directly or indirectly. One such factor is the transcriptional factor **Sox8**. *Sox8* belongs to group SoxE along with *Sox9* and *Sox10*. Unlike *Sox9, Sox8* exhibits an expression pattern similar to *Sox10* with occurrence in peNCSCs, eNCSCs and later confinement to enteric glia (Maka *et al.*, 2005). Although single *Sox8* mutant animals show no defects in ENS development (Sock *et al.*, 2001), *Sox8* functions as a modifier gene for *Sox10*-dependent ENS defects as it increases cell death in the peNCSC population leading to an increased severity of aganglionosis in *Sox10* heterozygous mice.

A number of genetic and cell culture studies have highlighted also the interaction between Sox10 and the signalling pathways EDNRB/endothelin-3 and RET/GDNF in

the regulation of the progenitor pool size. **EDNRB** is a G protein-coupled receptor for the intracellular messengers endothelin-1 (ET-1), ET-2, ET-3 (Inoue *et al.*, 1989). Among these peptides ET-3 is the only known ligand of EDNRB in the gut during mouse embryogenesis (Gershon, 1999; Leibl *et al.*, 1999). Analysis of spontaneous and targeted mutations in the genes encoding ET-3 (*Edn3)* and EDNRB (*Ednrb*) established the requirement of this signalling pathway in ENS development. In mice and rats lacking *Edn-3* or *Ednrb*, enteric ganglia are missing from the distal regions of the GI tract (Baynash *et al.*, 1994; Hosoda *et al.*, 1994). Genetic studies showed that the EDNRB/ET-3 signalling pathway interacts with Sox10 to promote the survival of peNCSCs as loss of *Ednrb* (Cantrell *et al.*, 2004) or *Edn3* (Stanchina *et al.*, 2006) increases severity of aganglionosis in *Sox10* heterozygous animals due to increased cell death of peNCSCs. Consistent with a genetic interaction between *Sox10* and *Ednrb* loci, Sox10 binding sites exist in an *Ednrb* enhancer region and are required for proper spatiotemporal expression of *Ednrb* in the ENS (Zhu *et al.*, 2004). Moreover, EDNRB/ET-3 signalling cooperates with Sox10 to maintain eNCSCs in an uncommitted, self-renewing state allowing a larger enteric neural precursor population to be built up, for later differentiation (Bondurand *et al.*, 2006; Hearn *et al.*, 1998; Nagy & Goldstein, 2006). Experiments performed in mouse and chick showed that via its receptor on crest cells, ET-3 produced by the gut mesenchyme, inhibits or retards neuronal differentiation (Hearn *et al.*, 1998; Wu *et al.*, 1999). Accordingly, in the absence of signalling via this pathway there is premature neuronal differentiation and a resultant smaller pool of undifferentiated cells. As a consequence, fewer progenitors are available to complete colonisation of the distal gut leading to distal aganglionosis as

observed in mice and humans with mutations in the genes encoding either ET-3 or EDNRB (Amiel & Lyonnet, 2001; Baynash *et al.*, 1994; Brooks *et al.*, 2005; Hosoda *et al.*, 1994). Furthermore, studies by Barlow et al. (2003) showed that EDNRB/ET-3 signalling enhances the proliferative response of eNCSCs to RET signalling.

RET is a transmembrane tyrosine kinase that acts as a receptor for the glial cell line-derived neurotrophic factor (GDNF) and the other members of the GDNF family of ligands (GFLs), neurturin, artemin and persephin (Baloh *et al.*, 2000; Saarma, 2000). The interaction between GFLs and RET is mediated by glycosyl-phosphatidyl-inositol (GPI)-linked cell-surface glycoproteins (GFRα1-4) and these determine RET ligand specificity (Airaksinen *et al.*, 1999; Baloh *et al.*, 2000).

The gene that encodes RET (*c-Ret*) was originally identified as a transforming gene. Dominant, gain-of-function mutations in this locus lead to the familial cancer syndromes multiple endocrine neoplasia type 2A (MEN2A) and 2B (MEN2B) and familial medullary thyroid carcinoma (FMTC) (Grieco *et al.*, 1990; Mulligan *et al.*, 1993). These syndromes are defined by tumors of the endocrine glands arising from neural crest derivatives and result from an increased kinase activity and aberrant stimulation of downstream pathways.

Besides its role in oncogenesis, RET is essential for proper development of diverse neuronal populations of the PNS, including enteric, sympathetic, parasympathetic and sensory neurons (Durbec *et al.*, 1996; Schuchardt *et al.*, 1994). In the ENS, *c-Ret* is expressed in peNCSCs when they first reach the vicinity of the gut at E9-9.5 and continues to be expressed as these cells migrate within the foetal gut (Durbec *et al.*, 1996; Pachnis *et al.*, 1993; Tsuzuki *et al.*, 1995). In the differentiated lineages of the

ENS, *Ret* expression is maintained exclusively in the neuronal population (Young *et al.*, 2003). As suggested by this expression pattern, RET is essential for the development of the ENS in vertebrates. For instance, humans heterozygous for germline mutations at the *c-RET* locus have increased susceptibility to Hirschsprung's disease (HSCR), a congenital disorder characterized by the absence of enteric ganglia in the distal colon (intestinal aganglionosis; (Edery *et al.*, 1994; Romeo *et al.*, 1994). Mice with null mutations in this gene (Ret^{k-}) die soon after birth and display complete intestinal aganglionosis (Durbec *et al.*, 1996; Schuchardt *et al.*, 1994). A similar phenotype is found in mice deficient for either g*dnf* or *gfrα1*, highlighting the functional interaction between these molecules (Cacalano *et al.*, 1998; Pichel *et al.*, 1996).

Gene targeting studies in mice have provided valuable insight into the role of RET in ENS development. A detailed analysis of the *Ret*-deficient embryos showed that in the absence of RET, eNCSCs undergo apoptosis and fail to enter the midgut suggesting that the RET signalling pathway provides survival signals to the majority of the progenitors of the ENS (Durbec *et al.*, 1996; Taraviras *et al.*, 1999). In addition to this early function, the RET receptor has also been implicated in eNCSCs proliferation. *In vitro* assays have demonstrated that activation of the RET receptor by exogenous GDNF added to the culture medium enhanced cell proliferation and induced an increase in the number of neural precursors (Chalazonitis *et al.*, 1998; Hearn *et al.*, 1998; Heuckeroth *et al.*, 1998; Taraviras *et al.*, 1999). Furthermore, studies by Gianino et al. (2003) suggested that GDNF critically determines the proliferative capacity of eNCSCs and that the extent of their proliferation ultimately determines the number of enteric neurons in the adult gut. Interestingly, the response of eNCSC to GDNF changes as a function of

developmental age. Chalazonitis et al. (1998) reported that even though at E12 GDNF has a mitogenic effect on eNCSCs, during the post-migratory stages of enteric neurogenesis (in the mouse after E14) GDNF preferentially induces the differentiation of these cells into neurons. The EDNRB/ET-3 signalling system seems to be critical in the balance between the mitogenic and differentiation effect of RET/GDNF signalling by counteracting the differentiation role of GDNF without affecting its effect on proliferation (Hearn *et al.*, 1998).

Maintenance of the eNCSCs population is also dependent on the mitogenic role of the epidermal growth factor (EGF) as highlighted in a recent study (Fuchs *et al.*, 2009). Fuchs et al. (2009) showed that, once in the gut, eNCSCs acquire responsiveness to EGF through upregulation of the EGF receptor (**EGFR)**. This receptor acts through two small GTPases, Rac1 and Cdc42. Conditional deletion of *Rac1* and *Cdc42* in eNCSCs results in the reduction of the self-renewal and proliferative abilities of eNCSCs which suggests that, acting via Rac1 and Cdc42, EGFR signaling promotes self-renewal and proliferation of eNCSCs.

Another factor involved in maintenance of the progenitor pool is **Notch**. The Notch receptors are transmembrane proteins activated by Delta and Jagged ligands (Yoon & Gaiano, 2005). The role of Notch signalling in the CNS and PNS is complex and context-dependent. Overexpression of Notch pathway components has been shown to either promote the maintenance of undifferentiated progenitor cells or gliogenesis. In the CNS, overexpression of Notch1 or its target genes increases the formation of radial glia in the telencephalon (Yoon *et al.*, 2004) and instructs adult hippocampus progenitors to become astrocytes in culture (Tanigaki *et al.*, 2001). In contrast, the absence of Notch or

the Notch transcriptional effectors Hes1 or Hes5 in mice leads to the loss of neuroepithelial cells and premature neuronal differentiation due to the upregulation of proneural genes such as *Mash1* (mammalian achaete-scute homologue 1–Ascl1; (Hatakeyama *et al.*, 2004; Hitoshi *et al.*, 2002). Whether Notch acts to maintain undifferentiated progenitors and, subsequently to promote gliogenesis, or whether the defects in gliogenesis are secondary to a premature depletion of the progenitor pool is still under debate.

Evidence for the role of Notch signalling in the maintenance of eNCSCs in an undifferentiated state came from the study of mice exhibiting neural crest-specific defects in Notch signalling (Okamura & Saga, 2008; Taylor *et al.*, 2007). Taylor et al. (2007) showed that conditional deletion of *Rbpsuh*, which encodes a DNA binding protein (RBP/J) required for canonical signalling by all Notch receptors, results in severe deficits in the number of eNCSCs. Consistent with a role for the Notch pathway in the maintenance of the progenitor pool, *Rbpsuh*$^{-/-}$ eNCSCs exhibited low efficiency in colony formation precluding analysis of a potential requirement for Notch signalling in ENS gliogenesis (Taylor *et al.*, 2007). These results were further confirmed by Okamura and Saga (2008), who showed that in the absence of Notch signalling there is loss of Sox10 expression suggesting that Notch is required for the maintenance of Sox10 in eNCSCs, maybe by repressing expression of *Mash1* (Chen *et al.*, 1997). Downregulation of Sox10 is accompanied by reduced proliferation and premature neuronal differentiation leading to the depletion of the progenitor pool.

Another transcription factor essential for the survival of eNCSCs is **Phox2b**. This paired box homeodomain transcription regulator is expressed by autonomic neural crest

derivatives (sympathetic, parasympathetic and enteric ganglia) as well as by some cranial ganglia and hindbrain nuclei (Pattyn *et al.*, 1999). In the PNS, Phox2b is required for the activation of the neuronal programme by inducing expression of proneural genes such as *Mash1* and also for the early specification of the neuronal phenotype in noradrenergic neurons of the PNS (Pattyn *et al.*, 1999; Pattyn *et al.*, 2000). In the ENS, peNCSCs induce expression of Phox2b upon arrival to the dorsal aorta and its expression is maintained in essentially all differentiated neurons and also a subpopulation of glial cells (Corpening *et al.*, 2008; Young *et al.*, 1998; Young *et al.*, 2003). In the absence of *Phox2b*, eNCSCs cells arrive at the foregut but fail to migrate further and undergo apoptosis, which leads to aganglionosis. Furthermore, eNCSCs fail to express the receptor tyrosine kinase RET (Pattyn *et al.*, 1999). This phenotype suggests an essential role for Phox2b in the early survival of eNCSCs, probably through the regulation of *Ret* expression.

1.3.2.Molecular regulation of enteric neuronal differentiation

Mutant mice in which myenteric neuron density is increased (Taketomi *et al.*, 2005) or decreased (Roberts *et al.*, 2008) exhibit motility defects, and thus eNCSCs must generate correct numbers of enteric neurons for the formation of an intact, functional ENS. Studies over the past years showed that the number and type of neurons produced by neural stem cells is modulated by changes in the expression of neurogenic signals in the environment as well as by cell-intrinsic changes that affect the way stem cells respond to these neurogenic factors (Qian *et al.*, 2000; White *et al.*, 2001). Within the ENS analogous regulatory mechanisms have been identified. Earlier *in vitro* studies on chick, mouse and rat suggested that the RET/GDNF signalling pathway can stimulate

differentiation of eNCSCs into neurons and promote neurite growth (Chalazonitis *et al.*, 1998; Hearn *et al.*, 1998; Heuckeroth *et al.*, 1998; Taraviras *et al.*, 1999). Interestingly, Chalazonitis et al (1998) reported that GDNF inhibits expression of the glial marker S100β, thus favouring neuronal but not glial differentiation. Despite these studies, the role of the RET/GDNF signalling pathway in eNCSCs differentiation *in vivo* is still poorly understood. Conditional ablation of *gfrα1* or *c-Ret* suggested that, at late stages of development, RET signalling is required for the survival of enteric neurons in the colon (Uesaka *et al.*, 2007; Uesaka *et al.*, 2008). However, the effects of abrogating RET signalling pathway in eNCSCs and how that affects cell commitment and differentiation was not addressed in this study. Evidence supporting a potential association between RET signalling and differentiation of eNCSCs into specific cellular phenotypes came from the study of the monoisoformic *Ret* mutants, which express only one of the two RET isoforms, RET9 or RET51 (de Graaff *et al.*, 2001). In contrast to RET9, the RET51 isoform is not sufficient to support normal embryonic and postnatal development as mutant mice that express only RET51 ($Ret^{51/51}$) exhibit distal aganglionosis. Further analysis of these mutants revealed a significant reduction in the number of enteric neurons (Dipa Natarajan, personal communication), thus suggesting that RET signalling is required for the generation of neurons in proper numbers. The role of the RET receptor in neuronal differentiation is further supported by the analysis of **Sprouty2**-deficient mice (Taketomi *et al.*, 2005). Sprouty proteins are evolutionary conserved inhibitors of tyrosine kinase signalling. Studies by Taketomi et al. (2005) showed that Sprouty2 functions as a negative regulator of RET/GDNF signalling as its

absence results in hyper-responsiveness to the RET/GDNF system and consequently, enteric neuron hyperplasia.

Neurturin is another functional ligand of the RET receptor important for ENS differentiation. The RET/NRTN pathway has a minimal effect on enteric neuron survival or proliferation but it is involved in the development of cholinergic neurons, not only in the gut but also in parasympathetic ganglia, since *neurturin*-/- and *gfrα2*-/- mice have reduced numbers of cholinergic neurons in the cranial and enteric ganglia (Heuckeroth *et al.*, 1999; Rossi *et al.*, 1999).

Another class of signalling molecules that influence neuronal differentiation is the bone morphogenetic proteins (**BMPs**). The BMPs instruct neuronal differentiation of NCSCs by inducing expression of proneural genes, such as *Mash1* (Shah *et al.*, 1996; Shah & Anderson, 1997). However, the response of NCSCs to these neurogenic signals changes over time influencing the number and type of neurons that are generated (White *et al.*, 2001). Within the ENS, BMPs are also involved in the regulation of enteric neuron number as shown by the study of transgenic mice over expressing the BMP inhibitor noggin. Noggin antagonism of BPM signalling resulted in an increase of the total number of enteric neurons, however, enteric neuron subtypes were affected differently; the number of early-born neuronal subpopulations increased whereas late-born neuronal subtypes decreased. The effects of noggin over expression are, thus, correlated with cell cycle exit suggesting that BMP signalling may regulate enteric neuronal phenotypic diversity by promoting the exit of precursors from the cell cycle (Chalazonitis *et al.*, 2004; Chalazonitis *et al.*, 2008).

A number of transcription factors involved in enteric neuronal differentiation have also been identified, including Mash1 and Hand2. **Mash1** is a transcription factor that belongs to the basic helix-loop-helix (bHLH) family. In the CNS, *Mash1* is expressed transiently during embryogenesis in neuron-committed progenitors and it is involved in promoting neurogenesis by activating the expression of a cascade of neuron-specific genes as well as inhibiting the expression of glia-specific genes (Guillemot *et al.*, 1993; McNay *et al.*, 2006; Nieto *et al.*, 2001; Parras *et al.*, 2002; Tomita *et al.*, 2000). In the ENS, *Mash1* is also expressed transiently; expression begins in neural precursor cells but it is extinguished as they differentiate into neurons. An earlier report on the *in vivo* role of Mash1 in the ENS showed that deletion of this bHLH transcription factor results in a transient delay in the migration and differentiation of early neural crest cells. This delay is eventually overcome but the selective elimination of the transient catecholaminergic-serotonergic neuronal lineage persists suggesting that Mash1 is required for the development of this particular subtype of neurons (Blaugrund *et al.*, 1996).

More recent studies have identified the transcription factor **Hand2** as an important player in neuronal differentiation. Hand2 is a member of the twist family of bHLH transcription factors (Firulli, 2003) and it is expressed in the peripheral autonomic nervous system, including the enteric ganglia (Cserjesi *et al.*, 1995; Dai *et al.*, 2004; Morikawa *et al.*, 2005). In the ENS, *Hand2* expression is developmentally regulated; it peaks at E12.5 and then at E14 declines to a lower level that is maintained throughout adulthood. The effect of the conditional deletion of *Hand2* in eNCSCs has been analysed (D'Autreaux *et al.*, 2007; Hendershot *et al.*, 2007). A study by D'Autreaux et

al. (2007) showed that loss of Hand2 leads to a dramatic reduction in the differentiation capacity of eNCSCs without affecting their ability to colonise the gastrointestinal tract or differentiate into glial cells. Furthermore, eNCSCs that enter the neuronal lineage are unable to complete neuronal differentiation and acquire subtype-specific markers. In a similar study, Hendershot et al. (2007) suggested that *Hand2* expression is required for the differentiation of specific neuronal subtypes, namely TH^+ and VIP^+ neurons.

1.3.3.Molecular control of enteric glia differentiation

A fundamental question in developmental neurobiology concerns the mechanisms that regulate the transition from neurogenesis to gliogenesis in vertebrates. In the ENS, neurons appear prior to glial cells as evidenced by the detection of specific markers for both cell types (Baetge & Gershon, 1989; Baetge *et al.*, 1990a; Young *et al.*, 2003). This suggests that, as in other parts of the nervous system, enteric glial differentiation lags behind neural differentiation. However, formation of new neurons persists after the appearance of glial cells indicating that neurogenesis and gliogenesis co-exist in the gut of mouse embryos. Although substantial progress has been made towards understanding environmental and cell intrinsic signals that promote ENS formation in general, very little is known about the mechanisms that regulate gliogenesis in the gut.

The continuous expression of **Sox10** in peripheral glia, including enteric glial cells, points towards a role for this transcription factor in glial fate specification and differentiation. Such function has already been verified in the DRGs. In mice homozygous for a targeted mutation in *Sox10*, neuronal cells are able to form in the DRGs but Schwann cells or satellite cells are not generated (Britsch *et al.*, 2001). Also, $Sox10^{-/-}$ NCCs from mouse embryos cultured in gliogenic promoting conditions fail to

produce any glia (Paratore *et al.*, 2001). This is thought to involve ErbB3 and Notch1 as its expression in glial progenitors is lost in the Sox10 mutant DRGs (Britsch *et al.*, 2001; Paratore *et al.*, 2001). Indeed, another important player in glia development is the receptor tyrosine kinase **ErbB3**. This receptor interacts with the neuronal-derived neuregulins (NRGs), in particular with the soluble NRG1 isoform, which strongly suppresses the neuronal fate while promoting differentiation of peripheral glia (Marchionni *et al.*, 1993; Shah *et al.*, 1994).

Notch is another factor that has been implicated in gliogenesis (Kopan & Ilagan, 2009). More than just inhibiting neuronal differentiation, Notch signalling has been shown to participate in the regulation of binary fate decisions during neural development by instructing gliogenesis (Morrison, 2001). Studies by Tanigaki et al. (2001) showed that Notch activation in CNS stem cells of the adult hippocampus leads to astrocytic differentiation (Tanigaki *et al.*, 2001). Also, overexpression of Notch1 or its target genes promotes acquisition of glial fates *in vivo* in the postnatal retina (Hojo *et al.*, 2000). Consistent with the idea that Notch can send a positive signal that promotes glial lineage determination in vertebrates, RBP/J (a DNA-binding protein that interacts with the intracellular domain of activated Notch to regulate transcription) can directly bind the promoters of glial genes and activate transcription (Anthony *et al.*, 2005; Ge *et al.*, 2002). The role of Notch signalling in gliogenesis has also been examined in the peripheral nervous system (Morrison *et al.*, 2000; Taylor *et al.*, 2007). Morrison et al. (2000) showed that exposure of NCSCs of the DRG to Notch ligand delta-like1 (Dll1) instructs glial differentiation and causes an irreversible loss of neurogenic potential. In light of these results, the authors suggested that the transient expression of Notch ligands

by nascent autonomic neurons lead NCSCs to loose their neurogenic potential and differentiate into glial cells triggering the switch between neurogenesis and gliogenesis during development. Also, i*n vivo* studies using mice with neural crest-specific defects in the Notch pathway showed that Notch signalling is required for gliogenesis in sensory and sympathetic ganglia (Taylor *et al.*, 2007). In the developing ENS, Notch signalling was shown to be involved in the maintenance of normal numbers of NCSCs (Okamura & Saga, 2008; Taylor *et al.*, 2007) but its role in gliogenesis was not examined.

1.4. Lineages of the ENS

The mature ENS is comprised of a vast number of different neuronal subtypes plus an even higher number of enteric glial cells. Generation of specific neuronal subtypes with a defined projection pattern and the supporting glial cells is essential for the assembly of functioning neural circuits within the ENS.

1.4.1.Phenotype of neural crest-derived cells that form the ENS

Differentiation of NCSCs into enteric neurons and glia is thought to follow a series of cell commitment and differentiation steps orchestrated by a highly regulated pattern of gene expression. Thus, after delamination from the neural tube peNCSCs express general neural crest cell markers, including the HMG-containing transcriptional regulator **Sox10** and the low-affinity nerve growth receptor **p75** (Anderson *et al.*, 2006b; Young *et al.*, 1998; Young *et al.*, 1999). Upon arrival at the dorsal aorta, peNCSCs induce expression of the transcription regulator **Phox2b** and the receptor tyrosine kinase **RET** and invade the foregut mesenchyme (Durbec *et al.*, 1996; Lo & Anderson, 1995;

Pattyn *et al.*, 1999). At this point, a subpopulation of eNCSCs starts to express the transcription factor **Mash1** (Blaugrund *et al.*, 1996; Lo *et al.*, 1997).

From the foregut, eNCSCs initiate a well-orchestrated rostro-caudal migration culminating in the uniform colonization of the entire gastrointestinal tract (Kapur *et al.*, 1992; Young & Newgreen, 2001). As eNCSCs migrate through the gut, they proliferate extensively, while subsets of eNCSCs initiate neuronal and glial differentiation (**Fig.1.4**; (Heanue & Pachnis, 2007). In a study by Young et al. (2003) the phenotype of eNCSCs as they differentiate into neurons and glia was examined (Young *et al.*, 2003). This showed that differentiation of eNCSCs into neurons takes place shortly after invasion of the foregut and it is accompanied by the expression of pan-neuronal markers (molecular characteristics common to all neurons) such as neuron class III β-tubulin (**Tuj1**) and **HuC/D** (Marusich *et al.*, 1994; Wakamatsu & Weston, 1997). Along with these markers, differentiating neurons express also RET, Phox2b but not Sox10. As for the glial precursors, these are firstly identified behind the migratory wave front, at E11.5, by the expression of Sox10, p75 and brain-specific fatty acid binding protein (**BFABP**;(Kurtz *et al.*, 1994; Veerkamp & Zimmerman, 2001). Besides Sox10, p75 and BFABP, adult glial cells express **S100β** (Ferri *et al.*, 1982) from E14.5 onwards and glial fibrillary acidic protein (**GFAP**; (Jessen & Mirsky, 1980; Jessen & Mirsky, 1983) after E16.5 (Young *et al.*, 2003).

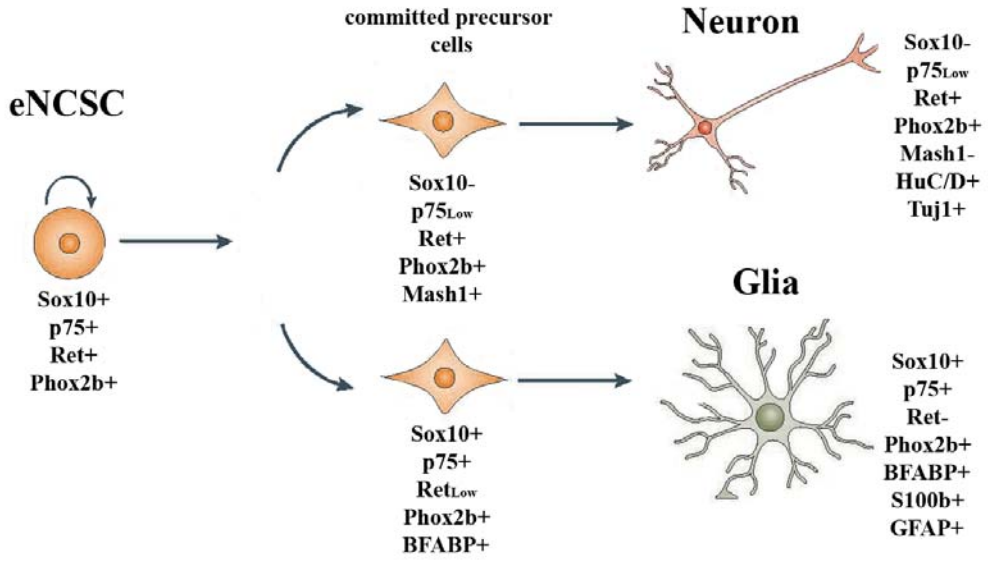


Figure 1.4. ***Differentiation of eNCSCs into neurons and glia follows a highly regulated programme of gene expression.*** eNCSCs that enter the foregut express the transcription factors Sox10 and Phox2b, p75, and the receptor tyrosine kinase RET. A fraction of eNCSCs induce expression of Mash1 and these are thought to be committed precursors of the neuronal lineage. These cells do not express Sox10 and they exhibit low levels of p75. Cells that differentiate into neurons downregulate Mash1 and p75, maintain RET expression, and upregulate pan-neuronal markers such as Tuj1 and HuC/D. Similar to eNCSCs, gliogenic precursor cells express Sox10 and p75 but they downregulate RET expression and upregulate the glial specific marker BFABP. Differentiated glial cells express BFABP and upregulate other glial markers such as S100β and GFAP. Adapted from (Heanue & Pachnis, 2007).

1.4.2.Enteric neuronal cells

Neuronal diversity in the adult ENS

Enteric neurons are very diverse in terms of cell morphology, function, type of neurotransmitters and neuropeptides produced and patterns of axonal projection. Initially, enteric neuron subtypes were classified based on morphological characteristics (Furness, 2006). More recently, a combination of morphology, neurotransmitter/neuromodulator content, electrophysiology, target tissue and direction and length of axon projection have been used to classify enteric neurons. In the small

intestine of the guinea pig about 20 distinct subtypes of myenteric neurons and 4-5 subtypes of submucosal neurons have been identified. These include excitatory and inhibitory motor neurons (which target the muscle layers), ascending and descending interneurons (that connect enteric neurons in different ganglia), sensory neurons (which monitor the state of the lumen and gut wall), secretomotor neurons and vasodilator neurons (project to the mucosa and to the local blood vessels) (Brookes, 2001; Costa *et al.*, 1996; Furness, 2000). A similar number of enteric neuron subtypes is also present in the mouse gut (Qu *et al.*, 2008).

Developmental appearance of enteric neuronal subtypes

During differentiation, enteric neurons first express pan-neuronal markers and then acquire markers of terminally differentiated neurons (Baetge & Gershon, 1989; Branchek & Gershon, 1989; Rothman & Gershon, 1982; Young *et al.*, 1999). As in most parts of the nervous system, different types of enteric neurons (identified by the combined expression of specific neurotransmitters and the enzymes responsible for their synthesis) develop at different developmental stages (**Table1.1**; (Epstein *et al.*, 1983; Rothman & Gershon, 1982). The first neurons of the developing ENS express the cathecolamine synthetic enzyme, tyrosine hydroxylase (TH), and they are found only transiently in the mouse gut. TH is expressed by all developing enteric neurons from E10 to E15 (Baetge & Gershon, 1989; Baetge *et al.*, 1990a; Young *et al.*, 1999). However, expression of this marker is downregulated by immature enteric neurons (Pisano & Birren, 1999) and so, in the mature ENS only a small population of neurons (less than 0.5% of myenteric neurons) are TH^+ and these do not arise from the transiently TH^+ cells that are present early in embryonic development (Li *et al.*, 2004).

Table1.1 The earliest detection of subtype-specific markers and birthdates of myenteric neurons in the mouse small intestine

Neuronal Subtype	Developmental appearance	Birthdate (myenteric plexus)	References
5-HT	E12*	E8-E14	(Pham *et al.*, 1991; Rothman & Gershon, 1982)
NOS	E12	E12.5-P1	(Branchek & Gershon, 1989; Chalazonitis *et al.*, 2008)
cholinergic	E10$^{\delta}$	E8-E15	(Pham *et al.*, 1991; Rothman & Gershon, 1982)
NPY	E13-E13.5	E10-E18	(Branchek & Gershon, 1989; Pham *et al.*, 1991)
VIP	E14	E10-P5	(Pham *et al.*, 1991; Rothman *et al.*, 1984)
CGRP	E17	E10-P3	(Branchek & Gershon, 1989; Pham *et al.*, 1991)

* Radioactively labeled 5'HT can be detected in the gut at E12 but immunoreactivity to 5'HT is not found until E18

$^{\delta}$ Synthesis of radioactively labeled Ach from ^{3}H-choline is detected from E10 onwards but immunoreactivity to acetylcholine transporter (ChAT) cannot be detected until E18.5.

As neuronal precursors loose their catecholaminergic phenotype they acquire properties of cholinergic, serotoninergic, nitregic or peptidergic terminally differentiated neurons. This has been suggested to involve changes in the microenvironment as the original catecholaminergic phenotype is maintained when the gut is cultured *in vitro* (Baetge *et al.*, 1990b). Terminally differentiated enteric neurons can first be detected in the mouse gut on day E12. Among these are the serotoninergic neurons that express 5'-hydroxytryptamine (5-HT) and nitrergic neurons, identified by the expression of nitric oxide synthase (NOS; (Branchek & Gershon, 1989; Rothman & Gershon, 1982). Using the synthesis of ^{3}H-acetylcholine (Ach) as a marker, cholinergic neurons were first detected from E10 to E12 (Rothman & Gershon, 1982) although immunoreactivity to acetylcholine transporter (vChAT) cannot be detected until E18.5 (Vannucchi & Faussone-Pellegrini, 1996). In embryonic mice, expression of neuropeptide Y (NPY) is, firstly, found at E13-13.5 (Branchek & Gershon, 1989), vasoactive intestinal peptide

(VIP) at E14 (Rothman *et al.*, 1984) and calcitonin gene-related peptide (CGRP) at E17 (Branchek & Gershon, 1989).

Phenotypic characteristics of enteric neurons in the adult ENS

In adult mice, NOS-immunoreactive neurons are inhibitory motor neurons to the muscle or descending interneurons and they represent around 29% of all neurons (Qu *et al.*, 2008; Sang & Young, 1996). Most NOS^+ neurons express VIP and a subset of these is also NPY^+ (Sang & Young, 1997). 5-HT expression is restricted to 1% of all neurons and these are descending interneurons that also express acetylcholine (Ach; (Sang & Young, 1996; Sang & Young, 1997). Ach is expressed in excitatory motor neurons, which comprise approximately 35% of all neurons, and in interneurons. Intrinsic sensory neurons represent about 26% of all neurons and express Ach, CGRP and calbindin (Furness, 2006; Qu *et al.*, 2008).

1.4.3.Enteric glial cells

Morphology, localization and quantification of enteric glia

In addition to neurons, the ENS contains a large number of supporting glial cells. These are small, star-shaped cells with numerous processes of various length and shape. Enteric glial cells are found in both myenteric and submucosal ganglia and in the interconnecting nerve strands. In the nerve strands, glial processes ensheat multiaxonal bundles whereas in the ganglia, glial cells are in close association with neurons (**Fig.1.5**). Unlike the satellite cells of sympathetic ganglia, enteric glia do not envelop neurons completely. Indeed, a characteristic feature of the enteric plexus is that the glial sheet that surrounds ENS neurons is incomplete and, as a result, portions of axons or

neuronal cell bodies can make direct contact with the extraganglionic connective tissue (Gabella, 1972).

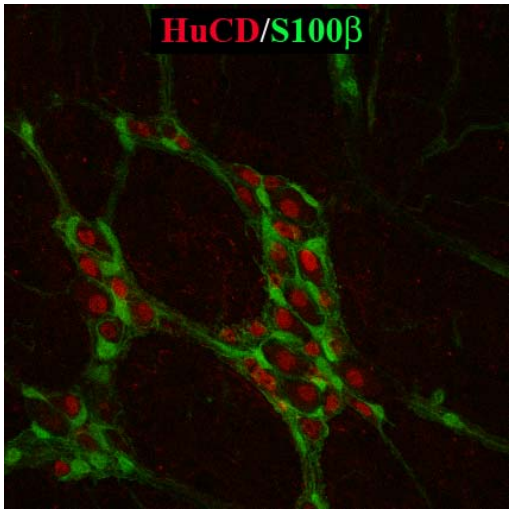


Figure 1.5. ***Ganglia of the myenteric plexus from the mouse small intestine.***
Confocal microscope image (x400 magnification) of a whole mount preparation of the myenteric plexus immunostained for the neuronal marker HuC/D (red) and the glial marker S100β (green). Glial cells are found in close association with neurons and in the interconnecting nerve strands between ganglia.

Glial cells are more abundant than neurons. Quantification of these cells showed that the glia index, which describes the ratio of glial cells to neurons, increases with species size from 1.1 or 0.6 in mice to 7 or 1.9 in humans, in the myenteric and submucosal ganglia respectively (Gabella & Trigg, 1984; Hoff *et al.*, 2008). The number of enteric glia decreases, however, with age. Age-related glial loss is accompanied by a proportional loss of enteric neurons but it is unclear whether enteric glia decay precedes or follows neuronal loss (Phillips *et al.*, 2004). Changes in glial cell numbers were further observed in animal models of inflammation (Bradley *et al.*, 1997; Bush *et al.*, 1998) as well as in patients with inflammatory bowel disease (Geboes & Collins, 1998; Storsteen *et al.*, 1953).

Phenotypic characteristics of enteric glia

Glial precursors are first identified in the developing ENS at E11.5 by the expression of BFABP (Veerkamp & Zimmerman, 2001; Young *et al.*, 2003). BFABP is found

exclusively in glial cells and its expression starts even before glial differentiation and so this is considered as a commitment marker for glial lineage (Young *et al.*, 2003). Other markers that identify differentiated enteric glia are S100β (Ferri *et al.*, 1982) and GFAP (Jessen & Mirsky, 1980; Jessen & Mirsky, 1983), both of which are typical of CNS astrocytes. S100 proteins are small, acidic Ca^{2+}-binding proteins, localized in the cytoplasm of glial cells where they regulate cytoskeletal structure and function as well as Ca^{2+} homeostasis. In the gut of embryonic mice, S100β expression can be first detected at E14.5, exclusively in glial cells (Gershon & Rothman, 1991; Young *et al.*, 2003). GFAP identifies the intermediate filament found in cells of astroglial lineage. In the gut, GFAP starts to be expressed at E16.5 and it is a specific glial marker.

Physiological roles of enteric glia

For some time, glial cells were seen as mere mechanical support elements, holding the various components of the ENS together. Indeed, during the mechanical activity of the muscularis externa there are changes in width and thickness of the ganglia as well as changes in shape of the individual neurons. Morphological data suggest that, by being firmly anchored to the surface of enteric ganglia and nerve strands through GFAP filaments, enteric glial cells stabilize the ENS (Gabella, 1981; Gabella, 1990; Hanani & Reichenbach, 1994). In addition, enteric glia actively respond to mechanical stimulation with enhanced expression of *c-fos* (Sharkey *et al.*, 1999) and a rise in intracellular Ca^{2+} levels (Zhang *et al.*, 2003) which suggests that these cells may adjust for structural and metabolic changes resulting from mechanical stress in the gut wall.

Besides providing structural support, increasing evidence suggest that enteric glia are actively involved in the control of gastrointestinal functions. The fundamental role

of enteric glia in the maintenance of ENS integrity was demonstrated by the study of transgenic mouse models in which enteric glial cells were conditionally ablated (Bush *et al.*, 1998; Cornet *et al.*, 2001). In these mouse models, ablation of enteric glia led to the disruption of the intestinal epithelium and vascular integrity resulting in fulminant intestinal inflammation, hemorrhage and necrosis. The breakdown of the intestinal epithelial barrier in the absence of enteric glia suggests that these cells are involved in maintaining mucosal barrier integrity. Also, the occurrence of early vascular lesions in the intestinal mucosa and submucosa following selective depletion of enteric glia indicates that these cells may be involved in the regulation of the vascular function of the gastrointestinal tract. Indeed, enteric glial processes can be found in the mucosal crypts and tips of the villi where they are in close proximity to epithelial cells and blood vessels but it is still unclear whether the direct contact between glial cells/processes and epithelial cells and blood vessels is required for maintaining epithelial and vascular integrity. Besides their role as a potential component of the mucosal defence system through the maintenance of the intestinal epithelial barrier, enteric glia have been shown to be involved in mucosal immune responses. Studies by Ruhl et al., (2001) showed that enteric glia can synthesise cytokines and respond to inflammatory insults (Ruhl *et al.*, 2001a). In accordance, an enhanced GFAP expression and increased proliferative capacity of glial cells was observed in animal models of intestinal inflammation and tissue samples from patients with inflammatory bowel disease (Bradley *et al.*, 1997; Storsteen *et al.*, 1953). Whether this proliferative response represents a phenomenon comparable to the neuroprotective reactive gliosis observed in the CNS is not known.

Ablation of enteric glia in genetically modified animals indicates that these cells may also be crucial for neuronal maintenance as their absence results in neuronal degeneration (Bush *et al.*, 1998) and changes in the neurochemical coding and function of enteric neurons (Aube *et al.*, 2006; Nasser *et al.*, 2006). It has been proposed that enteric glia may promote neuronal survival by directly regulating substrate supply and extracellular homeostasis (Cabarrocas *et al.*, 2003). As for the role of glia in the control of neurochemical phenotypes, is still unknown whether the actual presence of glia is required or whether enteric glia secrete factors which stabilize the neurochemical composition of the ENS but there is some evidence that neurotrophins may be produced by enteric glia to modulate neuronal gene expression and, eventually, enteric neurophenotypes (Hoehner *et al.*, 1996). The crosstalk between glial cells and neurons is further underlined by recent studies demonstrating that glial cells respond to electrical stimulation or depolarization of enteric neurons with an increase of intracellular Ca^{2+} (Gomes *et al.*, 2009; Gulbransen & Sharkey, 2009).

Despite the complex and diverse roles of enteric glial cells there is no gastrointestinal disorder for which an underlying glial defect has been established. Nonetheless, it is possible that subtle changes in glial function may be involved in the etiopathogenesis of gastrointestinal disorders.

(Dis)similarities between peripheral extraenteric glia, enteric glia and CNS astrocytes

Enteric glial cells share certain characteristics with two other distinct glial cell populations, namely Schwann cells and CNS astrocytes (**Table1.2**). Like Schwann cells, enteric glia are derived from the neural crest and are involved in the structural support of

Table1.2 Comparison of the properties of Schwann cells, enteric glia and astrocytes

Phenotypic and functional characteristics	Schwann cells	Enteric glia	CNS astrocytes
Origin	Neural crest	Neural crest	Neuroectodermal
Morphology	Regular shape; ensheath axons individually[A]	Small star-shaped cells; extension of processes ensheathing bundles of axons[A]	Small star-shaped cells
Markers	Ran-1[B]; SMP[C]; S100 β	GFAP, Ran-2; vimentin, S100β[D]	GFAP, Ran-2; vimentin, S100β[D]
Response to injury	*de novo* expression GFAP[E]	Hyperplasia; upregulation of GFAP[F]	Hypertrophy, hyperplasia, upregulation of GFAP and vimentin

A (Gershon & Rothman, 1991); **B** (Jessen & Mirsky, 1983); **C** (Dulac *et al.*, 1988); **D** (Cabarrocas *et al.*, 2003); **E** (Jessen & Mirsky, 1992); **F** (Bradley *et al.*, 1997); SMP, Schwann cell myelin protein; GFAP, glial fibrillary acidic protein.

peripheral nerve fibers. However, whereas in most peripheral nerves and ganglia Schwann cells envelop neurons and axons individually, in the ENS a single glial cell unsheathes several densely packed enteric neurons (Gershon & Rothman, 1991). In addition to these structural differences, enteric glia are phenotypically and morphologically distinct from Schwann cells. Enteric glia do not express characteristic markers of Schwann cells, such as Schwann cell myelin protein (SMP; (Dulac *et al.*, 1988) and Ran-1, and they exhibit a smaller cell body and more irregular shape (Gabella, 1981).

Despite having a different origin, enteric glia display many morphological similarities with CNS astrocytes. Furthermore, these two subtypes share a number of molecular characteristics; just like CNS astrocytes, enteric glial cells express S100β (Ferri *et al.*, 1982), glutamine synthase (Jessen & Mirsky, 1983), Ran-2 (Jessen & Mirsky, 1985) and GFAP (Jessen & Mirsky, 1980; Jessen & Mirsky, 1983). The

morphological and phenotypic resemblance between enteric glia and CNS astrocytes has led to the hypothesis that the two cell types may be functionally related.

In the adult CNS, distinct astrocyte cell types have been identified. The neurogenic astrocytes are the direct descendants of embryonic radial glia cells (Merkle *et al.*, 2004; Merkle *et al.*, 2007) and reside within rare germinal niches where they function as neural stem cells, continually renewing the granular and periglomerular interneurons of the olfactory bulb (Doetsch *et al.*, 1999) and granular neurons of the adult hippocampus (Seri *et al.*, 2001). In addition to neurogenic astrocytes, fully differentiated postmitotic astrocytes can also assume a stem-like profile in response to injury. These cells, called reactive astrocytes, are found within and surrounding a brain lesion and they are characterized for being highly proliferative and for exhibiting an enhanced expression of GFAP, nestin and other stem cell markers (Buffo *et al.*, 2008; Lin *et al.*, 1995). *In vitro* these proliferative astrocytes behave as multipotent self-renewing neural stem cells suggesting that brain injury might induce astrocytes to resume highly controlled developmental programs for neurogenesis (Buffo *et al.*, 2008). Similar to reactive astrocytes, an increased proliferation of enteric glial cells has been documented in an inflammation mouse model (Bradley *et al.*, 1997) and in patients with inflammatory bowel disease (Geboes & Collins, 1998; Storsteen *et al.*, 1953). Using Brd-U to identify cycling cells, in the myenteric ganglia, Bradley et al., (1997) showed that a few proliferative cells are normally present in the ganglia of adult guinea pig and that these are glial cells. These observations expand the observations of Gabella (1990) that enteric glia can divide for some time after birth even when neurons have ceased to do so. However, the number of proliferative glia increases significantly with inflammation

suggesting that glial cells are able to respond to inflammatory mediators. Indeed, Ruhl and colleagues showed that the immunosuppressive cytokine IL-10 enhances proliferation of enteric glia (Ruhl *et al.*, 2001b). Which cytokines are involved in the glia response to inflammation is unknown. In addition, the biological significance of this response is still unclear.

1.4.4.Enteric neural stem cells

Identification of enteric neural stem cells

There are two main types of neural stem cells, CNS stem cells and NCSCs. CNS stem cells are defined by their ability to give rise to the neurons, astrocytes and oligodendrocytes of the brain (Temple & Alvarez-Buylla, 1999). NCSCs are defined by their ability to give rise to neurons and glia of the PNS (Morrison *et al.*, 1999; Stemple & Anderson, 1992). As part of the PNS, the ENS is derived from NCSCs.

Most, if not all, the cells that invade the foregut mesenchyme are multipotent self-renewing NCSCs (Lo & Anderson, 1995; Rothman *et al.*, 1990). At this point, eNCSCs express a number of markers that are valuable for their identification. Analysis of the expression profile of eNCSCs from E10.5-E13.5 mice showed that these cells express Sox10, Phox2b, p75 and RET (Anderson *et al.*, 2006b; Young *et al.*, 1999). However, expression of these markers is not exclusive of eNCSCs. For example, Sox10, Phox2b and p75 are expressed in enteric glial cells whereas RET expression is maintained in enteric neurons (Corpening *et al.*, 2008; Young *et al.*, 2003). The lack of specific and unique markers for eNCSCs makes their positive identification *in vivo* rather difficult. For that reason, the presence of these cells has been ascertained by their behaviour in culture. A number of different strategies have been used to isolate eNCSCs (**Table1.3**).

For instance, self-renewing multipotential progenitors of the ENS have been isolated from the gut of foetal, newborn and adult rodents using cell-surface markers, such as RET (Lo & Anderson, 1995; Natarajan *et al.*, 1999) or p75 (Bixby *et al.*, 2002; Kruger *et al.*, 2002). *In vitro* clonogenic assays and *in vivo* engraftment of these cells showed that they are capable of generating both neurons and glial cells. eNCSCs have also been identified by culturing either a mixed population of gut cells (Schafer *et al.*, 2003; Suarez-Rodriguez & Belkind-Gerson, 2004) or myenteric ganglion cells in isolation from the surrounding non-neuronal tissues (Saffrey *et al.*, 2000; Schafer *et al.*, 1997; Silva *et al.*, 2008), in conditions that favour growth of cells of interest. In these studies, the presence of eNCSCs in culture was suggested by the expression of the neural progenitor marker nestin (Silva *et al.*, 2008; Suarez-Rodriguez & Belkind-Gerson, 2004) and the formation of neurospheres (floating spherical colonies characteristic of neural stem cells) (Schafer *et al.*, 2003); Silva *et al.*, 2008) Using an approach that does not depend on expression of cell surface or intracellular markers, Bondurand et al. (2003) isolated eNCSCs using cultures of dissociated gut or outer muscle layers and retrovirus-mediated gene transfer. *In vitro*, these cells were shown to undergo progressive differentiation thereby generating glial cells and different neuronal subtypes as indicated by the expression of TH, VIP, NPY and CGRP. eNCSCs have also been isolated from neurospheres obtained from foetal and postnatal bowel of surgical or post-mortem specimens in humans (Rauch *et al.*, 2006). Despite the contribution of these reports to the better understanding of the properties of eNCSCs, the lack of markers and genetic lineage tools to follow the fate of these cells efficiently has hindered the study of this population *in vivo*.

Table1.3 Summary of strategies used to isolate eNCSCs.

Source	Selection method	Multipotency	Self-renewal capacity	References
Embryonic mouse gut	Sort of RET^+ cells	yes	ND	(Lo & Anderson, 1995; Natarajan *et al.*, 1999)
Embryonic/post natal rat gut	Sort of $p75^+\alpha integrin4^+$ cells	yes	+	(Bixby *et al.*, 2002)
Embryonic/post natal mouse gut	Culture of dissociated gut	ND	formation of neurospheres	(Schafer *et al.*, 2003)
Embryonic/post natal mouse gut	Infection with GFP-containing retrovirus	yes	yes	(Bondurand *et al.*, 2003)
Postnatal rat gut	Culture of isolated myenteric ganglia	ND	Nestin+ cells; formation of neurospheres	(Silva *et al.*, 2008)
Postnatal/adult mouse gut	Culture of dissociated gut	ND	Nestin+ cells; formation of neurospheres	(Suarez-Rodriguez & Belkind-Gerson, 2004)
Postnatal/adult rat gut	Sort of $p75^+$ cells	yes	yes	(Kruger *et al.*, 2002)
Infant human gut	Culture of isolated myenteric ganglia	ND	Nestin+ cells; formation of neurospheres	(Rauch *et al.*, 2006)

ND, not determined; + positive

Temporal changes in the properties of enteric neural stem cells

A number of studies have highlighted the changes in the function of stem cells and other progenitors with age in diverse tissues, including the haematopoietic system, muscle and brain (Wagers & Weissman, 2004). These age-related differences have been associated with changes in the microenvironment but also with cell-intrinsic changes in the stem cells. In the case of neural stem cells, changes in the proliferative abilities, self-renewal and differentiation potential have been documented (Kruger *et al.*, 2002; White *et al.*, 2001). Clonal analysis of p75-sorted cells showed that eNCSC isolated from postnatal and adult animals generate colonies with fewer cells than the colonies from embryonic

eNCSCs. Moreover, cell cycle analysis revealed that cultures derived from the gut of postnatal mice contain less mitotically active cells than cultures from the gut of E14.5 embryos (Kruger *et al.*, 2002). Similar results were obtained when eNCSCs were isolated using a retroviral approach (Bondurand *et al.*, 2003). Selecting for proliferating cells, Bondurand et al. (2003) reported that ENS cells isolated from postnatal gut give rise to smaller neurosphere-like bodies, containing almost half of the proliferating cells present in the neurospheres from embryonic gut.

Besides changes in proliferation, NCSCs also undergo temporal changes in self-renewal and neuronal subtype potential (Kruger *et al.*, 2002; Qian *et al.*, 2000; White *et al.*, 2001). Kruger et al. (2002) cultured $p75^{+}$ eNCSCs isolated from E14.5 and P22 mice and showed that E14.5 eNCSCs exhibit a higher self-renewal potential than P22 eNCSCs. In terms of neurogenic potential, cultured postnatal eNCSCs were shown to be less responsive to the neurogenic factor BMP4 than E14.5 eNCSCs. Conversely, eNCSCs become more responsive to the gliogenic effects of soluble Notch ligand Delta-Fc with increasing time after birth. This change in the responsiveness to factors in the microenvironment is likely to occur *in vivo* since, upon transplantation into developing peripheral nerve, E14.5 eNCSCs generate mainly neurons (Bixby *et al.*, 2002) whereas postnatal eNCSCs generate mainly glia (Kruger *et al.*, 2002).

Adult CNS and ENS neural stem cells

An increasing body of evidence has accumulated in recent years supporting the existence of adult stem cells in a large number of tissues. Interestingly, these cells are not only present in the hematopoietic system, intestine and skin, which have rapid physiological turnover rates, but also in organs with slow physiological cell turnover

rates that would be expected to have extremely poor ability to regenerate, including the CNS (Morrison & Spradling, 2008).

During development, CNS neuroepithelial cells become radial glia by acquiring some glial features. This cell type was long thought as specialized glia that guides neuronal migration along the radial fibers, but recent studies revealed that these are embryonic neural stem cells (Alvarez-Buylla *et al.*, 2001; Anthony *et al.*, 2004). Radial glia undergo many rounds of asymmetric cell divisions forming one radial glial cell and one neuron from each cell division. After production of neurons, radial glial cells finally give rise to glial cells such as astrocytes and oligodendrocytes. From this point onwards, active neurogenesis becomes restricted to two discrete regions: the subventricular zone (SVZ) of the lateral ventricles in the forebrain and the subgranular layer (SGL) of the hippocampal dentate gyrus (Alvarez-Buylla *et al.*, 2001). The identity (or identities) of adult neural stem cells in the CNS has been under debate. The prevailing model based on a series of genetic tracing, pharmacological ablation, and morphological and immunocytochemistry analysis suggests that special astrocytes are the adult NSCs in the SVZ and SGL (Alvarez-Buylla *et al.*, 2001). These astrocytes are the direct descendants of embryonic radial glia (Merkle *et al.*, 2004) and they exhibit radial glial properties. For example, they express GFAP and, unlike mature cortical astocytes, they maintain a thin process tethering them to the lateral ventricular wall and ventricular cavity. Despite sharing a number of features with radial glial cells, NSCs of the adult CNS show different developmental properties. Indeed, adult neurogenesis exhibits a more prolonged time course of neuronal maturation (Ge *et al.*, 2006) and, unlike postnatal

neurogenesis, is dynamically regulated by stimuli that modulate the activity of the existing neuronal circuitry (Parent *et al.*, 1997).

Even though, ongoing neurogenesis in the intact mammalian brain is restricted to the SVZ and SGL, multipotent NSC capable of long term self-renewal and generating multiple neural lineages have been derived from regions throughout the adult CNS (Ming & Song, 2005). Whether and to what extent active neurogenesis occurs in these regions under physiological conditions is still unknown, but injuries and pathological stimuli such as stroke appear to activate the neurogenesis program outside of neurogenic regions (Ming & Song, 2005). For example, Carlen et al. (2009) showed that, upon injury $CD133^{+}$ ependymal cells lining the ventricles in the mammalian forebrain can be activated to function as adult neural stem cells. Also, postmitotic astrocytes have been shown to resume stem cell properties in response to injury. Buffo et al. (2008) showed that following brain damage, mature astrocytes, referred to as reactive astrocytes, resume proliferation and undergo a number of morphological and phenotypic changes, such as hypertrophy of their somata and processes (Wilhelmsson *et al.*, 2006), increased synthesis of GFAP and re-expression of the progenitor markers vimentin and nestin (Lin *et al.*, 1995; Sofroniew, 2005). These changes in astrocytes are accompanied by the acquisition of stem cell properties as astrocytes acquire the capacity to form multipotent and self-renewing neurospheres *in vitro*. *In vivo*, reactive astrocytes do not contribute to the neuronal population, probably due to the antineurogenic environment present in the adult brain parenchyma (Hampton *et al.*, 2007; Yamamoto *et al.*, 2001). These studies have contributed to strengthen the hypothesis that, after brain injury or disease, the release of growth factors, cytokines and other factors from at-risk or dying cells, in

addition to vascular- and immune-related elements might support a reactive cyto- or neurogenesis. Also, these studies suggest that distinct cells in the adult CNS can serve as NSC in mediating neurogenesis under normal conditions or after dramatic injuries as highlighted by the identification of different cell types with neural stem cell properties.

In the PNS, the presence of adult neural stem cells has been demonstrated in mammalian olfactory neuroepithelium (Calof *et al.*, 1998) and in the neural crest-derived carotid body (Pardal *et al.*, 2007). Studies by Pardal et al. (2007) showed that the carotid body harbours cells that are quiescent under normoxia but that can be activated in the context of the homeostatic adaptative response to chronic hypoxia. Thus, upon exposure to hypoxia, the carotid body that does not support ongoing neurogenesis increases in size due to the generation of new neurons. *In vivo* neurogenesis in this organ was shown to depend on the activation of a resident population of glial cells that in culture form multipotent self-renewing colonies. In response to hypoxia, these $GFAP^+$ glia cells are converted into actively proliferating $GFAP^-$ $nestin^+$ intermediate progenitors that give rise to mature neurons.

In the adult ENS, the presence of neural stem cells *in vivo* has never been demonstrated and active neurogenesis is not known to occur under physiological conditions. However, eNCCs capable of long term self-renewal and of generating multiple neural lineages have been derived from the gut of adult animals suggesting that the adult ENS harbours progenitor cells (Kruger *et al.*, 2002; Suarez-Rodriguez & Belkind-Gerson, 2004). This is further corroborated by evidence from injury models suggesting that enteric neural precursors that are probably quiescent in the adult gut may be stimulated to proliferate and differentiate in response to stimuli, such as injury,

contributing to the regeneration of the adult ENS (Filogamo & Cracco, 1995; Hanani *et al.*, 2003; Ramalho *et al.*, 1993). The identity of these cells and whether they constitute a permanent undifferentiated pool or are recruited in a regular basis is unknown. In addition, the potential role of these cells in normal intestinal physiology and in states of disease or injury is still unclear.

1.5. Neurogenesis in the Enteric Nervous System

Enteric neurons appear early during ENS development, soon after invasion of the foregut mesenchyme by eNCSCs, as evidenced by the detection of pan-neuronal markers (Baetge & Gershon, 1989; Young *et al.*, 1999). However, different types of neurons appear at different stages of development (Branchek & Gershon, 1989); (Rothman & Gershon, 1982; Rothman *et al.*, 1984) and some of them only at postnatal stages (Matini *et al.*, 1997; Vannucchi & Faussone-Pellegrini, 1996). Furthermore, there is an increase in the number of enteric neurons until well after birth (Ali & McLelland, 1979; Gabella, 1971; Liu *et al.*, 2009). eNCSCs are, thus, thought to maintain their neurogenic potential for a long period but little is known about the rate and extent of neurogenesis in the ENS.

1.5.1.Neurogenesis in the embryonic and postnatal ENS

The timing of neurogenesis in the ENS has been documented in two birth-dating studies (**Table1.1**; (Chalazonitis *et al.*, 2008; Pham *et al.*, 1991). Unlike CNS neuronal precursors, enteric neuroblasts continue to divide after expressing pan-neuronal markers (Baetge *et al.*, 1990a; Young *et al.*, 2005) and so the birth date of an enteric neuron is defined as the stage at which the neuronal precursor exits the cell cycle. Pham et al.

(1991) determined the birth-date of specific subclasses of enteric neurons by providing a pulse of tritiated thymidine to timed pregnant or postnatal mice (Pham *et al.*, 1991). Later, labelled nuclei were sought in different neuronal subtypes of the ENS. This revealed that the birth date of the different neuronal subtypes does not reflect the developmental stage at which they express specific markers at detectable levels for the first time. The time window during which each neuronal subtype exits the cell cycle is long, with major overlaps between the different types of neurons, thus suggesting that many neurons of all types are ceasing mitosis and differentiating simultaneously in the same gut region and probably in the same ganglia. Nevertheless, the birth of enteric neurons of different phenotypes occurs in sequential waves suggesting that generation of a specific class of enteric neurons may influence the formation of subsequent ones. It is presently unclear whether this pattern is due to a systematic change in the potential of eNCSCs/neuronal precursors during enteric neurogenesis or to the presence of multiple distinct pools of progenitors/precursors each generating a particular neuronal subtype.

Interestingly, some enteric neuronal precursors, more specifically cholinergic and serotoninergic neuronal precursors, were shown to exit the cell cycle as early as E8, before invasion of the foregut mesenchyme (Pham *et al.*, 1991). Peptidergic neurons were shown to be firstly born at around day E10, as the cells first enter the gut whereas NOS, calbindin and (GABA)ergic neurons withdraw from the cell cycle from E12.5 onwards (Chalazonitis *et al.*, 2008). All subclasses of myenteric neurons are born during fetal life except for VIP- and CGRP- expressing neurons, which were shown to be born during the first postnatal weeks. In fact, the last cells to withdraw from the cell cycle in the mouse myenteric plexus were VIP-expressing neurons, born at P5 (in the myenteric

plexus) or at P14 (in the submucosal plexus). These findings suggest that new neurons are added to the enteric plexuses after birth, when the gut has already become functional and, thus, maturation of the ENS is likely to continue long after embryogenesis. Because the mentioned birth dating studies are based on the incorporation of markers by enteric neuroblasts little is known about the population of multipotent, undifferentiated cells of the ENS and so the question of how long eNCSCs persist in the gut and how their neurogenic potential changes over the course of ENS development remains unanswered.

1.5.2.Neurogenesis in the adult ENS

Neurogenesis in the CNS was thought to be restricted to embryogenesis. However, recent studies showed unequivocally that neurons are born throughout life, particularly in the SVZ of the lateral ventricles and SGL of the dentate gyrus of the hippocampus (Alvarez-Buylla & Lim, 2004). Unlike the SVZ and SGL, active neurogenesis has not been detected in the adult mammalian ENS under physiological conditions. Birth-dating studies were unable to demonstrate neurogenesis in the ENS of adult mice and so the birth of enteric neurons is thought to occur exclusively during embryonic and early postnatal stages (Chalazonitis *et al.*, 2008; Pham *et al.*, 1991). Nevertheless, a number of cell culture studies showed that self-renewing progenitors capable of generating enteric neurons and glia can be isolated not only from embryonic gut but also from post-neurogenic adult intestine (Kruger *et al.*, 2002; Suarez-Rodriguez & Belkind-Gerson, 2004). Using flow-cytometry, Kruger et al. (2002) isolated the 1-2% of cells that expressed highest levels of p75 from the gut of P5 to P110 rats. In culture, these cells were able to self-renew, proliferate and differentiate into neurons, glia and myofibroblasts suggesting the persistence of a population with neural stem cell

properties in the adult gut. Similar results were obtained by Suárez-Rodriguez and Belkind-Gerson, who found that *in vitro*, multipotent, proliferating cells isolated from the ENS of lactating and adult mice express stem cell markers, such as vimentin and nestin. The neurogenic potential of these cells *in vivo* and their functional significance in the adult gut is still unknown. However, these studies raise the possibility that a population of eNCSCs may persist in the adult ENS but become dormant due to cell-intrinsic changes or extrinsic factors in the progenitor microenvironment.

Changes in the niche following injury have been shown to activate neurogenesis in a number of systems (Carlen *et al.*, 2009; Czaja *et al.*, 2008; Ooto *et al.*, 2004). In the adult ENS, new enteric neurons have been shown to be generated and added to enteric ganglia when 5-HT agonists are provided (Liu *et al.*, 2009). Liu et al. (2009) demonstrated that there is a physiological increase in the number of enteric neurons during the first 4 months after birth and this is 5-HT dependent. From that time point onwards there is an age-dependent decline in the number of neurons. However, activation of 5-HT receptors by appropriate agonists in adult mice is able to promote generation of new neurons that are incorporated into enteric ganglia. Interestingly, the progenitors of such newly generated neurons are located in germinal niches that lie in the vicinity but outside the myenteric plexus. The identity of such progenitors and the mechanisms that drive their neuronal maturation and their migration towards the enteric ganglia are still unknown.

Neurogenic changes have also been reported in several injury models (Filogamo & Cracco, 1995; Hanani *et al.*, 2003; Jew *et al.*, 1989; Luck *et al.*, 1993; Ramalho *et al.*, 1993; Tokui *et al.*, 1994). Incomplete stenosis of the gut has been shown to result in

smooth muscle hypertrophy and a significant increase in the size and number of ganglionic cell bodies of the myenteric and submucosal plexus (Filogamo & Cracco, 1995). This increase was reported to occur in the absence of mitotic nerve cells and so it was attributed to the differentiation of previously dormant postmitotic cells. Interestingly, Tokui et al. (1994) showed that after partial resection and reanastomosis of the intestine of the guinea pig, there is a time-dependent increase in the number of extraganglionic neurons in the areas adjacent to the lesion. Eventually, these ectopic neurons reach the lesion area where they form small clusters. Whether these ectopic neurons emerge *de novo* or migrate from adjacent ganglia is unclear. Another experimental model that has suggested neurogenesis in the adult ENS consists of the denervation of the gut using benzalkonium chloride (BAC; (Hanani *et al.*, 2003; Ramalho *et al.*, 1993). BAC is a cationic detergent that can cause severe cell damage. When high concentrations of BAC are applied to the intestinal serosa it causes generalized tissue damage, including disruption of the smooth muscle, lymphocytic infiltration, intestinal perforation and death. At lower concentration, this detergent can be used to selectively destroy the myenteric plexus without damaging the submucosal plexus or the smooth muscle (Fox *et al.*, 1983; Ramalho *et al.*, 1993; Sato *et al.*, 1978). BAC has been used to generate animal models of aganglionosis (Sakata *et al.*, 1979; Sato *et al.*, 1978; Yoneda *et al.*, 2002) and also to study the regenerative capacity of the ENS (Cracco & Filogamo, 1993; Hanani *et al.*, 2003; Luck *et al.*, 1993; Ramalho *et al.*, 1993). A time course analysis of the morphological changes in the myenteric plexus after BAC application was carried out by Hanani et al. (2003). This showed that, 2 days after BAC treatment, neurons are efficiently ablated from the treated area. However, by

day 7, the denervated region is invaded by regenerating nerve fibers, closely associated with glial cells. Moreover, from day 30 onwards, undifferentiating cells were identified around the treated area by electron microscopy. In light of these results, the authors postulated that quiescent eNCSCs persist in the adult ENS and may be stimulated to proliferate and differentiate in response to stimuli, such as injury. This is further supported by studies of Williams and Jew (1991), who proposed that the differentiation of neural crest-derived precursors can be triggered by diffusible factors originated from the intestinal wall treated by the BAC where profound degenerative and regenerative processes take place. The existence of diffusible factors that stimulate neuronal differentiation can be deduced also by the observation that the PC12 cells transplanted in the intestinal wall of BAC treated animals rapidly give rise to long filaments and differentiate into mature neurons (Filogamo & Cracco, 1995).

Further evidence for neurogenesis in the adult ENS comes from clinical studies. In ulcerative colitis and Crohn's disease, for example, neurodegeneration as well as an increase in both the size and number of neurons in the adult ENS have been described (Davis *et al.*, 1955; Nadorra *et al.*, 1986; Siemers & Dobbins, 1974; Storsteen *et al.*, 1953). Whether the increased number of neurons is related to the underlying cause of inflammation or is a secondary phenomenon due to the altered environment produced by inflammation is not known but it is interesting that serotonin availability, which has been shown to increase in inflammation murine models (Linden *et al.*, 2003) has recently been linked to neurogenesis in the adult ENS (Liu *et al.*, 2009).

1.5.3.Changes in enteric neurons with aging

Unlike other parts of the nervous system, the ENS undergoes non-pathological age-related neurodegeneration. Age-associated neuronal loss has been described in humans and rodents and it occurs in both the myenteric and submucosal plexus (Cowen *et al.*, 2000; de Souza *et al.*, 1993; Gabella, 1989). In a detailed study carried out in rats, Philips and Powley (2001) found a linear rate of loss of myenteric neurons with increasing age starting in 12 month old animals. This decrease was dependent on the diet as rats maintained on an unrestricted diet lost about 50% of the myenteric neurons, whereas 4 and 24 month-old rats on a restricted diet had the same number of neurons (Cowen *et al.*, 2000). Age-related loss of enteric neurons has been linked to an increase in the intraneuronal levels of reactive oxygen species (ROS; (Thrasivoulou *et al.*, 2006). Thrasivoulou et al. (2006) further showed that neurotrophic factors, in particular GDNF, protect neurons against ROS-induced cell death by reducing ROS levels, an effect that is enhanced in diet restriction conditions. Interestingly, evidence suggests that age-associated neuronal loss does not affect all neuronal subtypes equally. In fact, the loss of myenteric neurons is selective and it affects predominantly cholinergic neurons while nitrergic neurons are spared (Phillips *et al.*, 2003; Wade & Cowen, 2004). The mechanisms underlying this selective vulnerability are poorly understood. In addition, it is still unclear how age-related changes in intestinal innervation affect gastrointestinal function but it is likely that these changes may compromise its effectiveness and maybe account, to some extent, for the gastrointestinal disorders that frequently affect the elderly, such as dysphagia, gastrointestinal reflux and constipation.

1.6. Diseases of the Enteric Nervous System

The ENS is involved in monitoring fundamental activities of the gut wall and so abnormalities in this system lead invariably to a more or less severe gastrointestinal dysfunction. Enteric neuropathies form the basis for a large proportion of digestive diseases (Di Nardo *et al.*, 2008; Furness, 2008); they can be congenital or acquired and the underlying defect can range from subtle changes in the cellular composition of ganglia or connectivity of enteric neurons to complete absence of the enteric plexi from entire segments of the bowel.

1.6.1.Hirschsprung's disease and other congenital enteric neuropathies

The most common and best understood congenital enteric disorder is Hirschsprung's disease (HSCR). HSCR affects approximately 1 in 5000 newborns (Chakravarti, 2001) and it is associated with the absence of enteric ganglia in the most distal part of the intestine, although the extent of the aneural region is very variable. The functional characteristic of the disease is intestinal obstruction caused by the localized inability of the gut to transmit a motile relaxation and contraction (peristaltic) wave. As a consequence, the segment of the bowel proximal to this becomes grossly distended by faecal accumulation and this is termed megacolon.

In a significant proportion of HSCR cases, abnormalities of ENS structure or function are not restricted to the aganglionic region. The area proximal to the aganglionic segment is called the transition zone and it can exhibit hypoganglionosis (White & Langer, 2000) or, conversely, hyperganglionosis (Schmittenbecher *et al.*, 1999; Szabolcs *et al.*, 1996). Abnormalities in the transition zone, such as decrease in

the density of myenteric neurons and changes in neurotransmitter expression, have been reported also in mouse models of HSCR (Roberts *et al.*, 2008; Sandgren *et al.*, 2002).

Infants with HSCR often exhibit severe constipation, growth failure and are at risk of dying from the complications of toxic megacolon (Swenson, 2002). The current treatment for HSCR is surgical resection of the aganglionic segment of the bowel but intestinal dysfunction may persist after surgery ranging from severe constipation to faecal incontinence (Thapar, 2009). It is presently unclear whether the post-operative morbidity is related to the surgery or to the potentially abnormal function of the residual ENS associated with the primary defect that initially caused aganglionosis.

HSCR is a complex trait with a strong genetic component and great phenotypic variability pointing to considerable influences from environment and modifying genetic factors. Several genes have been found to be mutated in association to this intestinal disorder. Some of the identified mutations are sporadic, others are inherited, in which case inheritance in non-mendelian, suggesting that HSCR is a multifactorial disease. In most cases it occurs as an isolated trait (70%; (Amiel & Lyonnet, 2001) but it can also occur as part of a number of syndromes consisting of various congenital abnormalities, such as congenital central hypoventilation syndrome and a variant of Shah-Waardenburg syndrome (WS4). These abnormalities usually involve systems developmentally related to the neural crest and so HSCR is regarded as a neurocristopathy (Bolande, 1997).

Animal models of HSCR have contributed significantly to the identification and functional analysis of genes underlying HSCR. Indeed, HSCR-like phenotypes have been reported in a wide array of organisms, including horse, pig, rat and mouse. In each of these cases, a single-gene mutation is the cause of congenital megacolon and the

extent of ENS absence varies between the different animal models. Mutations in the *c-Ret* gene that lead to inactivation of the receptor tyrosine kinase RET have been shown to be central to the genesis of HSCR. Homozygous *Ret*-null mice show total absence of the ENS from all regions of the gastrointestinal tract except the esophagus. Similarly, mutations in other components of the RET signalling cascade such as *gdnf* also contribute to HSCR (Moore *et al.*, 1996; Pichel *et al.*, 1996; Sanchez *et al.*, 1996). In humans, the *c-Ret* gene is the most frequently affected (Edery *et al.*, 1994). Heterozygous mutations in this gene alone or in combination with alterations in other loci account for 15-35% of patients with sporadic HSCR and approximately half of all congenital HSCR cases (Brooks *et al.*, 2005; Chakravarti, 2001). Furthermore, recent analysis of non-coding mutations in *RET* suggests that mutations in this gene are more frequent than previously thought and these may confer susceptibility in all HSCR cases (Chakravarti, 2001; Emison *et al.*, 2005; Griseri *et al.*, 2007).

Another signalling pathway that is known to have a key role in ENS development is the EDNRB/ET-3 system. Mice carrying mutations in *EdnrB* or *Edn3* (gene that encodes ET-3) exhibit aganglionosis and pigmentation defects (Baynash *et al.*, 1994; Hosoda *et al.*, 1994). Similarly, heterozygous mutations in *EDN3* and *EDNRB* have been identified in HSCR patients and these comprise approximately 5% of all HSCR cases (Amiel & Lyonnet, 2001).

HSCR can also be caused by alterations in transcription factors or co-factors in eNCCs. For example, mice carrying *Sox10* mutations exhibit aganglionosis and hypopigmentation (Kapur, 1999; Southard-Smith *et al.*, 1998). In humans, mutations in this locus are associated with HSCR in Waardenburg syndrome (Waardenburg-Shan

syndrome; WS4; (Pingault *et al.*, 1998; Southard-Smith *et al.*, 1998) and accounts for less than 5% of HSCR cases.

HSCR is the easiest congenital enteric neuropathy to diagnose because of the complete absence of enteric neurons from affected regions. However, other congenital disorders may involve a more restricted neuron loss or even loss or phenotype change of specific subtypes of neurons (De Giorgio *et al.*, 2004). A peculiar neuropathological pattern with restricted neuronal loss is observed in hypertrophic pyloric stenosis (Vanderwinden *et al.*, 1992). This disease is associated with the specific loss of inhibitory innervation of the pyloric sphincter. In contrast, some congenital disorders are characterized by an increase in the number of neurons. For example, grossly enlarged ganglia (ganglioneuromatosis) and increased number of ganglion cells (King *et al.*, 2006; Smith *et al.*, 1999) is observed in tissue samples of patients with Multiple Endocrine Neoplasia type 2B (MEN 2B). MEN2A and MEN2B are rare hereditary syndromes associated with mutations in *c-RET* (Grieco *et al.*, 1990; Mulligan *et al.*, 1993). Most patients exhibit mucosal neuromas, pheochromocytomas and medullary thyroid carcinoma, and some also experience gastrointestinal motility problems, including constipation and intestinal obstruction (King *et al.*, 2006). Another congenital bowel condition that is functionally consistent with a developmental disturbance of ENS formation is Intestinal Neural Dysplasia type B (INDB). INDB has been described as a condition in which there is an increase in the number of neurons per submucosal ganglion, an increase in the number of submucosal neurons and ectopic ganglia in the muscularis mucosae and lamina propria (Meier-Ruge *et al.*, 2004). Currently, diagnosis, treatment, and even existence of INDB as a distinct clinical identity, are controversial

and this is largely related to the difficulties associated with diagnosing quantitative changes in the ENS given the variability in ENS density between different regions of the intestine and different individuals (Cord-Udy *et al.*, 1997; Koletzko *et al.*, 1999). Interestingly, inflammatory cells have been described in the intestinal plexus of INDB patients. In CNS injury responses, inflammatory cells are known to secrete brain-derived neurothophic factor (BDNF) and GDNF, which are active in promoting nerve growth (Batchelor *et al.*, 2000). Whether a similar mechanism accounts for hyperplasia in the ENS in the context of INDB is unknown.

1.6.2.Other ENS disorders

Besides the developmental defects, the ENS can also be subject to injury. In fact, unlike the highly protected CNS, throughout life the ENS is exposed to pathogens, toxins, allergens and other potential insults that may result in ENS damage.

10 to 20% of adults suffer from diseases involving the ENS, most notably irritable bowel syndrome (IBS; Ehrenpreis, 2005). IBS is a functional disorder associated with hyperactivity of the sensory nerve fibers and altered bowel motility (Camilleri & Choi, 1997; Drossman, 1999). Even though IBS is not an inflammatory disease, a high proportion of cases can be traced back to an inflammatory episode (Neal *et al.*, 1997; Spiller, 2003). Furthermore, the incidence of IBS increases following inflammation suggesting that inflammatory process can cause long-term changes in neuromuscular function that persist beyond the period of inflammation and may contribute to the symptoms of IBS (Sharkey & Kroese, 2001). The relation between inflammation and changes in the number, phenotype and/or function of enteric neurons is further confirmed by the documented effects of inflammation on enteric neurons in the context

of Inflammatory Bowel Diseases (IBD; (Davis *et al.*, 1955; Geboes & Collins, 1998; Lomax *et al.*, 2005; Nadorra *et al.*, 1986; Siemers & Dobbins, 1974).

IBD (i.e., ulcerative colitis and Crohn's disease) are recurrent inflammatory disorders of the gastrointestinal tract. Early histopathological studies in adult Crohn's ileitis patients, reported hypertrophy and hyperplasia of neurons and nerve fibers (Davis *et al.*, 1955; Nadorra *et al.*, 1986; Siemers & Dobbins, 1974). In contrast, in murine models of acute colitis, neuronal loss (Lin *et al.*, 2005; Sanovic *et al.*, 1999) and proliferation of glial cells (Bradley *et al.*, 1997) has been reported in association with inflammation. In addition to changes in neuronal numbers, alteration of the neurochemical coding of enteric neurons have also been shown in Crohn's disease patients (Boyer *et al.*, 2007; Kimura *et al.*, 1994) and in animal models of inflammation (Galeazzi *et al.*, 2000; Lin *et al.*, 2005).

The ENS can also be damaged in the context of some forms of chronic disease, such as Parkinson's disease (Micieli *et al.*, 2003; Wakabayashi & Takahashi, 1997) and diabetic neuropathy (Vinik *et al.*, 2003).

1.6.3.Transplantation models

Given the often unsatisfactory outcome of the available treatments for ENS defects, in particular HSCR, a number of investigators have suggested that therapeutic strategies, based on the replacement of missing or malfunctioning enteric ganglia, could play an important role in restoring functionality of aganglionic bowel (Thapar, 2009). Early avian grafting experiments have demonstrated the potential for cells to emigrate from grafts of embryonic gut tissue and to colonise and differentiate in the host gut (Rothman *et al.*, 1990). Since then, a series of transplantation studies have been carried out.

Natarajan et al. (1999) grafted Ret^+ cells isolated from dissociated embryonic mouse gut into the wall of the stomach anlagen of cultured embryonic guts from wild-type and Ret-null mice. The grafted cells were shown to proliferate extensively and to generate neurons and glia (Natarajan *et al.*, 1999). Also using bowel explants, Bondurand et al. (2003) reported that transplanted enteric progenitor cells have the potential to generate differentiated progeny that localized within the intrinsic ganglionic plexus (Bondurand *et al.*, 2003). Similarly, $p75^+$ eNCSCs cells microinjected into the trunk neural crest migratory route of developing chick embryos (Kruger *et al.*, 2002) or into wild-type or aganglionic HSCR mouse model intestine (Kruger *et al.*, 2003) gave rise to neurons and glia. More recently, NCSCs were also successfully transplanted into the peritoneal space of postnatal *Sox10* mutant animals (Martucciello *et al.*, 2007). Whether transplanted cells can be integrated in the endogenous enteric functional circuits is not clear. However, recent findings suggest that transplanted cells may be able to contribute to gastrointestinal function (Liu *et al.*, 2007; Micci *et al.*, 2001; Micci *et al.*, 2005). Using CNS-derived stem cells, Micci et al (2005) grafted the gastric antrum of NOS-deficient mice, which display gastroparesis (partial paralysis of the stomach muscles) due to the lack of nitric oxide that relaxes gastric muscles. One week after implantation, transplanted cells were shown to have differentiated into NOS^+ neurons and this was accompanied by a significant improvement in gastric transit. Restored function of denervated gut was also reported following transplantation of neuroepithelial stem cells into a BAC-treated colonic region in rats (Liu *et al.*, 2007). Altogether, these studies suggest that cell therapy via transplantation may be of therapeutic benefit in pathological gastrointestinal conditions associated with the loss of neurons.

1.7. Aims of the work presented in this thesis

The presence of stem cells in the migrating neural crest has long been established. Some of these NCSCs invade the gut during embryogenesis and give rise to the ENS. To form a mature functional ENS, eNCSCs must self-renew and generate correct numbers of neurons and glia in a spatiotemporal controlled manner. So far, the presence of eNCSCs and their properties has only been ascertained by their behaviour in culture. The study of this population *in vivo* has been hindered by the paucity of specific markers and genetic lineage tools to efficiently identify and follow eNCSCs.

The overall aim of this work was to identify and characterise eNCSCs *in vivo* over the course of ENS development. At the outset, we focused on studying the lineage of *Sox10*-expressing cells in the ENS using the Cre-loxP genetic system. The purpose of this analysis was to determine whether *Sox10*-expressing cells give rise to both enteric neurons and glia. Secondly, a strategy to selectively label *Sox10*-expressing eNCSCs at particular developmental stages was devised and the neurogenic potential of temporally identified $Sox10^{+}$ progenitors *in vivo* was investigated. Next, we focused on elucidating whether $Sox10^{+}$ cells from the adult gut, the glial cells, possess neural stem cell potential. The goal of this part of the work was to establish the identity of eNCSCs that have been isolated from the adult gut and study their potential physiological function *in vivo*.

Finally, as part of the study of eNCSCs *in vivo* we wished to further our understanding of the molecular mechanisms underlying cell fate decisions during enteric neurogenesis. Towards this end, a genetic strategy was developed to test the hypothesis

that RET is part of the molecular switch between the neuronal and glial lineage being able to divert differentiation of eNCSCs towards the neuronal fate.

Chapter II

Materials & Methods

2.1. General materials and equipment

2.1.1.General chemicals, buffers and solutions

The following commonly-used buffers were prepared in-house at the MRC National Institute for Medical Research (NIMR) using reagents from Sigma or Fisher Scientific. All components are stated at final working concentration (1x). Specialized reagents are described in the relevant section in which they were first used.

PBS	phosphate buffered saline pH7.4 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4 in dH2O
TAE	Tris-acetate EDTA, pH8.3 40 mM Tris-acetate, 1 mM EDTA in dH_2O
TBE	Tris-borate EDTA pH8.0 100 mM Tris-borate, 5 mM EDTA in dH_2O
SSC	sodium chloride-citrate buffer pH7.0-7.2 150 mM NaCl, 15 mM trisodium citrate, in dH_2O
LB (Agar)	Luria Broth (Agar) 1% [wt/vol] Bacto tryptone, 0.5% [wt/vol] yeast extract, 1% [wt/vol] NaCl, (2% [wt/vol] agar).

2.1.2.Centrifuges

All routine centrifugation was performed in a Heraeus Pico microcentrifuge (Thermo Scientific) fitted with 24x 1.5/2.0 ml rotor (Sections **2.6.1, 2.6.2, 2.6.7**).

For plasmid and PAC DNA isolation, large scale cultures were pellet using a Beckman J6-HC refrigerated centrifuge, fitted with rotor JS 5.2 or a Sorvall RC5C refrigerated centrifuge fitted with rotor SLA-1500. Further centrifugation of bacterial supernatants was performed using the same centrifuge fitted with rotor SS34 (Sections **2.6.2** and **2.6.7**).

Primary cell cultures were pellet in an IEC Centra-4R refrigerated centrifuge fitted with rotor IEC 215A (Section **2.8.1** and **2.8.2**). ES cells were centrifuged in a MSE Centaur II centrifuge (Section **2.8.4**).

2.1.3.Polymerase Chain Reaction (PCR) machines

PCR analysis was carried out in a DNA Engine Dyad Thermal Cycler Chassis (Biorad) or in a Hybaid Omn-E dryblock thermocycler (Thermo Scientific).

2.1.4.Other equipment

An INNOVA 4200 incubator shaker (New Brunswick Scientific) was used to grow all bacterial cultures at 32ºC or 37ºC with 225-250 rpm rotary shaking (Sections **2.2**, **2.6.2**, **6.4**, **6.5.3**, **6.6.1**, **6.6.4**, **6.6.5**). A LEEC incubator was used for growing colonies on plates (Sections **6.2**, **6.4**, **6.5.3**, **6.6.1**, **6.6.4**, **6.6.5**). A Hybaid Mini 10 oven (Thermo Scientific) was used for digestion of mouse biopsies in tail buffer (section **6.1.1**), for the crosslink of DNA to the Southern Blot membrane (section **6.3.2**) and for RNA *in situ* experiments (section **7.4**). A Hybaid Shake 'n' Stack Hybridization oven (Thermo Scientific) was used for the hybridization of radiolabelled probes for Southern blotting (section **6.3.2**).

2.2. Generation of the Sox10iCreER^{T2} construct and transgenic mice

2.2.1.Generation of the targeting vector

An AscI-SalI (460 base pairs - bp) fragment was used as the 5' homology and a SpeI-PacI (440 bp) fragment was used as the 3' homology. Both fragments were amplified by PCR as outlined in Section **2.6.8.1**, using the Sox10 phage artificial chromosome (PAC) as template and the oligonucleotide primers P1-P4 (see **Table 2.1**). PCR products were

run in a 1% agarose gel in TAE and purified as described in Sections **2.6.6.1**. The 5' and 3' homologies were cloned into pCR®II-TOPO® (Invitrogen K4600-01) and electroporated into XL1-Blue competent cells (Stratagene 200268; Section **2.6.6.3**) which were plated onto LB-agar-Ampicilin-IPTG-XGal for blue/white selection (Section **2.6.6.3**). Single white colonies were picked, grown in LB-Ampicilin at 37ºC overnight (O/N) and then DNA was isolated as described in Section **2.6.2**. Finally, 5' and 3' homologies were sequenced to confirm the homology between the PCR-amplified fragments and the template.

A 3.3 Kb fragment containing the coding sequence of the iCreER^{T2} recombinase followed by the chloramphenicol resistance gene (Cm^{R}), flanked by *frt* sites, was used as the targeting cassette (kindly provided by N. Kessaris; UCL, UK).

5' and 3' homologies were released from the vector by AscI-SalI digestion and SpeI-PacI digestion, respectively. The targeting cassette was isolated by SalI-SpeI digestion. DNA fragments were run on a 1% gel agarose in TAE and purified as described in Section **2.6.6.1**. The targeting vector was generated by subcloning the AscI-SalI 5' homology, the SalI-SpeI targeting cassette and the SpeI-PacI 3' homology into the PacI-AscI cloning sites of the vector pBlueScript PacAsc (pBlueScript with a modified MCS to introduce PacI and AscI, kindly provided by N. Kessaris; UCL, UK) in a four-way ligation (**Fig.4.2**). Once generated, the targeting cassette was electroporated into XL1-Blue competent cells (Section **2.6.6.3**) and these were plated onto LB-agar-Ampicilin-IPTG-XGal for blue/white selection. Single white colonies were picked and grown in LB-Ampicilin at 37ºC, O/N. DNA was isolated, as described in Section **2.6.6.1,** and then

digested with restriction endonucleases to confirm success of the ligation (Section **2.6.6.2**).

LB (-agar-) Ampicilin: LB, (agar), Ampicilin at a final concentration of 100 µg/ml (Sigma A9518).

LB-agar-Ampicilin-IPTG-XGal: LB-agar, 100 µg/ml of Ampicilin, 0.1 M isopropyl-1-thio-β-D-galactopyranoside (IPTG) and 8 mg/ml of 2% 5-bromo-4-chloro-3-inodlyl-β-D-galactopyranoside (X-Gal; 40 mg/ml stock in n,n'-dimethylformamide –DMF) were spread on solidified LB-agar plates 30 minutes prior to plating the transformations.

2.2.2.Phage Artificial Chromosome (PAC) isolation and modification

A genomic PAC (RP21-529-I6 from RPCI mouse PAC library 21, HGMP Resource Centre, UK) spanning 170 kilobases (Kb) around the Sox10 locus (Matsuoka *et al.*, 2005) was kindly provided N. Kessaris (UCL, UK) (**Fig.4.2)**. PAC DNA was isolated and propagated as described in Sections **2.6.7**. PAC modification was carried out in a bacterial system as previously described (Lee *et al.*, 2001; see Sections **2.6.7.4** and **2.6.7.5**). Once modified, PAC DNA was isolated (Sections **2.6.7.2**) and then checked for recombination events by restriction analysis followed by Pulse Field Gel Electrophoresis (PFGE) and Southern blot using the 3' homology fragment as a probe (Sections **2.6.7.3** and **2.6.3.2**). Finally, the modified PAC insert was linearized by NotI digest and purified away from the vector pPAC4 backbone before microinjection into the pronuclei of fertilized mouse eggs as described in Section **2.6.7.6** (**Fig.4.2**).

2.2.3.Generation of Sox10iCreER^{T2} transgenics

For the generation of the SOX10iCreER^{T2}, superovulated females were mated with (CBA/CAxC57BL/10)_F1 fertile males for the production of fertilized eggs. Typically,

4-6 female mated yielding a total of 100-200 fertilized eggs. Pseudopregnant mice were prepared by mating 6 weeks-4 months old (CBA/CA_C57BL/10)_F1 females in natural estrus with vasectomized males the day before implantation. After microinjection of DNA constructs into the pronuclei of fertilized mouse eggs, embryos were re-implanted into the oviducts of pseudopregnant foster mother for development. After weaning at 3-4 weeks of age, the pups were genotyped (Section **2.6.8.3**) and all transgene-positive offspring were kept to develop stable lines. At all times, animals were handled in compliance with the Animal (Scientific Procedures) Act 1986.

2.3. Generation of *R26*^{RET9} and *R26*$^{RET9(MEN2A)}$ constructs and transgenic mice

2.3.1.Construction of the transgenes

The RET9-IRES-GFP and RET9^{MEN2A}-IRES-GFP cassettes were constructed as follows. A 161 bp ($_F$A) and 153 bp ($_F$B) fragments were amplified from the RET9 cDNA by PCR, using the primers P5-P8 (Section **2.6.8.1**; **Table 2.1**). $_F$A was run on a 1% gel agarose in TAE, purified (Section **2.6.6.1**) and cloned into the KpnI-XhoI double-digested pBlueScript KS (pBS KS) generating pBS KS+$_F$A. $_F$B was run on a 1% gel agarose in TAE, purified (Section **2.6.6.1**) and ligated to the HindIII-XhoI double-digested pBS KS+$_F$A. A 3 Kb fragment of RET9 or RET9^{MEN2A} cDNA ($_F$C') was released by AatII-NsiI digestion and cloned into pBS KS+$_F$A+$_F$B. NheI digestion of pBS KS+$_F$A+$_F$B+$_F$C released a 3.2 Kb fragment which was then cloned into the NheI-digested pBigT vector (kindly provided by S. Srinivas, University of Oxford, UK; Srinivas *et al.*, 2001) (**Fig.7.1 A**). This plasmid includes a PacI site 5' to the adenovirus splice acceptor sequence, the IRES-GFP cassette and an AscI site 3' to the bovine

growth hormone polyadenylation sequence. These restriction sites were used to excise the RET9^{MEN2A}-IRES-GFP cassette and clone it into the pROSA26PA (a kind gift from S. Srinivas, University of Oxford, UK; Srinivas *et al.*, 2001). This plasmid contains genomic ROSA26 sequences for homologous recombination and a diphtheria toxin gene for negative selection in ES cells (**Fig.7.1 B**). Finally, the *R26*$^{RET9(MEN2A)}$ targeting vector was linearized by SfiI digest, separated from the plasmid backbone by gel electrophoresis and eluted as described in Section **2.6.3.1** and **2.6.6.1**.

2.3.2.Generation of transgenic mice

The targeting vector was electroporated into the E14G2A Embryonic Stem (ES) cell line (kindly provided by A. Smith, Institute for Stem Cell Research, UK) as described in Section **2.8.4.3**. ES clones carrying the transgene were identified by PCR (Section **2.6.8.2**; **Table 2.1**) and were further verified by Southern blot analysis (Section **2.6.3.2**) using a 692 bp 5' external probe (kind gift of A. Smith, Institute for Stem Cell Research, UK). Chimeric mice were generated by microinjection of the selected ES clones (heterozygous for the *R26*$^{RET9(MEN2A)}$ transgene) into C57BL/6 blastocysts and transfer of the injected embryos into the uterus of pseudopregnant female mice. The chimeras were mated with C57BL/6 wild type mice and the offspring examined for germ line transmission of ES cell DNA, as determined by the presence of agouti coat colour, indicating the successful transmission of ES cell DNA through the germ line. Agouti offspring were genotyped by PCR as described in Section **2.6.8.3**, using primers listed in **Table 2.1**. At all times, animals were handled in compliance with the Animal (Scientific Procedures) Act 1986.

2.4. Generation of Sox10Cre; R26ReYFP

Sox10Cre transgenic mice were kindly provided by N. Kessaris (UCL, UK) and have been described (Matsuoka *et al.*, 2005). For lineage analysis, Sox10Cre transgenic animals were crossed with the reporter line R26ReYFP (a kind gift from S. Srinivas, University of Oxford, UK; Srinivas *et al.*, 2001). The Cre and R26ReYFP alleles were identified by PCR as described in Section **2.6.8.3**. Timed matings for embryonic studies were generated by crossing Sox10Cre; R26ReYFP mice with Parkes (outbred) mice. At all times, animals were handled in compliance with the Animal (Scientific Procedures) Act 1986.

2.5. Generation of hGFAPCreER^{T2}; R26ReYFP

The hGFAPCreER^{T2} transgenic mice were kindly provided by F. Guillemot (NIMR, UK) and were generated as described by (Ganat *et al.*, 2006). hGFAPCreER^{T2} mice were crossed with the reporter line R26ReYFP to generate hGFAPCreER^{T2}; R26ReYFP animals. The Cre and R26ReYFP alleles were identified by PCR as described in Section **2.6.8.3**. At all times, animals were handled in compliance with the Animal (Scientific Procedures) Act 1986.

2.6. Molecular Techniques

2.6.1.Genomic DNA Isolation

2.6.1.1.DNA extraction from mouse biopsies

Genomic DNA was extracted from tail snips/ear biopsy or yolk sacs using 500 µl of Proteinase K Tail Lysis Buffer and incubating the mixture at 55ºC, O/N. The DNA was precipitated by adding 500 µl of isopropanol and then centrifuged at 13200 rpm for 10

minutes, at room temperature (RT). The DNA pellet was air dried and ressuspended in 50 µl of distilled H_20 (dH_2O).

Proteinase K Tail Lysis Buffer: 100 mM Tris.HCl pH8.0, 5 mM EDTA pH8.0, 0.2% SDS (Biorad 1610418), 200 mM NaCl, 0.1 mg/ml Proteinase K (Roche 3 115 879).

2.6.1.2.DNA isolation from ES cells

In a confluent 48-well plate, cells were washed and trypsinized using 100 µl of Trypsin/EDTA solution/well. Following trypsinization, 100 µl of PBS (Ca^{2+}/Mg^{2+}-free, Invitrogen 14190-094) were added and cells were transferred to eppendorf tubes. Cells were centrifuged at 13000 rpm for 1 minute at RT, incubated in 500 µl of Proteinase K Tail Lysis Buffer O/N, at 55ºC and then 140 µl of 5 M NaCl were added. After shaking well, samples were centrifuged at 8000 rpm for 20 minutes at 4ºC; the supernatant was collected in a clean eppendorf and centrifuged again until no debris was visible. DNA was precipitated by adding 1ml of cold 100% ethanol, centrifuged at 8000 rpm for 10 minutes, at 4ºC and then washed three times with cold 80% ethanol. Finally, the DNA pellet was air dried and dissolved in 10 µl of TE.

TE: 5 mM Tris pH7.5, 1 mM EDTA pH8.0.

2.6.2.Plasmid DNA isolation

Plasmid DNA was isolated using QIAprep Spin Miniprep Kit (Cat. No. 27106) or QIAGEN Plasmid Maxi Kit (Cat. No. 12163), following manufacturer's instructions.

2.6.3.Analysis of DNA

2.6.3.1.Restriction enzyme digestion and agarose gel electrophoresis

Restriction enzymes were supplied by New England Biolabs and Roche. Digestions were carried out using the appropriate buffer systems supplied with the enzyme and following manufacturer's protocols. Conventional electrophoresis was performed in MultiSub gel apparatus. Horizontal minigels were prepared by dissolving molecular biology grade multi-purpose agarose (Roche 11 388 991 001) in either 1x TAE or 1x TBE. Typically, 0.6-2% (w/v) gels were used, depending on the molecular weight of the DNA of interest. DNA samples were mixed in a 9:1 ratio with 10x DNA Loading buffer before loading and electrophoresed in 1x TAE or 1x TBE running buffer at 120V. Nucleic acids were stained by the addition of 1 µg/ml ethidium bromide (VWR 429912N) to the molten agarose prior to casting. In applications where the DNA product underwent downstream processing, staining was performed only upon completion of electrophoresis by soaking the gel in a 10 µg/ml solution of ethidium bromide in dH_2O for 5-10 minutes. The size of nucleic acids was determined by comparison against a 1 Kb DNA ladder (Invitrogen 1561 016). The DNA was visualized in a BioDoc-IT lightproof cabinet containing an ultraviolet transilluminator at 302nm (UVP) and a CCD camera.

10x DNA Loading buffer: 20% Ficoll, 0.1 M EDTA, 1% SDS, 0.25% Orange G.

2.6.3.2.Southern Analysis

Probe synthesis and labelling

Probes were synthesized by random priming using the Rediprime II DNA Labelling System (Amersham Pharmacia Biotech RPN1633). Unincorporated radioactivity was

removed by passing through a Microspin S-200HR column (Amersham Pharmacia Biotech 27-5120). Both kits were used following the manufacturer's instructions. Probes were denatured by heating (95ºC, 5 minutes) and snap cooling (5 minutes, on ice) to prevent re-annealing.

Southern Blotting

Genomic DNA (8-10 μg) was digested O/N with restriction endonucleases and run in a 1% agarose gel (90V) for 6-7 hours (Section **6.3.1**). The gel was photographed and then incubated sequentially in Depurination solution (2x 5 minutes, RT), Denaturation solution (2x 30 minutes, RT) and in Neutralization solution (2x 30 minutes, RT). The gel was rinsed with dH_2O between each solution. DNA was transferred O/N on a Hybond-N^+ membrane (Amersham Pharmacia Biotech RPN303B), using 20x SSC. After transfer, the DNA was fixed on the membrane either by baking (2 hours, 80ºC) or by using a UV crosslinker (UV Stratalinker 2400). The membrane was placed on a piece of nylon mesh whenever the ends of the membrane overlap, rolled up into a tight roll in 2x SSC and placed in a hybridization bottle. 2x SSC was replaced by 20 ml of pre-hybridization solution in which the membrane was incubated for 2 hours at 65ºC and then by hybridization solution containing the denatured probe. Following hybridization O/N at 65ºC, the membrane was washed in two solutions of increased stringency: 2x SSC, 0.1% (w/v) SDS (2x 15 minutes, 65ºC) and in 0.5x SSC, 0.1% (w/v) SDS (2x 15 minutes, 65ºC). Excess liquid was blotted off the radioactive membranes, which were then wrapped in Saranwrap and exposed to BioMax MS film (Kodak 832 6836) in a light-sealed cassette with 2 intensifier screens at -80ºC. Exposure time varied according to the intensity of the signal.

Depurination solution: 0.25 N HCl

Denaturation solution: 1.5 M NaCl, 0.5 N NaOH

Neutralization solution: 1.5 M NaCl, 0.5 M Tris.HCl pH7.5

Pre-hybridization/Hybridization Solution: 3x SSC, 10x Denhardt's (50x Denhardt's Sigma D2532), 10% Dextran Sulphate (Amersham Pharmacia Biotech 17-0340), 0.1% SDS, 100 mg/ml denaturated salmon sperm DNA (Biochemika 31149).

2.6.3.3.Quantification of DNA

The concentration of DNA was assayed by submitting an aliquot of the DNA to agarose gel electrophoresis. The brightness of the band was compared to the 1 Kb DNA ladder (Invitrogen 1561 016) to have an estimate of the amount of DNA present. In parallel, concentration of DNA was determined using a NanoDrop ND-1000 spectrophotometer.

2.6.4.Bacterial Strain, growth and storage

Plasmids were maintained in *E. coli* strain One Shot® ([F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*Δ139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G]; Invitrogen C4040-06) or XL1-Blue strain ([*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*qZΔ*M15* Tn*10* (Tetr)]; Stratagene 200268) . PAC DNA was maintained in *E. coli* strain EL250 (DH10B [λ*cI857(cro-bioA)*<> *ara*C-P_{BAD}*flpe*), *a* modified DH10B strain [GIBCO] containing defective lambda prophage (Yu *et al.*, 2000) and arabinose-inducible *flpe* gene (Lee *et al.*, 2001). Bacteria grew in LB or LB-agar plates containing the appropriate antibody at 32ºC (EL250) or 37ºC (One Shot® and XL1-Blue). For long-term storage of bacterial strains and clones, glycerol

was added to O/N cultures at a final concentration of 15% (v/v), and the stocks were stored in 1 ml aliquots at -70ºC.

2.6.5. Colony lifts

Colony lifts were performed by placing a nitrocellulose filter over the plate with colonies and then pressing it against a new wet filter. After the transfer, bacteria were grown on the filter at 37ºC, O/N. Filters were then incubated sequentially in 0.5 N NaOH (5 minutes, RT), 1 M Tris (pH7.5) (5 minutes, RT), 1 M Tris (pH7.5)/1.5 M NaCl (10 minutes, RT). Finally, the filter was transferred to 2x SSC/0.1% SDS solution, rubbed with gauze and washed with 2x SSC. After baking for 2 hours at 80ºC, the filter was processed for Southern blot analysis as described in Section **2.6.3.2**.

2.6.6.Cloning techniques

2.6.6.1.DNA fragment isolation

DNA fragments were isolated from agarose gels using QIAquick Gel Extraction Kit (Cat. No. 28706), following manufacturer's instructions.

2.6.6.2.Ligation of DNA fragments

Ligations here described involved DNA fragments digested with restriction enzymes and PCR products. Ligation of DNA fragments was performed using TaKaRa DNA Ligation Kit (TaKaRa 6022), following manufacturer's instructions. Ligation of PCR products was carried out using pCR®II-TOPO® Cloning according to manufacturer's instructions.

2.6.6.3.Transformation of competent bacteria

Ligation constructs were transformed into chemically-competent One Shot® cells or into electro-competent XL1-Blue cells. PAC DNA was transformed into electro-competent EL250 cells.

Transformation of chemically-competent One Shot® cells was performed according to the manufacturer's protocol. Briefly, plasmid DNA was added to 50 µl of competent cells. Reactions were incubated on ice for 30 minutes, followed by 30 seconds heat-shock at 42ºC and further incubation on ice for 2 minutes. 250 µl of LB medium was added to each transformation reaction and incubated at 37ºC for 1 hour. 100 µl of each transformed culture was plated on LB-agar plates containing the appropriate antibody and grown O/N at 37ºC.

For the transformation of electrocompetent cells (XL1-Blue and EL250), DNA was added to the cells and these were transferred to a chilled disposable electroporation cuvette (BioRad 1652088). Electroporation was performed using a Bio-Rad gene pulser set at 2.5KV (electroporation of plasmid DNA into XL-Blue strain) or 1.8KV (electroporation of PAC DNA into EL250 strain). Electroporated cells were transferred to an eppendorf in 500µl of LB medium and incubated at 37ºC (XL-Blue strain) or 32ºC (EL250 strain) for 30 minutes before plating.

When using the XL-Blue strain, blue/white selection was used to distinguish recombinant plasmids from non-recombinant ones. Bacteria carrying the insert generate white colonies in the presence of the LacZ substracts IPTG and X-Gal due to the disruption of the *LacZ* gene of the cloning vector by the insert.

2.6.7.PAC techniques

2.6.7.1.PAC isolation and propagation

To isolate single colonies, the PAC clone was streaked onto LB-agar-Kanamycin and incubated at 32ºC, O/N. Single colonies were picked and grown in LB-Kanamycin at 32ºC, O/N.

LB (-agar-) Kanamycin: LB (agar), Kanamicin at a final concentration of 12.5 µg/ml (Sigma K0879).

2.6.7.2.Small and large scale preparation of PAC DNA

Small Scale PAC DNA isolation (MINIPREP)

PAC bacterial colonies were grown in 2 ml of LB-Kanamycin, at 32ºC, O/N. Bacteria were harvested by centrifugation for 1 minute at 13000 rpm, resuspended in 100 µl of Solution I. Bacteria were lysed with 200 µl of Solution II and neutralized with 150 µl of Solution III. After centrifugation at 13000 rpm for 10 minutes, PAC DNA was precipitated by adding 900 µl of cold 100% ethanol and then washed in 70% ethanol. The dry DNA pellet was ressuspended in TE. Cut tips were used in all steps of the protocol to avoid shearing PAC DNA.

Solution I: 50 mM Glucose, 25 mM Tris pH8.0, 10 mM EDTA pH8.0.

Solution II: 0.2 N NaOH, 1% SDS.

Solution III: 3 M KOAc, 1 N Glacial Acetic Acid.

Large Scale PAC DNA isolation (MAXIPREP)

PAC bacterial colonies were grown in 500 ml of LB containing 12.5µl/ml kanamycin O/N, at 32ºC. Bacteria were harvested by centrifugation for 10 minutes at 3750 rpm,

resuspended in 16 ml of Solution IV and incubated for 5 minutes at RT. Bacteria were lysed with 16 ml of Solution V for 5 minutes at RT, neutralized with 16 ml of cold Solution VI and then incubated for 1 hour at 4ºC. After centrifugation at 3750 rpm for 15 minutes at 4ºC, PAC DNA was precipitated with ice-cold isopropanol, incubated for 5 minutes at RT and then centrifuged at 3750 rpm for 15 minutes at 4ºC. DNA pellet was ressuspended in TE and then extracted using phenol:chloroform:isoamylacohol (25:24:1; Sigma P2069) and chloroform, followed by 100% ethanol precipitation (1:1), and an ethanol 70% wash. The dry DNA pellet was ressuspended in TE and kept at 4ºC until use. Cut tips were used in all steps of the protocol to avoid shearing PAC DNA.

Solution IV: 50 mM Tris pH8.0, 10 mM EDTA, pH8.0, 100 µg/ml RNase A.

Solution V: 0.2 N NAOH, 1%SDS.

Solution VI: 3 M KOAc, 1 N Glacial Acetic Acid pH5.5.

2.6.7.3.PAC analysis by pulse field gel electrophoresis

Linear double-stranded DNA molecules longer than ~40 Kb in length migrate through agarose gels at the same rate making fractionation impossible. In PFGE, DNA molecules are subjected to an electrical field that switches periodically between two different directions. This permits fractionation of smaller molecules, which can reorient quicker and hence move faster, from larger molecules. PFGE was carried out in 0.5x TBE buffer (pH 8.0) at 4ºC using a countour-clamped homogenous electric field apparatus (Bio-Rad) with fluid circulation. PAC DNA was run in 1% agarose gel in 0.5x TBE at 5.8V/cm, initial switch 2.1 seconds and final switch 10 seconds, for 12.5 hours. Pulse Marker Ladder (Sigma D2291) was used. The gel was stained in ethidium

bromide diluted in water for 30 minutes and then washed in water, except when isolating PAC DNA for micro-injection.

2.6.7.4.Homologous recombination in bacteria

PAC modification was carried out as previously described (Lee *et al.*, 2001). Electrocompetent EL250 *E. coli* were transformed with PAC DNA by electroporation as described in Section **2.6.6.3.** Transformed bacteria cells were plated onto LB-agar-Kanamycin plates and incubated O/N, at 32ºC. Overnight cultures containing the PAC were grown from single colonies, diluted 50-fold in LB-Kanamycin and grown in a 32ºC shaking incubator to an OD_{600}=0.5-0.8. Induction was performed by transferring 10 ml of the growing culture into 125 ml flask and placed it in a water-bath at 42ºC shaking for 15 minutes. Immediately after induction the flask was placed into ice bath slurry and shaken for 5 minutes to cool down. A non-induced culture was kept at 32ºC, placed into the ice bath slurry and used as a negative control. Cells were then centrifuged for 8 minutes at 1000g at 4ºC and washed with 1 ml ice-cold sterile water three times. After the final wash the cell pellet was resuspended in 100 µl of ice-cold sterile water. Immediately prior to electroporation, 100-300 ng of the targeting vector was mixed with 50 µl of the competent cells (from induced and non-induced culture) and transferred into an electroporation cuvette. Electroporation was performed as described in Section **2.6.6.3**. Pulsed bacterial cells were incubated in LB in a 32ºC shaking incubator for 1.5 hours before plating on appropriate selective agar media (LB-agar-Ampicilin plates) and non-selective agar media (LB-agar-Kanamicin plates). Single colonies were picked and grown in LB-Kanamycin at 32ºC, O/N.

2.6.7.5.Arabinose-inducible "FLPE"

Removal of the cloramphenicol resistance cassette (Cm^R) was achieved by L-arabinose induction of *flp* recombinase present in the EL250 strain (Lee *et al*., 2001). For this 300 µl of EL250 bacterial culture containing the recombinant PAC was inoculated in 15 ml of fresh LB and grown at 32ºC to OD_{600}=0.5-0.8 shaking. Sterile L-arabinose was then added (0.2%). After 1 hour at 32ºC, 1 ml of the bacterial culture was taken to inoculate 10 ml of fresh LB and incubated for 2 hours at 32ºC before plating on appropriate selective agar media (LB-agar-Ampicilin) and non selective agar media (LB-agar-Chloramphenicol) using a 1:100 dilution. Incubation was carried out at 32ºC, O/N. Removal of the selection marker was confirmed by Southern blot using the 3' homology fragment as a probe and recombinant PAC DNA prior recombination as a negative control.

LB-(agar)-Chloramphenicol: LB, cloramphenicol at a final concentration of 15 µg/ml (Sigma C7795).

2.6.7.6.PAC linearization for microinjection

The modified PAC DNA was linearized by restriction digest and isolated away from the vector by PFGE (Section **2.6.7.3**). The marker lanes were cut off and stained in dH_2O containing 0.5 µg/ml ethidium bromide for 10 minutes. These were used to localize the position of the PAC and vector bands in the unstained gel and excise them. The linear PAC DNA was concentrated in a 4% low-melting point (LMP) agarose gel in TBE by running it at 50V for approximately 9 hours at 4ºC. Considering the position of the vector DNA, the PAC DNA was localized in the 4% LMP agarose gel and 0.5-0.8cm block was excised. The block was equilibrated on a rotator in 12.5 ml of TENPA buffer

with 30 μM Spermine (Sigma S-1141) and 70 μM Spermidine (Sigma S-2501) for 1.5 hours. To melt the agarose, the block was incubated at 68ºC for 3 minutes, pulse spun and incubated at 68ºC for 5 minutes. After agarose melting, 2 units of β-agarase 1000U/ml (New England Biolabs M0392S) was added to the molten gel slice and incubated at 42ºC for 3 hours. For dialysis of the PAC DNA, 3 membranes (Millipore VSWP01300, pore size 0.025 μm) were placed floating on microinjecton buffer containing fresh 30 μM Spermine and 70 μM Spermidine. 50 μl of the PAC DNA was put on top of each membrane and left at RT for 1 hour. The PAC DNA was then recovered from the top of the dialysis membranes and stored at 4ºC. DNA concentration and integrity was checked by running the purified, dialysed DNA by PFGE (Section **6.6.3**). PAC DNA was diluted in microinjection buffer with 3 mM Spermine and 7 mM Spermidine before injection into the pronuclei of fertilized mouse eggs.

TENPA buffer: 10 mM Tris.HCL pH7.5, 1 mM EDTA pH8, 100 mM NaCl.

Microinjecton buffer: 10 mM Tris.HCl pH7.5, 0.1 mM EDTA pH8, 100 mM NaCl

2.6.8.Polymerase Chain Reaction (PCR) analysis

PCR cycling parameters and primers sequence are listed in **Table 2.1.**

2.6.8.1.PCR amplification of cloning DNA fragments

5' and 3' homologies of the targeting vector pBS-iCreER^{T2}-*frt*-Cm^{R}-*frt* were amplified form the Sox10 PAC using the Expand High Fidelity PCR System (Roche 03 310 256). Primers P1 and P2 amplify a 460 bp fragment; primers P3 and P4 amplify a 440 bp fragment.

Fragments $_{F}$A and $_{F}$B were amplified from the RET9$^{(MEN2A)}$ cDNA using Expand High Fidelity PCR System (Roche 03 310 256) and the primers P5-P8. Primers P5 and P6 amplify a 161 bp fragment; primers P7 and P8 amplify a 153 bp fragment.

2.6.8.2.PCR screen of ES cell clones

ES cell clones carrying the transgene were identified by PCR as previously described (Philippe Soriano, 1999). Primers F1_PS and R1-PS identify the recombinant allele by amplifying a 1.2 Kb fragment, primers F1_PS and R2_PS identify the wild-type allele by amplifying a 1.4 Kb fragment. PCR reaction was prepared using 1x Modified Gitschier Buffer (MGB), 10% DMSO, 0.5% 2-mercaptoethanol, 0.1 μM each primer, 0.5 mM each dNTP and 0.025 U/μl Taq DNA polymerase (Invitrogen 18038042).

MGB: 670 mM Tris pH8.8, 166 mM Ammonium Sulphate, 65 mM $MgCl_2$, 0.1% gelatin.

2.6.8.3.PCR genotyping of transgenic strains

PCR reactions were prepared with reagents obtained from Invitrogen (Taq polymerase, 10x buffer, $MgCl_2$) and Promega (dNTP's).

The genotype of Sox10Cre and hGFAPCreER^{T2} mice were determined using primers CreF and CreR which amplify a 550 bp fragment. Standard PCR conditions were used, adding $MgCl_2$ at a final concentration of 1.5 mM.

The genotype of Sox10iCreER^{T2} was determined using primers Cre250 and Cre880 which amplify a 630 bp fragment. Standard PCR conditions were used, adding $MgCl_2$ at a final concentration of 1.5 mM.

Table 2.1. Oligonucleotide primers and PCR cycling parameters

	Primer Sequence (5'-3')	PCR settings												
		Step 1 1 cycle		Step 2 # cycle			Step 3 # cycle			Step 4 # cycle			Step 5 1 cycle	
		°C	T	°C	T	#	°C	T	#	°C	T	#	°C	T
5'*Sox10* hom	**(P1)**GCGGCGCCCTGTCAGAGCAGACGAGGGG (**P2**)GGCGAC*CAT*GGTGCTCCCGGCCGCCGCCGCTTCG	94	4'	94 61 72	30'' 50'' 90''	5	94 59 72	30'' 50'' 90''	5	94 59 72	30'' 50'' 90''	31	72	10'
3'*Sox10* hom	(**P3**)ACTAGTAGGGGCCCTGTCACCACCAGTGCC (**P4**)TTAATTAAAAGATCAGAGTGTCCCACCCTGC	94	4'	94 61 72	30'' 50'' 90''	5	94 59 72	30'' 50'' 90''	5	94 59 72	30'' 50'' 90''	31	72	10'
RET9 $_F$A	**(P5)**CAGGGTACCGCTAGCGCCACCATGGCGAAGGCGACGTCCGGTG **(P6)**CCGGCCGCCTGGTCCACATACAGC	94	4'	94 61 72	30'' 50'' 90''	5	94 59 72	30'' 50'' 90''	5	94 59 72	30'' 50'' 90''	31	72	10'
RET9 $_F$B	**(P7)**GAGGAGGAGACACCGCTGGTGG **(P8)**CCAAAGCTTGCTAGCAGTGCAGAGGGGACAGCGGTGC	94	4'	94 61 72	30'' 50'' 90''	5	94 59 72	30'' 50'' 90''	5	94 59 72	30'' 50'' 90''	31	72	10'
R26$^{RET(MEN2A)}$	(**F1_PS**)CCTAAAGAAGAGGCTGTGCTTTGG (**R1_PS**)CATCAAGGAAACCCTGGACTACTG (**R2**)CATACTGTAGTAAGGATCTCAAGC	93	2'	93 55 65	30" 30" 3'	40	-	-	-	-	-	-	65	10'
Sox10Cre	(cre**F**)ATCCGAAAAGAAAACGTTGA (cre**R**)ATCCAGGTTACGGATATAGT	94	3'	94 53 72	30'' 1' 90''	35	-	-	-	-	-	-	72	5'
Sox10iCre ER^{T2}	(cre250)GAGGGACTACCTCCTGTACC (cre880)TGCCCAGAGTCATCCTTGGC	94	3'	94 53 72	30" 1' 90"	35	-	-	-	-	-	-	72	5'
hGFAPCre ER^{T2}	(cre**F**)ATCCGAAAAGAAAACGTTGA (cre**R**)ATCCAGGTTACGGATATAGT	94	3'	94 53 72	30'' 1' 90''	35	-	-	-	-	-	-	72	5'
R26ReYFP	(**F**)GCTCTGAGTTGTTATCAGTAAGG (**R1**)GCGAAGAGTTTGTCCTCAACC (**R2**)GGAGCGGGAGAAATGGATAGT	95	6'	95 55 72	30'' 30'' 45''	35	-	-	-	-	-	-	72	10'

P1, P3, P5, P7, F1-PS, CreF, Cre250, F – forward primer; **P2, P4, P6, P8, R1_PS, R2, CreR, cre880, R** – reverse primer; **°C** – temperature in Celsius degrees; **T** – incubation time; **#** - number of cycles, **bp** – base pairs. Restriction sites in primers P1-P8 are underlined.

The genotype of heterozygous and homozygous R26eYFP was established by PCR analysis using primers F, R1 and R2. Primers F and R1 identify the recombinant allele by amplifying a 350 bp fragment, primers F and R2 identify the wild-type allele by amplifying a 500 bp fragment. Standard PCR conditions were used, adding $MgCl_2$ at a final concentration of 2mM.

PCR products were electrophoresed through agarose gels containing ethidium bromide as described in Section **2.6.3.1**.

2.6.9.Riboprobe synthesis

Riboprobes were generated by in vitro transcription reaction with RNA polymerase, 5x Transcription Buffer (Promega P1181), 10 mM DTT (Promega P1171), 10x DIG Labelling Mix (Roche 1 277 073), RNasin (Promega N2111) and 1-1.5 µg/µl linearized plasmid. Samples were treated with DNase I (Promega M610A) for 30 minutes at 37ºC. After DNAse I treatment, probes were precipitated using LiCl precipitation (25 minutes, -20ºC), re-dissolved in 50 µl of dH_20 and stored at 20ºC.

The probe for Cre was derived from a plasmid pBS-$CreER^{T2}$ (gift from N. Kessaris). The plasmid was linearized with NotI and transcribed with T7 RNA polymerase to generate an antisense cRNA probe.

The probe for the mouse Sox10 gene was derived from plasmid pBS-Sox10 containing an 800 bp fragment of the rat Sox10 cDNA cloned into pBluescript (gift from Michael Wegner). The plasmid was digested with EcoRI and transcribed with T7 RNA polymerase to generate an antisense RNA probe.

2.7. Tissue Manipulation Techniques

2.7.1.Dissection of embryos and embryonic gut

E12.5 and E16.5 embryos were harvested from pregnant females sacrificed by a Schedule One Method. The day at which vaginal plug was found was considered to be E0.5.

Dissection of embryos was done in L15 medium (PAA Laboratories E15821), on ice. Embryos were dissected out of the uterus and isolated from the placenta and extra-embryonic membranes. When required, a portion of the tail was collected to isolate DNA for genotyping. Whole embryos were fixed in 4% paraformaldehyde (PFA) in PBS for 2 hours at 4ºC and then washed in PBT (3x, 30 minutes) prior to any other treatment.

Embryonic gut was isolated from the remaining tissue and processed for culture or fixed in 4% PFA in PBS for 2 hours at 4ºC and then washed in PBT (3x, 30 minutes).

PBT: 1x PBS, 0.1% Triton X-100 (Sigma X100).

2.7.2.Harvest of gut muscle strips with adherent myenteric plexus (MS-MP)

Adult (P84 or older) Sox10iCreER^{T2}; R26ReYFP and hGFAPCreER^{T2}; R26ReYFP transgenic mice were sacrificed by a Schedule One Method. The small intestine was dissected and cut into smaller pieces. Using a 1ml pipette the pieces of small intestine were stretched. A cotton bud soaked in PBS was used to strip the inner and outer enteric muscle layers, together with the myenteric plexus, away from the submucosa of the gut. Muscle strips with adherent myenteric plexus (MS-MP) were then stretched and pinned flat onto sylgard-coated 6-well plates using insect pins. Once pinned, muscle strips were

either processed for immunostaining (Section **2.7.3.4**) or cultured (Sections **2.8.2** and **2.8.3**).

2.7.3.Immunohistochemistry (IHC)

Primary and secondary antibodies are listed in **Tables 2.2** and **2.3**, respectively.

2.7.3.1.Fluorescence IHC on cryostat sections

Embryos were fixed for 2 hours at 4ºC in 4% PFA in PBS, cryo-protected in 30% sucrose in PBS, O/N at 4ºC, embedded in 15% sucrose; 7.5% gelatine in PBS and then frozen in Tissue-Tek OCT on dry ice. Cryosections were acquired on a Microm HM 560 CryoStar cryostat (Thermo Scientific) at a thickness of 12 µm. For immunostaining, sections were post-fixed in 4% PFA in PBS for 10 minutes at RT, incubated in Blocking solution I for 2 hours and then incubated with the primary antibody diluted in Blocking solution I, O/N, at 4ºC. After washing with PBT (3x, 5 minutes) the sections were incubated with the secondary antibody diluted in Blocking solution I for 2 hours at RT. After extensive washes with PBT, preparations were mounted in Vectashield™ Mounting Medium containing DAPI (Vector Labs H-1200).

2.7.3.2.Fluorescence IHC on embryo and gut whole-mount preparations

Embryos and guts were fixed for 2 hours at 4ºC in 4% PFA in PBS, washed in PBT three times for 5 minutes and then incubated in Blocking solution I for 2 hours at RT. Specimens were incubated with primary antibodies diluted in Blocking solution I, O/N, at 4ºC. After three 5 minutes washes with PBT, secondary antibodies diluted in Blocking solution I were added for 2 hours at RT. After extensive washes with PBT, preparations were mounted in Vectashield™ Mounting Medium containing DAPI (Vector Labs H-1200).

2.7.3.3.Fluorescence IHC on cell cultures

Cultures were fixed in 4% PFA in PBS for 10 minutes, at RT. After washing twice in PBT, cells were incubated with Blocking solution II for 2 hours, at RT. Primary antibodies were diluted in Blocking solution II and applied to cells for 5 hours, at RT or O/N, at 4ºC. After three 5 minutes washes with PBT, secondary antibodies diluted in Blocking solution II were added for 2 hours, at RT. Cells were washed with PBT extensively and then mounted in Vectashield™ Mounting Medium containing DAPI (Vector Labs H-1200).

Blocking solution II: 1x PBS, 0.1% Triton X-100, 1%BSA, 0.15%glycine.

2.7.3.4.Fluorescence IHC on muscle strips

Muscle strips were fixed in 4% PFA in PBS for 30 minutes, at RT. After fixation, muscle strips were unpinned and transferred to 24-well plates. Free-floating muscle strips were then washed in PBT three times for 5 minutes and incubated in Blocking solution I for 2 hours at RT. Tissue was incubated with primary antibodies diluted in Blocking solution I O/N, at 4ºC, followed by three washes in PBT for 5 minutes. Secondary antibodies were diluted in Blocking solution I, and the tissue was incubated for 2 hours at RT. Muscle strips were then extensively washed in PBT. If cell nuclei were required to be detected, TOTO-3-iodide (Molecular Probes T-3604) was applied for 15 minutes at RT followed by three 5 minutes washes in PBT. Finally, muscle strips were flat mounted in Vectashield™ Mounting Medium containing DAPI (Vector Labs H-1200).

Table 2.2. Primary antisera used for immunofluorescence

Antiserum	Host	Dilution	Source
B-FABP	rabbit	1:1000	Thomas Muller
Brd-U	rat	1:1000	Oxford Biotechnology OBT0030CX
DIG		1:5000	Roche 11 093 274 910
GFP*	rabbit	1:1000	Molecular Probes A11122
GFP*	rat	1:1000	Nacalai Tesque 0440484
GFAP	rabbit	1:300	DAKO Z0334
HuC/D	mouse	1:500	Molecular Probes A21271
Mash1	mouse	1:500	François Guillemot
nNos	rabbit	1:200	Zymed 617000
NPY	rabbit	1:50	Biogenesis 6730-0004
Phox2b	rabbit	1:200	Christo Goridis
SMA	rabbit	1:250	Sigma A2547
Sox2	rabbit	1:500	ISL GT15098
Sox10	goat	1:200	St Cruz Biotechnology sc-17343

Table 2.3. Secondary antisera used for immunofluorescence

Primary specificity	Host	Fluorochrome	Dilution	Source
anti-rat	donkey	Alexa Fluor 488	1:500	Molecular Probes A21208
anti-rabbit	goat	Alexa Fluor 488	1:500	Molecular Probes A11034
anti-rat	goat	Alexa Fluor 568	1:500	Molecular Probes A11077
anti-rabbit	goat	Alexa Fluor 568	1:500	Molecular Probes A11011
anti-mouse	goat	Alexa Fluor 568	1:500	Molecular Probes A11004
anti-goat	donkey	Alexa Fluor 568	1:500	Molecular Probes A11057
anti-rabbit	donkey	Cy5	1:500	Jackson ImmunoResearch 711176152
anti-mouse	donkey	Cy5	1:500	Jackson ImmunoResearch 715175150

2.7.4.RNA in situ hybridization (ISH)

In situ hybridization was performed using digoxigenin-labeled cRNA probes on coronal cryosections of whole embryos or on whole-mount embryos as previously described (Schaeren-Wiemers & Gerfin-Moser, 1993).

2.7.4.1.ISH on fixed sections

Sections were fixed in 4% PFA in PBS for 10 minutes at RT. After three 5 minutes washes in PBT, sections were incubated in acetylation solution for 10 minutes, at RT and washed again in PBT. Sections were incubated in pre-hybridization solution for 4 hours at RT and then in hybridization solution O/N at 72ºC (100 µl of Hybridization solution/slide). Slides were immersed in 5x SSC to remove coverslips, washed in 0.2x SSC (1 hour at 72ºC and then 5 minutes at RT) and equilibrated in B1 buffer for 5

minutes at RT. After pre-blocking in B2 buffer for 2 hours at RT, slides were incubated with the antibody anti-DIG diluted in B2 buffer O/N at 4ºC. Sections were washed in B1 three times for 5 minutes and then equilibrated in B3 buffer for 10 minutes at RT. Staining was performed using NBT/BCIP Section staining solution (in the dark) after which slides were washed and mounted with Glycergel Mounting Medium (DAKO C0563).

PBT: PBS, 0.1%Tween-20 (Sigma P1379).

Acetylation Solution: In 295 ml dH20 add 4 ml Triethanolamine (Fluka 90279) and 525 µl HCl. After mixing on stirrer and just before immersing the slides, add 750 µl acetic anhydride.

Pre-hybridization: 50% (v/v) de-ionized Formamide, 5x SSC, 5x Denhardt's (Invitrogen 750018), 250 µg/ml yeast $_t$RNA (Sigma R6750), 500 µg/ml herring sperm DNA (Promega D1816).

Hybridization Solution: Pre-hybridization solution containing 200-400 ng/ml DIG-labelled RNA probe.

B1 buffer: 150 mM NaCl, 100 mM Tris.HCl pH7.5.

B2 buffer: 10% heat inactivated sheep serum in B1.

B3 buffer: 100 mM NaCl, 100 mM Tris.Hcl pH9.5, 50 mM $MgCl_2$.

NBT/BCIP Section staining solution: In 2 ml B3 add 6.75 µl NBT (Sigma N6639) solution (0.075g NBT in 70% DMF) and 5.25 µl BCIP (Sigma B1026) solution (0.050g BCIP in dH_20).

2.7.4.2.Whole-mount ISH on mouse embryos

Whole-mount in situ hybridization was performed as previously described (Wilkinson, 1992). Embryos were fixed in 4% PFA in PBS, for 2 hours at 4ºC, washed twice in PBT, dehydrated and rehydrated in 25%-50%-75% Methanol in PBT, washed twice in PBT and bleached with 6% H_2O_2 in PBT for 1 hour. After three washes in PBT, embryos were treated with 10 µg/ml of Proteinase K in PBT for 30 minutes. Samples were then washed with 2 mg/ml glycine in PBT for 10 minutes to stop the Proteinase K reaction, post-fixed with 0.1% glutaraldehyde/4% PFA in PBT for 20 minutes and washed twice in PBT. Embryos were incubated in pre-hybridization buffer for at least 1 hour at 70ºC and then the hybridization solution was added. Samples were kept in hybridization solution O/N at 70ºC, washed twice for 30 minutes at 70ºC with solution I, washed twice in solution II at 65ºC and then washed three times in TBST at RT for 5 minutes. After incubation in Blocking solution for 2.5 hours at RT, samples were incubated with the antibody anti-DIG (Roche 11 093 274 910) diluted in Blocking solution, O/N, at 4ºC. Embryos were washed extensively in TBST and then in NTMT. Staining was performed using NBT/BCIP Section staining solution (in the dark) after which embryos were washed extensively.

Pre-hybridization: 50% (v/v) de-ionized Formamide, 1.3x SSC, 5 mM EDTA, 0.2% Tween-20, 50 µg/ml yeast $_t$RNA (Sigma R6750), 10% CHAPS, 100 µg/ml heparin (Sigma 3393).

Hybridization Solution: Pre-hybridization solution containing 0.2 µg/ml DIG-labelled RNA probe.

Solution I: 50% (v/v) de-ionized Formamide, 5x SSC pH4.5, 1%SDS.

Solution II: 50% (v/v) de-ionized Formamide, 2x SSC pH4.5.

TBST: 137 mM NaCl, 27 mM KCl, 25 mM Tris.HCl pH7.5, 0.1% Tween-20, 2 mM Levamisole.

NTMT: 100 mM NaCl, 100 mM Tris.HCl pH9.5, 50 mM $MgCl_2$, 0.1% Tween-20, 2 mM Levamisole.

2.8. Cell Culture Techniques

2.8.1.Short-term cultures of embryonic dissociated guts

Whole gut from E16.5 embryos was dissected in L15 medium (PAA E15821), washed with PBS (Ca^{2+}/Mg^{2+}-free; Invitrogen 14190-094) and digested for 3 minutes with 1 mg/ml dispase/collagenase (Roche 296638) at RT. Dissociated tissue was washed with PBS and plated onto fibronectin-coated (20 μg/ml; Sigma F-1141) SONIC-SEAL slide wells (BDH 402 032350) in OptiMEM (GIBCO 31985) supplemented with L-glutamine (1 mM, Invitrogen 25030-024) and penicillin/streptomycin antibiotic mixture (Invitrogen 15140-122). Cultures were maintained for up to 4 hours in an atmosphere of 5% CO_2.

2.8.2.Culture of dissociated MS-MP strips

MS-MP strips were washed in 0.1% Penicillin/Streptomycin in PBS, cut into small pieces and then incubated in 1 mg/ml Collagenase (Sigma C0130), 100 μg/ml DNase I (Sigma D4513) for 1 hour, at 37ºC. After two washes in PBS, dissociated tissue was resuspended in NCSC medium and centrifuged at 900 rpm for 5 minutes, at RT. Cells were plated onto fibronectin-coated (20 μg/ml; Sigma F-1141) LabTek 8-well slide

chambers (VWR 734 2126) in NCSC medium. Cultures were maintained in an atmosphere of 5% CO_2.

Neural Crest Stem Cell (NCSC) medium: in 50ml of DMEM, add 15% CEE, 20 ng/ml FGF (R&D System 233FB025), 20 ng/ml EGF (Merk Biosciences 324831), 1% N2 supplement (Invitrogen 17502), 2% B27 supplement (Invitrogen 17504-044), 1% Penicillin/Streptomycin (Invitrogen 15140-122), 50 µM β-mercaptoethanol (Sigma M3148), 35 ng/µl Retinoic Acid (Sigma R2625).

2.8.3.*Ex vivo* culture of MS-MP strips

MS-MP strips were kept pinned flat onto sylgard-coated 6-well plates and cultured in the different cell culture media: NCSC, Dulbecco's Modified Eagle's Medium (DMEM; Sigma D5671), DMEM_F, DMEM_E, DMEM_G. Cultures were maintained for up to 7 days in an atmosphere of 5% CO_2.

DMEM_F: DMEM supplemented with 20 ng/ml FGF (R&D System 233FB025)

DMEM_E: DMEM supplemented with 20 ng/ml EGF (Merk Biosciences 324831)

DMEM_G: DMEM supplemented with 20 ng/ml GDNF (Peprotech 450-10)

2.8.4.Mouse ES cell culture protocols

2.8.4.1.ES cells media

ES cells were grown in DMEM/high glucose (Sigma D5671) supplemented with 15% fetal bovine serum (HyClone SH30070.030E), 1 mM sodium pyruvate (Sigma S8636), 0.1 mM non-essential amino acids (Invitrogen 11140-035), 2 mM L-glutamine (Invitrogen 25030-024), penicillin/streptomycin antibiotic mixture (100U/ml penicillin, 100 µg/ml streptomycin; Invitrogen 15140-122), 0.1 mM β-mercaptoethanol (Sigma

M7522) and 10^3U/ml of leukaemia inhibitory factor–LIF (Chemicon ESG1107). Fresh medium was added every 24h.

2.8.4.2.Standard ES cell culture procedures

Gelatin treatment

Cell culture dishes were pre-gelatinized with 5 ml of PBS with gelatin (Invitrogen 041-94000M) for 30 minutes at RT.

Trypsinization

Cells were re-fed 2-4 hours before trypsinization. Culture medium was removed and cells were washed twice with 5 ml of PBS (Ca^{2+}/Mg^{2+}-free; Invitrogen 14190-094). To trypsinize cells, 500 µl of trypsin/EDTA solution were added for 2 minutes at 37ºC. Cells were vigorously resuspended in 4 ml of ES cell medium and transferred to a 15 ml Falcon tube. After centrifugation (800 rpm) for 5 minutes at RT, cells were ressuspended in fresh medium and plated in pre-gelatinized plates.

Typsin/EDTA solution: 0.05% Trypsin, 0.02% EDTA in PBS (Ca^{2+}/Mg^{2+}-free).

Freezing ES cells

Cells were trypsinized as described above and then ressuspended in freezing medium in a cell density of 0.5-1.0x10^7 cells/ml. Aliquots of 1 ml were kept at -70ºC in isopropanol container for 24h and then transferred to liquid N_2.

Freezing medium: 90% FBS (Hyclone SH30070.030E), 10% DMSO (Sigma D2650).

Thawing ES cells

ES cells were thawed for 2-3 minutes, at 37ºC. Once thawed, cells were transferred to a 15 ml Falcon tube in 9 ml of pre-warmed ES cell medium and centrifuged at 800 rpm, for 5 minutes, at RT. After centrifugation, the medium was aspirated, the cell pellet was

resuspended in fresh medium and then plated in a pre-gelatinized dish. Cells were re-fed the following day to remove dead cells and debris.

2.8.4.3.ES cell electroporation and selection of clones

In each experiment, approximately $1x10^7$cells were electroporated with 30 µg of DNA. Cells were trypsinized as described and resuspended in 0.9 ml of DMEM/high glucose (Sigma D5671). DNA was diluted in 0.1 ml of dH_2O and added to the cell suspension. Electroporation was performed by applying to successive shocks at 240V and 230V in a Gene Pulser X cell (Biorad) set to 500µF capacitance. After electroporation, cells were left on ice for 20 minutes and then plated with ES cell medium in pre-gelatinized 90-mm^3 dishes. Transfected cells were selected for 10 days in medium containing G418 (300 µg/ml; Invitrogen 10131019) added to cells 24h after electroporation.

2.8.4.4.Picking, expanding and analysing ES cell clones

At the end of the selection period, resistant clones were isolated. Plates were washed with PBS to remove any remaining debris and 10 ml of PBS were added. Resistant clones were picked using a P200 Gilson pipette under the microscope and transferred to 96-well plates containing 50 µl of dilute tripsyn/EDTA solution (1:3 in PBS). After trypsinization form 2 minutes at 37ºC, cells were dispersed using a multi-pipette and transferred into pre-gelatinized 96-well plates containing 120 µl of ES cell medium/well. Cells were re-fed every 24 hours. Once wells were confluent, cells were trypsinyzed and plated in duplicate onto pre-gelatinized 48-well plates. One set of ES clones was frozen at -70ºC for subsequent aggregation with embryos and the other set was used to extract DNA for genotyping by Southern analysis. To freeze ES cell clones, cells from confluent 48-well plates were trypsinized using 100 µl of Trypsin/EDTA

solution and then 0.9 ml of Freezing Mix (8 volumes FBS: 1 volume DMSO) was added. Cells were resuspended and transferred to freezing vials.

2.9. Tamoxifen Treatment

4-hydroxytamoxifen (4-OHT; Sigma H-6278) was dissolved in ethanol/sunflower oil (1:9) mixture at the concentration of 10 mg/ml and stored at 4ºC for 2 weeks or at -20ºC for months.

For embryonic studies, pregnant Parkes females crossed with Sox10iCreER^{T2}; R26ReYFP males were injected intraperitoneally with 0.2 mg/g of 4-OHT. To analyse neurogenesis in postnatal and adult ENS, 0.2 mg/g of 4-OHT was administered by intraperitoneal injection to SER26; R26ReYFP animals.

To label single Sox10-expressing cells *in vivo*, SER93; R26ReYFP animals were injected intraperitoneally with 2 μg/g, 4 μg/g, 5 μg/g, 15 μg/g or 25 μg/g of 4-OHT.

hGFAPCreER^{T2}; R26ReYFP animals were injected intraperitoneally with 0.2 mg/g of 4-OHT. Control mice were not injected or injected with ethanol/sunflower oil (1:9) mixture.

2.10. BAC Treatment

SER26; R26ReYFP and hGFAPCreER^{T2}; R26ReYFP double transgenics, P84 or older were injected intraperitoneally with 0.2 mg/g of 4-OHT. All animals were operated upon general isoflurane anesthesia. A laparotomy with a midline incision was performed and a portion of the small intestine was exposed out of the abdomen. Paper gauze soaked in 0.1% of BAC (Sigma B1383) in 0.9% Krebs solution was wrapped around 1-cm of the exteriorized intestine delineated with two serosal suture tags. The treatment was carried

out for 5 minutes after which the paper gauze was removed and the treated area and the peritoneum were thoroughly flushed with 0.9% saline. The wound was then sutured in two layers. After surgery, animals were allowed to recover, housed under controlled temperature conditions and allowed free access to water and food.

The animals were sacrificed 3, 30, 80 days after treatment. The treated segment of bowel and untreated segments orad and aborad to the treated area were harvested from each animal and MS-MP strips were isolated for fluorescence immunohistochemistry.

2.11. Brd-U labelling *in vitro*

For *in vitro* Brd-U labelling, specimens were incubated with 30 µg/ml of Brd-U solution in culture medium for up to 6 hours. Proliferating cells were identified by fluorescence immunohistochemistry, using rat anti-Brd-U antibody (Section **2.7.3** and **2.8.3**).

2.12. Image processing and cell counting

eYFP expression in Sox10Cre; R26ReYFP and Sox10iCreER^{T2}; R26ReYFP double transgenic embryos and guts was analyzed using a Zeiss M2-Bio stereofluorescent microscope. Images were acquired with Hamamatsu ORCA ER Digital Camera and Openlab software (Improvision). Immunostained cultures and muscle strips were examined with a Zeiss epifluorescent Axioplan and Openlab software (Improvision) or with a Bio-Rad/Radiance 2100 confocal laser scanning microscope and Lasersharp software (Biorad), using standard excitation and emission filters for visualizing DAPI, Alexa Fluor 488, Alexa Fluor 568 and Alexa Fluor 647. All images were processed with Adobe Photoshop CS 8.0 (software Adobe Systems, San Jose, CA).

Cell counts are presented as mean±SEM and are derived from at least three animals in three independent experiments.

Chapter III

Lineage analysis of *Sox10*-expressing cells in the ENS

3.1. Introduction

The identification of eNCSCs and the lineages they produce is fundamental to understand ENS organogenesis. So far, the lack of specific markers and lineage tools to accurately identify eNCSCs and their progeny has hampered the study of this population *in vivo*.

The transcriptional regulator Sox10 is the earliest neural crest marker to be found in the developing ENS. It is first detected in NCCs shortly after delamination from the neural tube and it continues to be expressed while these cells migrate and, eventually, invade the foregut (Anderson *et al.*, 2006; Kuhlbrodt *et al.*, 1998). *Sox10* plays a critical role in ENS development as null mutations in this locus lead to a complete absence of enteric ganglia due to apoptosis of NCCs before reaching the gut (Kapur, 1999; Southard-Smith *et al.*, 1998). This phenotype and the early onset of Sox10 expression in NCCs that enter the gut have led to the widely held belief that Sox10 is a marker for eNCSCs that give rise to both enteric neurons and glia. However, this conclusion is not entirely justified because Sox10 may function non-autonomously for ENS development, i.e., $Sox10^{+}$ progenitors may be required for the survival of adjacent ($Sox10^{-}$) precursor cells. Moreover, despite *in vitro* studies demonstrating that *Sox10*-expressing undifferentiated progenitors generate both enteric neurons and glia, in the adult gut expression of Sox10 is associated exclusively with mature glial cells (Bondurand *et al.*, 1998; Bondurand *et al.*, 2003; Young *et al.*, 2003). This raises the question whether *in vivo* both enteric neurons and glia derive from *Sox10*-expressing eNCSCs or whether there are two different progenitor populations: a $Sox10^{+}$ progenitor population that gives

rise to the glial lineage ($Sox10^{+}$) and $Sox10^{-}$ progenitor cells that give rise to the neuronal lineage ($Sox10^{-}$).

The lineage relationship between progenitor cells and their derivatives can be examined using lineage tracing analysis whereby cells are labelled or marked so that their progeny can be reliably identified throughout development. Earlier studies of cell lineage have employed either microinjection of lineage tracer dyes or infection by recombinant retrovirus carrying a marker gene (Stern & Fraser, 2001). These methods have the disadvantage of marking cells indiscriminately and, in most tissues, they cannot label specific sub-populations. Another approach is to genetically mark progenitor cells using endogenous gene expression patterns. Valuable insight into ENS development has emerged from the analysis of transgenic lines in which expression of an easily detectable marker is under the control of a promoter of interest. For instance, regulatory regions of dopamine β-hydroxylase linked to β-gal (Mercer *et al.*, 1991) have been used to visualise a subpopulation of ENS cells (Kapur *et al.*, 1992; Stewart *et al.*, 2003). Also, the migratory paths and behaviour of these cells have been studied by knocking into the first exon of the *Ret* locus, a transgene that drives expression of GFP (Anderson *et al.*, 2006; Enomoto *et al.*, 2001). More recently, transgenic lines in which expression of a reporter is driven from the *Sox10* (Deal *et al.*, 2006) or the *Phox2b* (Corpening *et al.*, 2008) promoter have also been described. The drawback of these transgenic approaches is that any progeny of the labelled cells that cease to express the chosen protein cannot be followed and so its usefulness for fate-mapping analysis is limited. Such limitation can be overcome using a genetic fate mapping system. This system makes use of two genetically engineered alleles to selectively and irreversibly mark cells

and their progeny. In one allele, tissue specific promoters drive expression of site-specific recombinases, such as Cre, which acts on *loxP* sites or Flp, which acts on *frt* sites. In the other allele, tissue-specific or ubiquitous promoters drive expression of a reporter gene (e.g. lacZ, (Soriano, 1999); human alkaline phosphatase, (Lobe *et al.*, 1999); enhanced green fluorescent protein, (Srinivas *et al.*, 2001)). In the absence of the site-specific recombinase, a *loxP*- or *frt*- flanked transcriptional terminator sequence located upstream of the reporter gene prevents its expression. However, in double transgenic animals the *loxP*- or *frt*- flanked sequence is excised following site-specific recombination. This leads to the permanent and heritable expression of the reporter gene in the targeted cells. Thus, by using tissue-specific promoters to drive expression of site-specific recombinases, progenitor cells can be selectively and irreversibly marked permitting detection of progeny cells that no longer express the gene of interest.

Here, we have investigated the lineage of *Sox10*-expressing cells in the ENS, *in vivo*, using genetic fate mapping. The purpose of this analysis was to determine whether both enteric neurons ($Sox10^-$) and glia ($Sox10^+$) arise from *Sox10*-expressing cells.

3.2. Results

3.2.1. Cre efficiently mediates recombination in the ENS of Sox10Cre; R26ReYFP transgenics

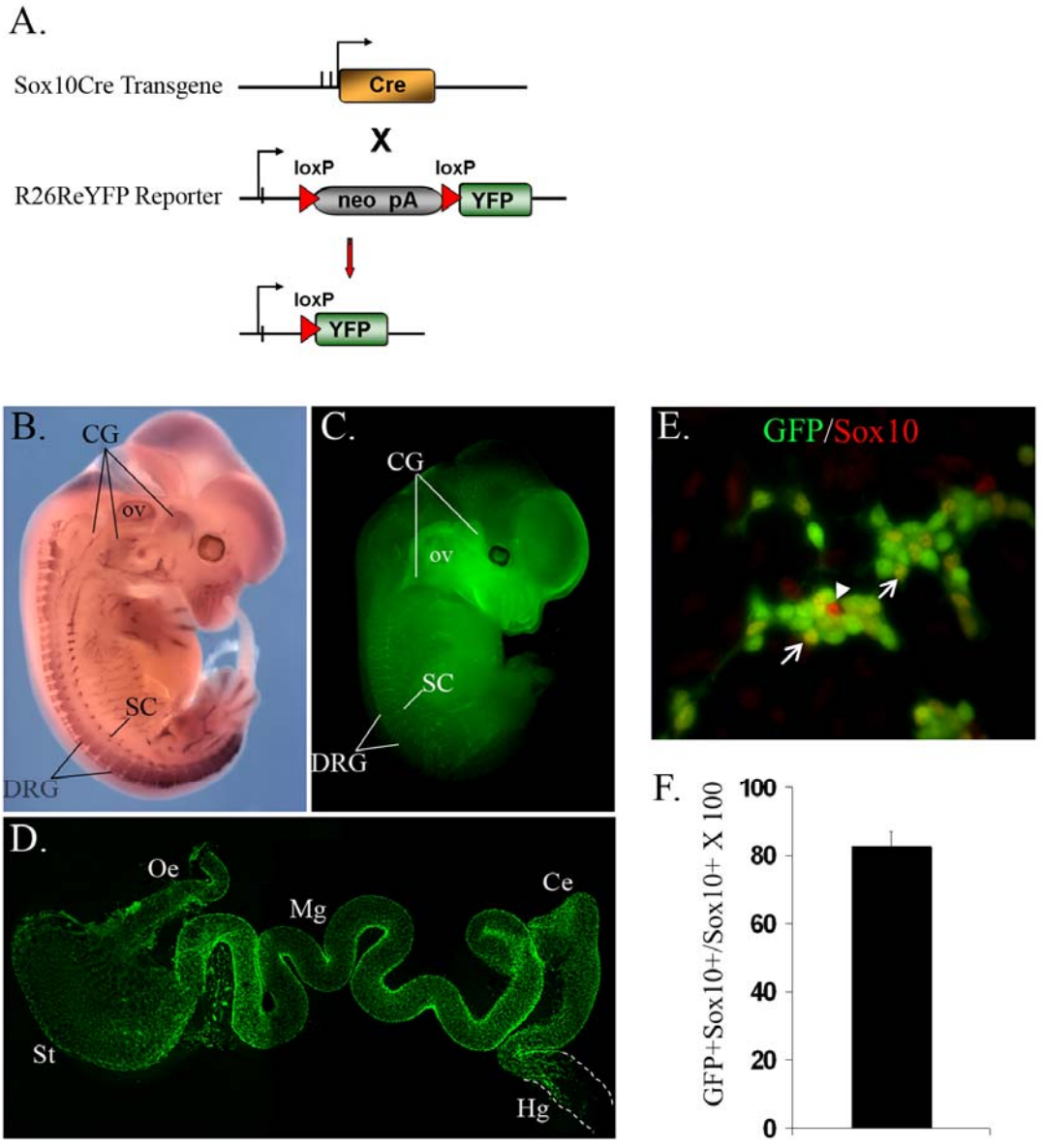
To examine the lineage relationship between *Sox10*-expressing cells and mature enteric neurons and glia, we lineally marked the progeny of $Sox10^+$ cells using the *Cre-loxP* system. Transgenic mice expressing *Cre* under the transcriptional control of *Sox10* (Matsuoka *et al.*, 2005) were crossed with the reporter line R26ReYFP (Srinivas *et al.*, 2001). In offspring carrying both alleles, the transcriptional terminator sequence flanked

by *loxP* sites is excised specifically in *Sox10*-expressing cells leading to permanent expression of *YFP* in these cells and all their descendants (**Fig.3.1 A**). To establish that the *Sox10Cre* transgene confers expression on the *YFP* reporter analogous to that of endogenous *Sox10*, whole-mount preparations of E12.5 Sox10Cre; R26ReYFP embryos were immunostained for the reporter YFP using anti-GFP antibodies. The pattern of GFP fluorescence in double transgenics was then compared to *Sox10* mRNA expression in wild-type embryos as detected by *in situ* hybridization using a Sox10 riboprobe. This analysis revealed appropriate expression of GFP within the *Sox10* expression domain and in derivatives of $Sox10^+$ cells. In accordance, GFP fluorescence was detected in the cranial sensory ganglia, dorsal root ganglia and sympathetic chain (**Fig.3.1 B, C**). We also found GFP fluorescence within the ENS. The spatial distribution of GFP fluorescence in the gut of double transgenic embryos followed the pattern previously described for eNCCs as they colonise the gut (Kapur *et al.*, 1992; Young *et al.*, 1998; Young & Newgreen, 2001). Thus, in the bowel of E12.5 transgenics GFP-labelled eNCCs were observed throughout the stomach, midgut and past the caecum (**Fig.3.1 D**).

To determine the efficiency of Cre-mediated recombination in the ENS of Sox10Cre; R26ReYFP mice, short-term cultures of dissociated gut from E16.5 embryos were immunolabelled with antibodies for GFP and Sox10 (**Fig.3.1 E**). GFP expression was detected in 83±4.2% (391 cells total from three mice) of the $Sox10^+$ cells (**Fig.3.1 F**).

Figure 3.1. ***Sox10Cre; R26ReYFP transgenic line was used to lineally mark the progeny of Sox10-expressing cells in the ENS.***

(A) Top: Schematic of the *Sox10Cre* transgene generated by replacing *Sox10* coding region (exon 3 to exon 5; exons are represented as black vertical lines) with that of Cre recombinase (orange box; Matsuoka *et al.*, 2005). Middle: The Sox10Cre transgenic line was crossed with the reporter line R26ReYFP (Srinivas *et al.*, 2001) in which the *YFP* sequence (green box) is disrupted by a transcriptional terminator cassette (a phosphoglycerate kinase promoter-neomycin selectable marker followed by a transcriptional stop sequence; grey box) flanked by *loxP* sites (red triangles). Bottom: Expression of *YFP* requires excision of the transcriptional terminator cassette by Cre-mediated recombination at the *loxP* sites. **(B)** *Sox10* expression pattern in an E12.5 embryo as shown by whole mount *in situ* hybridization using a Sox10 riboprobe; x16 magnification. **(C)** Whole mount GFP immunostaining of an E12.5 Sox10Cre; R26ReYFP double transgenic embryo showing expression of the transgene within the *Sox10* expression domain; x12.5 magnification. **(D)** Whole mount GFP immunostaining of the gut of an E12.5 double transgenic embryo showing fluorescent eNCCs; x50 magnification. **(E)** Short-term cultures of dissociated gut from E16.5 double transgenics were immunostained for GFP (green) and Sox10 (red). Most $Sox10^+$ cells undergo Cre-mediated recombination and, therefore, express GFP (arrows). Arrowhead indicates a $Sox10^+$ cell that does not express GFP. x400 magnification. **(F)** Quantification of $Sox10^+$ cells that undergo Cre-mediated recombination and therefore express GFP. Graph error bars, s.e.m. CG, cranial ganglia; ov, otic vesicle; DRG, dorsal root ganglia; SC, sympathetic chain; Oe, oesophagus; St, stomach; Mg, midgut; Ce, caecum; Hg, hindgut.


A.
Sox10Cre Transgene
Cre
X
R26ReYFP Reporter
loxP
neo pA
loxP
YFP
loxP
YFP
B.
CG
ov
SC
DRG
C.
CG
ov
SC
DRG
E.
GFP/Sox10
D.
Oe
Mg
Ce
St
Hg
F.
GFP+Sox10+/Sox10+ X 100
100
80
60
40
20
0

3.2.2.A common pool of *Sox10*-expressing cells gives rise to enteric neurons and glia

To follow the fate of *Sox10*-expressing cells in the ENS, we analysed co-expression of GFP and lineage markers in short-term cultures of dissociated gut from E16.5 Sox10Cre; R26ReYFP transgenic embryos or whole-mount preparations of muscle strips with adherent myenteric plexus (MS-MP) from P30 animals of the same genotype.

At E16.5, 28±2.9% (523 cells from three mice) of the GFP^+ cells expressed the glial marker BFABP (Kurtz *et al.*, 1994; Young *et al.*, 2003) whereas a fraction of the GFP^+ cells expressed the pan-neuronal marker Tuj1 (47±4.7%; 514 cells from three mice). Approximately 25% of GFP^+ cells in the ENS of double transgenic mice were not labelled by either glial or neuronal markers (**Fig.3.2 A, B, G**). At P30, the majority of GFP^+ cells co-expressed the glial marker S100β (64±2.3%; 1981 cells from three mice) indicating that they are glial cells (Gershon & Rothman, 1991; Young *et al.*, 2003), while 34±3.7% of GFP^+ cells expressed the neuron-specific RNA binding protein HuC/D (2180 cells from three mice), demonstrating that they represent enteric neurons (Marusich *et al.*, 1994; Wakamatsu & Weston, 1997). At this stage, only approximately 1% of GFP^+ cells present in the gut of double transgenic mice were not labelled with either the glial or neuronal marker (**Fig.3.2 D, E, G**). Finally, no co-localization between GFP and the myofibroblast marker smooth muscle actin (SMA; Morrison *et al.*, 1999) was detected in the gut of Sox10Cre; R26ReYFP animals (644 cells from three E16.5 mice; 527 cells from three P30 mice; **Fig.3.2 C, F, G**).

Figure 3.2. ***Both enteric neurons and glial cells derive from a common pool of Sox10-expressing progenitors.***

(A-G) Short-term cultures of dissociated gut from E16.5 Sox10Cre; R26ReYFP transgenic embryos **(A-C)** and whole mount preparations of MS-MP strips from P30 animals of the same genotype **(D-F)** were immunostained for GFP (green) and lineage markers (glial markers: BFABP **[A]**, S100β **[D]**; neuronal markers: Tuj1 **[B]**, HuC/D **[E]**; myofibroblast marker: SMA **[C, F]**; red). Arrows indicate double positive cells. x400 magnification. **(G)** Quantification of glial cells ($BFABP^+$ or $S100\beta^+$), neurons ($Tuj1^+$ or HuC/D^+) and myofibroblasts (SMA^+) in the GFP^+ population. Graph error bars, s.e.m.

A. GFP/BFABP
B. GFP/Tuj1
C. GFP/SMA
D. GFP/S100β
E. GFP/HuC D
F. GFP/SMA
E16.5
P30
G.
% of differentiated cells in the GFP+ population
100
80
60
40
20
0
E16.5
P30
Muscle cells
Neurons
Glial cells

3.3. Discussion

In vitro experiments have suggested that *Sox10*-expressing eNCSCs give rise to both the $Sox10^+$ (glial cells) and the $Sox10^-$ (neurons) lineages of the ENS (Bondurand *et al.*, 2003). However, the lineage relationship between *Sox10*-expressing cells and the neurons and glia of the ENS *in vivo* is currently unclear. To address this issue directly, we followed the fate of enteric $Sox10^+$ cells using a genetic fate mapping system that combines a *Sox10Cre* transgene (Matsuoka *et al.*, 2005) with the Cre-dependent R26ReYFP reporter (Srinivas *et al.*, 2001).

The *Sox10Cre* transgene has been shown to be correctly expressed in the lineages of the cranial neural crest (Matsuoka *et al.*, 2005). However, expression of this transgene within the developing and adult gut was not previously characterized. We used the R26ReYFP reporter (Srinivas *et al.*, 2001) to monitor Cre-mediated recombination. Analysis of Sox10Cre; R26ReYFP embryos confirmed that the *Sox10Cre* transgene confers expression of the *YFP* reporter in *Sox10*-expressing cells and its derivatives, including the NCCs that colonise the gut. Moreover, we found that Cre efficiently mediates recombination in the ENS as the vast majority of $Sox10^+$ cells (83%) in the gut were GFP-labelled. These results establish the Sox10Cre; R26ReYFP transgene combination as a valuable tool for fate-mapping studies in the ENS.

By following the progeny of $Sox10^+$ cells, we demonstrated that these cells have the potential to give rise to both enteric neurons and glia *in vivo*. The results from this direct cell lineage analysis eliminate uncertainties associated with the use of gene co-expression as a lineage marker or the cell autonomy questions left open by the analysis of mutant phenotypes (see Introduction) and unequivocally establish *Sox10*-expressing

cells as the progenitors of both enteric neurons and glia. Since our lineage analysis did not resolve the multipotency of a single $Sox10^+$ cell but rather traced the fates of $Sox10^+$ cell populations, it is possible that *Sox10*-expressing cells are a mixture of neuronal and glial progenitors. Alternatively, our results may reflect the possibility that single $Sox10^+$ cells have the potential to generate both neurons and glia. Clonal cell fate analysis *in vivo* will be required to directly address the differentiation potential of single *Sox10*-expressing cells.

We also examined directly the lineage relationship between *Sox10*-expressing cells and myofibroblasts *in vivo*. Previous cell culture studies suggested that eNCSCs have the potential to give rise to myofibroblasts as identified by the expression of SMA (Bondurand *et al.*, 2003; Kruger *et al.*, 2002; Suarez-Rodriguez & Belkind-Gerson, 2004). Bondurand et al. (2003) reported the presence of SMA^+ cells in the neurospheres generated by culturing dissociated gut cells from wild-type embryos and newborn mice. Similarly, Suarez-Rodriguez and Belkind-Gerson (2004) established adherent cultures from dissociated intestine and observed an increase of the expression levels of SMA transcripts throughout the culture period. In these studies, the lineage relationship between eNCSCs and SMA^+ cells was not addressed and so it is possible that the SMA^+ cells found in culture corresponded to contaminating muscle cells. Another possibility is that eNCSCs were able to generate myofibroblasts as in the case of other NCSCs (Joseph *et al.*, 2004; Morrison *et al.*, 1999). In accordance with this hypothesis, Kruger et al. (2002) found that single $p75^+$ cells isolated from the gut generate colonies that contain neurons, glia and myofibroblasts. In our study, we did not find co-localization between GFP and SMA in the ENS of E16.5 or P30 double transgenics, thus indicating

that Sox10$^+$ cells do not have the potential to give rise to myofibroblasts *in vivo*. The discrepancy between our findings and Kruger et al. observations may be explained by the possibility that Sox10$^+$ cells represent a population of neural stem cells different from the p75$^+$ cell population that includes myofibroblast progenitors. Alternatively, acquisition of a muscle cell phenotype by eNCSCs in culture may be associated with broadening of their developmental potential *in vitro* in ways that do not occur under physiological conditions. Such differences in the developmental potential exhibited in culture and *in vivo* have been reported for other progenitor cells (reviewed in Joseph & Morrison, 2005).

Because in our lineage tracing system *Sox10*-expressing cells and their progeny are irreversibly labelled by GFP, the relative frequency of neurons and glia in the ENS at a given time can be examined by determining the frequency of GFP$^+$ neurons and glia in the GFP-labelled population. We found that at E16.5, 47% of the GFP$^+$ cells expressed the neuronal marker Tuj1, thus indicating that at this stage the eNCC population is comprised mainly of neurons. This is consistent with a previous report showing that at the same developmental stage the neuronal population corresponds to 50% of the eNCCs as identified by the expression of the transcription factor Phox2b (Young *et al.*, 2003). In contrast, at P30, 64% of the GFP$^+$ cells co-expressed the glial marker S100β indicating that glial cells become relatively more abundant than neurons. This observation is in accordance with previous reports and suggests that enteric glia are the most abundant cell type in the ENS of adult animals (Gabella, 1981; Hoff *et al.*, 2008). Since our fate analysis did not distinguish between the potential of Sox10$^+$ cell populations and Sox10$^+$ single cells, these results raise two possible interpretations. One

possibility is that from embryogenesis to adulthood $Sox10^+$ gliogenic progenitors become predominant over neuronal progenitors. Alternatively, it is possible that $Sox10^+$ cells become biased to generate glial cells as development proceeds. Our data are consistent with findings of Kruger et al. who showed that *in vitro* eNCSCs exhibit reduced neurogenic potential and become biased to generate glia with increasing age. The authors further suggested that such changes in the potential of eNCSCs are associated with temporal differences in their responsiveness to lineage-determination factors present in the cellular micro-environment. Whether eNCSCs undergo similar changes *in vivo* and whether signals present in the extracellular environment can account for the shift between neurogenesis and gliogenesis is still unclear.

Based on the assumption that undifferentiated, multilineage progenitors do not express neuronal or glial markers, Young et al. (2003) estimated that 2% of the cells in the myenteric ganglia of newborn animals are eNCSCs. However, the neural crest origin of the non-neuronal non-glia cells found in this study was not confirmed. In our study, most $Sox10^+$ cells and their progeny are permanently labelled and so the relative frequency of eNCSCs at a given time can be inferred by calculating the proportion of GFP^+ cells that do not express neuronal or glial markers. We estimated that eNCSCs correspond to 25% of all ENS cells at E16.5 and less than 1% in the adult ENS, thus suggesting a dramatic reduction in the number of eNCSCs with increasing age. This is in agreement with previous studies proposing a reduction in the size of the multipotent progenitor pool from embryonic to adult stages (Kruger *et al.*, 2002; Young *et al.*, 2003).

Taken together, our data demonstrated that during gut organogenesis both neurons and glia of the ENS derive from a common pool of *Sox10*-expressing cells.

Chapter IV

Generation and Analysis of Sox10iCreER^{T2} transgenics

4.1. Introduction

As eNCSCs migrate away from the gut entry points and spread uniformly through the gastrointestinal tract, they respond to strong proliferative signals which increase their numbers dramatically (Gianino *et al.*, 2003). In parallel, subsets of eNCSCs undergo sequential lineage restriction before differentiating into a vast array of phenotypically diverse neurons and glial cells. Thus, the development of the ENS is a complex, asynchronous process that relies on the temporal regulation of eNCSCs migration, proliferation and differentiation. It is still largely unknown how such cellular processes are appropriately controlled and integrated temporally in the context of the gastrointestinal tract, which is itself undergoing growth and morphogenesis. Previous reports suggested that this depends on temporal changes in both the environment and cell-intrinsic properties of eNCSCs over time (Bixby *et al.*, 2002; Chalazonitis *et al.*, 1998; Chalazonitis *et al.*, 2008; Gershon *et al.*, 1993; Kruger *et al.*, 2002). In the absence of specific markers and tools to identify and follow eNCSCs *in vivo*, changes in the properties of these cells (i.e self-renewal capacity, neurogenic potential) over the course of ENS development have only been examined in culture, by studying the behaviour of prospectively identified cell populations (Bondurand *et al.*, 2003; Kruger *et al.*, 2002). Therefore, the question as to whether and how the properties of eNCSCs change over time *in vivo* in a manner consistent with the changing demands of tissue growth and maintenance remains unanswered.

The study of temporally defined cell populations and their progeny *in vivo* can be achieved using an inducible genetic fate mapping system. This system makes use of a fusion protein in which the catalytic domain of Cre recombinase is fused with a mutated

ligand-binding domain of the human (ER^T; ER^{T2}) or mouse (ER^{TM}) estrogen receptor gene (Danielian *et al.*, 1998; Feil *et al.*, 1996; Feil *et al.*, 1997). The mutated ligand-binding domain of the chimeric CreER fails to bind the naturally occurring ER ligand 17β-estradiol but has high affinity for the synthetic ligand tamoxifen (TM) and 4-hydroxytamoxifen (4-OHT), a liver-generated metabolite of TM that induces Cre activity as effectively as TM (Danielian *et al.*, 1993; Danielian *et al.*, 1998). Unlike other Cre recombinases, CreER lacks a nuclear localization sequence and is sequestered in the cytoplasm in an inactive form by heat shock protein 90 (Hsp90). In the presence of TM/4-OHT, the fusion protein undergoes a conformational change that releases it from Hsp90 and allows it to be transported to the nucleus where it catalyzes recombination of a floxed transcriptional STOP signal, thereby allowing expression of a reporter encoded by an unlinked locus (**Fig. 4.1**). The recombination event that activates the reporter allele occurs within 6-12 hours after TM/4-OHT administration and continues for up to 36 hours (Hayashi & McMahon, 2002), consistent with TM pharmacokinetics *in vivo* (Robinson *et al.*, 1991). Because of this 48 hour window of TM/4-OHT activity, cells expressing CreER at a specific developmental stage can be selectively labelled. Thus, this type of recombination can be used to follow the progeny of cells that express the gene of interest at defined developmental stages, including postnatal growth and during regeneration.

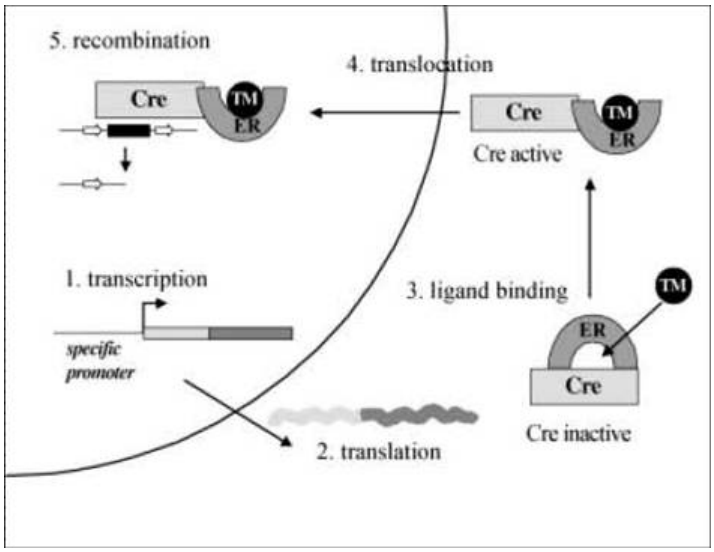


Figure 4.1. ***Schematic of tamoxifen-induced Cre activity.*** The CreER fusion protein is transcribed under the control of a tissue specific promoter (1) and translated in the cytoplasm (2). Cre is retained in the cytoplasm until ligand binding (3) at which time the activated protein translocates to the nucleus (4) where it mediates recombination at the *loxP* sites in the genome (5) (adapted from (Zhang *et al.*, 2005).

A major limitation in mouse functional genetics is that it involves the generation of complicated targeting and selection constructs. Generation of such constructs is often restricted by the availability of appropriate restriction enzyme cleavage sites in both cloning vectors and genomic DNA. In the past years, highly efficient phage-based homologous recombination systems in *Escherichia coli* have been developed (Lee *et al.*, 2001; Yu *et al.*, 2000). These systems make use of a defective λ prophage to supply functions that protect and recombine electroporated linear DNA. Most commonly, the defective λ prophage is integrated into the *E. coli* chromosome and its expression is driven by the strong P_L promoter which itself is under the tight control of the temperature-sensitive repressor *cI857*. Expression of λ-encoded recombination functions and, consequently, homologous recombination is induced by shifting the bacterial cultures from 32ºC to 42ºC. The prophage-based recombination system has the advantage of being proficient with homology sequences as short as 30-50 bp. Thus, PCR-amplified fragments can be used to generate the targeting cassette and modify,

without using restriction enzymes or DNA ligase, large segments of genomic DNA, such as those carried on phage artificial chromosomes (PACs).

Here, we have used a phage-based homologous recombination system to generate a novel Sox10iCreER^{T2} transgenic line which allowed us to selectively label *Sox10*-expressing cells at particular developmental stages.

4.2. Results

4.2.1.Generation of the Sox10iCreER^{T2} transgenic line

We generated transgenic mouse lines in which a tamoxifen-inducible Cre recombinase is expressed under the control of the *Sox10* regulatory sequences. To this end, a 170 Kb PAC spanning the *Sox10* locus plus, 62 Kb upstream and 57 Kb downstream of the *Sox10* gene, was isolated from the RCPI 21 PAC library (from UK HGMP Resource Centre; (Matsuoka *et al.*, 2005); **Fig.4.2 A**). The Sox10 PAC was modified using a phage-based homologous recombination system in *E. coli*, as previously described (Lee *et al.*, 2001; Yu *et al.*, 2000). First, the Sox10 PAC was electroporated into the recombinogenic bacterial strain EL250, a modified DH10B strain containing the defective λ prophage and an arabinose-inducible *flpe* gene (Lee *et al.*, 2001). Transformed bacterial colonies were selected based on their resistance to kanamycin conferred by a kanamycin-resistance (Kan^{R}) gene in the pPAC4 vector backbone (see Chapter II).

To modify the Sox10 PAC, a targeting vector was built as follows (**Fig.4.2 B**). A cassette containing the coding sequence of *iCreER^{T2}* (a second generation inducible Cre that is 4- to 10-fold more sensitive to 4-OHT than CreER^{T}; (Indra *et al.*, 1999) followed

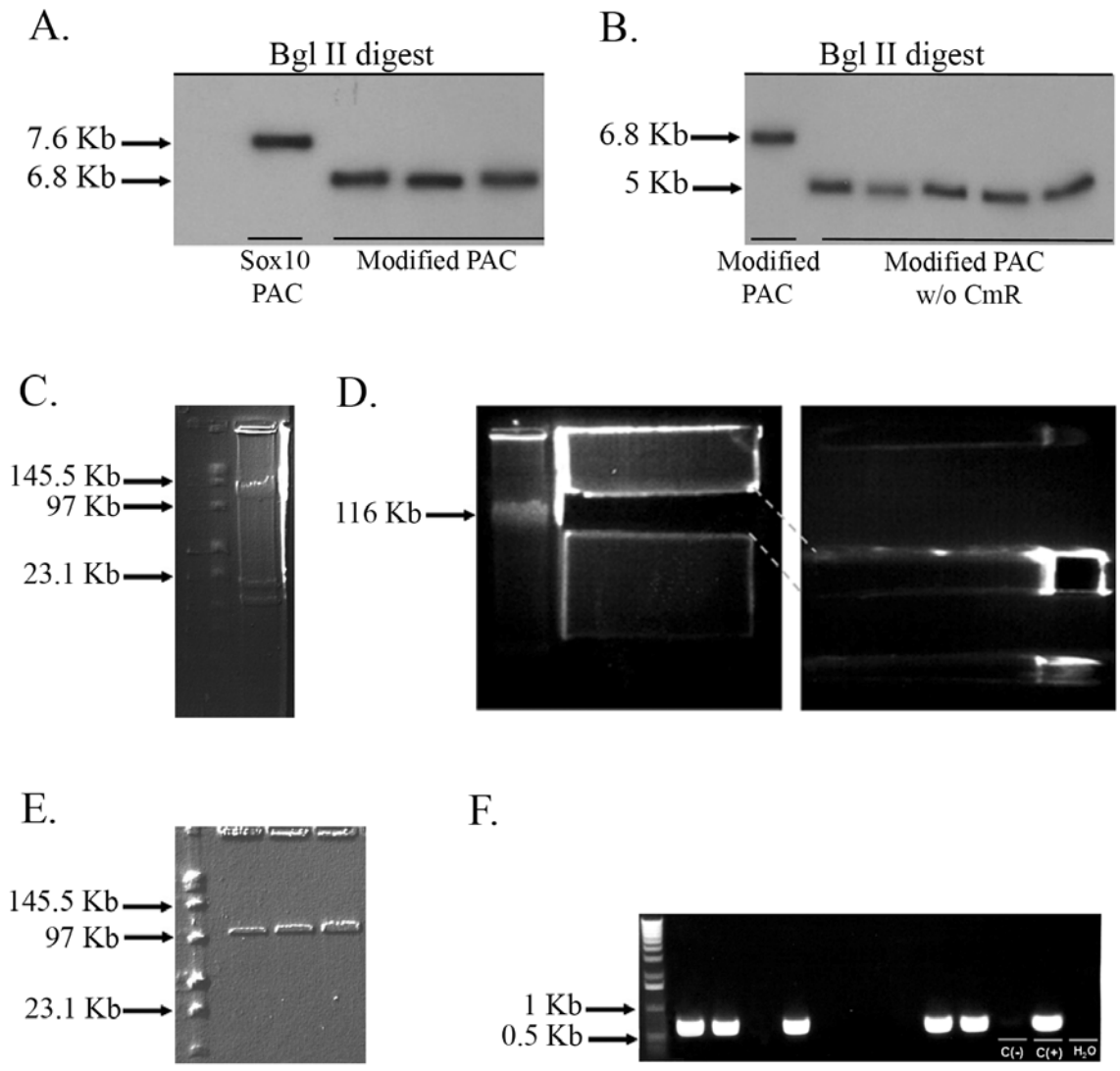
by the chloramphenicol-resistance gene (Cm^R) was isolated from the vector backbone by SalI-SpeI digestion. The selection marker Cm^R, used to identify the correctly targeted clones, was flanked by *FRT* sites which allow its removal, prior to pronuclear microinjection of the PAC DNA, by *flpe*-mediated recombination (see below and Chapter II). To introduce the iCreER^{T2}-*frt*-Cm^R-*frt* targeting cassette at the correct location, it was flanked with DNA fragments homologous to the 5' and 3' regions of the targeting site. More specifically, we used the Sox10 PAC as a template to amplify by PCR, an AscI-SalI (460bp) and a SpeI-PacI (440 bp) fragments corresponding to the 5' and 3' homology regions, respectively. The targeting vector was then generated by subcloning the AscI-SalI 5' homology, the SalI-SpeI targeting cassette and the SpeI-PacI 3' homology into the PacI-AscI cloning sites of the vector pBlueScript PacAsc (pBlueScript with a modified MCS to introduce PacI and AscI, kindly provided by N. Kessaris; UCL, UK) in a four-step ligation (see Chapter II) .

Targeting of the PAC clone was carried out by shifting EL250 cells carrying the PAC from 32ºC to 42ºC to induce expression of the phage-encoded recombination functions. EL250 cells were then electroporated with the targeting cassette. By homologous recombination, the targeting cassette was inserted between exon 3 (coding for the translation initiation site ATG) and exon 5 of the *Sox10* locus in the PAC, thereby replacing the coding sequence of *Sox10* with that of the *iCreER^{T2}* (**Fig.4.2 C**). To test whether the PAC DNA was correctly modified by homologous recombination, Southern blot analysis was performed. PAC DNA isolated from chloramphenicol-resistant bacterial colonies was digested with BglII and the 3' homology of *Sox10* gene was used as a probe. A difference of 800 bp between modified (6807 bp) and

unmodified (7622 bp) Sox10 PAC was seen as expected for all the colonies tested (**Fig.4.3 A**).

Next, the Cm^R selectable marker was removed to prevent its possible interference with *iCreER*^{T2} expression. Besides homologous recombination functions (the λ prophage genes), the EL250 strain carries also site-specific recombination functions, namely an arabinose-inducible *flpe* gene which encodes for the genetically enhanced recombinase Flp. In the presence of arabinose, the *flpe* gene is expressed leading to the removal of the *frt*-flanked selection marker from the targeted locus by Flp-mediated recombination. Removal of the selection marker was confirmed by Southern blot analysis of the modified PAC DNA digested with BglII using the 3' homology fragment as a probe. A difference of 1.8 Kb between modified PAC with and without the Cm^R selectable marker confirmed correct targeting in all colonies tested (**Fig.4.2 C**, **Fig.4.3 B**).

Finally, the modified PAC DNA was linearized by restriction digest with NotI endonuclease generating 116 Kb, 16.7 Kb and 13.8 Kb bands (**Fig.4.3 C**). The 116 Kb band corresponding to the modified *Sox10* locus was isolated, concentrated in a 0.5-0.8 cm agarose gel block (**Fig.4.3 D**), purified and checked by PFGE before injection into the pronuclei of fertilized mouse eggs (**Fig.4.3 E**; see ChapterII). 190 pups were born from injected eggs and genotyped for the presence of the transgene. Sixteen founder transgenic mice (8 males; 8 females) carrying the *Sox10iCreER*^{T2} transgene were identified based on genotyping by PCR (**Fig.4.3 F**; see Chapter II). Four males showed germline transmission of the transgene and these were bred with the R26ReYFP reporter line.


A.
Bgl II digest
7.6 Kb
6.8 Kb
Sox10 PAC
Modified PAC
B.
Bgl II digest
6.8 Kb
5 Kb
Modified PAC
Modified PAC w/o CmR
C.
145.5 Kb
97 Kb
23.1 Kb
D.
116 Kb
E.
145.5 Kb
97 Kb
23.1 Kb
F.
1 Kb
0.5 Kb
C(-)
C(+)
H2O

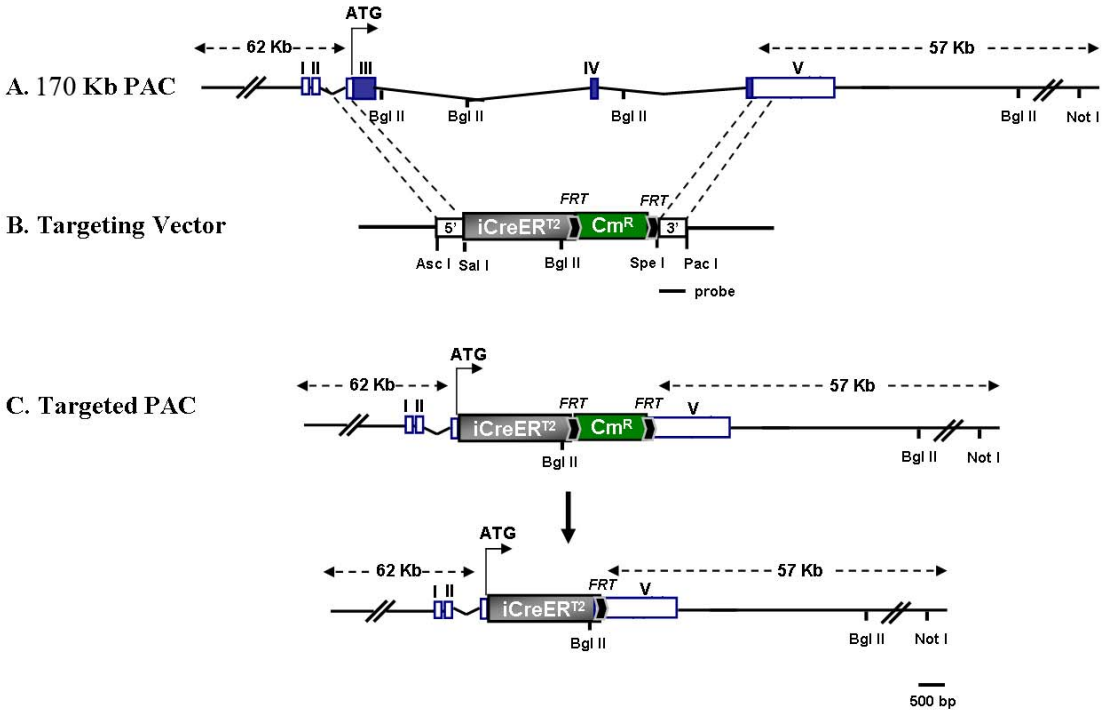


Figure 4.2. ***Strategy used to generate Sox10iCreER^{T2} PAC transgenic mice.***

(A) Schematic representation of the 170 Kb PAC indicating the intron-exon structure of the *Sox10* locus (exons - white/blue rectangles; introns – black lines), initiator ATG, extent of the genomic regions upstream and downstream of the *Sox10* locus in the PAC insert and relative locations of BglII and NotI restriction sites. **(B)** A gene cassette comprising 400-500 bp 5' and 3' homology regions (white boxes), *CreER^{T2}* coding sequence (grey box) and chloramphenicol resistance (Cm^r) expression cassette (green box) flanked by *FRT* sites (black arrows) was used as the targeting vector for homologous recombination. Relative locations of AscI, BglII, SalI, SpeI and PacI restriction sites and the location of the 3' UTR probe used to hybridize to Southern blots of BglII-digested DNA are depicted. **(C)** Schematic representation of the PAC vector after removal of the *Sox10* locus (top) and the Cm^R cassette (bottom) by homologous recombination.

Figure 4.3. ***Generation of the Sox10iCreER^{T2} PAC transgenic mice.***

(A) *Targeting of the Sox10 locus by homologous recombination.* Southern blot analysis of BglII-digested PAC DNA from modified Sox10 PAC using as a probe the 3' homology of *Sox10* gene (3 mini-preps plus the negative control -Sox10 PAC DNA- are shown). The expected fragment sizes are indicated on the left. **(B)** *Removal of the Cm^R cassette by homologous recombination.* Southern analysis of BglII-digested PAC DNA from modified Sox10 PAC with and without the selection marker *Cm^R*, using the 3' homology of *Sox10* gene as a probe (5 mini-preps plus the negative control -targeted Sox10 PAC- are shown). The expected fragment sizes are indicated on the left. **(C-E)** *Isolation of PAC DNA prior to microinjection into the pronuclei of fertilized eggs.* Targeted PAC DNA was purified away from the vector backbone by restriction digest with NotI (**C**), concentrated in a 0.5-0.8 cm agarose gel block (**D**) and quantified by PFGE (**E**; see Chapter II). Shown on the left are the molecular weights in kilobases. **(F)** Identification of founders by PCR genotyping using Cre primers (Table 2.1) and tail DNA. Representative samples depicting the presence of a single 550 bp fragment corresponding to a positive genotype. A 550 bp fragment was amplified from the modified PAC, used as positive control [C(+)]. Lanes corresponding to the negative control DNA [C(-)] and water were blank. The molecular weights in kilobases are shown on the left.

4.2.2. *Sox10iCreER^{T2}* transgene is appropriately expressed within the *Sox10* expression domain

To establish that expression of the *Sox10iCreER^{T2}* transgene faithfully recapitulates expression of endogenous *Sox10*, we performed *in situ* hybridization using Sox10 and Cre riboprobes on adjacent coronal sections of E12.5 double transgenic embryos treated with 0.2 mg/g of 4-OHT at E9.5 (**Fig.4.4 A** and **C**). In parallel, embryo serial sections were also immunostained for GFP to confirm that the transgene is capable of inducing recombination on the reporter *R26ReYFP*. In all four independent transgenic lines, expression of the transgene *Sox10iCreER^{T2}* and GFP fluorescence were detected exclusively in *Sox10*-expressing neural crest derivatives, such as the dorsal root ganglia (DRG), the sympathetic chain ganglia and the ganglia of the oesophagus, stomach and intestine (**Fig.4.4 B, D**). Three transgenic lines (*SER26*, *SER66*, and *SER87*) exhibited consistent and comparable patterns of transgene expression and GFP fluorescence (**Fig.4.4 B**). In the remaining mouse line (*SER93*) the transgene and, consequently, GFP were expressed only in a subset of *Sox10*-expressing cells (**Fig.4.4 D**). Transgenic lines *SER26* and *SER93* were selected for subsequent analysis.

Appropriate expression of the *Sox10iCreER^{T2}* transgene was further confirmed by analysing the GFP fluorescence pattern in whole embryos and dissected guts of E12.5 SER26; R26ReYFP double transgenics treated with 4-OHT at E9.5 (**Fig.4.4 E ii-viii** and **F ii, iv**). Appropriate expression of GFP was found in the cranial ganglia (**Fig.4.4 E ii, vi**), dorsal root ganglia (**Fig.4.4 E ii, iii**) and sympathetic chain (**Fig.4.4 E ii, iii**). We also observed expression of GFP very distinctly in the nerve fibers leaving the spinal cord area (**Fig.4.4 E ii, iv**), in the radial and ulnar nerves of the limbs (**Fig.4.4 D v**) and

in the otic vesicle (**Fig.4.4 E ii).** Coronal sections of E12.5 double transgenics confirmed GFP expression in cranial ganglia and in the heart (**Fig.4.4 E vii-viii**). Finally, GFP expression was clearly visible in eNCCs in gut whole mount samples of E12.5 embryos (**Fig.4.4 F ii, iv**). This expression pattern is consistent with *in situ* hybridization analysis of Sox10 mRNA in mouse (**Fig.3.1 B**; (Kuhlbrodt *et al.*, 1998) as well as with GFP expression pattern in the *Sox10Cre, R26ReYFP* trangenics (**Fig.3.1 C**; see Chapter III) and X-gal staining pattern in Sox10βGeoBAC transgenic line (Deal *et al.*, 2006).

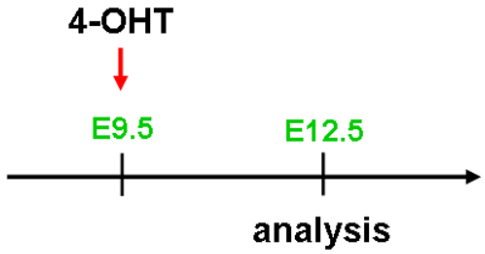
4.2.3.Sox10iCreER^{T2} –mediated recombination is inducible

To examine whether the *Sox10iCreER^{T2}* transgene is capable of inducing a temporally controlled recombination of the reporter *R26ReYFP*, we compared the pattern of GFP immunoreactivity in E12.5 Sox10iCreER^{T2}; R26ReYFP transgenic embryos collected from females treated with 4-OHT 9.5 days post-coitum or from control (uninjected or corn oil-injected) females. GFP was detected only in neural crest derivatives in embryos from treated pregnant females and was essentially absent from their untreated counterparts (n=8; **Fig.4.4 E i, ii and F i, ii** -SER26; R26ReYFP-**; Fig.4.4 F iii, iv** -SER93; R26ReYFP-). More specifically, we observed that upon administration of 4-OHT at E9.5, 88±8.6% of the Sox10^{+} cells of SER26; R26ReYFP double transgenic embryos (1177 cells total from three mice) expressed the YFP reporter, as assessed by immunostaining for GFP, and Sox10. In contrast, only 3±1.1% (1219 cells total from three mice) of the Sox10^{+} cells were GFP^{+} in control embryos (**Fig.4.4 F vi**). In the SER93; R26ReYFP transgenic line, upon administration of 4-OHT at E9.5, 8±0.6% of all Sox10^{+} cells (953 cells total from three mice) were GFP^{+} whereas no Sox10^{+} cells were GFP^{+} in the absence of 4-OHT (1460 cells total from five mice) (**Fig.4.4 F v**).

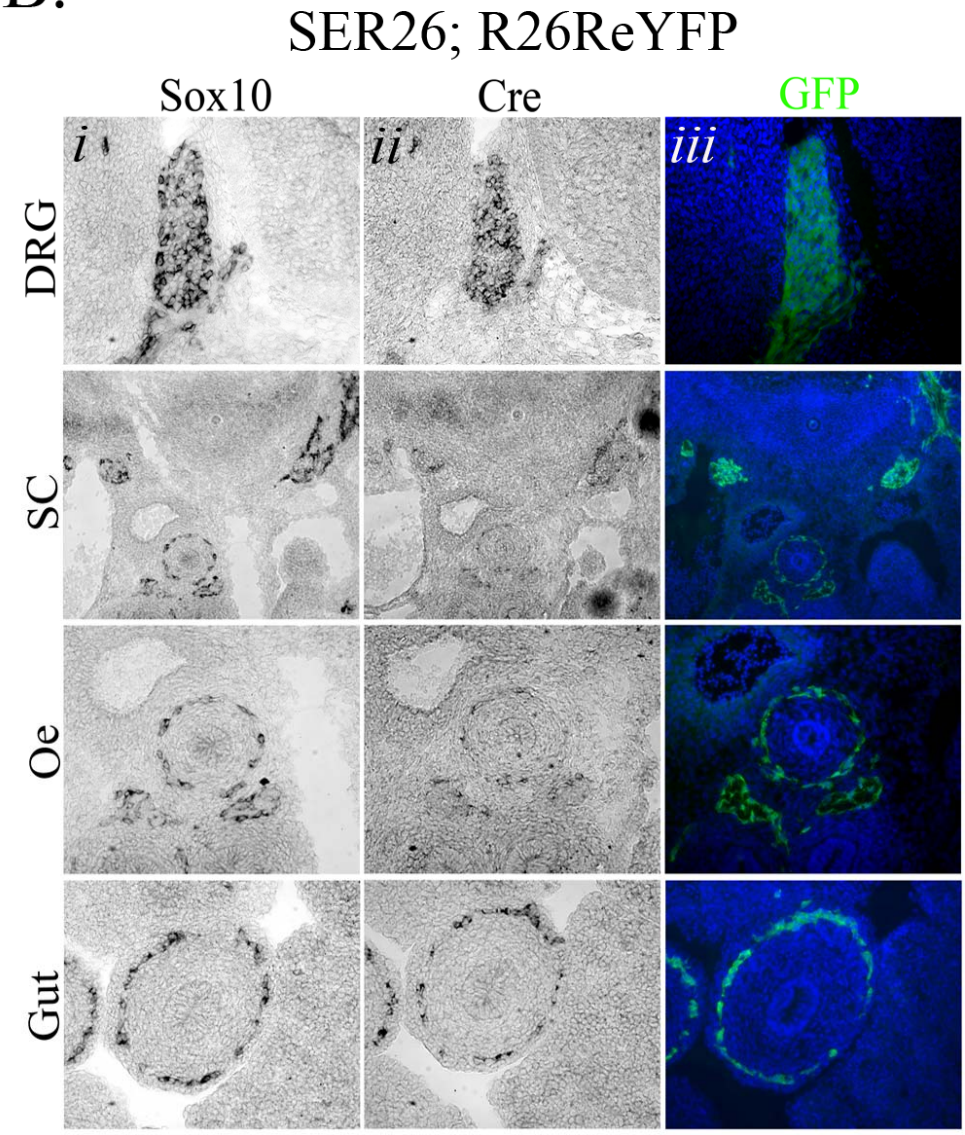
Figure 4.4. ***Expression of YFP in R26ReYFP embryos carrying the transgene Sox10iCreER^{T2} recapitulates expression of endogenous Sox10 and is inducible.***

(A and **C)** Pregnant females were injected with 4-OHT (0.2 mg/g) 9.5 days post-coitum as indicated by the red arrow. Sox10iCreER^{T2}; R26ReYFP embryos were harvested and analysed at E12.5. **(B, D)** Adjacent coronal sections through SER26; R26ReYFP **(B)** and SER93; R26ReYFP **(C)** double transgenic embryos were used for *in situ* hybridization using Sox10 (**i**) and Cre (**ii**) riboprobes and for GFP immunostaining (**iii**). Images show specific expression of the transgene *iCreER^{T2}* and the reporter within the *Sox10* expression domain, namely, in the dorsal root ganglia (DRG), sympathetic chain (SC), oesophagus (Oe) and loops of the gut (Gut). Arrows indicate *CreER^{T2}*-expressing cells (B-ii) and GFP^{+} cells (B-iii). All sections were 12µm. **(E-i, ii)** GFP whole-mount immunostaining of SER26; R26ReYFP double transgenic embryos harvested from an uninjected female (control) (**i**) or a female exposed to 4-OHT (**ii**). Arrows in (**ii**) point to nerve fibers leaving the spinal cord area. **(iii-v)** Magnification of boxed area in (ii) showing DRGs and sympathetic ganglia (**iii**), magnification of the nerves fibers sprouting from the spinal cord area (**iv**) and the radial and ulnar nerves of the limb (**v**). **(vi-viii)** GFP immunostaining of coronal sections of SER26; R26ReYFP transgenic embryo showing expression of YFP in the cranial ganglia **(vi** and **vii)** and heart **(viii)**. **(F)** GFP immunostaning of guts from SER26; R26ReYFP **(i, ii)** and SER93; R26ReYFP **(iii, iv)** double transgenic embryos showing minimal or no induction of YFP in the absence of 4-OHT **(i, iii),** whereas, in the presence **(ii, iv)** of 4-OHT iCreER^{T2} efficiently mediates recombination in enteric cells. Inset: Higher magnification (x400) of ENS cells at the neural crest front of migration. Quantification of the number of GFP-labelled cells in the Sox10^{+} population in the gut of SER26; R26ReYFP and SER93; R26ReYFP embryos is shown in the graphs **vi** and **v**, respectively. Graphs error bars, s.e.m; Cg, cranial ganglia; SC, sympathetic chain; DRG, dorsal root ganglia; Oe, oesophagus; St, stomach; Mg, midgut; Ce, caecum; Hg, hindgut. i and ii; x16; iii and iv, x40; v, x32; vi-viii, x100; (F) i-iv, x50.

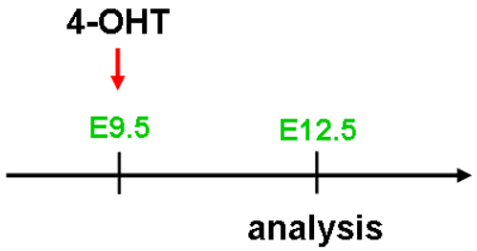
A.


4-OHT
E9.5
E12.5
analysis

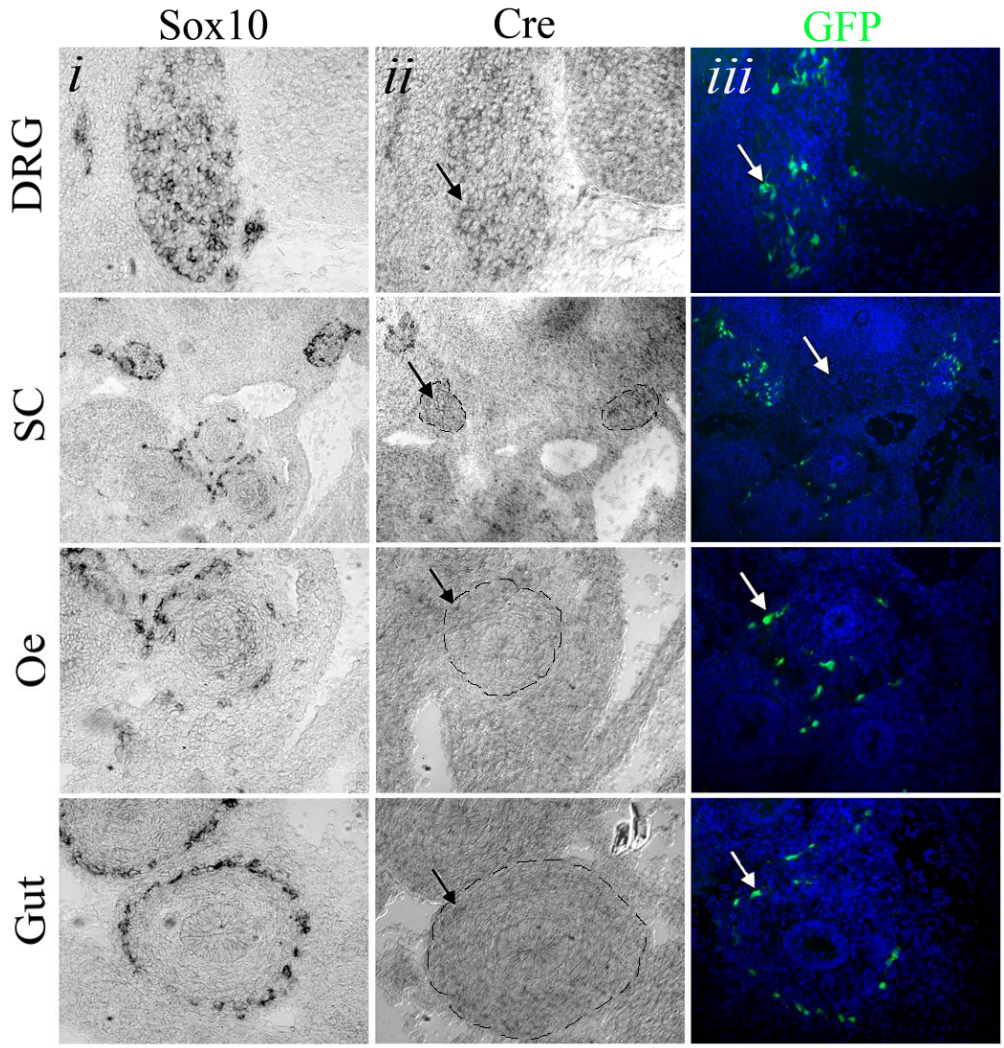
B.

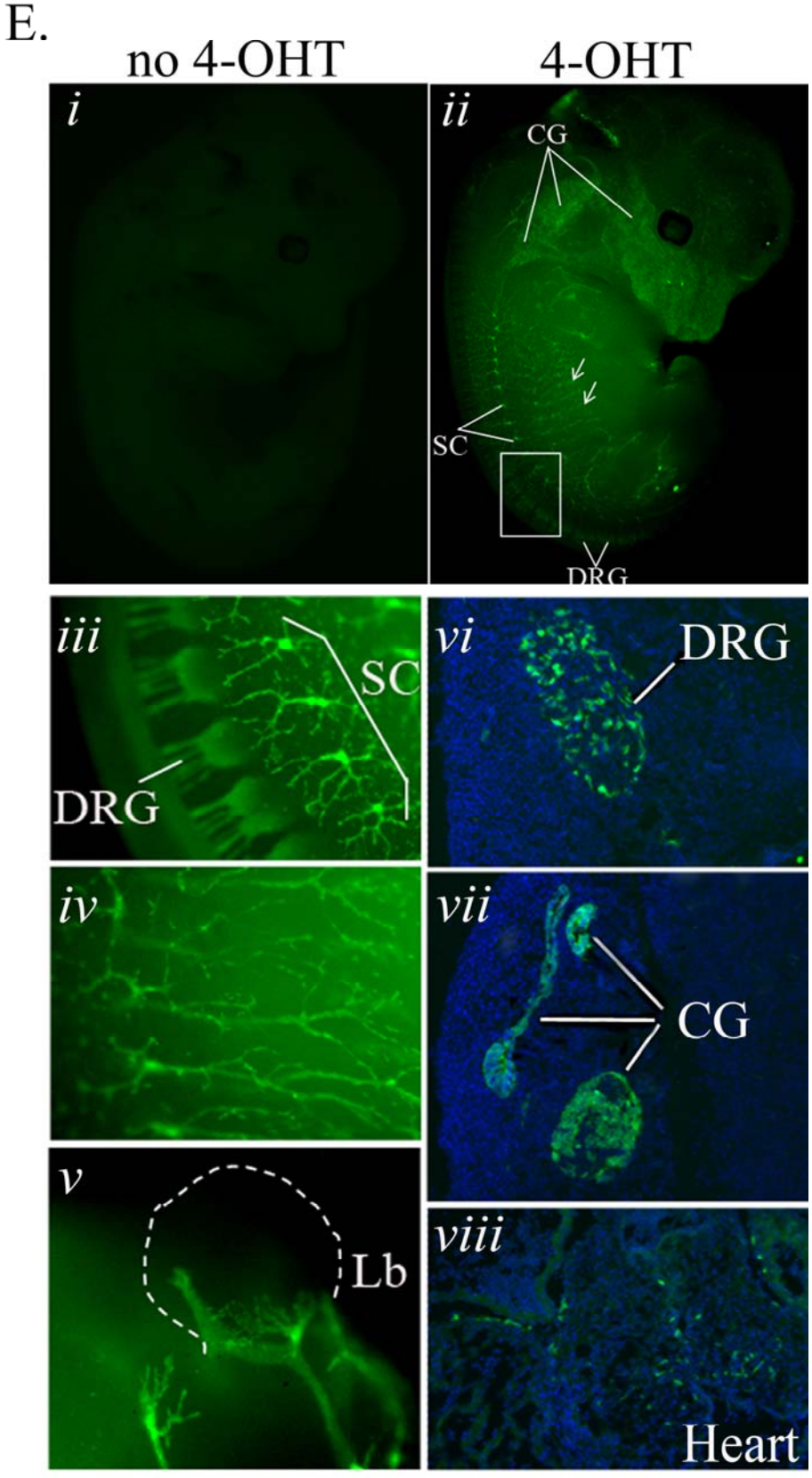

SER26; R26ReYFP
Sox10
Cre
GFP
i
ii
iii
DRG
SC
Oe
Gut

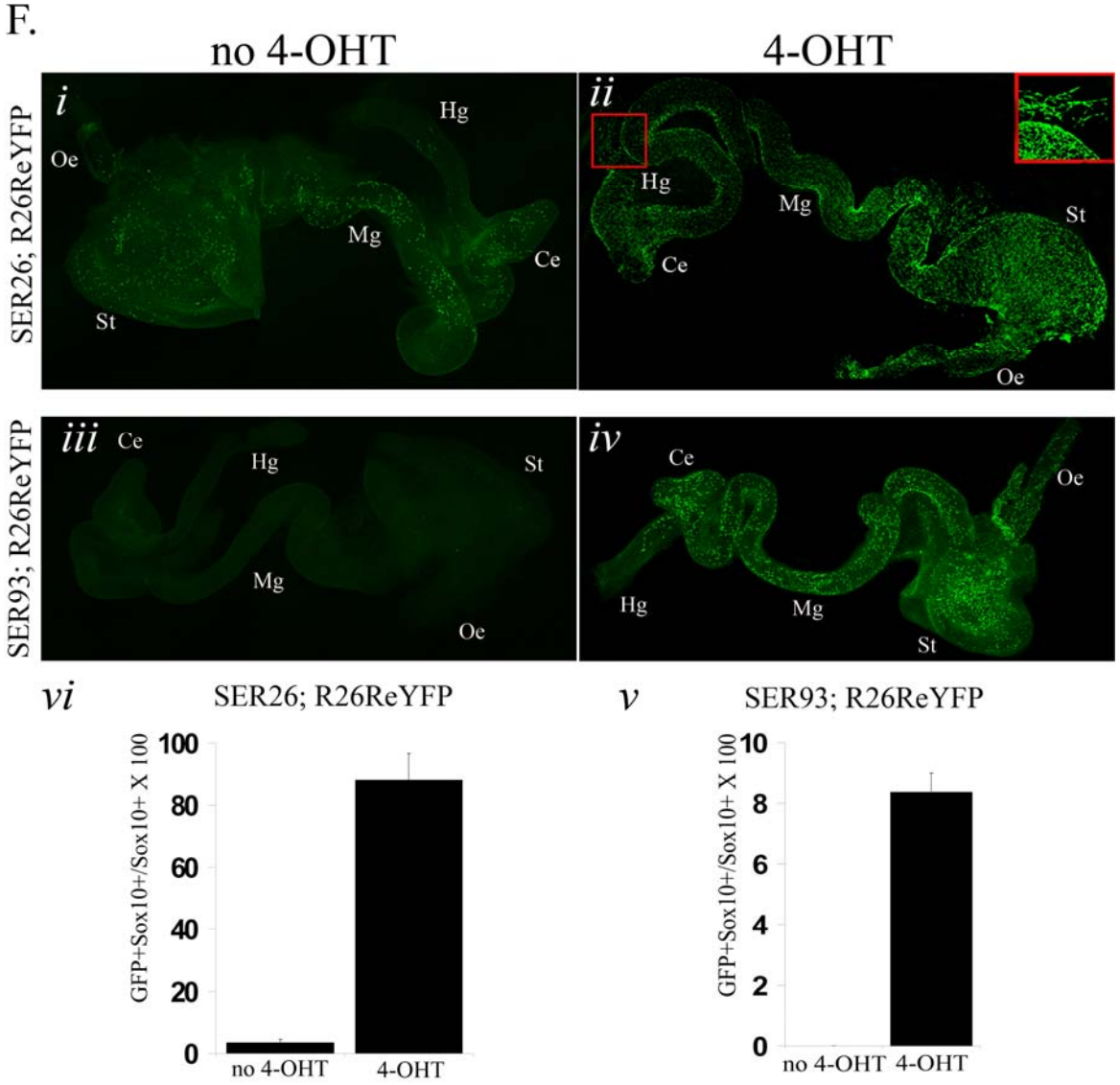
C.


4-OHT
E9.5
E12.5
analysis

D.


SER93; R26ReYFP
Sox10
Cre
GFP
i
ii
iii
DRG
SC
Oe
Gut


E.
no 4-OHT
4-OHT
i
ii
CG
SC
DRG
iii
SC
DRG
vi
DRG
iv
vii
CG
v
Lb
viii
Heart

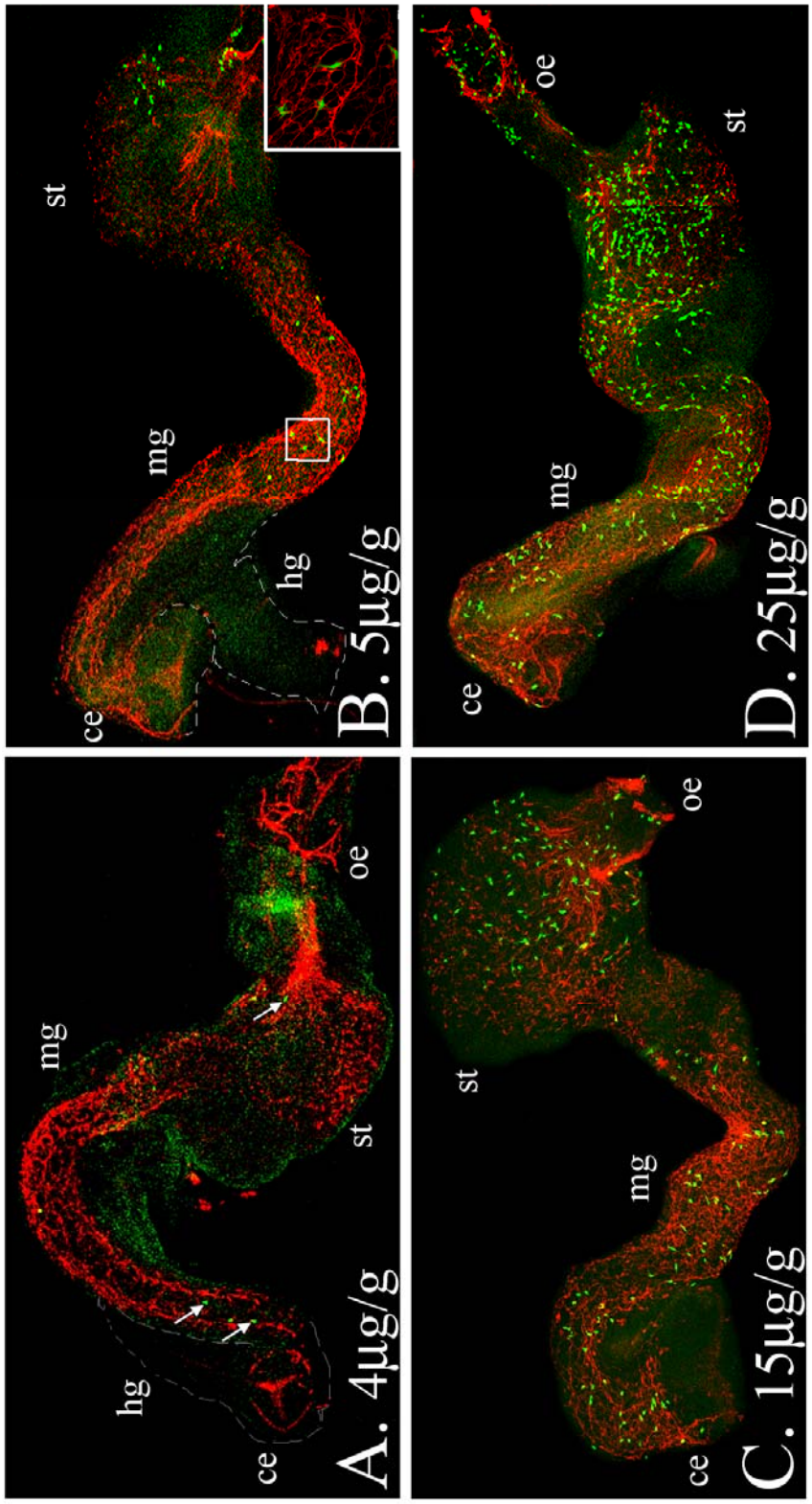

F.
no 4-OHT
4-OHT
SER26; R26ReYFP
SER93; R26ReYFP
i
ii
iii
iv
v
vi
Hg
Oe
Mg
Ce
St
SER26; R26ReYFP
SER93; R26ReYFP
GFP+Sox10+/Sox10+ X 100
100
80
60
40
20
0
10
8
6
4
2
no 4-OHT
4-OHT

4.2.4. *In vivo* labelling of single *Sox10*-expressing cells

To establish a system of single cell-labelling, we examined reporter activation in the gut of E12.5 SER93; R26ReYFP transgenic embryos treated with different dosages of 4-OHT at E9.5. We consistently found absence of GFP^+ cells in the gut of double transgenics upon treatment with 2 μg/g of 4-OHT, indicating that this dose is below the threshold for activation of the $Sox10iCreER^{T2}$ recombinase (n=14; data not shown). In contrast, we found scattered isolated GFP^+ cells along the gut of double transgenic embryos treated with 4 μg/g of 4-OHT (n=6; **Fig.4.5 A**). Progressively higher dosages of 4-OHT led to a reproducible increased frequency of GFP^+ gut cells, thus suggesting a dose-dependent response of the extent of recombination in eNCCs (**Fig.4.5 B-D;** 5 μg/g, n=8; 15 μg/g, n=5; 25 μg/g, n=4).

Figure 4.5. ***Single-cell labelling of Sox10-expressing cells.***

(A-D) Immunostaining of dissected guts from E12.5 SER93; R26ReYFP double transgenic embryos for GFP (green) and the pan-neuronal marker Tuj1 (red) showing GFP fluorescence in small clusters of eNCCs. Embryos were treated with 4 µg/g **(A)**, 5 µg/g **(B)**, 15 µg/g **(C)** or 25 µg/g **(D)** of 4-OHT. x50 magnification. Inset in B: Higher magnification (x400) of single GFP-labelled eNCCs. Representative embryos are shown. oe, oesophagus; st, stomach; mg, midgut; ce, caecum; hg, hindgut.


A. 4µg/g
B. 5µg/g
C. 15µg/g
D. 25µg/g
ce
hg
mg
st
oe

4.3. Discussion

Our current understanding of the changes eNCSCs undergo over time and the mechanisms that regulate their orderly differentiation into distinct lineages is based mainly on *in vitro* observations (Bondurand *et al.*, 2003; Kruger *et al.*, 2002). The study of the properties of eNCSCs *in vivo* relies on the ability to positively identify and follow them. One way to study eNCSCs as they exist *in vivo* and follow these cells during ENS development, homeostasis and regeneration is to use the Cre-loxP lineage-labelling system to indelibly mark the descendants of specific cell types. However, because in this system Cre is constitutively expressed, cells are marked from the time when the promoter driving Cre expression is first turned on in development. Marked progeny accumulate as development proceeds and new cells activate Cre. Thus, it is not possible to follow the progeny of cells that express the gene of interest at defined developmental stages. The inducible CreER-loxP overcomes such limitations by allowing temporal control of recombination.

Aiming at exploring the temporal changes in the developmental potential of ENS progenitor cells *in vivo* during pre- and post-natal stages and having established that *Sox10* is expressed by ENS progenitor cells, we developed mouse lines transgenic for the tamoxifen-inducible Sox10iCreER^{T2} cassette. To this end, we used PAC transgenics. PACs are vectors derived from the P1 bacteriophage that can carry large fragments of genomic DNA (100-300 Kb in size). Given their large size, PACs have a high probability of harbouring most, if not all, regulatory elements of the gene of interest. This is particularly important in light of recent studies demonstrating that the regulation of *Sox10* is not strictly controlled by proximal promoter elements associated with the

start site of transcription but by cis-elements situated at a great distance (Antonellis *et al.*, 2006; Deal *et al.*, 2006). For instance, Antonellis et al. (2006) showed that a 16 Kb region localised 47 Kb upstream of the *Sox10* gene is required for *Sox10* expression in early neural-crest progenitors. Taking those observations into account, we chose a large PAC clone (170 Kb) spanning the *Sox10* locus plus 62 Kb upstream and 57 Kb downstream of the *Sox10* gene. The Sox10 PAC was modified employing established PAC modification techniques thereby inserting the coding sequence of *iCreER*^{T2} in frame with the initiating ATG of the *Sox10* locus in the PAC (Lee *et al.*, 2001). By rigorously comparing expression of *Sox10iCreER*^{T2} transgene with the expression pattern of endogenous *Sox10*, we found appropriate expression of the transgene in all four independent transgenic lines generated. We never observed expression of the transgene in regions other than cell types and tissues originated from *Sox10*-expressing cells, thus ruling out any potential ectopic expression of the transgene due to position effects. This is consistent with accumulating evidence from analysis of PAC transgenics, which suggest that the extensive flanking regions incorporated in such constructs make PAC transgenics less susceptible to position effects and more likely to confer position-independent transgene expression (Deal *et al.*, 2006; Wells & Carter, 2001).

Using a Rosa26-based reporter system we demonstrated that besides being appropriately expressed, the *Sox10iCreER*^{T2} transgene was able to mediate recombination leading to YFP expression exclusively in *Sox10*-expressing neural crest derivatives. More specifically, we observed expression of YFP in the intrinsic ganglia of various embryonic organs, including the lungs, the heart and intestinal tract. This is in accordance with previous studies reporting expression of Sox10 in the peripheral nerves

in the lung (Burns & Delalande, 2005), autonomic nerves of embryonic chick heart (Montero *et al.*, 2002) and gut (Paratore *et al.*, 2002; Young *et al.*, 2003). Outside the peripheral nervous system, we observed expression of YFP in regions where Sox10 expression has been documented, such as the nerves of the limbs, brain, spinal cord and otic vesicle (Britsch *et al.*, 2001; Kuhlbrodt *et al.*, 1998). Taken together, our results suggest that the extensive flanking regions carried in the Sox10 PAC clone include the necessary regulatory elements to direct faithful and tissue-specific expression of the *Sox10iCreER*^{T2} transgene.

To induce Cre-mediated recombination in double transgenic embryos 0.2 mg/g of 4-OHT were administered to pregnant females. Based on macroscopic and histological evaluation, exposure to the dose of 4-OHT used did not reveal any developmental defects nor did it affect embryonic viability or delivery of the pups. This is in contrast to other studies reporting that the dose of ligand required to activate Cre recombinase is close to that which interferes with the maintenance of pregnancy (Danielian *et al.*, 1998). Furthermore, we demonstrated that administration of 0.2 mg/g reliably induced recombination in R26ReYFP embryos carrying the *iCreER*^{T2} transgene. In the SER26; R26ReYFP transgenic line, we observed a very low percentage (3%) of Sox10^{+} cells within the gut that underwent recombination in the absence of 4-OHT thus, indicating slight "leakiness" of the *iCreER*^{T2} transgene. In contrast, when 4-OHT was provided at E9.5, the time point when NCSCs invade the gut, almost all (88%) Sox10^{+}cells in the gut of SER26; R26ReYFP transgenic line were labelled which demonstrates the feasibility of this system for fate-labelling ENS progenitors.

Unlike SER26; R26ReYFP transgenics, the SER93; R26ReYFP transgenic line did not exhibit Cre-mediated recombination in the absence of 4-OHT. Interestingly, we observed that administration of 0.2 mg/g of 4-OHT to pregnant females induced low-level recombination as only 8% of the $Sox10^{+}$ cells in the gut of SER93; R26ReYFP were GFP-labelled. Furthermore, our results showed that the extent of recombination in eNCCs of SER93; R26ReYFP embryos was dose-dependent. By lowering the dose of 4-OHT administered, we were able to control the efficiency of recombination in target cells in such a way that single cells or small populations of cells were labelled. The ability to target small populations of cells at a specific time point can be a considerable advantage. In a similar approach to that used in genetic analysis of *Drosophila* development, this transgenic line can potentially be used to study gene function by generating genetically mosaic animals in which a particular mutation (gene inactivation or overexpression) is restricted to a small population of cells, at a desirable time, in an otherwise wild-type background (see Chapter VII). Moreover, this system can be exploited to study cell morphology and visualize wiring patterns in the ENS; to isolate progenitor cells and their progeny for gene expression analysis to investigate the mechanisms of cell fate, specification and differentiation and to perform lineage analysis at the single-cell level. For instance, our data demonstrated that *Sox10*-expressing cells are multipotent at the population level (Chapter III), however, the potential of single $Sox10^{+}$ cells to generate both neurons and glia and to differentiate into various neuronal subtypes is still unknown. By lowering the dose and changing the time of 4-OHT administered to SER93; R26ReYFP transgenics it is possible to label $Sox10^{+}$ cells present in the gut at different developmental stages and then trace their fates clonally *in*

vivo. Further refinement to this analysis can be added by combining the SER93; R26ReYFP transgenic line with a Brainbow-like reporter system (Livet *et al.*, 2007), thereby labelling genetically with different colors, cell clusters derived from different single $Sox10^{+}$ cells. Identification of the cell types present in such clusters will provide valuable insight into the clonal relationship between neurons and glia, as well as between different enteric neuron subtypes.

In summary, our results demonstrated a 4-OHT-dependent and cell type-specific activation of Cre recombinase in *Sox10iCreER^{T2}; R26ReYFP* mice and established this as a valuable genetic system in which *Sox10*-expressing progenitor cells can be lineally marked as a population (SER26; R26ReYFP) or as single cells (SER93; R26ReYFP) at specific pre- and post-natal stages *in vivo*.

Chapter V

Temporal regulation of the neurogenic potential of *Sox10*-expressing cells in the ENS

5.1. Introduction

The mature ENS contains a vast number of neurons that differ in neurotransmitter expression, morphology, electrophysiology and function (Furness, 2000; Gershon *et al.*, 1993). Generation of correct numbers and types of neurons by eNCSCs is crucial for the development of a functional ENS. In accordance, motility defects have been reported in mutant mice in which myenteric neuron density is increased (Taketomi *et al.*, 2005) or decreased (Roberts *et al.*, 2008).

Previous studies have suggested that enteric neurogenesis extends through a considerable period of time. Based on the expression of neuron-specific markers, differentiating neurons are found amongst the first wave of eNCCs that invade the foregut at E9.5 (Baetge & Gershon, 1989; Young *et al.*, 1999) and, by E10.5-E11.5, they comprise 10-15% of the eNCCs within the bowel (Young *et al.*, 1999). As development proceeds, there is an increase in the absolute number of differentiating neurons, which continues until well after birth (Ali & McLelland, 1979; Gabella, 1971; Liu *et al.*, 2009). Furthermore, differentiation of neuronal precursors into different neuron subtypes (identified by the combined expression of specific neurotransmitters and the enzymes responsible for their synthesis) occurs at different stages of development and some of them are only found in the gut at postnatal stages (Branchek & Gershon, 1989; Rothman & Gershon, 1982; Rothman *et al.*, 1984; Matini *et al.*, 1997; Vannucchi & Faussone-Pellegrini, 1996). Analysis of the time window during which neuronal precursors differentiate into different neuron subtypes showed that myenteric neurons are born from E8 to P5 whereas submucosal neurons are born from E8 to P14 (Chalazonitis *et al.*, 2008; Pham *et al.*, 1991). Therefore, eNCSCs are thought to maintain their

neurogenic potential during embryogenesis and early postnatal life. However, little is known about the developmental changes these cells undergo during maturation of the ENS in terms of neurogenic potential, and how this affects the rate and extent of neurogenesis in the ENS.

Reports over the past years showed that neural stem cells change their intrinsic properties over time in a way that influences the number and type of neurons they can generate (Qian *et al.*, 2000; White *et al.*, 2001). Analysis of the behaviour of cell populations isolated from the gut, in culture, suggested that this might be the case also for eNCSCs (Kruger *et al.*, 2002). $p75^{+}$ cells isolated from postnatal gut were shown to exhibit a reduced sensitivity to neurogenic factors in comparison to their embryonic counterparts. Such changes in the differentiation potential of these cells with age were further observed upon transplantation of $p75^{+}$ cells into developing chick nerves. Whether, *in vivo,* eNCSCs undergo significant changes in their neurogenic potential with time, as suggested by *in vitro* studies, remains unknown.

Here, we have examined the temporal changes in the neurogenic potential of *Sox10*-expressing cells *in vivo* using the *Sox10iCreER^{T2}; R26ReYFP* transgene combination to label temporally distinct populations of eNCSCs.

5.2. Results

5.2.1. Generation of neurons from *Sox10*-expressing progenitor cells decreases progressively with time

To address the temporal regulation of the neurogenic potential of *Sox10*-expressing progenitor cells, 0.2 mg/g of 4-OHT was administered to SER26; R26ReYFP animals at

different developmental stages that broadly correspond to specific stages of enteric neurogenesis. Previous studies have shown that activation of Cre and recombination of target *loxP* sequences occurs within 48 hours after 4-OHT administration (Hayashi & McMahon, 2002; Robinson *et al.*, 1991). Therefore, $Sox10^+$ cells present within double transgenic animals at or shortly after the time of 4-OHT administration will be marked by the permanent induction of YFP. The identity of their progeny can be established by double immunolabelling of adult gut MS-MP for GFP and appropriate lineage markers. For our present studies, 4-OHT was administered at E7.5, E8.5, E12.5, P0, P7, P30 and P84. The population of enteric neurons generated from *Sox10*-expressing ENS progenitors present in the gut at a given developmental stage was determined as the proportion of HuC/D^+ cells that co-expressed GFP. Two or more segments of MS-MP from the small intestine of P84 (for the E7.5-P30 stages) or P140 (for the P84 administration) animals were examined (**Fig.5.1 A)**.

For all the time points considered, the difference in the proportion of GFP-labelled cells in the HuC/D^+ population between different regions of the small intestine was consistently less than 2.5% (**Fig.5.1 J**) and was statistically not significant ($p>0.05$). In the absence of 4-OHT, 0.6±0.8% of HuC/D^+ cells (1589 cells total from three mice) were co-labelled for GFP, indicating low background levels of Cre activity (**Fig.5.1 B, K**). When 4-OHT was administered to double transgenics at E7.5, 23.5±1.5% of the HuC/D^+ neurons co-expressed GFP (1280 cells total from three animals), indicating that almost a quarter of the enteric neurons in P84 mice are generated from *Sox10*-expressing cells after E7.5 (**Fig.5.1 C, K**). From E8.5 onwards the neurogenic potential of *Sox10*-expressing cells decreased progressively. Thus, 39.5±10.6% (3013 cells total from five

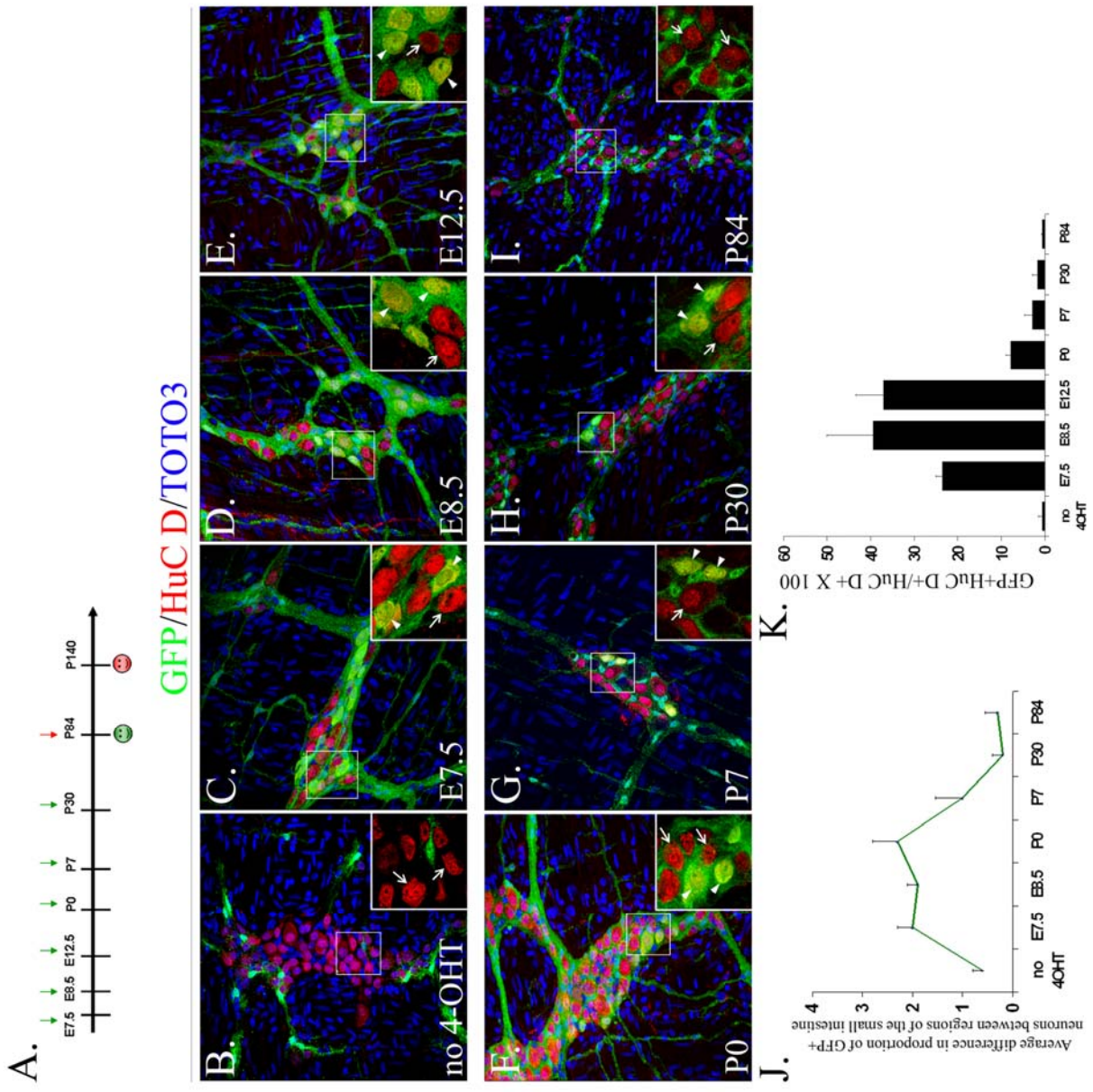
animals) of the neurons in P84 animals were generated after E8.5 (**Fig.5.1 D, K**), 36.9±6.4% (1606 cells total from four animals) after E12.5 (**Fig.5.1 E, K**) while only 7.9±1% (2748 cells total from four animals) at around P0 (**Fig.5.1 F, K**). An even smaller fraction of enteric neurons was generated from $Sox10^+$ progenitors that persist at postnatal stages: 2.9±1.7% (2471 cells total from three animals) were generated from progenitors after P7 (**Fig.5.1 G, K**) while 1.7±1.1% (3579 cells total from four animals) from progenitors present in the gut at P30 (**Fig.5.1 H, K**). Finally, animals treated with 4-OHT at P84 (0.6±0.2%; 3457 cells total from four animals) showed no GFP-labelled enteric neurons above background levels at P140 (**Fig.5.1 I, K**), indicating that by this stage the number of *Sox10*-expressing cells that differentiate into enteric neurons is negligible.

5.2.2.All $Sox10^+$ cells in the ENS of P84 and older animals are glial cells

The MS-MP strips of adult double transgenic mice treated with 4-OHT from P84 onwards was triple immunolabelled for GFP, the neuronal-specific marker HuC/D and the glial marker S100β. No GFP^+HuC/D^+ cells were found above background levels. Moreover, 100% of the GFP^+ cells co-expressed the glial marker S100β (3081 cells total from four animals) (**Fig.5.2**). This data indicates that all the GFP^+ cells in the myenteric plexus of P84 or older double transgenic mice represent glial cells.

Figure 5.1. ***The neurogenic potential of Sox10-expressing ENS progenitor cells decreases progressively over time.***

(A) SER26; R26ReYFP transgenic animals were treated with 4-OHT (0.2 mg/g) at different developmental stages (arrows) and their myenteric plexus was analysed (☺) at P84 (animals treated up to P30) or P140 (for animals treated at P30). (**B-I**) Confocal microscope images of representative whole mount preparations of the MS-MP strips from P84 or P140 transgenic mice not treated (**B**) or treated with 4-OHT at E7.5 (**C)**, E8.5 **(D)**, E12.5 **(E)**, P0 **(F)**, P7 **(G)**, P30 (**H)** or P84 (**I**). Whole mount preparations of the MS-MP strips were immunostained for GFP (green) and HuC/D (red) and nuclei were labelled with TOTO3. x400 magnification. Insets show higher magnifications (x1080) of the boxed areas. Arrowheads point to GFP^+/HuC D^+ double labelled cells which correspond to neurons generated after 4-OHT administration. Arrows indicate GFP^-/HuC D^+ neurons. (**J**) Quantification of the difference in the proportion of GFP^+/HuC D^+ cells in the HuC/D^+ population between different regions of the small intestine. (**K**) Quantification of GFP^+ cells in the HuC/D^+ population is shown in the graph. The error bars indicate s.e.m.


A.
E7.5
E8.5
E12.5
P0
P7
P30
P84
P140
GFP/HuC D/TOTO3
B.
no 4-OHT
C.
E7.5
D.
E8.5
E.
E12.5
F.
P0
G.
P7
H.
P30
I.
P84
J.
Average difference in proportion of GFP+ neurons between regions of the small intestine
no 4OHT
E7.5
E8.5
P0
P7
P30
P84
K.
GFP+HuC D+/HuC D+ X 100
no 4OHT
E7.5
E8.5
E12.5
P0
P7
P30
P84

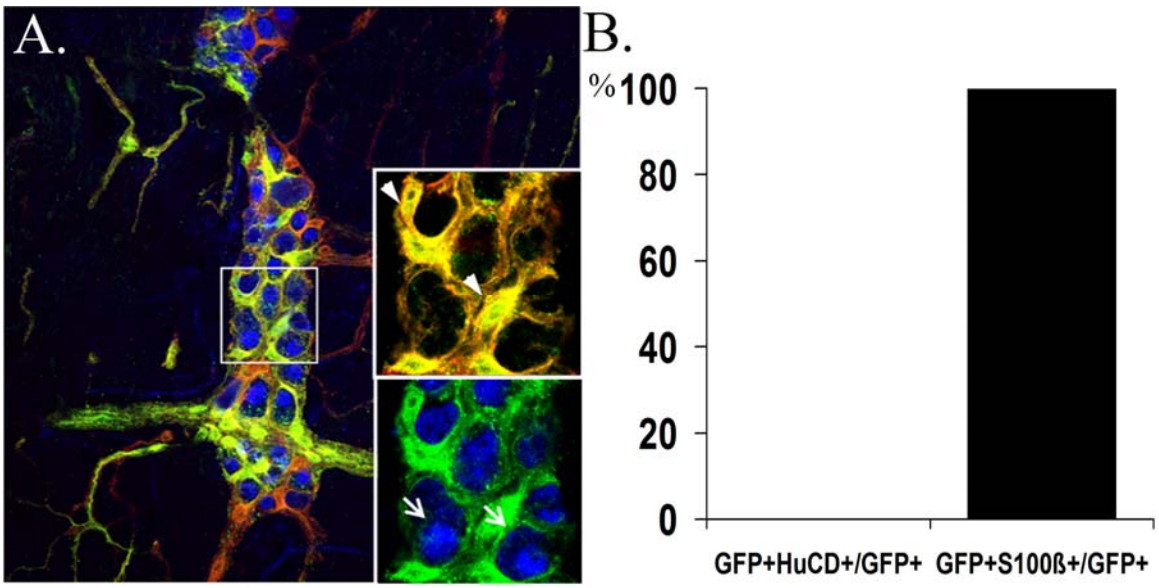


Figure 5.2. ***All Sox10^{+} cells in the ENS of P84 or older animals are glial cells.***

(**A**) MS-MP whole mount preparations from P140 SER26; R26ReYFP mice exposed to 4-OHT at P84 and immunostained for GFP (green), HuC/D (blue) and S100β (red); x400 magnification. Inset shows higher magnification (x1080) of the boxed area: arrowheads point to GFP^{+}/HuC D^{-}/S100β^{+}cells while arrows indicate GFP^{-}/HuC D^{+} cells. (**B**) Graph depicts quantification of the percentage of HuC/D^{+} and S100β^{+} cells in the GFP^{+} cell population of P140 animals treated at P84. The error bars indicate s.e.m.

5.3. Discussion

To form the mature ENS eNCSCs must generate correct numbers of neurons and glia in a developmentally appropriate manner. The neurogenic potential of eNCSCs in response to lineage determination factors was analysed *in vitro* (Kruger *et al.*, 2002). However, the temporal regulation of neurogenesis *in vivo* during ENS development and maturation remained unknown.

Here, we employed the *Sox10iCreER^{T2}; R26ReYFP* transgene combination to examine the neurogenic potential of Sox10^{+} progenitors over the course of ENS development *in vivo*. At the outset, we determined the background levels of iCreER^{T2} activity in the neuronal population of adult SER26; R26ReYFP mice. Our results demonstrated that the *Sox10iCreER^{T2}* transgene can mediate recombination in the myenteric plexus of adult double transgenics in the absence of 4-OHT, as previously shown in double transgenic embryos (Chapter IV). Nevertheless, we found that less than 1% of the myenteric neurons were GFP-labelled, confirming that 4-OHT-independent recombination occurs at low levels in the neuronal progeny of Sox10^{+} cells.

Earlier reports showed that Sox10 expression is first detected in NCCs at E8.5 (Anderson *et al.*, 2006; Kuhlbrodt *et al.*, 1998). As 4-OHT is active in injected mice for around 48 hours (Hayashi & McMahon, 2002), by administering this compound to double transgenics at E7.5 we expected to label most of the *Sox10*-expressing ENS progenitors. However, administration of 4-OHT at E7.5 resulted in GFP-labelling of only a quarter of all neurons (23%). One possible explanation for this result is the fact that the chorio-allantoic placental connection is only established after E9 which may lead to inefficient delivery of 4-OHT prior to this stage and, consequently, low levels of

iCreER^{T2} activity to mediate recombination. Moreover, as shown previously (Chapter IV), recombination mediated by the *Sox10iCreER^{T2}* transgene following administration of 0.2 mg/g of 4-OHT is insufficient to induce all the CreER^{T2+} cells to undergo excision. Higher doses of 4-OHT, however, could not be administered without toxic side effects. Finally, another possible explanation for the incomplete labelling observed is that some NCCs may have started to express *Sox10* when 4-OHT was no longer active (from around E9 onwards).

We analysed the neurogenic potential of enteric *Sox10*-expressing cells *in vivo* by labelling temporally distinct Sox10^{+} subpopulations and following their progeny. Analysis of the proportion of GFP-labelled neurons in different regions of the small intestine of adult double transgenics revealed no differences for any of the developmental stages considered, which strongly suggests that the rate of neurogenesis is equivalent throughout the small intestine. In contrast, we found that the neurogenic potential of Sox10^{+} cells is temporally regulated. The proportion of lineally marked neurons in the myenteric plexus of adult double transgenics was highest when 4-OHT was administered at midgestation (E8.5-12.5) and decreased progressively at postnatal stages. Consistent with our results, Pham et al. (1991) reported that the rate of birth of most neuronal subtypes was higher during embryogenesis. The reason for the progressive reduction of the neurogenic potential of *Sox10*-expressing cells with increasing developmental age is unclear. It is possible that changes in the microenvironment, for example, in signal availability, account for the changes in developmental potential here reported. Alternatively, these findings can also reflect differences among progenitor cells at different ages in terms of their competence to

respond to specific environmental cues. In support of the latter is work published recently emphasizing that neural stem cells change their cell-intrinsic properties over time in a manner that influences cell fate determination. For example, radial glial cells of the cerebral cortex isolated from later embryonic stages were shown to give rise to fewer neurons before initiating gliogenesis than their earlier counterparts (Qian *et al.*, 2000). Similarly, eNCSCs from postnatal gut were shown to generate neurons less efficiently than embryonic eNCSCs upon transplantation into the same environment (the neural crest migratory pathway of chick embryos; Kruger *et al.*, 2002). The molecular basis for these cell intrinsic changes is still not clear but analysis of the behaviour of eNCSCs *in vitro* suggested that it might involve changes in the sensitivity to lineage determination factors present in the microenvironment. In comparison to embryonic eNCSCs, eNCSCs from postnatal animals were shown to exhibit decreased sensitivity to the neurogenic effects of BMP4 and increased sensitivity to the gliogenic effects of the soluble Notch ligand Delta-Fc and neuregulin (Kruger *et al.*, 2002). It is still unknown whether similar mechanisms and cues account for reduction of the neurogenic potential of eNCSCs *in vivo*. Also, it remains uncertain how this reduction in neurogenesis relates to the timing of gliogenesis.

As in other parts of the nervous system, in the ENS glial cells appear after neurons. However, formation of new neurons persists after the appearance of glial cells suggesting that neurogenesis and gliogenesis co-exist for some time. Given that Sox10 expression is maintained in glial cells throughout adulthood (Kuhlbrodt *et al.*, 1998), administration of 4-OHT from E11.5 (time point at which expression of the glial marker BFABP is first detected in the gut) onwards labelled eNCSCs as well as glial cells. For

that reason, changes in the gliogenic potential of $Sox10^{+}$ progenitors over the course of ENS development *in vivo* could not be addressed with our experimental strategy.

A significant finding of our lineage tracing analysis was the generation of new neurons in adult animals after P30. Previously, neurons were thought to be generated during embryogenesis and early postnatal life (Chalazonitis *et al.*, 2008; Pham *et al.*, 1991). However, from postnatal to adult stages, the ENS enlarges massively as the gut grows in length and diameter, thus suggesting that neurons continue to be generated. By administering 4-OHT to P30 double transgenic animals we found 2% of GFP-labelled cells in the neuronal population, indicating that new myenteric neurons are added to the mature ENS. In contrast with our results, a previous study of enteric neuronal birthdays was unable to detect neurogenesis in the myenteric plexus beyond P5 (Pham *et al.*, 1991). In this report, radiolabelled-thymidine was administered to pregnant females and to P0 to P21 mice for a 24 hour period. Such a regime would, however, be unlikely to detect neurogenesis if the cell cycle length of adult neuroblasts is long; to detect low rate neuronal birth days with a single systemic injection of radiolabelled-thymidine, a terminal mitosis would have to occur precisely during the period of time that radiolabelled-thymidine is available which would be a rare event. Similarly, serial injections of Brd-U for 7 days failed to detect generation of new neurons in 6 week old animals, further supporting the idea that adult eNCSCs undergo mitosis infrequently (Liu *et al.*, 2009). In our lineage tracing system, *Sox10*-expressing progenitor cells and all their progeny were irreversibly labelled independently of their cell cycle length allowing the identification of newly generated neurons from the time point of 4-OHT administration.

Importantly, our results demonstrated that after P84 the number of *Sox10*-expressing cells that differentiate into enteric neurons is negligible. 4-OHT administration at P84 failed to label enteric neurons above the background level, thus suggesting that subsequent to this stage, the adult ENS does not support neurogenesis under physiological conditions. Interestingly, cells isolated from the adult ENS have been shown to exhibit neurogenic potential *in vitro* (Kruger *et al.*, 2002). This may reflect reprogramming of adult ENS cells in culture in such a way that they acquire neurogenic potential. Indeed, there are several well-documented examples of cells that change their developmental potential in culture in ways that do not appear to occur under physiological circumstances (reviewed in Joseph & Morrison, 2005). Alternatively, it is possible that $Sox10^{+}$ progenitor cells in the adult ENS retain neurogenic potential but only reveal it under conducive conditions (e.g. in culture) remaining quiescent otherwise. In support of this hypothesis, Liu et al. (2009) suggested that failure to detect neurogenesis even after long-term exposure to BrdU results from the fact that generation of new neurons in the adult ENS occurs *in vivo* at a rate too low to be detected unless it is increased by a stimulus, such as activation of 5-HT receptors. If quiescent $Sox10^{+}$ progenitor cells remain in the adult ENS we would expect to find undifferentiated GFP-labelled cells in the myenteric plexus of adult animals treated with 4-OHT after P84. Instead, we found that all GFP-labelled cells in the myenteric plexus of these animals were $S100\beta^{+}$, which strongly suggests that all *Sox10*-expressing cells in the adult ENS are glia. These results raise the possibility that the pool of *Sox10*-expressing eNCSCs with neurogenic potential becomes depleted as development proceeds. In support of this

view, the self-renewal capacity of eNCSCs *in vitro* has been shown to decline with increasing age (Kruger *et al.*, 2002).

Taken together, our data demonstrated that the neurogenic potential of *Sox10*-expressing cells of the mammalian gut is temporally regulated. $Sox10^{+}$ progenitors showed strong neurogenic potential during embryogenesis which was progressively lost as development proceeded. In the adult, virtually all *Sox10*-expressing cells were identified as glia.

Chapter VI

Identification of neural stem cells in the adult ENS

6.1. Introduction

An increasing body of evidence has accumulated in recent years supporting the existence of adult stem cells in a large number of tissues, including the nervous system (Morrison & Spradling, 2008). Contrary to previous beliefs, the adult CNS is now known to contain neural stem cells that sustain ongoing neurogenesis in two discrete neurogenic niches, the SVZ of the lateral ventricles and the SGL in the hippocampus (Alvarez-Buylla & Lim, 2004). Besides SVZ- and SGL-resident neural stem cells, cells capable of long-term self-renewal and multipotency have been derived from regions throughout the adult CNS and neurogenesis has been shown to be activated outside of the CNS neurogenic niches, in response to injuries and pathological stimuli (Ming & Song, 2005). *In vivo* neurogenesis has also been reported in the adult PNS, more specifically, in the rat nodose ganglia, following capsaicin-mediated destruction of existing neurons (Czaja *et al.*, 2008) and in the neural crest-derived carotid body in response to hypoxia (Pardal *et al.*, 2007).

Another important development that has had major impact in our understanding of adult neurogenesis was the realisation that glia can perform dual functions of providing homeostatic support and participating in neurogenesis (Alvarez-Buylla *et al.*, 2001; Doetsch *et al.*, 1999). A variety of approaches demonstrated that ongoing neurogenesis in the CNS is ensured by GFAP-expressing progenitors with the characteristic morphological appearance of astrocytes (Doetsch *et al.*, 1999; Seri *et al.*, 2001). Moreover, glial cells are the source of new neurons in the injured retina (Ooto *et al.*, 2004) and in the neural crest-derived carotid body (Pardal *et al.*, 2007) of adult mammals.

With regard to the adult ENS, neural stem cells have not been identified *in vivo* and constitutive neurogenesis is not known to occur under physiological conditions. Long-term exposure to BrdU has failed to identify newly generated neurons in the intact adult mammalian ENS (Liu *et al.*, 2009) and our data using a genetic labelling system suggested that *Sox10*-expressing progenitor cells loose their neurogenic potential at some point between P30 and P84 (ChapterV, **Fig.5.2**). Nevertheless, self-renewing progenitors can be identified in cultures of post-neurogenic adult intestine (Kruger *et al.*, 2002; Suarez-Rodriguez & Belkind-Gerson, 2004). Moreover, a recent report has provided evidence for enteric neurogenesis in adult animals upon pharmacological stimulation (Liu *et al.*, 2009), strengthening the possibility that cells with neurogenic potential persist in the adult gut. This is further corroborated by studies using injury animal models. Experimental intestinal obstruction, for example, has been shown to lead to a significant increase in the number of enteric neurons in the regions proximal to the affected area (Filogamo & Cracco, 1995). Similar neurogenic changes have been reported in rodents treated with the cationic detergent BAC (Hanani *et al.*, 2003; Luck *et al.*, 1993; Ramalho *et al.*, 1993). BAC has been shown to efficiently destroy the myenteric plexus (Fox *et al.*, 1983; Sato *et al.*, 1978) and so it has been used to study the regenerative capacity of the ENS (Hanani *et al.*, 2003; Ramalho *et al.*, 1993). In this context, it was demonstrated that BAC treatment results in an initial reduction in the number of myenteric neurons followed by regeneration of enteric nerve fibers and re-population of the lesioned area by neurons and glia. In light of these results, it has been suggested that quiescent eNCSCs may persist in the adult ENS and be stimulated to proliferate and differentiate in response to stimuli, such as injury. The identity of adult

eNCSCs and whether they constitute a permanent undifferentiated pool or are recruited in a regular basis is still unclear. The identification of these cells and their potential role in ENS homeostasis and intestinal physiology can have major implications for the understanding of ENS development and maintenance and the treatment of its diseases.

We have demonstrated that, during gut organogenesis, *Sox10*-expressing cells give rise to neurons and glia (Chapter III, **Fig.3.2**) but, eventually, these cells loose their neurogenic capacity *in vivo* (ChapterV, **Fig.5.1**). Furthermore, our data suggested that all *Sox10*-expressing cells in the mature ENS are glia (ChapterV, **Fig.5.2**). This raises the possibility that enteric glia may be the source of ENS progenitors identified in cultures of post-neurogenic adult intestine and potentially involved in injury-induced neurogenesis. The experiments described in this chapter address such a possibility.

6.2. Results

6.2.1. Glia-derived GFP$^+$ cells of the adult SER26; R26ReYFP show neural stem cell properties *in vitro*

To directly test whether enteric glia could be a source of neural stem cells *in vitro*, we genetically labelled enteric glia and followed their fate in culture. To this end, 4-OHT was administered to adult (>P84) SER26; R26ReYFP animals, which results in expression of GFP exclusively in enteric glia, as previously shown (Chapter V, **Fig.5.2**). MS-MP strips from 4-OHT-treated double transgenic mice were then isolated, dissociated enzymatically and cultured in a medium that supports proliferation and self-renewal of ENS progenitors (NCSC medium; Morrison *et al.*, 1999; **Fig.6.1 A**). Triple immunostaining of such cultures for GFP, the glial marker GFAP and the neuronal marker Tuj1 showed that, 24 hours after plating, all GFP$^+$ cells express GFAP (100%;

428 cells total from three mice), confirming that GFP^+ cells present within MS-MP strips represent glial cells. Expression of Tuj1 was found exclusively in GFP^- cells (**Fig.6.1 B, F**).

After 4 days *in vitro* (DIV), we observed a dramatic increase in the number of GFP^+ cells relative to the number present in the cultures shortly after plating. These GFP^+ cells were found as part of small individual colonies, morphologically similar to the colonies generated by embryonic eNSCCs (Bondurand *et al.*, 2003; Stemple & Anderson, 1992). The colonies contained between 3 to 29 GFP^+ cells (average 9.6±6.3% of GFP^+ cells per colony) and the majority of these (73.2±11.3%) co-expressed GFAP, indicating that they are glial cells. Interestingly, we found that 26.5±10.9% of the GFP^+ cells did not express glial (GFAP) or neuronal (Tuj1) markers (246 cells total from three mice; **Fig.6.1 C, F**). This raised the possibility that these $GFP^+Tuj1^-GFAP^-$ cells represent glial-derived eNCSCs. To test this hypothesis, we further characterized the expression profile of $GFP^+Tuj1^-GFAP^-$ cells in day 4 GFP^+ colonies for expression of different eNCSCs markers. eNCSCs have been shown to express the transcription factor Sox10 (Anderson *et al.*, 2006). However, Sox10 is not a specific marker of eNCSCs as it is also expressed in glial cells (Young *et al.*, 2003). Therefore, to examine whether DIV4 colonies contained non-glial $Sox10^+$ cells, triple-label immunofluorescence experiments were performed using antisera for GFP, Sox10 and GFAP. As shown in **Fig6.1 G**, we found that the majority of the GFP^+GFAP^- cells expressed Sox10. We also found that a fraction of GFP^+ cells in DIV4 colonies expressed $Phox2b^+$, another marker of eNCSCs (**Fig.6.1 H**). Phox2b expression is also maintained in a subpopulation of glial cells (Corpening *et al.*, 2008; Young *et al.*, 1999), however, analysis of Phox2b expression in

the non-glial GFP^+ population was precluded because Phox2b and GFAP antibodies were raised in the same species. Besides Sox10 and Phox2b, we examined GFP^+Tuj1^- $GFAP^-$ cells for the expression of the HMG transcription factor Sox2. Sox2 has been extensively used to label CNS neural stem cells and it has been shown to be expressed in the ENS (Heanue & Pachnis, 2007). By performing triple-labelling experiments, we found that the majority of $GFP^+Tuj1^-GFAP^-$ expressed Sox2 (**Fig.6.1 I**). These data suggest that GFP^+ glial cells are able to generate, *in vitro*, cells that exhibit an expression profile similar to embryonic ENS progenitor cells.

Next, we sought to test whether glia-derived GFP^+ cells are able to activate a neurogenic programme. For this, GFP^+ cells present in 4-day cultures were analysed for the expression of the pro-neural transcription factor Mash1. Mash1 is expressed transiently in a subset of enteric neuron precursors but it is extinguished as they differentiate into neurons (Blaugrund *et al.*, 1996). We found that a fraction of GFP^+ cells in DIV4 colonies expressed Mash1 (**Fig.6.1 J**) suggesting that, after 4 days in culture, a subset of the glia-derived GFP^+ cells had already taken a step towards neural maturation. Despite the presence of GFP^-Tuj1^+ neurons 24 hours after plating no neurons could be found in 4 day cultures, consistent with the idea that differentiated neurons isolated from the gut do not survive past the first days of culture in NCSC medium (Bondurand *et al.*, 2003).

Next, we examined the neurogenic potential of glia-derived GFP^+ cells. To this end, 7 day cultures were shifted to differentiating conditions (laminin substrate and neurobasal medium). Three days later (10 DIV) the expression profile of GFP^+ cells in culture was assessed by triple immunofluorescence experiments using antisera for GFP,

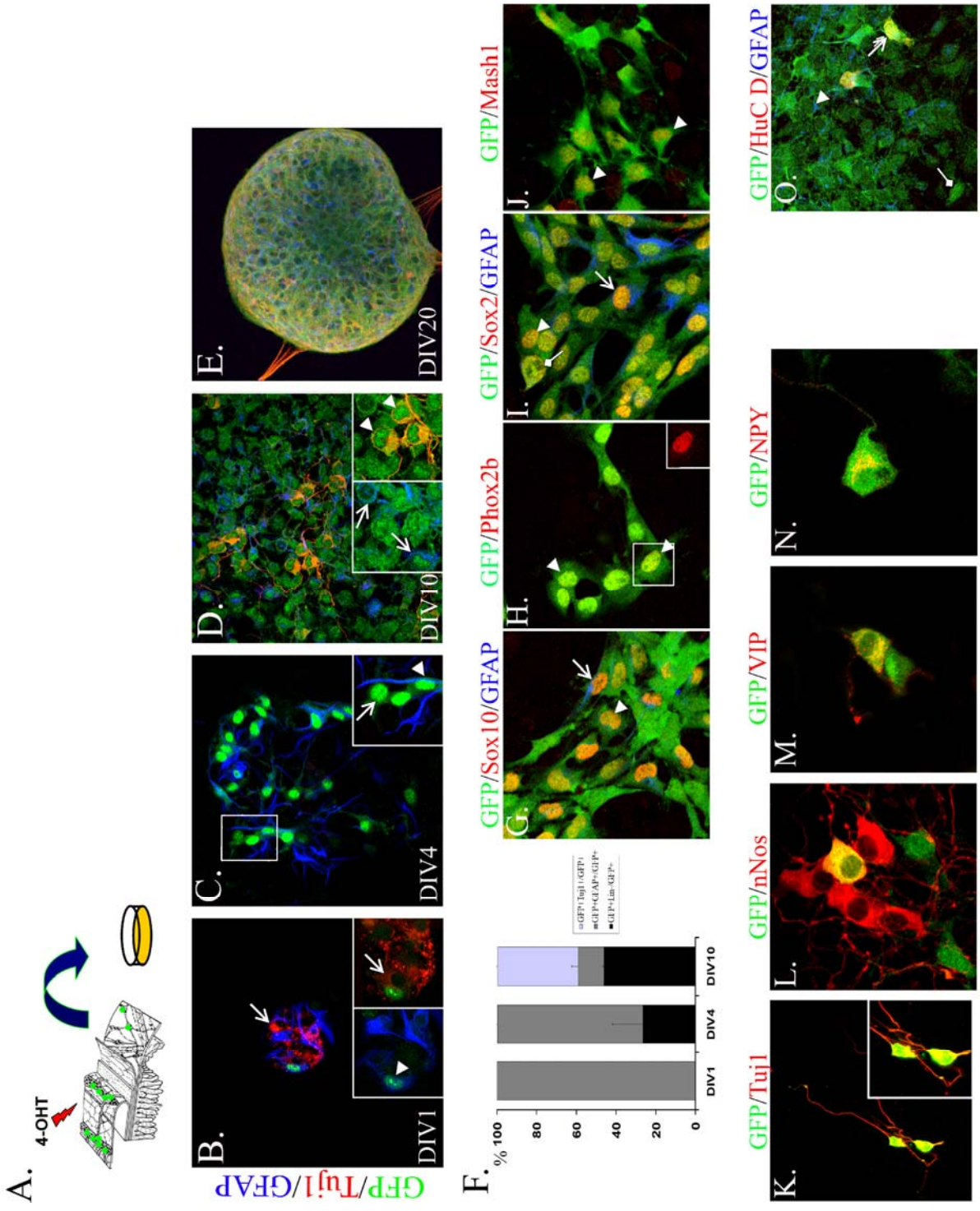
Tuj1 and GFAP. We identified 3 different subpopulations of GFP^+ cells within the colonies generated *in vitro*: 46.1±0.6% of the cells were negative for the neuronal marker Tuj1 and the glial marker GFAP, 11±3.3% co-expressed GFAP and 40.5±3.8% co-expressed Tuj1 or HuC/D (265 cells total from three mice; **Fig.6.1 D, K, F**). Subsequently, we examined the potential of GFP^+ cells to differentiate into distinct neuronal subtypes. For this, 10 day cultures were double-labelled with GFP and antibodies against Nitric Oxide Synthase (nNOS), Vasoactive Intestinal Peptide (VIP) and Neuropeptide Y (NPY). nNOS, VIP and NPY are all expressed by subsets of enteric neurons *in vivo* (Qu *et al.*, 2008). We found co-expression of GFP and all neuronal subtype markers considered, thus indicating that GFP^+ cells have the ability to generate neurons that express a variety of neurotransmitters normally found in the ENS (**Fig.6.1 L-N**).

Similar to cultures of dissociated embryonic gut (Bondurand *et al.*, 2003), after a few days in vitro GFP^+ cells started to pile up and, by day 20, most of these cells were found as part of neurosphere-like bodies (NLBs) which eventually detached and floated in the medium (**Fig. 6.1 E**). GFP^+ cells could be propagated without losing GFP expression for at least 25 passages and they were kept in culture for up to 6 months. Even in these late cultures, the GFP^+ population contained cells that were $GFP^+HuC\ D^-$ $GFAP^-$ and cells that expressed neuronal (Tuj1 and HuC/D) or glial markers (GFAP) (**Fig. 6.1 O**).

Taken together, our data suggest that, even though *Sox10*-expressing cells in the adult ENS loose their neurogenic capacity *in vivo*, *Sox10*-expressing glial cells can be activated *in vitro* to generate eNCSCs with self-renewal and neurogenic potential.

Figure 6.1. ***GFP$^+$ glial cells isolated from the ENS of adult SER26, R26ReYFP are the origin of in vitro neural stem cells.***

(A) MS-MP strips of adult (>P84) SER26, R26ReYFP transgenic animals exposed to 4-OHT at P84 were dissociated and cultured under standard conditions (Bondurand *et al.*, 2003). **(B-E)** Cell cultures were immunostained for GFP (green), the pan-neuronal marker Tuj1 (red) and the glial marker GFAP (blue) at day in vitro (DIV) 1 (**B**), DIV4 (**C**), DIV10 (**D**) and DIV20 (**E**). On DIV1, all the GFP$^+$ cells expressed GFAP (arrowhead in inset of panel B). Tuj1$^+$ neurons were all GFP$^-$ (arrow in inset of panel B). In DIV4 cultures, the GFP$^+$ population includes glial (GFP$^+$Tuj1$^-$GFAP$^+$) and non-glial non-neuronal (GFP$^+$Tuj1$^-$GFAP$^-$) cells (arrowhead and arrow in inset of panel C, respectively). After 10 DIV, GFP$^+$ population includes glia (arrows in insets of panel D), non-glial non-neuronal cells and also neurons (GFP$^+$Tuj1$^+$GFAP$^-$; arrowheads in insets of panel D). In DIV20 cultures, most GFP$^+$ cells are found in NLB's. (**F**) Quantification of the fraction of neurons and glia in the GFP$^+$ population in culture. **(G-J)** DIV4 cultures were immunostained for GFP (green), GFAP (blue) and the transcription factor Sox10 (red, **G**) or Sox2 (red, **I**). Sox10 and Sox2 were expressed by GFP$^+$GFAP$^+$ cells (arrows) as well as by GFP$^+$GFAP$^-$ cells (arrowheads). Note the presence of a GFP$^+$ dividing cell on a DIV4 colony (diamond arrow in panel I). Alternatively, DIV4 cultures were immunostained for GFP (green) and Phox2b (red, **H**) or Mash1 (red, **J**). Arrowheads in panels H and J indicate double positive cells. **(K-N)** Immunostaining of DIV10 cell cultures for GFP (green) and Tuj1 (red, **K**) or the neuronal subtype markers nNos (red, **L**), VIP (red, **M**) and NPY (red, **N**) showed that glial cells can give rise to nNos$^+$, VIP$^+$ and NPY$^+$ neurons. (**O**) Immunostaining of late cultures (6 months in culture) for GFP (green), the neuronal marker HuC/D (red) and the glial marker GFAP (blue). In late cultures the GFP$^+$ population included HuC/D$^+$ neurons (arrow), GFAP$^+$ glial cells (arrowhead) and HuC D$^-$/GFAP$^-$ cells (diamond arrow). B-D, x400; insets of B-D, x1080; E, x200; K and O, x400; L-N, x600.


A.
4-OHT
B.
DIV1
C.
DIV4
D.
DIV10
E.
DIV20
GFP/Tuj1/GFAP
F.
% 100
80
60
40
20
0
DIV1
DIV4
DIV10
GFP+Tuj1+/GFP+
GFP+GFAP+/GFP+
GFP+Lin-/GFP-
G.
GFP/Sox10/GFAP
H.
GFP/Phox2b
I.
GFP/Sox2/GFAP
J.
GFP/Mash1
K.
GFP/Tuj1
L.
GFP/nNos
M.
GFP/VIP
N.
GFP/NPY
O.
GFP/HuC D/GFAP

6.2.2.Glia-derived GFP$^+$ cells of the adult hGFAPCreER^{T2}; R26ReYFP show neural stem cell properties *in vitro*

By treating P84 SER26; R26ReYFP transgenics with 4-OHT we were able to genetically label only glial cells, however, with these experiments one cannot exclude the possibility that a small residual population of undetected Sox10$^+$GFAP$^-$ progenitors were the source of enteric neurons in culture. To provide further evidence that enteric glial cells are indeed the source of neural stem cells *in vitro*, we carried out a similar experiment using a transgenic line in which a tamoxifen-inducible form of Cre is expressed under the control of a 2.2 Kb region of the human GFAP promoter (hGFAPCreER^{T2}; Ganat *et al.*, 2006). The hGFAPCreER^{T2} transgene has been shown to be correctly expressed in the CNS (Ganat *et al.*, 2006). To confirm appropriate expression of the hGFAPCreER^{T2} transgene in the ENS, transgenic mice were bred with the R26ReYFP reporter line and double transgenics were assessed for *YFP* expression (using GFP antibodies) in response to 0.2 mg/g of 4-OHT. Analysis of MS-MP strips of P140 hGFAPCreER^{T2}; R26ReYFP animals after administration of 4-OHT at P84, revealed GFP expression within the myenteric plexus. More specifically, we found expression of GFP and the glial marker S100β in 19±7.4% (988 cells total from five animals) of the S100β$^+$ glial cells. No GFP-labeled cells expressed the neuronal marker HuC/D confirming that Cre-mediated recombination is restricted to the glial population (**Fig.6.2 A, B**).

Having established that the hGFAPCreER^{T2} transgene targets Cre recombination to enteric glial cells *in vivo*, we then tested whether GFP$^+$ glial cells isolated from MS-MP strips of 4-OHT-treated hGFAPCreER^{T2}; R26ReYFP mice could generate neural stem

cells *in vitro*. To this end, we cultured dissociated MS-MP strips isolated from P140 double transgenics treated with 4-OHT at P84. We found that, similar to SER26; R26ReYFP transgenics, GFP^+ glial cells from 4-OHT-treated hGFAPCreER^{T2}; R26ReYFP mice generated self-renewing $GFP^+Tuj1^-GFAP^-$ cells. In neuron-promoting culture conditions these cells were able to give rise to GFP^+ neurons that co-expressed pan-neuronal markers such as Tuj1 and also markers for different neuronal subtypes, namely, nNos, NPY (**Fig. 6.2 C-F**) and VIP (data not shown). These findings show that the GFP^+ glial cells isolated from the adult gut of SER26; R26ReYFP or hGFAPCreER^{T2}; R26ReYFP display properties of neural stem cells, including neurogenic potential.

6.2.3.GFP^+ glial cells generate neurons in *ex vivo* cultures of MS-MP

Next, we examined whether the neurogenic potential of enteric glia can be activated in the context of intact enteric ganglia. MS-MP strips were isolated from the small intestine of SER26; R26ReYFP transgenics treated with 4-OHT after P84 and cultured (without prior enzymatic dissociation) for 7 days in NCSC medium. This resulted in extensive proliferation of GFP^+ glial cells as assessed by Brd-U incorporation (**Fig.6.3 A, I**). Also, similar to the cultures of dissociated MS-MP strips, a large number of GFP^+HuC/D^+ cells was observed (**Fig.6.3 E, I**).

Understanding the environmental signals that trigger GFP^+ glial cells to acquire neural stem cell properties *in vitro* is instrumental for unravelling the mechanisms underlying adult neurogenesis in the ENS. For this, we tested whether exogenous growth factors are capable of stimulating glial cells to generate new neurons by replacing NCSC medium with DMEM or DMEM supplemented with EGF, FGF2 or GDNF (for experimental

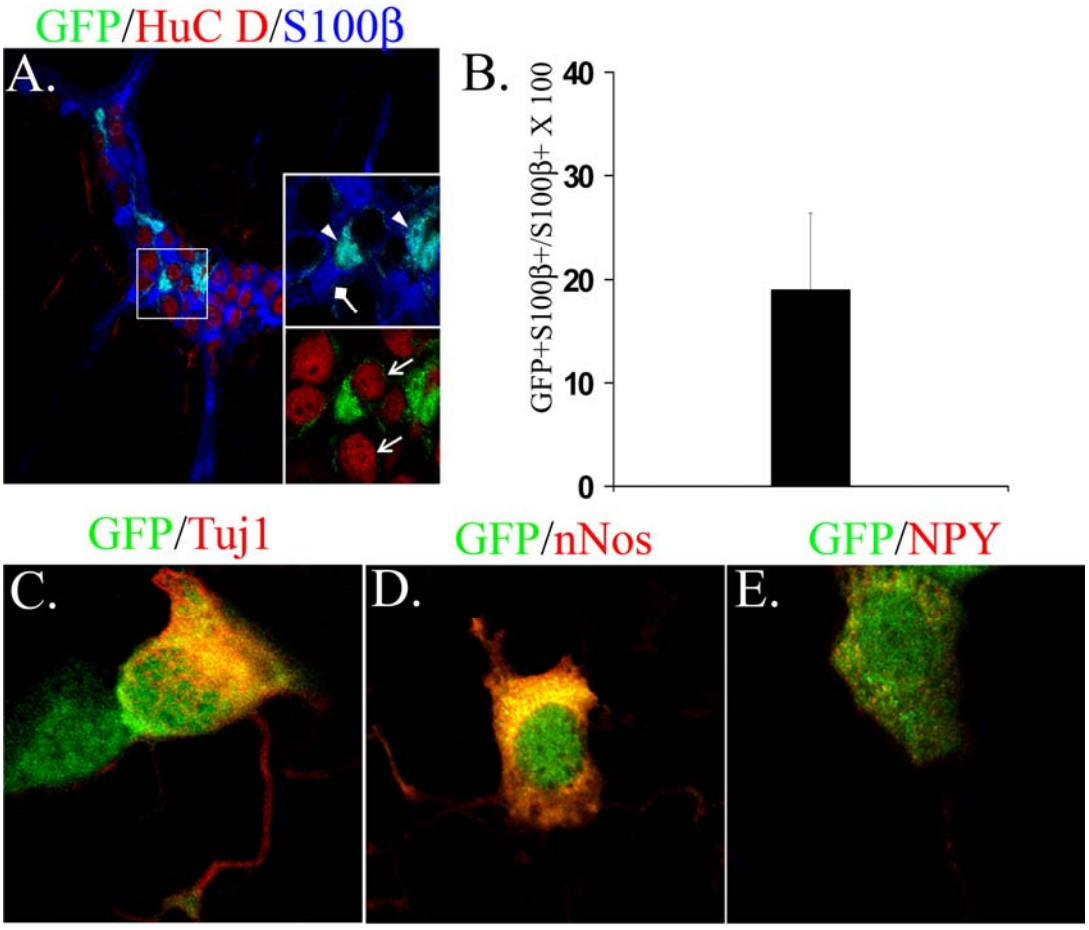


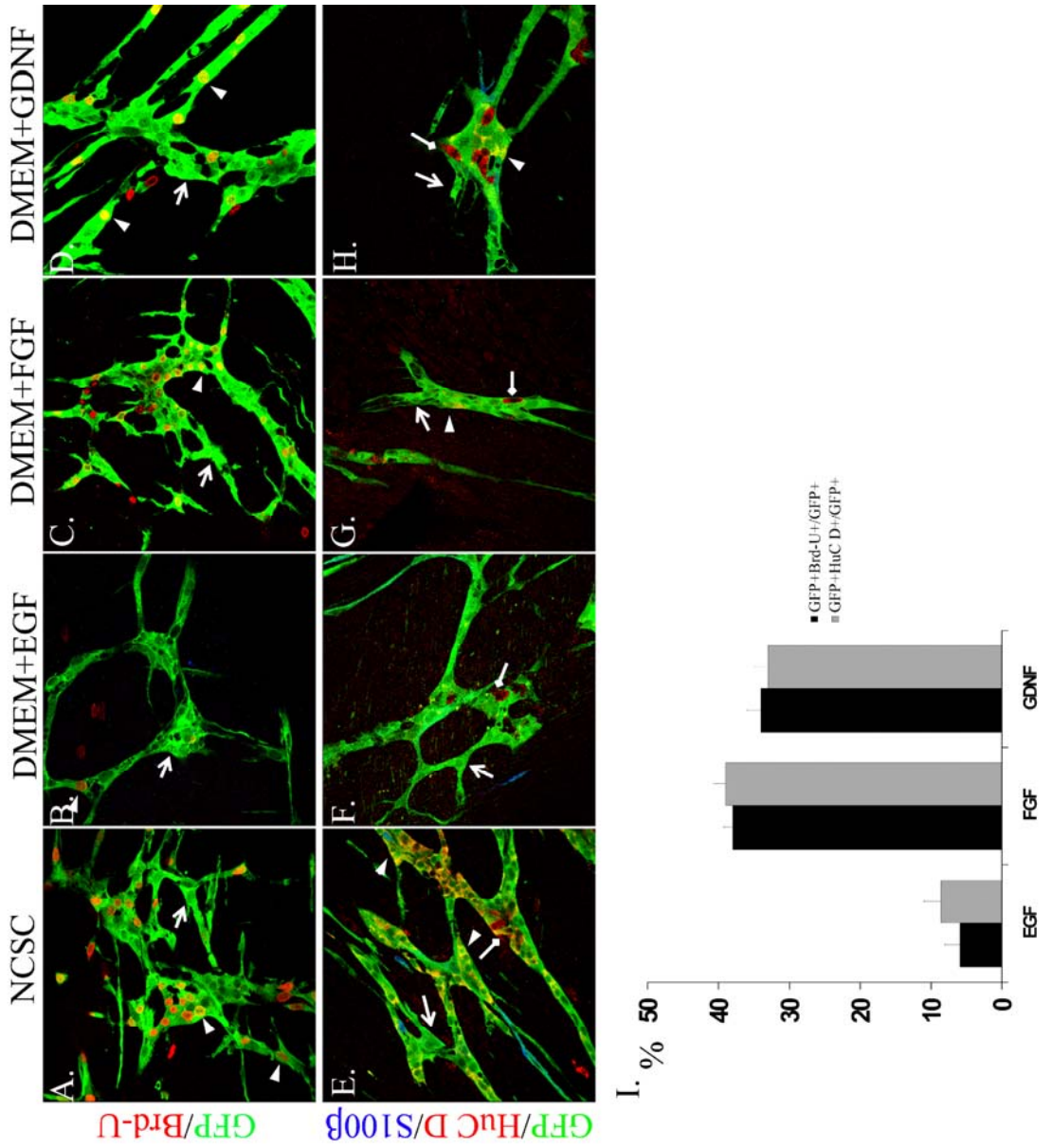
Figure 6.2. ***GFP$^+$ glial cells form hGFAPCreER^{T2}; R26ReYFP ENS show Self-Renewing, Multipotent NSC properties in vitro***

(A) hGFAPCreER^{T2}; R26ReYFP double transgenics were treated with 4-OHT (0.2mg/g) at P84 and then analysed at P140. Immunostaining of MS-MP whole mount preparations for GFP (green), HuC/D (red) and S100β (blue) showed that GFP expression is restricted to the S100β$^+$ population (arrowheads in inset of panel A). No GFP$^+$/HuCD$^+$ cells were found. Arrows and diamond arrow in insets of panel A point to GFP$^-$/HuCD$^+$/S100β$^-$ and GFP$^-$/HuCD$^-$/S100β$^+$ cells, respectively. **(B)** Quantification of the fraction of GFP$^+$ cells in the S100β$^+$ population. The error bars indicate s.e.m. **(C-F)** Cell cultures from dissociated MS-MP strips were immunostained for GFP (green), the pan-neuronal marker Tuj1 (red, **C**) or the neuronal subtype markers nNOS (red, **D**) and NPY (red, **E**). A, x400 (insets: x1080); C-E, x1080.

details see Chapter II). The mitogenic factors EGF and FGF2 are part of the NSCS medium and they have been shown to promote proliferation of embryonic eNCSCs (Fuchs *et al.*, 2009), whereas GDNF has been implicated in proliferation (Hearn *et al.*, 1998; Taraviras *et al.*, 1999) and neuronal differentiation (Chalazonitis *et al.*, 1998) during ENS development. MS-MP strips cultured in the absence of growth factors showed no GFP^+ cells or HuC/D^+ neurons suggesting that DMEM does not support the survival or differentiation of GFP^+ glial cells in culture (data not shown). In the presence of EGF a small number of GFP^+ cells could be found in the cultured myenteric ganglia. Despite its strong mitogenic effect on embryonic eNCSCs (Fuchs *et al.*, 2009), only 5.9±2.1% (543 GFP^+ cells total from 2 mice) of the GFP^+ cells present in adult MS-MP strips were found to incorporate Brd-U in the presence of EGF and only 8.6±2.4% (383 GFP^+ cells total from 2 mice) were HuC/D^+ neurons (**Fig.6.3 B, F, I**). In contrast, in MS-MP strips cultured in presence of FGF2 or GDNF most cells in the myenteric ganglia were GFP^+. Proliferation analysis showed that 38±1.2% (395 cells total from 2 mice) and 34±2% (443 cells total from 2 mice) of GFP^+ cells proliferated in FGF and GDNF supplemented medium, respectively (**Fig.6.3 C, D, I**). Also, we found that 39±1.7% (372 cells total from 2 mice) and 33±1.9% (403 cells total from 2 mice) of the GFP^+ cells co-expressed HuC/D^+ neurons in FGF and GDNF supplemented medium, respectively (**Fig.6.3 G, H, I**). These results suggest that FGF and GDNF signalling pathways might be implicated in triggering glial cells to proliferate and differentiate into enteric neurons.

Figure 6.3. ***GFP$^+$ glial cells generate neurons in ex vivo cultures of MS-MP strips***

(A-I) MS-MP strips were isolated from the small intestine of adult (>P84) SER26; R26ReYFP transgenics treated with 4-OHT after P84 and cultured without prior enzyme dissociation for 7 days in standard medium (Bondurand *et al.*, 2003) **(A, E)**, DMEM+EGF **(B, F)**, DMEM+FGF **(C, G)** or DMEM+GDNF **(D, H)** medium. (**A-D**) Cultured MS-MP strips were immunostained for GFP (green) and Brd-U (red). A fraction of the GFP$^+$ cells underwent cell division in culture (arrowheads). Arrows point to GFP$^+$ cells that did not incorporate Brd-U. (**E-H**) Alternatively, cultured peels were triple-labelled for GFP (green), HuC/D (red) and S100β (blue). Arrowheads point to GFP$^+$/HuC D$^+$ double labelled cells which correspond to newly-generated neurons. Arrows indicate GFP$^+$/Hu C D$^-$ and diamond arrows GFP$^-$/HuC D$^+$. x400 magnification. (**I**) Quantification of the fraction of Brd-U$^+$ cells and HuC/D$^+$ neurons in the GFP$^+$ population. Error bars in the graphs indicate s.e.m.


NCSC
DMEM+EGF
DMEM+FGF
DMEM+GDNF
A.
B.
C.
D
E.
F.
G.
H.
GFP/Brd-U
GFP/HuC D/S100β
I.
%
50
40
30
20
10
0
EGF
FGF
GDNF
GFP+Brd-U+/GFP+
GFP+HuC D+/GFP+

6.2.4.Neurons generated *in vitro* from GFP$^+$ glial cells express molecular markers associated with synaptogenesis

To get insight into the functional properties of glia-derived neurons, thus far identified by morphological and molecular criteria, we analysed GFP$^+$ neurons generated in culture for the expression of the synaptic marker synaptophysin. Synaptophysin is a synaptic vesicle glycoprotein present in virtually all neurons that participate in synaptic transmission and so its expression is an indicator for the formation of synapses (Vanden Berghe & Klingauf, 2007). Triple immunofluorescence experiments revealed co-expression of synaptophysin, GFP and the neuronal subtype marker nNos (**Fig.6.4**), thus suggesting that GFP$^+$ neurons generated *de novo* in culture are able to establish synaptic contacts.

6.2.5.GFP$^+$ glial cells generate neurons *in vivo* upon injury

Having established that enteric glial cells can generate new neurons in culture, we wished to address whether these cells also exhibited neurogenic potential *in vivo*. Since we could not detect neurogenesis in the ENS of adult (>P84) SER26; R26ReYFP animals (Chapter V, **Fig.5.2**), we hypothesized that the neurogenic potential of enteric glial cells may be revealed only upon disruption of tissue homeostasis. To test this premise, we used our genetic lineage marking system and explored the possibility that enteric glia can generate neurons *in vivo*, in response to injury that eliminates enteric neurons. The cationic chemical BAC has been shown to destroy the myenteric plexus in a dose-dependent manner when applied to the serosal surface of the intestine (Fox *et al.*, 1983). To generate a BAC injury model we first established a protocol for efficient

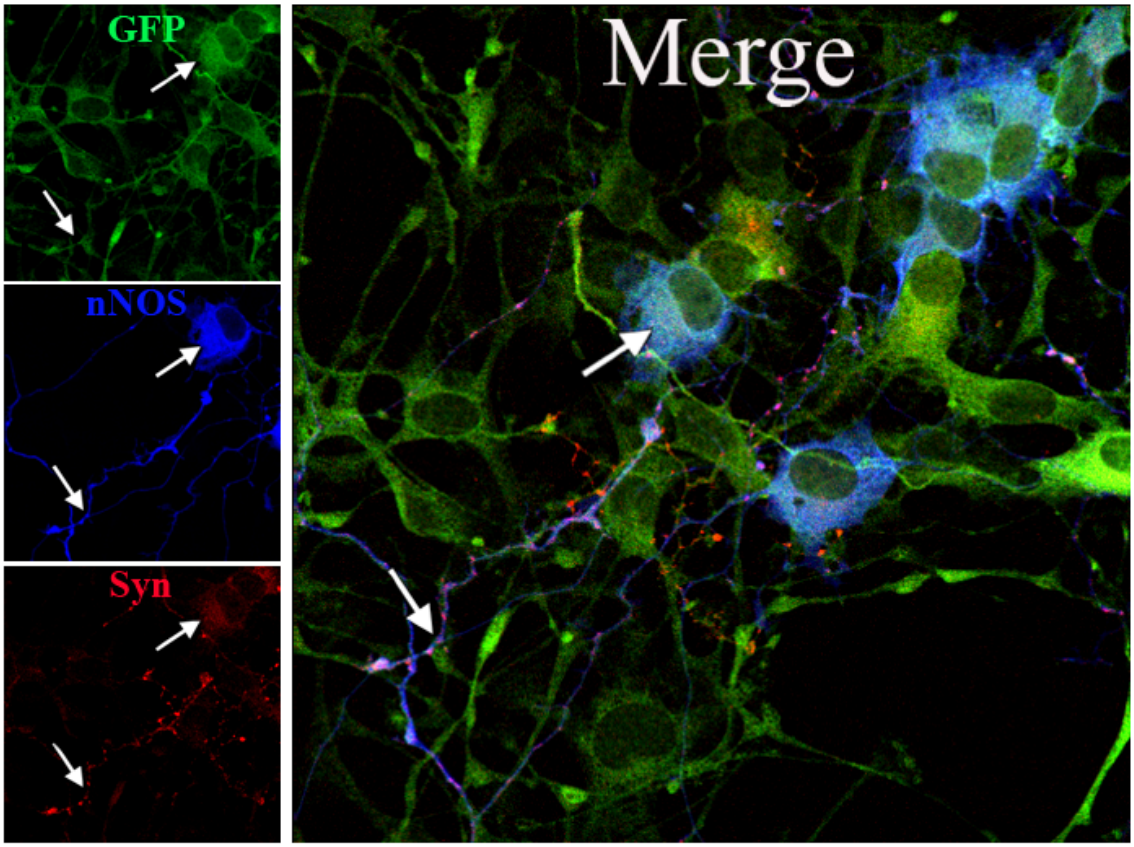


Figure 6.4. ***Neurons generated from GFP$^+$ glial cells express molecular markers associated with synaptogenesis***

Dissociated MS-MP strips of adult (>P84) SER26; R26ReYFP transgenic animals treated with 4-OHT at P84 were cultured under neuron-promoting conditions and immunostained for GFP (green), the neuronal marker nNOS (blue) and the synaptic marker synaptophysin (red). Arrows in panels point to the cell body and projection of a GFP$^+$nNOS$^+$ neuron that exhibit punctuate expression of the synaptic protein synaptophysin. x680 magnification.

ablation of myenteric neurons by applying different concentrations of BAC to a 1 cm segment of the small intestine of wild-type mice. 3 days after BAC treatment, MS-MP strips from the treated segments were immunostained for Tuj1 to highlight the myenteric plexus. This showed that application of 0.05% BAC for 5 minutes was insufficient to destroy the myenteric plexus (**Fig.6.5 A**). In contrast, application of 0.1% BAC for 20 minutes (n=4) (**Fig.6.5 B**) or 0.05% BAC for 30 minutes (n=2) (data not shown) led to effective ablation of enteric neurons. However, this was accompanied by generalized tissue damage, constriction of the treated segment and dilation of the adjacent proximal area (megacolon) and, in some instances, death. Treatment with 0.1% BAC for 5 minutes proved to efficiently destroy myenteric neurons without causing constipation or death of the animal (**Fig.6.5 C)**. Therefore, we used this regime in all following experiments.

To directly test whether GFP^+ glial cells are able to generate new neurons in response to neurodegeneration, we applied BAC to the small intestine of adult SER26; R26ReYFP and hGFAPCreER^{T2}; R26ReYFP mice (>P84) that had been previously treated with 4-OHT (to lineally mark glial cells). MS-MP strips from the treated and adjacent areas were analysed at different time points after BAC application for expression of GFP, the pan-neuronal marker HuC/D and the glial marker S100β. Consistent with previous reports (Hanani *et al.*, 2003; Ramalho *et al.*, 1993), three days after treatment, enteric ganglia were eliminated from the region of BAC application and only cellular debris could be found (n=3; **Fig.6.6 A**). Analysis of MS-MP strips isolated from the treated area 30 days after BAC treatment showed invasion of the aganglionic area by $GFP^+S100\beta^+$ glial cells, which formed network-like structures reminiscent of an

enteric plexus. No GFP^+ neurons were found in the aganglionic intestinal segment or in the area adjacent to the BAC-treated region, where most ganglia included GFP^+ glial cells but no neurons, as assessed by expression of HuC/D (n=3; **Fig.6.6 E, D**). Away from the treated area, we found morphologically normal ganglia containing GFP^- /$HuCD^+$ neurons and $GFP^+S100\beta^+$ glial cells (**Fig.6.6 C**). The absence of neurons within the BAC-treated area was maintained in both transgenic lines even 70-80 days after BAC application. However, in SER26; R26ReYFP transgenics, analysis of the area bordering the aganglionic area revealed a relatively large fraction of GFP^+/$HuCD^+$ cells (9±4%, 3197 HuC/D^+ scored from 4 animals) indicating that they are derived from GFP-expressing glial cells. The percentage of GFP^+ enteric neurons detected within enteric ganglia was inversely proportional to their distance from the treated area, ranging from 0.5% (background level at segments distant from treated region) to 9% (adjacent to the treated area) (**Fig.6.6 F-I**). In hGFAPCreER^{T2}; R26ReYFP mice, 1 of the two animals analyzed exhibited a single GFP-labelled HuC/D^+ neuron (377 HuC/D^+ scored; data not shown) suggesting that low recombination efficiency in this transgenic line may preclude detection of GFP^+ glial-derived neurons. These results strongly suggest that enteric glial cells possess neurogenic potential not only in culture but also *in vivo* being able to generate neurons in response to injury.

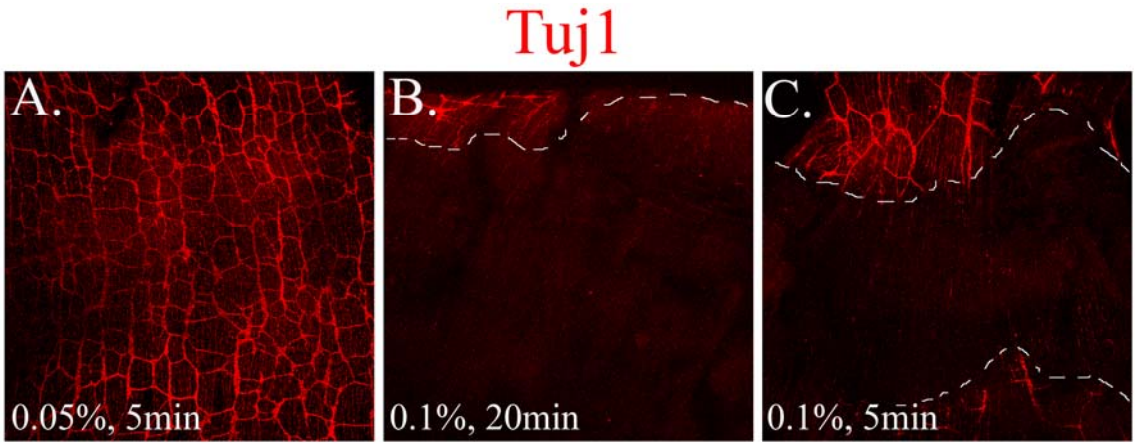
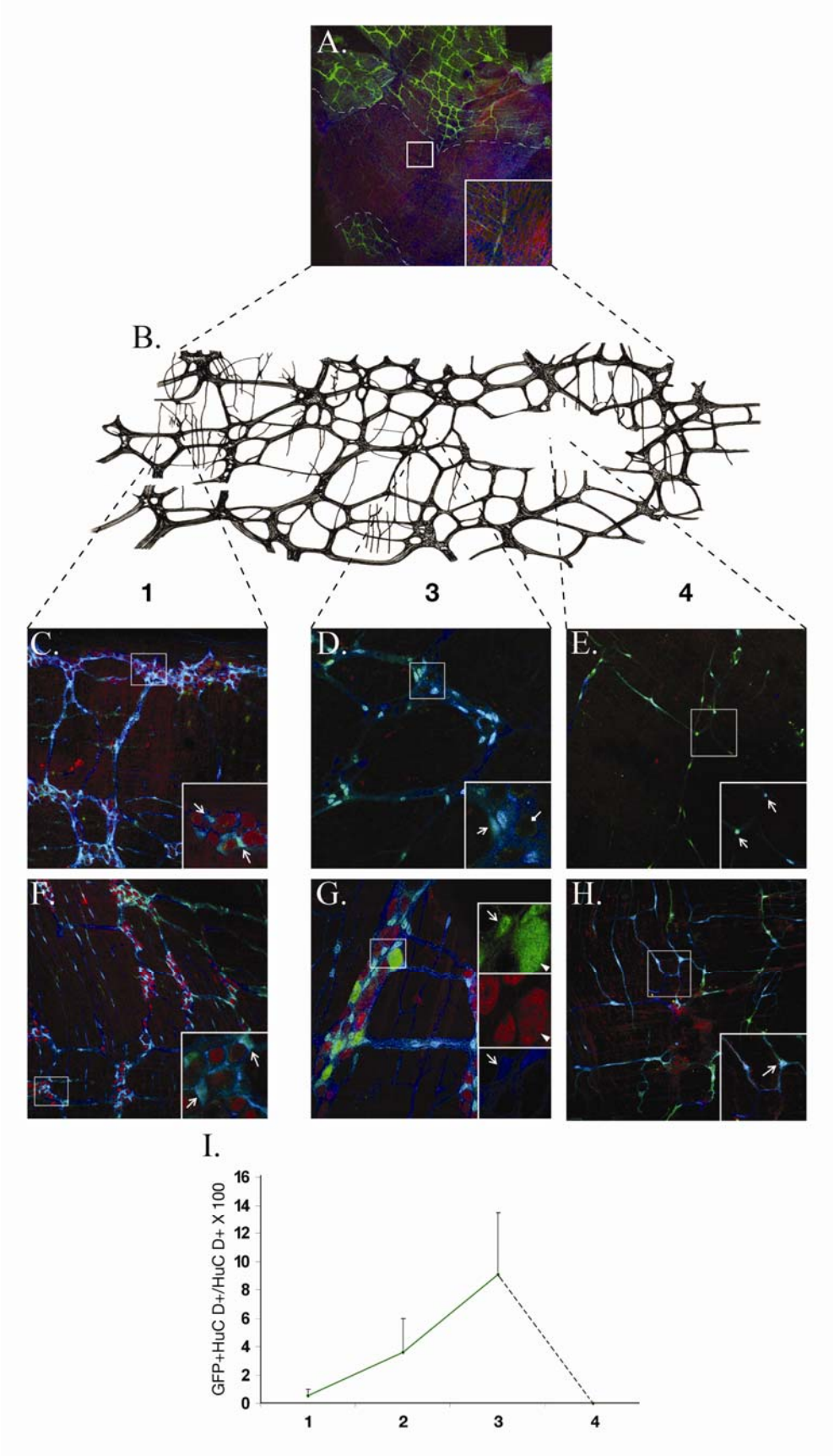


Figure 6.5. ***Acute destruction of myenteric neurons using the cationic detergent BAC***

(A-C) The cationic chemical BAC was applied to 1 cm segment of the small intestine of wild-type animals. Three days after BAC treatment MS-MP whole-mount preparations of the BAC-treated area were isolated and immunostained for Tuj1. Different regimes were tested regarding their efficiency to acutely destroy myenteric neurons: 0.05%, 5 minutes (**A**); 0.1%, 20 minutes (**B**); 0.1%, 5 minutes (**C**). x50 magnification.

Figure 6.6. ***GFP$^+$ glial cells from the ENS of adult SER26^2; R26ReYFP mice generate neurons, in vivo, upon injury.***

(A-I) SER26; R26ReYFP animals (P84 or older) were treated with 4-OHT and then BAC-treated a week later. (**A**) Confocal microscope images of MS-MP whole mount preparations from the BAC-treated area 3 days after the treatment. Inset shows absence of GFP$^+$, HuC/D$^+$ or S100 β$^+$ cells in the treated area. **(B)** Schematic representation of enteric plexus that includes an area which as a result of BAC treatment becomes aganglionic (area 4). **(C-E)** Confocal microscope images of MS-MP whole mount preparations from distant, adjacent and treated areas analysed 30 days (C, D and E, respectively) and 80 days (F, G and H, respectively) after BAC-treatment. MS-MP strips were immunostained for GFP (green), HuC/D (red) and S100β (blue). Arrowheads in insets of panel G point to a GFP$^+$/HuC D$^+$ double labelled cell which corresponds to a newly-generated neuron. Arrows indicate GFP$^+$/HuC D$^-$/S100β$^+$ cells. Diamond arrow in panel D point to spaces within the ganglia suggesting that neuron loss has occurred. (**I**) Quantification of the number of GFP-labelled HuC/D$^+$ cells in an area distant to the BAC-treated region (1), in the vicinity (2), at the border (3) and within the treated area (4). Note that the percentage of GFP$^+$ neurons detected within enteric ganglia was inversely proportional to their distance from the treated area. The error bars indicate s.e.m. A, x50 (inset: x200); C and F, x200 (inset: x600); D and G, x400 (inset: x1080); E and H, x200 (inset: x400).


A.
B.
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C.
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GFP+HuC D+/HuC D+ X 100
16
14
12
10
8
6
4
2
0
1
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6.3. Discussion

Enteric glia share many morphological and phenotypic characteristics with CNS astrocytes, which has led to the hypothesis that the two cell types may be functionally related. Despite recent reports highlighting an active participation of glial cells in the control of gastrointestinal functions (Bush *et al.*, 1998; Cabarrocas *et al.*, 2003), the potential role of enteric glia as a source of neurons had not been considered so far. We tested the possibility that, as SVZ and SGL astrocytes of the adult brain, enteric glial cells from the mature ENS can exhibit neural stem cell potential. This potential, however, is likely to remain dormant under the constraints of the non-neurogenic environment of the mature ENS and only become apparent when the enteric glia environment is altered, either by removing them from their niche (e.g culture) or perturbing the niche (e.g. injury). Here, we report that enteric glia isolated from the adult gut exhibit neural stem cell properties in culture. In addition, we provide direct evidence that enteric glial cells can generate new neurons both in culture and *in vivo* upon injury.

Using the Cre-loxP system, we lineally marked enteric glial cells isolated from the mature ENS and followed their fate *in vitro*. The results from these experiments demonstrated that enteric glia from the adult are a source of neural stem cells *in vitro*. Two previous cell culture studies have reported the isolation of enteric cells that in culture behave as neural stem cells. Suárez-Rodriguez and Belkind-Gerson (2004) cultured partially digested adult gut and reported the presence of different cell types *in vitro*, including glial cells, myofibroblasts and also nestin^{+} cells, to which neural stem cell properties were attributed. In this study, however, the lineage relationship between the different cell types in culture was not addressed. In another report, high level of p75

expression was used to select for eNCSCs (Kruger *et al.*, 2002). This approach was assumed to prospectively identify ENS progenitor cells *in vivo*, however, besides embryonic eNCSCs, p75 is also expressed in differentiated glial cells (Young *et al.*, 2003). Even though we cannot rule out the presence of different populations of neural stem cells in the mature ENS, in light of our results we propose that glial cells are the origin of neural stem cells identified *in vitro* previously.

Our fate-tracing experiments also provided some insight into the stepwise changes enteric glia undergo in culture. By performing a time course analysis of the fate of glial cells isolated from the mature ENS, we demonstrated that in culture these cells change their phenotypic profile. Glial cells were shown to loose GFAP expression while maintaining expression of markers that are common to embryonic eNCSCs and glial cells (Sox10 and Phox2b). Interestingly, we found that the majority of $GFAP^-$ lineage-labelled cells expressed Sox2, a transcriptional regulator that is expressed in CNS neural stem cells from embryogenesis through adulthood (Pevny & Placzek, 2005; Suh *et al.*, 2007). Although we cannot exclude direct conversion of glial cells into neurons, our time course analysis is consistent with a process whereby glial cells dedifferentiate to a more embryonic-like state prior giving rise to new neurons.

It is generally thought that epigenetic changes that underlie reprogramming events are most easily made during cell division. We observed that, in parallel to changes in the expression profile, GFP-labelled glial cells undergo extensive proliferation giving rise to colonies similar to those generated by embryonic eNCSCs (Bondurand *et al.*, 2003). Our experiments did not address this issue directly and so it is still unclear whether dedifferentiation of glial cells into eNCSCs involves obligatory proliferation steps but it

is possible that *in vitro* activation of cell proliferation is accompanied by conversion of glial cells into $GFAP^-$ progenitors that resume phenotypic features of embryonic eNCSCs.

Besides glia-derived eNCSCs, 4 day colonies included also a large number of GFP^+ glial cells. The origin of these cells is currently unclear. It is possible that GFP^+ glial cells have derived from proliferation of single GFP^+ glia present in culture on DIV1. Alternatively, glia-derived eNCSCs may retain differentiation potential to generate glial cells.

Importantly, our fate-mapping studies revealed that enteric glia have the potential to generate new neurons *in vitro*. We observed that, similar to embryonic eNCSCs, a fraction of the glia-derived neural stem cells expressed Mash1 as early as 4 days after plating. *Mash1* is expressed in neuron-committed progenitors and it has been shown to promote neurogenesis by activating the expression of neuron-specific genes, as well as to inhibit the expression of glia-specific genes (Guillemot *et al.*, 1993; McNay *et al.*, 2006; Nieto *et al.*, 2001; Parras *et al.*, 2002; Tomita *et al.*, 2000). Expression of Mash1 in a fraction of glia-derived neural stem cells is consistent with the acquisition of neurogenic potential by these cells. Indeed, we observed that in neuron-promoting conditions glia-derived neural stem cells efficiently differentiated along the neuronal lineage giving rise to different neuronal subtypes. We further demonstrated that glia-derived neurons generated *de novo* in culture expressed the pre-synaptic protein synaptophysin, a vesicle protein that has been implicated in the regulation of vesicle recycling (Evans & Cousin, 2005). Expression of this protein in glial-derived neurons suggests that besides exhibiting neuron-specific morphology and molecular features,

these cells were able to establish synaptic contacts in culture. Whole-cell recordings by patch-clamp will be required to further dissect the electrophysiological properties of these GFP^+ neurons.

We also examined whether the neurogenic potential of GFP^+ glial cells could be revealed in *ex vivo* cultures of MS-MP strips. In these cultures, we found that GFP^+ glial cells were activated to proliferate and generate new HuC/D^+ neurons, suggesting that the disruption of short-range cell-cell interactions through tissue dissociation is not required to activate the neurogenic potential of GFP^+ glial cells. Nevertheless, isolation of MS-MP strips is likely to involve resection of axons of subsets of enteric neurons and some perturbation of normal cell-cell interactions which may determine or, at least, contribute to trigger the neurogenic potential of glial cells.

Indeed, acute elimination of neurons has been shown to stimulate neurogenesis *in vivo* in a number of systems including the retina (Fischer & Reh, 2003), cortex (Magavi *et al.*, 2000) and nodose ganglia of the PNS (Czaja *et al.*, 2008). Neural regeneration following ablation of neurons in the adult ENS has also been reported (Hanani *et al.*, 2003; Luck *et al.*, 1993; Ramalho *et al.*, 1993). However, it remained unclear whether the neurons that repopulated the denervated area were actually newly generated neurons or cells that migrated from adjacent ganglia. By combining a lineage tracing strategy and an injury model, we demonstrate here that new neurons are generated *in vivo*, in response to injury. Furthermore, our results provide direct evidence that enteric glial cells can be a source of new neurons generated in the adult ENS.

In this study, acute destruction of myenteric neurons was achieved by applying to the serosal surface the cationic detergent BAC. As reported by other authors (Ramalho

et al., 1993; Sato *et al.*, 1978; Yoneda *et al.*, 2002), we found that BAC acts on a dose-dependent manner. A preliminary analysis was carried out using different concentrations of BAC solution for different periods of time. We successfully developed an experimental protocol in which we induced chemical ablation of the myenteric plexus producing an aganglionic segment without interfering with the survival of the animals. As early as 3 days after BAC treatment, we observed effective destruction of neurons and nerve fibers of the myenteric plexus. This is consistent with a previous study demonstrating that BAC leads to a reduction of approximately 90% of neurons 6 hours after application (Ramalho et al., 1993).

From day 3 to day 30 after BAC treatment, we observed invasion of the aganglionic area by GFP^+ glial cells. It is unclear at this stage whether these are cells that have migrated from the regions surrounding the lesion or newly-generated glial cells. This issue was not directly addressed but results from our lineage tracing analysis *in vitro* demonstrating generation of a large fraction of $GFAP^+$ glia are consistent with the latter case. Furthermore, previous studies have highlighted the proliferative capacity of glial cells *in vivo* in the adult ENS (Bradley *et al.*, 1997; Liu *et al.*, 2009). By administering short pulses of BrdU to adult rats, Bradley et al. (1997) showed that a small fraction of glial cells are able to proliferate under physiological conditions and similar results were obtained recently in adult mice (Liu *et al.*, 2009), thus indicating some resting mitotic activity of these cells. Interestingly and similar to CNS astrocytes, enteric glia display an increased proliferative capacity in response to intestinal inflammation (Bradley *et al.*, 1997). It is possible that the maintenance of mitotic quiescence of glial cells in the adult

ENS is perturbed upon injury leading to active proliferation and consequently, generation of new glial cells that invade the aganglionic area.

Interestingly, in the areas adjacent to the aganglionic segment, we found a number of morphologically abnormal ganglia containing GFP^+ glial cells but no HuC/D^+ neurons. This observation suggests that neuronal cell loss takes place also in the transition zone, next to the BAC-treated area. Whether and how acute destruction of neurons following BAC application triggers neuronal degeneration in adjacent ganglia is unclear. A possible scenario is that neurons in the transition area die secondarily due to the death of their post-synaptic targets in the BAC-treated region.

Despite our *in vitro* data suggesting that $GFAP^+$ glial cells give rise to $GFAP^-$ progenitors that resume phenotypic characteristics of embryonic eNCSCs, we could not find in the transition zone any GFP^+HuC/D^-S100^- cells. Thus, it remains unclear whether similar to what we observed in culture, glia-response to injury *in vivo* involves proliferation and parallel acquisition of neural stem cell-like properties. Noteworthy, electron microscopy studies by Hanani and colleagues (Hanani *et al.*, 2003) suggested the presence of undifferentiated cells in the ganglia surrounding the treated segment.

In contrast with results from Hanani et al. (2003) who found isolated $PGP9.5^+$ neurons in the treated area after 30 days, we could not identify any HuC/D^+ neuron in the aganglionic segment 30 or even 80 days after treatment. The reason for this discrepancy may be the severity of BAC treatment which, in our system, may lead to a longer recovery period. Nevertheless, 80 days after BAC application in SER26; R26ReYFP we were able to unequivocally identify glia-derived newly generated neurons in the areas adjacent to the aganglionic segment. The number of GFP^+ neurons

decreased progressively with increasing distance to the BAC-treated region. Whether the newly-generated neurons in the vicinity of the aganglionic segment eventually migrate and colonise the treated area, as suggested by Hanani's results, was not possible to address with the time points considered. In addition, although our data provide evidence for the generation of new neurons following BAC treatment in adults, there is as yet no evidence that the new neurons establish connections with previously innervated tissues. Functional experiments will be required to address whether newly generated neurons contribute to functional regeneration following denervation.

Unlike the BAC-treated SER26; R26ReYFP mice, we did not observe glia-derived newly generated neurons in hGFAPCreER^{T2}; R26ReYFP transgenics, even 80 days after BAC treatment. The fact that the *hGFAPCreER^{T2}* transgene targets only a small fraction of glial cells (19%) may contribute to this observation. An alternative possibility is that the *hGFAPCreER^{T2}* transgene is expressed exclusively in a non-neurogenic group of glial cells. Further experimentation will be required to address whether all or only a subset of enteric glia has the potential to form neurons.

Given that similar to CNS astrocytes, enteric glia express multiple stem cell/progenitor-specific genes, it may not be surprising that these cells carry out dual roles of: a) supporting neuronal functions; and b) participating in neurogenesis, providing that the environment allows it. Indeed, after a certain developmental point, the intact adult ENS does not support neurogenesis and so it is likely that the neurogenic potential of glial cells remains under the constraints of the non-neurogenic environment of the adult ENS. The mechanisms underlying activation of enteric glia are unknown but they are likely to involve cell-intrinsic and cell-extrinsic factors. One possible scenario

is that destruction of neurons may lead to the activation/abrogation of specific pathways that activate enteric glia to acquire neural stem cell properties and neurogenic potential. In the ENS, despite recent studies demonstrating neuron-glial cell communication (Gomes *et al.*, 2009; Gulbransen & Sharkey, 2009), little is known about the physiological role of these communication pathways, for example, in the proliferative ability of glial cells and maintenance of the glial identity. However, co-culture studies have demonstrated that CNS neurons can inhibit glial proliferation (Gomes *et al.*, 1999) and, in the adult *Drosophila* brain, neuronal programmed cell death has been shown to induce glial cell division (Kato *et al.*, 2009). How can the ablation of neurons activate glial cells to proliferate and acquire neural stem cell properties? A recent study showed that ependymal cells, that under physiological conditions are quiescent, in response to stroke loose their characteristic features, enter the cell cycle and give rise to neuroblasts and astrocytes. Interestingly, in this system, ependymal cell quiescence was shown to be actively maintained by canonical Notch signalling. In the ENS, Notch signaling has been implicated in the maintenance of embryonic eNCSCs (Okamura & Saga, 2008; Taylor *et al.*, 2007) but its role on the adult ENS has not been addressed yet. Nevertheless, expression of Notch and Notch ligands have been reported in the adult ENS (Sander *et al.*, 2003) thus suggesting a continuing role for this signalling pathway at later stages. Based on the fact that Notch ligands are produced by neurons (Sander *et al.*, 2003), we speculate that ablation of subsets of neurons may lead to abrogation of the canonical Notch signalling pathway in enteric glial cells prompting them to proliferate and acquire neural stem cell properties. This may be accompanied by acquisition of responsiveness to specific environmental signals that regulate their proliferation and

neurogenic potential. In accordance, we found that in *ex vivo* cultures of MS-MP strips, the growth factors FGF2 or GDNF can induce a response similar to that observed in dissociated cultures and *in vivo*. FGF and GDNF mediate their biological responses in the target cells by binding to the tyrosine kinase receptors FGFR and RET, respectively. FGF2 has been shown to have a mitogenic effect on eNCSCs during embryonic development. In the adult, expression of the FGF2 receptor, FGFR2, in postnatal ENS ganglia (Yoneda *et al.*, 2001) suggests a possible role for FGF signalling in the mature ENS. Such a role is supported by an *in vitro* study showing that FGF promotes proliferation of enteric glial cells from the adult ENS (Eccleston *et al.*, 1987). Whether *in vivo* this factor is involved in the proliferation of enteric glia in physiological conditions and/or in response to injury is unknown but FGF2 has been implicated in the reactive response of astrocytes to brain lesion and in neural regeneration in the retina (Chadashvili & Peterson, 2006; Fischer *et al.*, 2002).

GDNF also promotes proliferation of eNCSCs during development (Chalazonitis et al., 1998; Taraviras et al., 1999; Gianino et al., 2003). In addition to its mitogenic role, GDNF has a strong neurotrophic effect, as in its absence development of enteric neurons is impaired (Chalazonitis *et al.*, 1998; Taraviras *et al.*, 1999). The role of GDNF/RET signalling pathway in neuronal development is further supported by data showing that in the absence of the tyrosine kinase inhibitor Sprouty2, the GDNF/RET system leads to ENS hyperganglionosis (Taketomi *et al.*, 2005). We observed efficient generation of glia-derived neurons in the *ex vivo* cultures in the presence of GDNF. Since glial cells do not express *Ret* (Young *et al.*, 2003), the response to GDNF should not result from a direct effect of this factor on glial cells. Instead, it is likely that glial-derived eNCSCs

start expressing *Ret* as their embryonic counterparts and become responsive to GDNF and to its neuron-promoting effect. Further experiments will be required to dissect the role of both FGF and GDNF in adult neurogenesis in the ENS.

In conclusion, our data demonstrate for the first time that, in the adult mammalian gut, glial cells can generate neurons that become integrated into enteric ganglia. The role of glial cells in adult neurogenesis suggests that these cells may serve as a potential target for cellular manipulation for treating ENS degeneration. Further characterization of the genetic properties of adult glial cells and glial-derived neural stem cells should give insight into the mechanisms involved in development and remodelling of the ENS.

Chapter VII

Concluding Remarks

The mammalian nervous system comprises a vast number of neurons and glia and an astonishing diversity of neuronal subtypes. All neurons and glia are ultimately generated from neural stem cells which have the capacity for long-term self-renewal and generation of distinct differentiated neural lineages. The identification of neural stem cells and the characterization of their properties is crucial for understanding how these cells expand in number and undergo differentiation in an orchestrated manner to form a functional nervous system.

Significant progress has been made towards the identification and characterization of neural stem cells from the embryonic and mature brain. Outside the CNS, progenitors with neural stem cell properties have also been isolated from neural crest derivatives, in particular the ENS. These cells have been termed eNCSCs and were shown to have the capacity for self-renewal and differentiation into multiple lineages, such as neurons, glia and myofibroblasts, *in vitro* (Bondurand *et al.*, 2003; Kruger *et al.*, 2002; Morrison *et al.*, 1999; Silva *et al.*, 2008; Stemple & Anderson, 1992).

So far, the properties of eNCSCs *in vivo* have been deduced from studies on clonogenic cultures of cells isolated on the basis of molecular or functional characteristics (Bondurand *et al.*, 2003; Kruger *et al.*, 2002). Such studies have previously suggested that *Sox10*-expressing cells give rise to both the $Sox10^+$ (glial cells) and the $Sox10^-$ (neurons) lineages of the ENS (Bondurand *et al.*, 2003). However, the lineage relationship between $Sox10^+$ cells and enteric neurons and glia *in vivo* remained unclear. Using a genetic fate mapping system, we demonstrate here that $Sox10^+$ cells of the gut generate both enteric glia and neurons *in vivo* and therefore represent multilineage ENS progenitors.

At present, little is known about the mechanisms by which ENS progenitors integrate spatial and temporal cues that instruct them to generate the correct numbers of enteric neuron subtypes and glia at the right place and the right time. Some insight into such mechanisms has been provided, however, by examining other parts of the nervous system and two alternative models have been proposed. According to the first model, a single type of neural stem cells gives rise to both neurons and glia in a defined temporal sequence (neurons first and then glia - switching model); alternatively, distinct subpopulations of stem cells are intrinsically committed to generate one or the other cell type (segregating model). The segregating model is supported by a number of reports showing that neuron-restricted and glia-restricted progenitors can be isolated from the embryonic spinal cord and cerebral cortex (Qian *et al.*, 2000; Rao *et al.*, 1998). On the other hand, the switching model is consistent with neurogenesis and gliogenesis in *Drosophila* in which asymmetric division of neuroblasts can produce neurons, glial cells or both (Doe *et al.*, 1998). The fate map analysis in our studies has not resolved the issue of multipotency of single $Sox10^+$ cells but rather traced the fates of a $Sox10^+$ cell population. Therefore, it remains unclear whether in the ENS single *Sox10*-expressing cells have the potential to produce both neurons and glia via asymmetric division (switching model), or the $Sox10^+$ cells constitute a mixture of lineage committed neurogenic and gliogenic progenitors (segregating model). Lineage tracing at the clonal level *in vivo* will be crucial to directly address the differentiation potential of individual *Sox10*-expressing cells in the ENS and distinguish between these two differentiation models. A potential approach to address this issue is the administration of suboptimal doses of 4-OHT to SER93; R26ReYFP transgenic mice which, as our preliminary data

indicate, are capable of lineally marking a very small number of *Sox10*-expressing ENS progenitors.

Having developed the tools to genetically mark ENS progenitor cells *in vivo*, we have also determined how the neurogenic potential of these cells changes over the course of ENS maturation by following the fate of *Sox10*-expressing cells present in the gut at a given developmental stage. Results from these studies have established that the neurogenic potential of eNCSCs is temporally regulated and that the capacity of *Sox10*-expressing cells to generate enteric neurons is reduced over the course of gut organogenesis. A similar time course of neurogenic potential (high early, low later) has been observed in other parts of the nervous system. For example, neural stem cells isolated from E16 cortex generate fewer neurons before initiating gliogenesis relative to their E10 counterparts (Qian *et al.*, 2000). There are however, significant differences between the biological "timer" underlying the differentiation of CNS stem cells and eNCSCs. For instance, in the cerebral cortex differentiation of radial glial cells follows a precise temporal and spatial order: pyramidal neurons with distinct molecular and physiological characteristics are generated sequentially in an "inside-out" manner, with early-born neurons occupying the bottom cortical layers and late-born neurons residing in the most superficial layers. In contrast, the different subtypes of enteric neurons are not generated in spatially defined positions; instead, all enteric neuron subtypes and glial cells are born in an apparently uniform environment and they are, in general, evenly (and perhaps randomly) distributed throughout the gut as components of distinct ganglia. Furthermore, while genesis of neurons and glia in the CNS are temporally segregated, in the ENS there is considerable overlap between the period of neurogenesis

and gliogenesis. Thus, long after the first appearance of glial precursors in the developing gut at E11.5, eNCSCs continue to generate new neurons. Indeed this was documented in the present study in which we demonstrate that myenteric neurons continue to be generated in adult animals even after P30. These data indicate that neurogenesis and gliogenesis co-exist for a considerably long period and imply that new neurons continue to be integrated in the ENS circuitry long after it becomes functional.

How is the generation of a large array of enteric neuron subtypes (that are more or less evenly distributed throughout the gut) integrated in time and space? Are the number and type of neurons generated at various developmental stages dependent on temporal changes in the expression of neurogenic signals in the microenvironment of eNCSCs? Alternatively, do cell-intrinsic changes alter the competence of progenitor cells to respond to multiple co-existing signals? Of course, and as is often the case, these alternative mechanisms are not mutually exclusive and it is likely that both are implicated in enteric neurogenesis. *In vitro* experiments have revealed temporal differences in the responsiveness of eNCSCs to lineage determination factors, such as BMP4, suggesting that the differentiation potential of eNCSCs is controlled, at least partly, by cell-intrinsic changes in growth factor sensitivity (Kruger *et al.*, 2002). Such changes in the responsiveness to environmental cues may involve, for example, quantitative adjustments in the expression of cell-surface receptors (Lillien, 1995; Lillien & Wancio, 1998). In this context, it would be interesting to explore how changes in the expression levels of the RET RTK, which has been implicated in ENS differentiation, affect the neurogenic-gliogenic switch in the ENS. Also, the intrinsic epigenetic status of progenitor cells may modulate the response to extrinsic cues in a

way that is developmentally appropriate. For example, a recent study by Nahimira et al (2009) showed that in the developing brain the response of neural precursors to astrocyte-inducing cytokines depends upon a feedback signal whereby committed neuronal precursors and young neurons promote demethylation of astrocyte-specific gene promoters in neural precursor cells thus conferring gliogenic differentiation potential. Such a signal is mediated by the Notch pathway. Neuroblasts and young neurons express Notch ligands that are capable of activating the Notch signalling cascade in the neighbouring progenitor cells. Notch-activated neural precursors undergo demethylation of astrocytic-specific gene promoters (e.g gfap promoter) and acquire the ability to become astrocytes in response to astrocyte-inducing cytokines (Namihira *et al.*, 2009). Whether similar mechanisms are involved in the modulation of the differentiation capacity of eNCSCs *in vivo* is unknown. Insight into such mechanisms will be important not only in the understanding of normal developmental mechanisms but also they raise the possibility of manipulating mature and injured ENS with the aim of promoting regenerative neurogenesis.

Our lineage tracing analysis revealed that despite a long neurogenic period, enteric *Sox10*-expressing cells lose their neurogenic potential at some point between P30 and P84. This is in keeping with previous reports that have failed to identify newly generated neurons in the intact adult mammalian ENS (Liu *et al.*, 2009) and suggests that unlike the adult CNS, which is known to harbour neural stem cells that sustain neurogenesis throughout life, the mature ENS does not support constitutive neurogenesis. Despite these observations, self-renewing progenitors capable of generating enteric neurons and glia can be identified in cultures of post-neurogenic adult

intestine (Kruger *et al.*, 2002). Moreover, a recent report has provided evidence for enteric neurogenesis in adult animals upon pharmacological stimulation (Liu et al., 2009), reinforcing the idea that cells with neurogenic potential persist in the adult ENS and can be activated to generate new neurons under specific conditions. The identity of these cells and their physiological role, however, has remained elusive. Our results provide strong evidence that the elusive source of multilineage progenitors in cultures of adult ENS are the $GFAP^+$ enteric glial cells. Moreover, these mature glial cells can also be activated to generate neurons in an animal injury model of the ENS.

Once thought to be passive and merely supporting partners of neurons, glial cells have recently emerged as active regulators of many neuronal functions (Aoki *et al.*, 1991; Araque *et al.*, 2001). Furthermore, several reports have highlighted a close relationship between neural stem cells and glia. Ongoing neurogenesis in the CNS is ensured by GFAP-expressing progenitors with structure and molecular characteristics of astrocytes (Doetsch *et al.*, 1999; Seri *et al.*, 2001). Moreover, glial cells are the source of new neurons in the injured retina (Ooto *et al.*, 2004) and in the neural crest-derived carotid body (Pardal *et al.*, 2007) of adult mammals. The findings reported here support the idea that also the mature ENS harbours glial cells that carry out a dual role, supporting neuronal functions and participating in neurogenesis. The neurogenic potential of enteric glia is likely to remain dormant under the constraints of the adult ENS niche, however as our data indicates, such potential can be activated in specific conditions that are associated with the acute loss of enteric neurons.

Neuronal degeneration is a common feature of a number of gastrointestinal disorders, such as inflammatory bowel disease (IBD) and post-enteritis irritable bowel

syndrome. Interestingly, early histopathological studies reported that neuronal degeneration is often accompanied by other abnormalities, including ganglia and nerve bundles of increased size (hypertrophy) and/or number (hyperplasia), as well as changes to glial cells (hyperplasia) (Davis *et al.*, 1955; Nadorra *et al.*, 1986; Siemers & Dobbins, 1974; Storsteen *et al.*, 1953). The link between inflammation, neuronal degeneration and neurogenesis is further supported by increasing evidence pointing to mutual interaction between the ENS and the gastrointestinal immune system (Ruhl *et al.*, 2001a; Ruhl *et al.*, 2001b).

Our observations suggesting that glial cells are facultative stem cells ready to respond to neural injury raise a series of important questions: (1) Are the types of cells regenerated after damage dependent on the types of cells destroyed? (2) Do all or just a subset of glial cells have neurogenic potential? (3) Are the newly born neurons able to establish connections with previously innervated tissues and become integrated in the circuitry? (4) What are the signals responsible for activating the neurogenic potential of glial cells? Further insight onto these questions will be fundamental to better comprehend the plasticity and regenerative capacity of the ENS and may facilitate the design of novel therapeutic strategies for the treatment of conditions associated with congenital absence or acquired loss of enteric neurons, such as Hirschsprung's disease or gut inflammation.

Appendix

Development of a genetic strategy to address the role of RET in cell fate decisions during ENS development

Introduction

During development, receptor tyrosine kinases (RTKs) transduce extracellular signals into multiple cellular responses, such as growth and cell differentiation.

The RTK RET encoded by the *c-Ret* proto-oncogene, is the main signalling component of a receptor complex for the glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) – GDNF, neurturin, artemin and persephin (Baloh *et al.*, 2000). As a receptor tyrosine kinase, RET possesses an extracellular cysteine-rich ligand-binding domain, a transmembrane domain and a cytoplasmatic segment containing the catalytic tyrosine kinase domain (Yarden & Ullrich, 1988). Signalling through the RET receptor requires binding of GFLs to GFRα, a glycosyl phosphatidylinositol cell surface receptor which, in turn, binds to RET. This leads to the dimerization of the receptor and activation of the kinase domain, which results in autophosphorylation and phosphorylation of specific substrates that mediate intracellular signalling (Ullrich & Schlessinger, 1990).

Deregulation of the RET RTK function has been shown to be oncogenic. Dominant, gain-of-function mutations in the *c-RET* locus are associated with inherited cancer syndromes which involve cell types of neural crest origin (familial medullary thyroid carcinoma -FMTC- and multiple endocrine neoplasia -MEN- 2A and 2B; Jhiang, 2000). In the majority of MEN2B cases, a M918T mutation causes a change of RET substrate specificity which results in oncogenic transformation (Santoro *et al.*, 1995). In contrast, in most of MEN2A and FMTC cases mutations affect one of the cysteine residues in the cysteine-rich extracellular domain of *c-RET* leading to the formation of permanent disulfide-bonded homodimers and trans-auto-phosphorylation of the tyrosine kinase.

This results in the constitutive activation of the RET receptor independent of its ligands (Santoro *et al.*, 1995).

Besides its role in tumorigenesis, RET has been shown to be an important player in the development of the PNS, including the ENS. Mutations of *RET* in humans lead to absence of enteric ganglia from the distal colon (HSCR; Edery *et al.*, 1994; Romeo *et al.*, 1994), whereas mice homozygous for a targeted mutation in *Ret* (Ret^{k-}) die soon after birth and display complete intestinal aganglionosis (Durbec *et al.*, 1996; Schuchardt *et al.*, 1994). A similar phenotype is observed in mice in the absence of *Gdnf* or *Gfrα1* (Cacalano *et al.*, 1998; Enomoto *et al.*, 1998; Moore *et al.*, 1996; Sanchez *et al.*, 1996), highlighting the functional interaction between these molecules and the crucial role of the RET/GDNF/GFRα1 signalling pathway in ENS development.

During ENS development, *c-Ret* is expressed in NCCs when they first reach the vicinity of the gut at E9-9.5 and continues to be expressed as these cells colonize the entire gastrointestinal tract (Durbec *et al.*, 1996; Pachnis *et al.*, 1993; Tsuzuki *et al.*, 1995). In the differentiated lineages of the ENS, *Ret* expression is maintained exclusively in the neuronal population (Young *et al.*, 2003).

Early *in vitro* and *in vivo* studies have implicated *Ret* in numerous aspects of ENS development. Detailed analysis of *Ret*-deficient embryos revealed that ENS progenitors undergo apoptosis before reaching the midgut, thus suggesting a role for this gene in the early survival of ENS progenitors (Durbec *et al.*, 1996). Moreover, GDNF activation of RET via GFRα1 has been shown to be required for the migration and proliferation of ENS progenitors (Gianino *et al.*, 2003; Natarajan *et al.*, 1999; Taraviras *et al.*, 1999). Interestingly, a recent report has suggested that the physiological role of the RET

signalling pathway is regulated spatiotemporally (Uesaka *et al.*, 2007). Mice in which *Gfrα1* gene function is conditionally disrupted during midgestation (E11.5-13.5) display reduced proliferation in the ENS but not cell death. Thus, whereas at early stages of development RET signalling is required for the survival of ENS progenitors (Durbec *et al.*, 1996), at E11.5-13.5 this pathway plays a crucial role in cell proliferation but not cell survival. Impairment of RET signalling at late stages of development has also been addressed by conditionally ablating *Gfrα1* or *c-Ret*. At these stages, *Gfrα1* is expressed in both enteric neurons and glia whereas *c-Ret* is expressed in eNCSCs and neurons. The glial cell population does not express *c-Ret* and, so it is not affected by the loss of *Gfrα1* (Enomoto *et al.*, 2004). In contrast, enteric neurons in the colon undergo cell death in the absence of *Gfrα1* or *c-Ret*, thus suggesting that RET signalling is required for the survival of enteric neurons in the colon (Uesaka *et al.*, 2007; Uesaka *et al.*, 2008). However, the effects of abrogating the RET signalling pathway in ENS progenitors and how that affects cell commitment and differentiation was not addressed in this study.

Evidence supporting a potential association between signalling of the RET RTK in eNCSCs and their commitment and differentiation into specific cellular phenotypes came from an *in vitro* study by Chalazonitis and colleagues (Chalazonitis *et al.*, 1998). $p75^{+}$ cells from the gut of E16 rat embryos were cultured in the presence or absence of GDNF. This approach showed that in the presence of GDNF, $p75^{+}$ cells preferentially adopt a neuronal rather than a glial fate. Whether GDNF exerts an instructive effect on eNCSCs to follow the neuronal lineage is still unclear. A role for RET in cell fate decisions was further demonstrated by the study of the monoisoformic *Ret* mutants (de Graaff *et al.*, 2001). In mammals, *c-Ret* encodes for two isoforms, RET9 and RET51,

which are generated by alternative splicing and differ only in their carboxy-terminal sequences (Tahira *et al.*, 1990). RET isoforms have distinct signalling properties; whereas RET9 isoform is sufficient to support normal embryonic and postnatal development, the RET51 isoform is not. Mice mutants that express only the RET51 isoform ($Ret^{51/51}$) display absence of enteric ganglia in the colon, a phenotype that reproduces that of human aganglionosis (de Graaff *et al.*, 2001). Further analysis of these mutants showed that they exhibit differentiation defects as the fraction of enteric neurons in the gut of the $Ret^{51/51}$ mutants is significantly reduced relative to wild-type littermates (Dipa Natarajan, personal communication). Moreover, clonal cultures of enteric multipotential progenitors isolated from $Ret^{51/51}$ mutants contain a reduced number of enteric neurons (Dipa Natarajan, personal communication). Despite these studies, the role of RET signalling on cell commitment and differentiation is still poorly understood.

Here, we wished to explore the role of RET in cell fate decisions and test the hypothesis that sustained expression of *c-RET* is sufficient to divert differentiation of ENS progenitors away from the glial lineage and towards the neuronal cell fate *in vivo*.

Results

Generation of $R26^{RET9}$ and $R26^{RET9(MEN2A)}$ constructs

To elucidate the role of RET signalling in cell fate decisions during ENS development, we wished to generate novel transgenic lines in which a wild-type (*c-RET9*) or a mutated form of the RET9 isoform (Ret9-MEN2A) is knocked-in the *ROSA26 (R26)* locus ($R26^{RET9}$ and $R26^{RET9(MEN2A)}$, respectively).

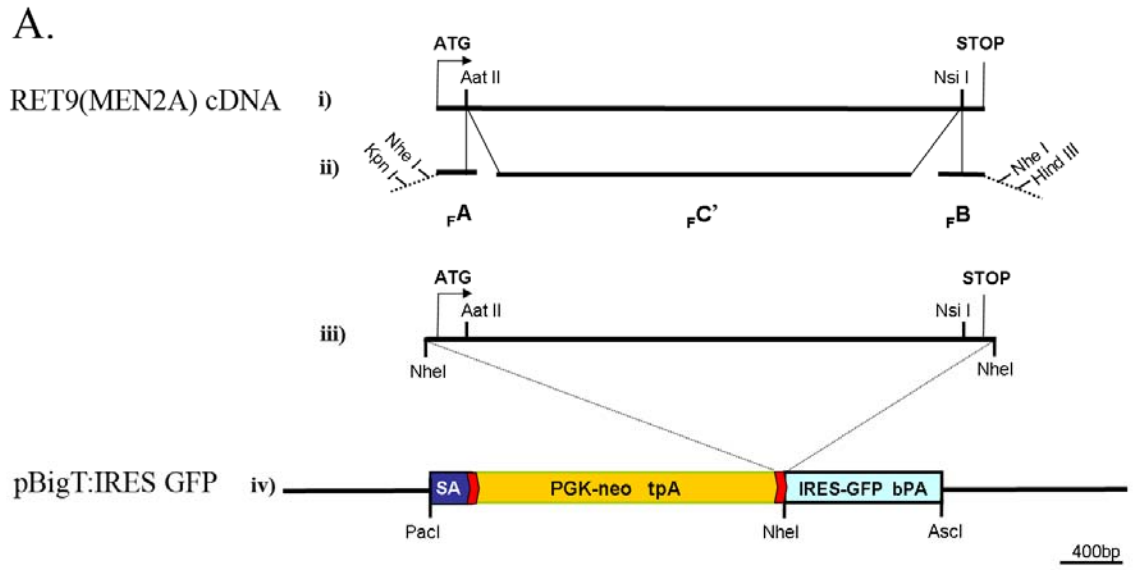
As a first step towards the generation of $R26^{RET9}$ and $R26^{RET9(MEN2A)}$ mouse lines, we constructed a *RET9-IRES-GFP* cassette. The *RET9/RET9^{MEN2A}* cDNA (3.2 Kb) could not be isolated from the vector pcDNA3 due to the lack of appropriate restriction enzyme sites. For that reason, *RET9/RET9^{MEN2A}* cDNA was reconstructed using a three-step strategy. First, fragments $_{F}$A (161 bp) and $_{F}$B (153 bp) were amplified by PCR using the RET9 cDNA as a template. Primers were designed to insert KpnI and XhoI restriction sites at the ends of $_{F}$A and XhoI and HindIII sites at the ends of $_{F}$B (**Fig.7.1 A-i, ii**; see Chapter II). $_{F}$A was subcloned into the pBlueScript KS (pBS KS) vector previously double-digested with KpnI/XhoI. Similarly, $_{F}$B was subcloned into XhoI/HindIII digested pBS KS+$_{F}$A construct, thus generating pBS KS+$_{F}$A+$_{F}$B. Secondly, a 3 Kb fragment of *RET9* cDNA with and without MEN2A mutation ($_{F}$C) was released by XhoI digestion and cloned into XhoI digested pBS KS+$_{F}$A+$_{F}$B. Because this is a non-directional cloning step, colony lifts and hybridization were used to perform a screen for positive transformed colonies using $_{F}$C as a probe. Then, DNA from the selected colonies was analysed following restriction digestion with KpnI to identify colonies carrying the $_{F}$C in the right orientation. All the positive colonies selected (50 for RET9 $_{F}$C and 36 for RET9^{MEN2A} $_{F}$C) carried $_{F}$C in the wrong orientation. In an effort to overcome this, we designed a new cloning strategy: *RET9/RET9^{MEN2A}* cDNA harbours two unique restriction enzyme sites (AatII and NsiI), which were used to release a 3 Kb fragment ($_{F}$C'). This fragment was then subcloned into pBS KS+$_{F}$A+$_{F}$B previously digested with AatII and NsiI thereby generating pBS KS+$_{F}$A+$_{F}$B+$_{F}$C (**Fig.7.1 A-iii**; see Chapter II). Finally, NheI digestion of pBS KS+$_{F}$A+$_{F}$B+$_{F}$C released the coding sequence of Ret9 and Ret9^{MEN2A} (3.2 Kb fragment) which was then inserted at a unique NheI site

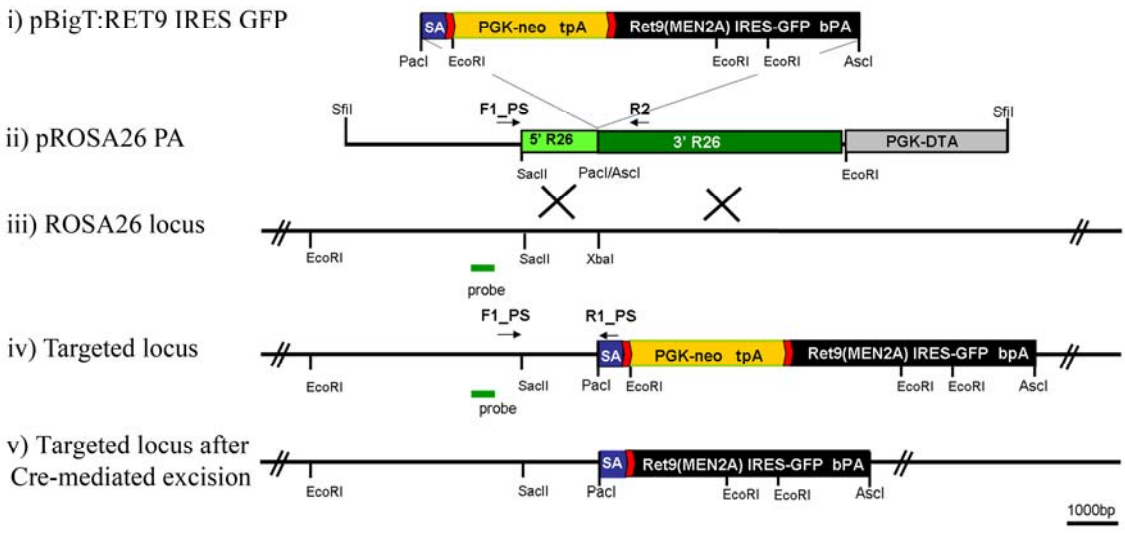
of the pBigT plasmid (kindly provided by S. Srinivas, University of Oxford, UK; Srinivas *et al.*, 2001). In this construct, a cassette containing the neomycin-coding gene under the control of the promoter for phosphoglycerate kinase (PGKneobpA) flanked by *loxP* sites lays 5' to a RET9/RET9^{MEN2A} coding sequence to prevent translation until Cre-mediated excision of the neo cassette. 3' to RET9/RET9^{MEN2A} coding sequence lays an IRES-GFP reporter construct to monitor expression of the transgene *in vivo* (**Fig.7.1 A-iv**; see Chapter II). Once again, colony lifts and hybridization were used to perform a screen for positive transformed colonies using $_{F}$C as a probe. DNA of the selected colonies was then digested with different restriction enzymes to assess for correct orientation of the cloned fragment. Restriction analysis revealed a wrong restriction pattern for all the colonies tested due to rearrangements of the pBigT vector. More specifically, we identified the loss of an 870 bp fragment from the PGK cassette of the pBigT vector following subcloning of RET9/RET9^{MEN2A} coding sequence. In an effort to overcome this unexpected rearrangement, pBigT-RET9 and pBigT-RET9$^{(MEN2A)}$ constructs were again subcloned into a non-rearranged pBigT vector and transformed into HB101 bacteria, grown at 28ºC.

Once generated, the RET9-IRES-GFP and the RET9^{MEN2A}-IRES-GFP cassettes were digested with PacI/AscI restriction enzymes and inserted into an AscI/PacI digested vector pROSA26PA (a kind gift from S. Srinivas, University of Oxford, UK; Srinivas *et al.*, 2001). This plasmid contains genomic *R26* sequences for homologous recombination and a diphtheria toxin gene for negative selection in ES cells (**Fig.7.1 B-ii**; see Chapter II).

Figure A1 ***Generation of the R26^{RET9} and R26$^{RET9(MEN2A)}$ constructs.***

(**A**) Map of the *RET9*/*RET9*^{MEN2A} cDNA indicating the relative locations of the AatII and NsiI restriction sites (**i**). Maps of $_{F}$C' and the PCR-amplified $_{F}$A and $_{F}$B, including the relative location of the added KpnI+NheI and HindIII+NheI restriction sites (**ii**). *RET9*/*RET9*^{MEN2A} were isolated by NheI digestion and then subcloned into pBigT:IRES-GFP vector (**iii**). Schematic representation of the pBigT:IRES-GFP vector indicating the adenovirus splice acceptor sequence (SA; blue) followed by a loxP site (red), neo expression cassette (PGK-neo tpA; orange), another loxP site (red), the NheI restriction site where *RET9*/*RET9*^{MEN2A} were subcloned into, the IRES-GFP cassette and the bovine growth hormone polyadenylation sequence (IRESGFP bPA; light blue) and the restriction sites PacI and AscI (**iv**). (**B**) Map of the pBigT:RET9:IRESGFP plasmid (**i**). Schematic of the pROSA26A vector showing the location of the 5' and 3' homology sequences for homologous recombination and the diphtheria toxin gene (PGK-DTA) for negative selection in ES cells (**ii**). Wild-type *R26* locus with the relative location of the BglI restriction sites and the location of the probe (**iii**). Structure of the targeted locus before (**iv**) and after (**v**) Cre-mediated excision of the *loxP*-flanked cassette.


A.
RET9(MEN2A) cDNA
i)
ii)
ATG
STOP
Aat II
Nsi I
Nhe I
Kpn I
Nhe I
Hind III
FA
FC'
FB
iii)
ATG
STOP
Aat II
Nsi I
NheI
NheI
pBigT:IRES GFP
iv)
SA
PGK-neo tpA
IRES-GFP bPA
PacI
NheI
AscI
400bp


B.
i) pBigT:RET9 IRES GFP
SA
PGK-neo tpA
Ret9(MEN2A) IRES-GFP bPA
PacI
EcoRI
EcoRI
EcoRI
AscI
ii) pROSA26 PA
SfiI
F1_PS
R2
SfiI
5' R26
3' R26
PGK-DTA
SacII
PacI/AscI
EcoRI
iii) ROSA26 locus
EcoRI
SacII
XbaI
probe
iv) Targeted locus
F1_PS
R1_PS
SA
PGK-neo tpA
Ret9(MEN2A) IRES-GFP bpA
EcoRI
SacII
PacI
EcoRI
EcoRI
EcoRI
AscI
probe
v) Targeted locus after
Cre-mediated excision
SA
Ret9(MEN2A) IRES-GFP bPA
EcoRI
SacII
PacI
EcoRI
EcoRI
AscI
1000bp

Both the targeting vectors were linearized with SfiI. We started by electroporating the targeting vector paROSA-RET9^{MEN2A}-IRES-GFP into E14G2A embryonic stem (ES) cells (kindly provided by A. Smith, Institute for Stem Cell Research, UK). Following G418 selection, 332 G418-resistant clones were picked. PCR analysis of the clones showed that 2 G418-resistant clones had correctly undergone homologous recombination (**Fig.A1 B-iv**; **7.2 A**). This result was further verified by Southern blot analysis of EcoRI digested DNA from the ES clones using a 5' external probe (692 bp fragment generated by PCR - kind gift of A. Smith, Institute for Stem Cell Research, UK; **Fig.A1 B-iii,iv**). The endogenous gene was detected as a 13 Kb band whilst the transgene was detected as 4.3 Kb band confirming correct targeting of the *R26* locus in both clones (**FigA2 B**).

GFP expression in targeted ES cell clones

To determine whether the GFP reporter in the R26^{RET9MEN2A} construct could be activated appropriately, the correctly targeted ES cell clones were electroporated with a plasmid encoding Cre recombinase under the control of the CAGGS promoter. Cre-mediated recombination leads to excision of the transcriptional terminator cassette and, consequently, expression of RET9^{MEN2A} and GFP (**Fig.A1 B-v**). Cells were monitored for GFP expression by immunostaining, which showed strong GFP expression exclusively in transfected ES cells (**Fig.A3**).

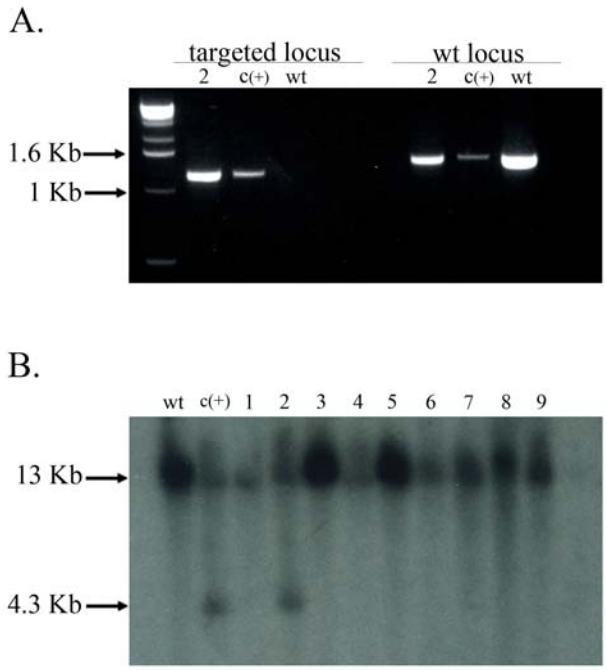


Figure A2 ***Targeting of the R26 locus.***

(**A**) ES cell clones were analysed by PCR using the set of primers F1-PS/R1-PS (amplify a 1.2 Kb diagnostic fragment of the targeted *R26* locus) and F1-PS/R2 (amplify a 1.4 Kb of the wild-type locus). The relative location of the primers is shown in Fig. 7.1 B. Sample 2 represents an ES cell clone carrying the targeted allele (identified by the diagnostic 1.2 Kb fragment) and the wild-type allele (1.4 Kb fragment). Genomic DNA from R26ReYFP transgenics and wild-type mice were used as positive [C(+)] and negative (wt) controls, respectively. As expected, lanes corresponding to the negative control and water were blank when the set of primers that recognizes the targeted locus were used. The molecular weights in kilobases are shown on the left. (**B**) Southern blot of DNA from 9 ES cell lines digested with EcoRI and hybridized with the probe indicated in Fig7.1 B. The 13 Kb band is the wild-type and the 4.3 Kb band represents the targeted allele. ES cell clones 1 and 3-9 are wild-type, while clone 2 is heterozygous for the targeted allele. Genomic DNA from R26ReYFP transgenics and wild-type mice were used as positive [C(+)] and negative (wt) controls, respectively.The expected fragment sizes are indicated on the left.

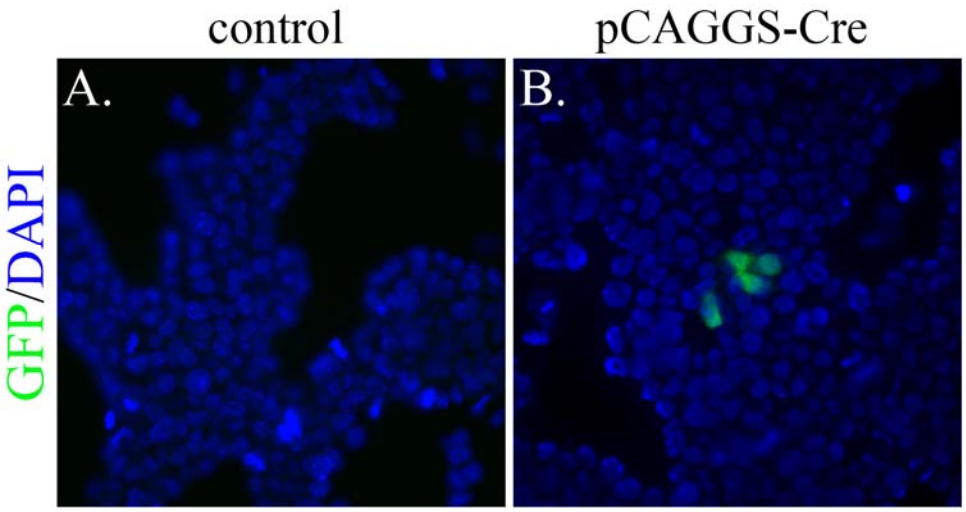


Figure A3 ***GFP is expressed in targeted ES cell clones***

(**A-B**) A correctly targeted ES cell clone was transiently transfected with a plasmid encoding Cre recombinase under the control of the CAGGS promoter. Immunostaining for GFP (green) revealed expression of this protein in the transfected ES cell clone (**A**) but not in the control (not transfected; **B**). Nuclei were labelled with DAPI. x400 magnification.

Generation of R26$^{RET9(MEN2A)}$ transgenics

The correctly targeted ES cell clones were microinjected into (C57BL/6) blastocysts and these were transferred into the uterus of pseudopregnant female [(CBA/CA_XC57BL/10)_F1] mice to produce chimeric mice. Twenty-one chimeras (19 males, 2 females) were generated, mated to F1 animals and the offspring examined for germ line transmission of ES cell DNA, as determined by the presence of agouti coat colour, indicating the successful transmission of ES cell DNA through the germ line. PCR analysis of tail DNA from the first 19 agouti pups has revealed no positive animals.

Discussion

In the ENS, progenitor cells divide during embryogenesis and postnatal stages but, at specific times throughout this period, some stop dividing to differentiate first into neurons, and later, into neurons and glia. Little is known about the mechanisms that regulate the timing and choice of differentiation in the ENS. Studies focusing mainly upon the developing CNS have shown that changes in signal availability are involved in cell type specification (Lillien, 1995; Miller & Gauthier, 2007). In addition, differences among progenitor cells in their competence to respond to extrinsic signals may also contribute to cell fate decisions. In the embryonic forebrain, for example, asymmetric distribution of the RTK EGFR during mitosis generates progenitors with different differentiation responses to the ligand EGF (Sun *et al.*, 2005). Similarly, a difference in the levels of expression of EGFR has also been implicated in biasing cell type choice in the mouse retina (Lillien, 1995). Thus, modulation of cell-surface receptor levels is one

of the molecular mechanisms that determine differences in responsiveness to extracellular cues and influences cell fate decisions.

The RTK RET has been identified as a crucial player in many aspects of ENS development, namely in cell survival, migration and proliferation (Durbec *et al.*, 1996; Natarajan *et al.*, 2002; Taraviras *et al.*, 1999). Whether at certain stages of development RET signalling also plays a role on cell commitment and differentiation is unclear. We wished to explore whether sustained expression of *RET* biases ENS progenitor cells to follow a neuronal fate at the expense of the glial lineage. To this end, we report here a strategy to attain persistent expression of RET in a spatiotemporal controlled manner, i.e in specific cell-types, at defined developmental stages.

To express *RET* ubiquitously and independently from the endogenous *Ret* locus, we targeted the *R26* locus. *R26* has been widely used for expression of various genes in a ubiquitous manner (Friedrich & Soriano, 1991; Soriano, 1999). Modification of the *R26* locus was carried out using established homologous recombination techniques in ES cells, thereby knocking-in the locus the coding sequence of the short isoform of the human c-*RET* gene (*RET9*). The RET9 isoform was chosen based on previous reports demonstrating that unlike RET51, RET9 is necessary and sufficient for normal development of the ENS (de Graaff *et al.*, 2001; Uesaka *et al.*, 2008). Besides wild-type *RET9,* a mutated form of *RET9* – $RET9^{MEN2A}$ - was also used. The MEN2A form of *c-RET9* harbours a single base-pair missense mutation that converts a cysteine residue into arginine (C634R mutation). Such a mutation results in the disruption of an intramolecular disulphide bond leading to ligand-independent dimerisation and constitutive activation of RET proteins. Thus, unlike the wild-type form of RET9,

activation of $RET9^{MEN2A}$ does not rely on the availability of GFLs at different stages of development (Santoro *et al.*, 1995).

Transgenic mice expressing *RET* with the common MEN2A point mutation C634R have been used to study the biological effect of MEN2A mutations in thyroid C-cell tumorigenesis (Michiels *et al.*, 1997; Reynolds *et al.*, 2001). In thyroid C-cells, expression of $RET9^{MEN2A}$ but not wild-type RET9 has been shown to result in the development of medullary thyroid carcinoma, a malignant tumour arising from thyroid C cells, in 8-12 month old mice. In our study, *RET9* and $RET9^{MEN2A}$ are expressed under the control of the ubiquitously expressed *R26* locus. However, expression of *RET9/*$RET9^{MEN2A}$ depends upon Cre-mediated excision of a *loxP*-flanked transcriptional terminator sequence (tpA). Thus, persistent expression of RET can be restricted to the specific cell types depending on the Cre lines used. For the purposes of this study $R26^{RET9}$/$R26^{RET9(MEN2A)}$ transgenic mice will be crossed with the inducible Cre line Sox10iCreER^{T2}. In the progeny of this cross, expression of *RET9/*$RET9^{MEN2A}$ will be restricted to *Sox10*-expressing cells and dependent on 4-OHT administration. Given that *Sox10* is expressed in thyroid C-cells (Deal *et al.*, 2006), development of C-cell neoplasia in Sox10iCreER^{T2}; $R26^{RET9(MEN2A)GFP}$ double transgenics cannot be ruled out. Nevertheless, this approach provides a valuable tool to attain persistent expression of *RET in vivo*, in a temporally and spatially controlled manner and assess its effect on ENS differentiation during development. In particular, it can be used to examine whether persistent expression of *RET* in *Sox10*-expressing progenitor cells prevents them from following a glial fate. For this purpose, analysis of SER93; $R26^{RET9}$/$R26^{RET9(MEN2A)}$ double transgenics may be particularly informative. We have

established a regime for 4-OHT administration that allows targeting of single or small populations of *Sox10*-expressing cells in the gut of SER93 transgenic animals (Chapter IV). By crossing SER93 and $R26^{RET9}/R26^{RET9(MEN2A)}$ transgenics we aim to generate genetic mosaics in which expression of RET9 or the constitutively active $RET9^{MEN2A}$ is restricted to a small population of *Sox10*-expressing cells in a wild-type background. The effect of persistently expressing RET in $Sox10^{+}$ progenitor cells can then be determined by tracing the fate of the targeted progenitors (GFP-labelled) clonally *in vivo*. Identification of the cell types present in each cell cluster will reveal the developmental potential of targeted progenitors. In light of our hypothesis, we predict that most multi-cell clusters derived from *Sox10*-expressing progenitors that persistently express $RET9/RET9^{(MEN2A)}$ will be mainly composed of neurons. Further analysis of these clusters for the presence of different neuronal subtypes will also be carried out. So far, little is known about the mechanisms that control the differentiation and subtype identity of enteric neurons. RET is known to be expressed in differentiated enteric neurons (Young *et al.*, 2003). However, expression of RET in the different enteric neuron subtypes and how this expression may be modulated during development has never been addressed. Therefore, the question as to whether RET signalling is involved in differentiation or maintenance of specific neuron subtypes remains unclear. The approach presented here can provide valuable insight onto the potential role of the RET signalling pathway in the generation of enteric neuron diversity.

Another unresolved question in stem cell biology is to what extent the differentiation programme is hardwired or reversible. This issue has received increased attention recently as the therapeutic value of inducing changes in cell identity and generating

patient-specific cell types has become apparent (Jaenisch & Young, 2008). Our findings that glial cells can generate eNCSCs *in vitro* and give rise to neurons under certain conditions raises the question as to how hardwired the glial identity actually is. During development, expression of *Ret* is downregulated as cells differentiate into glia (Young *et al.*, 2003). Whether downregulation of Ret expression is necessary for the maintenance of "glial identity" is unknown. The effect of persistently expressing *RET* in $GFAP^{+}$ glial cells in which *Ret* expression is normally downregulated can be addressed by following the fate of $GFAP^{+}$ cells *in vivo* in the gut of hGFAPCreER^{T2}; $R26^{RET9}$/$R26^{RET9(MEN2A)}$ double transgenics.

In summary, the genetic system described here aims at attaining persistent expression of *RET in vivo* in a spatiotemporally controlled manner. Analysis of this mouse model should give insight into the mechanisms underlying cell-fate decisions during ENS organogenesis, in particular, the role of RET in the enteric neurogenic-gliogenic switch.

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