SPECIFICATION OF ENTERIC NEURON SUBTYPES IN THE DEVELOPING GUT

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Stockholm 2019
“It is not birth, movement, or death, but specification, which is truly the most important decision in a cell's life.”

Eileen E. Furlong
modified from Lewis Wolpert
ABSTRACT

The enteric nervous system (ENS) is the biggest subdivision of the peripheral nervous system and harbors more neurons than the spinal cord. Its intricate network of ganglia is spanning the entire length of the gastrointestinal wall from where it controls peristalsis, secretion and blood flow. The ENS is able to comply with these functions due to its organization into a full reflex arc. In the adult ENS different subtypes of neurons are defined according to their morphology, electrophysiology, neurotransmitter expression and function. Yet, discrimination of subtypes according to marker gene expression is not reliable as different neurotransmitters are employed by several classes of neurons, and their expressions are not always conserved between species. Developmentally, the ENS arises from the neural crest, a transient cell population that gives rise to numerous cell types throughout the whole body. Neural crest cells find their way into the developing foregut from where they migrate, proliferate and colonize the complete length of the gut. In certain ENS disorders, though, neural crest cells fail to colonize the whole gut leading to a complete absence of neurons in parts of the colon. In other enteric disorders specific enteric neuron subtypes are lost or dysregulated.

The initial progression of neural crest migration and colonization as well as general aspects of neurogenesis and neuronal differentiation are well understood. How different enteric neuron subtypes are specified during development is, however, not resolved. This PhD thesis aspires to reduce this gap of knowledge.

In paper I we demonstrate that the transcription factor Ascl1 is needed for general neurogenesis and gliogenesis in addition to regulating CALB1, TH and VIP neuron subtype specification in the developing ENS.

In paper II we identified a vast array of transcription and signaling regulators expressed in the developing mouse and human ENS. We further show that the transcription factor Sox6 is indispensable for the development of gastric dopaminergic neurons and normal gastric motility.

In paper III we used high-throughput sequencing approaches to redefine the classification of small intestine myenteric plexus neurons. We established lineage trajectories for the identified enteric neuron classes during embryonic development and demonstrate the transcriptional regulation of subtype conversion from NOS1+ ENC2 neurons to CALB1+ ENC6 neurons via expression of Pbx3.

Taken together, the data collected in this thesis describe new regulatory mechanisms governing subtype specification in the developing ENS. This knowledge might help in future endeavors for finding molecular mechanisms in enteric neuropathies in which specific subtypes of enteric neurons are affected. Moreover, it will hopefully guide new cell-based regenerative approaches towards the generation of specific enteric neurons in vitro and in vivo.
ZUSAMMENFASSUNG


Zusammengenommen beschreibt diese Doktorarbeit neue molekularbiologische Mechanismen, die für die Differenzierung von enterischen Nervenzelltypen verantwortlich sind. Dieses Wissen kann in der Zukunft für die Entwicklung von zellbasiierenden Therapien genutzt werden, um abgestorbene Nervenzellen bei neuropathologischen Erkrankungen des ENS zu ersetzen.
LIST OF SCIENTIFIC PAPERS

Ascl1 is required for the development of specific neuronal subtypes in the enteric nervous system
**Journal of Neuroscience**, 2016 April, 36(15), 4339-4350

Transcription and signaling regulators in developing neuronal subtypes of mouse and human enteric nervous system
**Gastroenterology**, 2018 February, 154(3), 624-639

III. Knoflach V*, Morarach K*, Memic F, Linnarsson S, Marklund U
Diversification of enteric neuron lineages in the developing small intestine
Manuscript

*equal contribution
CONTENTS

1 Introduction................................................................................................................................. 1
  1.1 The Gastrointestinal Tract...................................................................................................... 1
  1.2 The Neural Crest .................................................................................................................. 2
  1.3 Cell Sources Contributing to the ENS .................................................................................. 3
    1.3.1 Vagal neural crest cells ................................................................................................. 3
    1.3.2 Sacral neural crest cells ............................................................................................... 3
    1.3.3 SCPs from extrinsic nerves .......................................................................................... 4
    1.3.4 Pancreatic endoderm tissue ......................................................................................... 4
  1.4 Molecular Regulators in Colonization of ENCCs ................................................................. 5
    1.4.1 Signaling pathways ......................................................................................................... 5
    1.4.2 Transcription factors ..................................................................................................... 6
    1.4.3 Extracellular matrix components .................................................................................. 6
  1.5 Development of Enteric Neurons and Glia ......................................................................... 7
    1.5.1 Transcriptional control of ENCC differentiation .......................................................... 8
    1.5.2 Signaling regulators during ENCC differentiation ......................................................... 8
    1.5.3 Gliogenesis .................................................................................................................... 9
    1.5.4 Transient expression of neurotransmitters ................................................................... 9
  1.6 Formation of Networks ......................................................................................................... 10
  1.7 Functionally Distinct Enteric Neurons and Glia ................................................................. 11
  1.8 Enteric Neuropathies ........................................................................................................... 13
    1.8.1 Hirschsprung Disease ..................................................................................................... 13
    1.8.2 ENS disorders with selective loss of NOS1+ neurons .................................................. 14
    1.8.3 Parkinson’s Disease ....................................................................................................... 14
  1.9 Future Treatments for Enteric Neuropathies ....................................................................... 15

2 Results and Discussion .............................................................................................................. 17
  2.1 Paper I ...................................................................................................................................... 17
  2.2 Paper II .................................................................................................................................... 18
  2.3 Paper III .................................................................................................................................. 19

3 Conclusion and Perspective ....................................................................................................... 21

4 Acknowledgements .................................................................................................................... 23

5 References ................................................................................................................................... 25
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>αSYN</td>
<td>αSynuclein</td>
</tr>
<tr>
<td>AIP</td>
<td>Anterior intestinal portal</td>
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<tr>
<td>CIP</td>
<td>Caudal intestinal portal</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<td>E</td>
<td>Embryonic day</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<td>ENC</td>
<td>Enteric neuron class</td>
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<td>ENCC</td>
<td>Enteric neural crest cell</td>
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<td>ENS</td>
<td>Enteric nervous system</td>
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<td>ESC</td>
<td>Embryonic stem cell</td>
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<td>GI</td>
<td>Gastrointestinal</td>
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<tr>
<td>HSCR</td>
<td>Hirschsprung Disease</td>
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<td>ICC</td>
<td>Interstitial cell of Cajal</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>IPAN</td>
<td>Intrinsic primary afferent neuron</td>
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<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cell</td>
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<tr>
<td>NCC</td>
<td>Neural crest cell</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<tr>
<td>PD</td>
<td>Parkinson’s Disease</td>
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<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>SCP</td>
<td>Schwann cell-derived precursor</td>
</tr>
<tr>
<td>scRNAseq</td>
<td>Single cell RNA sequencing</td>
</tr>
<tr>
<td>TC</td>
<td>Transiently catecholaminergic</td>
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<tr>
<td>wt</td>
<td>Wildtype</td>
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1 INTRODUCTION

The wall of the gastrointestinal (GI) tract is home to an extraordinary structure, called the enteric nervous system (ENS), which can assert most of its functions independently from any input of the central nervous system (CNS). Despite its classification as a subdivision of the peripheral nervous system (PNS), the ENS bares functional similarities to the CNS. The sum of neurons and glia in the ENS is greater than in the spinal cord. It is organized into full reflex arcs composed of sensory neurons, interneurons and motor neurons and utilizes the same neurotransmitters/peptides for signal transduction as the CNS. Consequently, the ENS got the popular name “brain in the gut” and is sometimes even denoted “second brain”.

The importance of the ENS is highlighted by the devastating outcomes any deficiency in this system have on our well-being. Gut disorders are affecting a huge amount of the world’s population and in some of these conditions treatment depends on the replacement of damaged or lost enteric neurons. In order to replenish enteric neurons though, we need to know how they are generated during normal development. And this is where we are running into troubled waters. How different subtypes of enteric neurons are generated during development is largely unknown. Therefore, the scope of this thesis was to investigate the specification of enteric neuron subtypes during ENS development.

In this chapter I will discuss the development of the ENS from its origin as neural crest to the final make-up of interconnected ganglia. I will describe identified signaling pathways and transcription factors responsible for the migration and differentiation of enteric progenitor cells as well as I will touch upon enteric neuropathies. Additionally, I will give an outlook on recent developments for cell-based treatment options for ENS disorders.

1.1 THE GASTROINTESTINAL TRACT

The GI tract is responsible for the nutrition and hydration of the body as well as modulation of hormone and immune responses. The formation of the GI tract is conserved among vertebrate species and starts during gastrulation. All three germ layers are involved in the development of the GI tract with the mesoderm giving rise to the smooth muscle layers, the endoderm making up the epithelium and the derivatives of the ectoderm colonizing the gut to form the ENS (Faure & de Santa Barbara 2011). Upon differential growth of the anterior and posterior definitive endoderm invagination is initiated and the anterior intestinal portal (AIP) and caudal intestinal portal (CIP), respectively, are formed (Romanoff 1960). The AIP lengthens posteriorly, whereas the CIP elongates anteriorly eventually leading to the fusion of both tubes. Differential expression of different members of the Hox gene family along the anterior-posterior (AP) axis (Roberts et al. 1995; Bayha et al. 2009) establishes the regionalization of the gut tube into the foregut including pharynx, esophagus and stomach, midgut (small intestine) and hindgut (large intestine). As the posterior foregut dilates and rotates the stomach is formed. The midgut
elongates extensively and rotates and where midgut and hindgut meet a tubular structure forms the caecum (Romanoff 1960).

### 1.2 THE NEURAL CREST

Upon re-arrangement of the three germ layers (ectoderm, endoderm and mesoderm) during gastrulation the dorsal ectoderm forms into the neural plate (Fig. 1A). At the border of the neural plate resides a transient embryonic cell population termed neural crest. Subsequent folding of the neural plate (Fig. 1B) will eventually lead to its fusion, thereby generating the neural tube (Fig. 1C). From this newly formed neural tube the regions of the brain and spinal cord are established (Sadler 2005). The pre-migratory neural crest cells (NCCs) remain at the border of the neural plate during its folding. Once the neural tube closes an epithelial-mesenchymal transition is induced and NCCs migrate away from the neural tube to colonize different regions of the body, including most of the PNS, connective tissue, cartilage, craniofacial bones and melanocytes (Fig. 1D) (Simões-Costa & Bronner 2015).

**Figure 1: Formation of the neural crest.** A) Upon completion of gastrulation the dorsal region of the ectoderm starts to thicken and flatten to form the neural plate. B) The edges of the plate move upwards thereby forming the neural folds. Between the neural plate and the non-neural ectoderm the neural crest forms. C) Eventually the neural folds fuse in the midline of the embryo to form the neural tube. The NCCs delaminate. D) NCCs migrate away from the neural tube to diverse destinations to give rise to distinct cell lineages. Modified from (Simões-Costa & Bronner 2015)

The migratory character of NCCs was demonstrated using the quail-chick marker system. In this system a specific region of an embryo of one species replaces the exact counterpart in a second embryo belonging to a different species. The cells of the two species can hereby be
discriminated due to differential staining properties of the nuclei of chick and quail cells (Le Douarin 1969). With this method a fate map was constructed showing the axial level-specific contribution of subpopulations of NCCs. Cranial NCCs migrate towards the head to form the facial skeleton and cranial sensory ganglia (Noden 1975; Noden 1978; Narayanan & Narayanan 1978). The ENS is derived from vagal and sacral NCCs (Le Douarin & Teillet 1973). The cardiac NCCs populate the pharyngeal arches and cardiac outflow tract (Le Douarin & Teillet 1974; Le Lièvre & Le Douarin 1975). Dorsal root ganglia and sympathetic ganglia are derivatives of the trunk NCCs (Le Douarin & Smith 1988), while parasympathetic ganglia arise from cranial and sacral NCCs. Melanocytes are not linked to a specific level and arise from NCCs from all axial levels (Le Douarin 1982).

Migrating NCCs are controlled by a complex gene regulatory network as they are exposed to constantly changing environments as well as they start their differentiation into diverse derivatives while still migrating. So far though, only a few transcriptional regulators have been identified in the migratory NCCs due to the difficulty in obtaining pure cell populations as the NCCs intermingle with other cell types while migrating to their distant locations. Simões-Costa and colleagues (2014) were recently able to isolate a pure SOX10+ population of migratory cranial NCCs in chick. When they compared the migratory cells with the whole embryo they could identify around 50 transcriptional regulators that were enriched in the migratory cell population.

1.3 CELL SOURCES CONTRIBUTING TO THE ENS

1.3.1 Vagal neural crest cells

The majority of the ENS is derived from vagal NCCs that enter the foregut at embryonic day (E) 9.5 in mice and after 4 weeks of gestation in humans. They migrate from the primitive foregut in a rostral to caudal direction to populate the entire length of the GI tract (Kapur et al. 1992). A recent study by Brunet and his team (2017) revealed two subpopulations of vagal NCCs with differential mode of migration and final destination. NCCs adjacent to somites 1 and 2 differentiated into Schwann cell-derived precursors (SCPs) and colonized the vagus nerve, which guided these cells to the foregut. Only very few cells coming from those SCPs migrated all the way to the colon; most cells only colonized the esophagus and stomach (Fig. 2). On the other hand, the NCC populations adjacent to somites 3-7 migrated along the ventral neural crest pathway colonizing the dorsal aorta and forming the sympathetic chain. A majority of the cells thereby overshoots the dorsal aorta and invades the esophageal wall. This NCC population colonized the entire length of the GI tract (Fig. 2) (Espinosa-Medina et al. 2017).

1.3.2 Sacral neural crest cells

Sacral NCCs (adjacent to somite 28 and lower) contribute to the ENS formation as well. These cells first colonize the pelvic autonomic ganglia at around E11.5 and from there enter the distal
gut to colonize the colon in a caudal to rostral direction (Fig. 2). They only start their migration into the gut mesenchyme once their vagal counterparts have arrived at E14.5. On their caudal-rostro migration they intermingle with their vagal counterpart and are indistinguishable from each other (Burns & Le Douarin 1998; Kapur 2000; R. B. Anderson et al. 2006; Wang et al. 2011). Sacral NCCs are able of normal colonization of the hindgut when vagal NCCs were ablated and even differentiated into neurons and glia in slightly higher numbers compared to control guts, but this increased cell differentiation was not able to make up for the absence of vagal NCC derived enteric neurons and glia (Burns et al. 2000).

The number of NCCs entering the gut is of crucial importance to ensure colonization along the entire length of the GI tract (Burns et al. 2000; Barlow et al. 2008). Once the NCCs reached the gut they are termed enteric neural crest cells (ENCCs). The mode of migration of the ENCCs through the outer mesenchyme is of chain-like characteristics with individual ENCCs being in close proximity to neighboring ENCCs. Different chains exhibit thereby variable speed as well as their trajectories change frequently. ENCCs typically follow the migration pattern of the chain ahead of them (Young et al. 2004). Behind the migratory front subpopulations of cells start to differentiate and slow down their speed before settling down (Baetge & Gershon 1989; Young et al. 1999). A second migration centripetally towards the mucosa leads to the colonization of the submucosal layer (Payette et al. 1984).

1.3.3 SCPs from extrinsic nerves

In 2015 Uesaka et al. demonstrated that SCPs migrating along extrinsic nerves are a major source for postnatal neurogenesis in the gut. These SCPs invaded the small intestinal gut mesenchyme at around E14.5 and migrated towards the submucosal layer (Fig. 2). In the colon invasion occurred two days later and both, the myenteric and submucosal plexus were populated. At this stage, SCPs were marked by SOX10 and BLBP (markers for glia) and did not express any neuronal markers. SCPs consequently differentiated into enteric neurons during postnatal stages. At one month of age up to 5% of submucosal plexus neurons in the small intestine were derived from SCPs, whereas up to 20% of all neurons in the myenteric and submucosal plexus of the colon originated from SCPs (Uesaka et al. 2015).

1.3.4 Pancreatic endoderm tissue

Brokhman et al. (2019) recently identified an endoderm cell population of pancreatic/duodenal epithelium origin, labeled by the marker Pdx1, that gave rise to neurons in the ENS (Fig. 2). Shortly after ENCCs colonized the foregut, PDX1+ cells from the developing pancreatic tissue and duodenal epithelia started their migration towards the gut mesenchyme, where they eventually differentiated into enteric neurons. The highest density of Pdx1-cell derived enteric neurons was detected in the duodenum with around 30% in the myenteric plexus. At more caudal regions the percentage of non-neural crest derived enteric neurons decreased to around
5%. Immunohistochemistry (IHC) analysis of Pdx1 progeny with glial markers were negative, indicating that Pdx1 lineage derivatives only generated enteric neurons.

**Figure 2: Contribution of different cell sources to the ENS.** Vagal NCCs adjacent to somites 1 and 2 enter the foregut via the vagus nerve and colonize the esophagus and stomach (yellow dots). NCCs from vagal levels 3-7 first migrate towards the dorsal aorta and from there invade the foregut and colonize the entire GI tract in a rostral to caudal direction (green dots). Sacral neural crest cells (somites 28 and below) enter the hindgut and migrate in an anterior direction (orange dots). Endoderm cells from the pancreatic/duodenal epithelium give rise to enteric neurons in the duodenum and small intestine (pink dots). Postnatal contribution to the ENS by SCPs migrating along extrinsic nerves innervating the gut (blue dots). FG, foregut; HG, hindgut; MG, midgut; PT, pancreatic tissue. Modified from (Heanue & Pachnis 2007; Espinosa-Medina et al. 2017)

### 1.4 MOLECULAR REGULATORS IN COLONIZATION OF ENCCS

Early markers for ENCCs include the transcription factors SOX10, PHOX2B and ASCL1 (formerly known as MASH1) and the signaling receptors RET and EDNRB. Moreover, components of the extracellular matrix (ECM) are crucial for the colonization of ENCCs.

#### 1.4.1 Signaling pathways

RET and its ligand GDNF, which is expressed in the gut mesoderm, are needed for the survival of ENCCs. Transgenic mice with mutations in either of the two genes display a complete absence of neurons (aganglionosis) in the gut (Schuchardt et al. 1994; Moore et al. 1996; Pichel et al. 1996; Sánchez et al. 1996). Furthermore, GDNF signaling enhances cell proliferation, and upon ablation results in a diminished pool of neural progenitors and eventually leading to hypoaganglionosis (reduced number of enteric ganglia) of the intestine (Gianino et al. 2003). GDNF also functions as a chemoattractant, first guiding the vagal NCCs into the gut and then inducing the rostral to caudal migration (Natarajan et al. 2002).

Another important signaling pathway controlling proliferation of ENCCs is EDNRB with its ligand EDN3. This pathway is critical for the maintenance of uncommitted progenitor cells. Ablation of either component of the pathway leads to colonic aganglionosis owing to premature neuron differentiation in the migratory wave front thereby diminishing the pool of migratory
ENCCs (Barlow et al. 2003). Furthermore, knock-out of Ednrb in NCCs reduced the speed of migration of ENCCs (Druckenbrod & Epstein 2009).

Notch signaling is another essential player in enteric neurogenesis as its expression keeps ENCCs in an undifferentiated state. Impairment of core components of the pathway led to a reduction in ENCC numbers and premature neurogenesis (Okamura & Saga 2008).

1.4.2 Transcription factors

*Sox10* is expressed in all ENCCs, keeps ENCCs in an undifferentiated state and promotes their survival. *Sox10<sup>dom</sup>* mutants show total gastrointestinal aganglionosis. This is due to the fact that *Sox10* is already expressed in premigratory NCCs, meaning without *Sox10* signaling NCCs die before entering the gut (Southard-Smith et al. 1998; Kapur 1999). Over-expression of SOX10 in ENCCs in vitro inhibits their differentiation while keeping their neurogenic and gliogenic potential (Bondurand et al. 2006). In vivo studies, however, revealed the intricate balance of ensuring correct expression levels of *Sox10*, as elevated levels of *Sox10* decreased the proliferation of ENCCs (Nagashimada et al. 2012).

*Phox2b* expression is upregulated shortly after *Sox10* expression in NCCs, continues to be expressed in enteric neuronal and glial progenitors and is even maintained in some mature enteric neurons and glia (Young et al. 1998; Corpening et al. 2008). *Phox2b* is needed for the expression of *Ret* (Leon et al. 2009) and the maintenance of *Ascl1* (Pattyn et al. 1999) in ENCCs. Without *Phox2b* expression ENCCs do enter the foregut, but die shortly after by massive apoptotic cell death (Pattyn et al. 1999).

The proneural gene *Ascl1* is expressed in all precursors of the sympathoadrenal and enteric sublineage and is subsequently downregulated once the cells leave the cell cycle and start expressing pan-neuronal markers (Lo et al. 1991). Loss of *Ascl1* eliminates all sympathetic neurons whereas in the gut only the esophagus is lacking neurons, while stomach and intestine have neurons, though at reduced numbers (Guillemot et al. 1993).

1.4.3 Extracellular matrix components

ECM components play a vital role in the colonization of the gut. Differential expression of ECM constituents along the rostrocaudal axis controls the migration of ENCCs. Fibronectin and tenascin C show higher levels of expression in the caecum and proximal hindgut shortly before ENCCs arrive at these regions. In vitro experiments indicated opposing effect of these ECM proteins with tenascin C inhibiting and fibronectin facilitating migration (Breau et al. 2009). During normal development these opposing effects temporally stall ENCCs when reaching the base of the caecum (Druckenbrod & Epstein 2005). Upregulation of laminin expression in the colon in later stages of development leads to a halt in the invasive character of ENCCs and their consequent differentiation (Druckenbrod & Epstein 2009). It was recently shown that ENCCs themselves secrete the ECM proteins Collagen 18 and agrin. Collagen 18
promoted thereby ENCC migration whereas agrin inhibited migration. Therefore, ENCCs seem to regulate to some extent their own migration (Nagy et al. 2018).

One important aspect of the colonization of the gut is cell-cell adhesion. One of the many cell adhesion molecules expressed on ENCCs is the glycoprotein L1. Blocking of L1 in in vitro cultures of ENCCs disrupted the chain migratory behavior resulting in incomplete colonization. In vivo ablation of L1 revealed only minor changes in cell migration indicating that other cell adhesion molecules account for chain migration as well (Anderson et al. 2006). In vivo studies with transgenic mice lacking β1 integrin showed a delay in normal migration of ENCCs through the gut and defects colonizing the caecum and hindgut leading to colonic aganglionosis (Breau et al. 2006; 2009).

1.5 DEVELOPMENT OF ENTERIC NEURONS AND GLIA

Enteric neurons are born continuously during the colonization of the gut and continue to mature in postnatal stages; up to 2 months in mouse (Laranjeira et al. 2011). Pan-neuronal markers can be seen as early as E10.5 in the developing gut (Baetge & Gershon 1989) and glial precursors are first detectable at E11.5 (Young et al. 2003). A recent study by Lasrado and colleagues (2017) uncovered that lineage traced Sox10+ progenitor cells gave rise to either clones containing both neurons and glia, only neurons or only glia, indicating that ENCCs can be separated into bipotential progenitors and fate-restricted progenitors with either glial or neuronal differentiation capacity. Lineage descendants from all clones could thereby be found in the myenteric plexus, but only clones from bipotential and glial-only progenitors were identified in the submucosal plexus (Lasrado et al. 2017). A new study suggests that neurogenesis is even ongoing in the adult intestine, as neurons that died via apoptosis were replenished by new neurons, keeping overall neuron numbers stable. When mice were treated with a pan-caspase inhibitor, total neuronal numbers in the myenteric plexus were increased. Up to 88% of the neuronal population was thereby replaced in a 2 week period (Kulkarni et al. 2017).

Differentiation into mature neurons is of asynchronous character where neurons expressing different neurotransmitters are born in sequential overlapping waves. Birth dating studies identified serotonergic and cholinergic neurons to be among the first neurons to leave the cell cycle at around E11.5, followed by Calretinin+, CGRP+, TH+ and NOS1+ neurons from E12.5 onwards and neurons expressing ENK, GABA, NPY and VIP have their peak time between E14.5 and E15.5. In general neurons in the submucosal layer are born one to two days later than their myenteric plexus counterparts (Pham et al. 1991; Chalazonitis et al. 2008; Bergner et al. 2014). These birth dating studies can, however, not reveal when specific enteric neuron subtypes are born, as most marker genes are expressed by a multitude of subtypes. The birthdate of certain subtypes of neurons can precede the expression of subtype specific markers by several days (Hao & Young 2009).
1.5.1 Transcriptional control of ENCC differentiation

As Sox10 keeps ENCCs in a proliferative state, it must be downregulated for cells to be able to differentiate (Young et al. 2003; Bondurand et al. 2006). This downregulation is achieved via the expression of Ascl1 and Phox2b, both repressing Sox10, thereby allowing cells to exit the cell cycle and up-regulate pan-neuronal markers (Kim et al. 2003; Nagashimada et al. 2012). In the colon of Sox10Dom mutant mice the proportion of neurons decreases and the proportion of glia increases with increasing extent of aganglionosis. Moreover, the number of CALB2+ neurons in the small intestine was increased in the Sox10 mutant, whereas CALB2+ neurons in the colon decreased. Contrary, NOS1+ neurons were increased in the colon in Sox10 mutant mice. This study indicates a role for Sox10 in ENS development beyond migration and proliferation (Musser et al. 2015).

Another important player in ENS development is Hand2. Over-expression of Hand2 in enteric neuron cultures increased neurogenesis, whereas blocking of Hand2 expression resulted in inhibition of neurogenesis (Hendershot et al. 2007; D’Autréaux et al. 2007; Lei & Howard 2011). Furthermore, Hand2 seems to regulate the patterning of the ENS, as in its absence no ganglia were formed and fewer glial cells were associated with fibers (Lei & Howard 2011).

1.5.2 Signaling regulators during ENCC differentiation

Besides the above-described functions of GDNF in cell survival and migration, the timing and location of GDNF expression influences the number and projections of certain neuronal subtypes, respectively. When GDNF was over-expressed in late stages of development the number of NOS1+ neurons increased, but neurons expressing CHAT or Substance P were unaffected. As CHAT+ neurons exit the cell cycle before E15, but NOS1+ neurons continue to be born until early postnatal stages, the experiment points towards a selective mitogenic effect of GDNF (Wang et al. 2010). Ret expression is needed for the terminal differentiation of ENCCs into neurons, as deletion of this gene resulted in higher numbers of ENCCs on the expense of neurons (Lasrado et al. 2017).

BMP signaling is involved in regulating the development of the ENS and is thought to act on similar cellular processes as GDNF. Over-expression of BMP2 and BMP4 increases the overall number of neurons but has differential effects on enteric neuron subtypes. Neurons that leave the cell cycle early during ENS development like 5-HT+ neurons were increased, whereas neuronal subtypes exiting the cell cycle later (CGRP+ neurons) were decreased (Chalazonitis et al. 2004; 2008).

The development of distinct neuronal subclasses is influenced by the interplay of intrinsic networks of transcription factors and extrinsic signaling factors. Besides these factors it was postulated by Li et al. that the system is to a great extend self-regulatory with early born neurons regulating the fate of later born neurons. Serotonergic neurons influenced the development of dopaminergic, GABAergic and CGRP-expressing enteric neurons in vitro and in vivo (Li et al. 2011).
Although several trophic factors, morphogens and transcription factors have been identified that control early events in ENCC migration, proliferation and differentiation, it is not clear how specific neuronal subtypes are generated and dispersed in the gut. The transcription factor Hand2 was so far identified to be necessary for the development of VIP\(^+\) and NOS1\(^+\) neurons (Hendershot et al. 2007; D’Autréaux et al. 2007; Lei & Howard 2011) and Ascl1 was suggested to regulate the generation of 5-HT\(^+\) neurons (Blaugrund et al. 1996). FGF2 signaling was correlated with the development of secretomotor neurons (Hagl et al. 2013).

1.5.3 Gliogenesis

Sox10 is maintained in enteric glia. Upregulation of Sox10 in ENCCs increased the numbers of glia cells and decreased the neuronal lineage (Nagashimada et al. 2012). In culture, SOX10 expressing adult glial cells can generate functional neurons. In vivo, SOX10\(^+\) glia only generated neurons upon injury. Taken together, these results indicate a neurogenic potential of adult glial cells (Laranjeira et al. 2011). Similarly to Sox10, Foxd3 is expressed in all ENCCs, ensuring their self-renewal and multipotent potential, turned down in cells undergoing neurogenesis and maintained in mature enteric glia (Mundell et al. 2012). Ablation of Foxd3 led to complete aganglionosis in the GI tract (Teng et al. 2008). Upon conditional deletion of Foxd3 in vagal NCCs progeny seemed to be healthy, but closer analysis revealed a severe reduction in glial cell numbers (Mundell et al. 2012).

Besides its maintenance function of the ENCC progenitor pool, Notch signaling is regulating gliogenesis as it promotes the continued expression of glial genes like Sox9 (Taylor et al. 2007). Exposure of ENCCs to the Notch ligand DLL1 switched the neurogenic program to gliogenesis. This switch was irreversible, even upon withdrawal of DLL1, cells continued with glial differentiation and did not acquire a neurogenic capacity again. Thus differentiating neuronal cells could mediate a feedback loop to neighboring undifferentiated ENCCs via the expression of Dll1 to induce gliogenesis (Morrison et al. 2000).

Furthermore, hedgehog signaling which regulates organogenesis of the GI tract as well as ENS development (Ramalho-Santos et al. 2000) is working in concert with Notch signaling. Upon Ptc\(h1\) deletion hedgehog signaling is induced, which in turn induced the expression of Dll1, leading to premature gliogenesis and a reduction of ENCCs (Ngan et al. 2011). A downstream target of Notch is the transcription factor Hes1. During neurogenesis Sox10 is down-regulated by Ascl1 whereas Ascl1 itself is repressed by Hes1. Therefore it is postulated that activation of Notch signaling induces Hes1 expression, which in turn represses Ascl1, thereby Sox10 stays activated and promotes gliogenesis (Charrier & Pilon 2017).

1.5.4 Transient expression of neurotransmitters

Certain neurotransmitters are expressed transiently by immature enteric neurons during ENS development, although the function of this expression is not clear. TH and DBH expression, together with the detection of catecholamine itself, in cells in the mesenchyme of the gut wall,
was first described in the late 70ies. The enzymes were first detected at E11.5 and disappeared after E13.5 (Cochard et al. 1978; Teitelman et al. 1978). Due to their catecholamine phenotype and disappearance at later developmental stages those cells were named transiently catecholaminergic (TC) cells (Teitelman et al. 1981). Although TC cells are proliferating, they do express pan-neuronal markers, indicating that they are immature neurons (Baetge & Gershon 1989; Baetge et al. 1990). Loss-of-function studies indicated a dependence of Ascl1 for the generation of TC cells and consequent serotonergic neurons but not dopaminergic or CGRP+ neurons. This led to the notion of two independent neuronal lineages, one lineage coming from ASCL1+ progenitors, generating TC cells and differentiating into early born neurons such as 5-HT+ neurons and an Ascl1-independent lineage, that does not go through a TC state and gives rise to later born neurons (Blaugrund et al. 1996). A later fate-mapping study revealed though, that only around 30% of serotonergic neurons arise from TC cells. A minor fraction of neurons such as calbindin, neurofilament M and nitrergic neurons arose from TC cells as well (Obermayr et al. 2013).

Another neurotransmitter with transient expression during development is nitric oxide (NO). Even though birth dating studies revealed that NOS1+ neurons leave the cell cycle earliest at E12.5 (Chalazonitis et al. 2008), NOS1+ neurons were detected already at E11.5 (Hao et al. 2010). These early expressing NOS1+ neurons were postmitotic and lineage tracing revealed that around 8% of neurons did express NOS1 transiently during development (Bergner et al. 2014). NOS1 is also transiently expressed at perinatal stages in the submucosal plexus. 50% of all neurons in the submucosal plexus at perinatal stages were immune-reactive for NOS1, whereas in the adult submucosal plexus only around 3% of neurons are NOS1+ (Young & Ciampoli 1998). It is not clear however, if this transient population only express NOS1 once they are in the submucosal plexus, or if they are NOS1+ before their migration from the myenteric to the submucosal plexus.

1.6 FORMATION OF NETWORKS

Individual neurons can be seen close to the migratory front whereas further behind the ratio of neurons to precursors increases rapidly. In this region neurons start to arrange themselves within small ganglia. These ganglia are regularly spaced and have similar cell numbers ranging between 10 to 35 neurons. Glial cells surround each ganglia and fibers with associated glial cells connect individual ganglia with each other (Hackett-Jones et al. 2011). The neurotransmitter composition in each ganglia is thereby evenly distributed throughout the gut (Sang & Young 1996).

Simultaneously with the aggregation of ENS ganglia the extracellular matrix and cells of the gut mesenchyme become concentrically oriented and smooth muscle layers appear (Duband et al. 1993; Newgreen & Hartley 1995). This leads to the formation of the outer longitudinal muscle layer and the inner circular muscle layer with the enteric ganglia sandwiched in the middle (Fig. 3). The resulting plexus is called myenteric plexus and consists of three components: primary plexus, secondary plexus and tertiary plexus. The ganglia and intermodal
strands build up the primary plexus. Branching nerve fibers from the intermodal strands make up the secondary plexus. The tertiary plexus consists of fine nerve bundles that weave in between the primary plexus. The ENCCs that migrated inwards to populate the submucosal layer are forming the submucosal plexus (Fig. 3). The ganglia and intermodal strands are smaller and finer than in the myenteric plexus. The submucosal plexus extends its ganglia over several layers, each containing different populations of neurons depending on the depth. The principal functions of the two plexuses are control of peristalsis (myenteric plexus) and regulating secretion, fluid exchange and blood flow (submucosal plexus) (Furness 2006).

**1.7 FUNCTIONALLY DISTINCT ENTERIC NEURONS AND GLIA**

In the adult small intestine we eventually end up with 16 to 20 enteric neuron subtypes each classified according to their morphology, physiological properties, neurotransmitter and peptide profile and innervation of target tissue. They can be grouped according to their function as either intrinsic primary afferent neurons (IPANs), interneurons or motor neurons (Sang & Young 1996; Sang et al. 1997; Qu et al. 2008; Mongardi Fantaguzzi et al. 2009). Classification of the different subpopulations of neurons in the colon (Sang & Young 1996; Lomax & Furness 2000) and stomach (Schemann et al. 1995; Pfannkuche et al. 1998; Michel et al. 2000) are less complete.

IPANs respond to distension, mechanical stimulation of the mucosa and luminal chemical stimuli, and can thus be seen as the intrinsic sensory neurons of the gut. About 20% of the neurons in the ENS belong to the group of IPANs (Furness et al. 1999). The first evidence for sensory neurons residing in the GI wall came from intracellular recordings in which acid was applied to the mucosa and the response from nearby neurons was recorded (Kunze et al. 1995). Soon after IPANs responding to stretch were identified (Kunze et al. 1998). Besides signaling to interneurons and motor neurons IPANs also assert their function through communication with interstitial cells of Cajal (ICCs). Upon the stimulation of IPANs calcium transients in nearby ICCs were increased, which in turn increased the electrical slow wave activity, both
orally and anally, thereby changing the propulsive activity of the muscle cells into a mixing motion (Zhu et al. 2014). Not long ago the microbiota was linked to motility in the gut. Germ-free mice displayed reduced excitability of IPANs compared to control animals. These effects were reversed when microbiota were introduced for 4 weeks (McVey Neufeld et al. 2013). The reduced excitability might be due to decreased numbers of CALB1+ IPANs in the myenteric plexus of germ-free mice (McVey Neufeld et al. 2015).

The majority of motor neurons have their cell bodies in the myenteric plexus from where they project their axons to the longitudinal and circular smooth muscle layer and are responsible for peristalsis. These neurons are either excitatory motor neurons, implementing their contractile force via the use of acetylcholine and tachykinin, or inhibitory neurons, utilizing a multitude of neurotransmitters including NO, VIP and ATP to relax the muscle (Sang & Young 1996; Sang et al. 1997; Qu et al. 2008). Excitatory motor neurons extend their axons orally and inhibitory motor neurons anally. The muscularis mucosae is innervated by motor neurons that have their cell bodies in the submucosal plexus. Additionally, the submucosal plexus harbors at least three classes of secretomotor neurons and one class of vasodilator neurons responsible for fluid exchange across the mucosa and blood flow, respectively (Furness 2006; Mongardi Fantaguzzi et al. 2009). For proper motor neurotransmission in the gut more cell types than motor neurons and muscle cells are needed. Analysis of mutant mice, in which soluble guanylyl cyclase, the main target of NO, was deleted in either ICCs or smooth muscle cells, revealed less pronounced relaxation effects. In double knock-out animals nitrergic response was completely abolished, indicating the necessity of both cell types for neurotransmission in the ENS (Groneberg et al. 2011; 2013; 2015).

Most interneurons reside within the myenteric plexus and can be grouped into ascending and descending interneurons. Their distribution in different regions of the gut varies greatly, though. Ascending interneurons mainly utilize acetylcholine and tachykinines (Johnson et al. 1996; 1998), whereas descending interneurons are labeled either by NOS, CHAT or both (Porter et al. 2002). ATP is employed as a neurotransmitter by descending interneurons and is co-released with NO (Ren & Bertrand 2008). Interestingly, Mazzuoli and Schemann (2009; 2012) discovered that mechanosensitive neurons were multifunctional, combining features of sensory neurons, interneurons and motor neurons.

Analysis of enteric glia using lineage tracing and single cell-labeling revealed the presence of four morphologically distinct subpopulations of glia with different physiological properties and distribution along the serosa-mucosal axis. This study also revealed that there is no pan-glial marker labeling all enteric glia, although SOX10 expression can be detected in most enteric glia (Boesmans et al. 2015). Rao et al. (2015) used RNA sequencing to compare enteric glia to other types of glia and found that enteric glia are very distinct from any other class of glia. Surprisingly enteric glia expressed many genes associated with myelinating glia, although myelination is not reported in the ENS. For a long time enteric glia were believed to only supply neurotrophic support and getting activated upon injury, but recent studies attest enteric glia an active role in neurotransmission (McClain et al. 2014). In the mouse adult ENS enteric glia and
neurons are present in similar numbers, whereas in humans 7 times more glia than neurons can be found in the gut (Gulbransen & Christofi 2018) indicating a more prevailing influence of glia on enteric neuron function in humans.

Together these different types of neurons and glia make up the intrinsic reflex circuit of the gut that is working to a great extent autonomously from the input of the CNS. There is, however, an extensive extrinsic innervation by neurons of the sympathetic and parasympathetic nervous system (via the vagus and pelvic nerves) as well as dorsal root ganglia sensory neurons (Uesaka et al. 2016).

Our lab recently generated RNA transcriptomes of 10 000 myenteric ENS cells that formed part of a mouse nervous system cell atlas in collaboration with Sten Linnarsson’s group (KI, Sweden). Our ENS neurons clustered into 9 subtypes when compared with neurons from for example brain, spinal cord, sympathetic ganglia and dorsal root ganglia (Zeisel et al. 2018). This analysis questions the previous categorization of enteric neurons, but further studies, including functional experiments, will be needed to elucidate how many functional enteric neuron types there are in the ENS. In this thesis we investigated those sequenced enteric neurons further (paper III).

1.8 ENTERIC NEUROPATHIES

Disorders that affect the ENS range from congenital and neurodegenerative to inflammatory diseases. These disorders can either lead to the total absence of neurons in distal parts of the gut (Hirschspring disease (HSCR)) or selective neuronal populations are lost (achalasia and hypertrophic pyloric stenosis). Furthermore, a huge proportion of the western population shows signs of irritable bowel syndrome, a disease that is not well understood yet, but most likely involves ENS malfunction.

1.8.1 Hirschsprung Disease

HSCR is the most well described congenital gut disorder identified in humans. This disease is characterized by the absence of neurons in the most distal part of the colon resulting in intestinal obstruction and distension of the colon (megacolon) preceding the aganglionic part. Two different severities of HSCR are defined: short-segment HSCR affecting the rectum and sigmoid colon (80% of cases) and long-segment HSCR in which the aganglionic part extends to various lengths of the proximal colon with 5% of the patients showing total colonic aganglionosis. It affects 1 in 5 000 live births, with a male:female ratio of 4:1 for short-segment HSCR, but approaching a ratio of 1:1 the longer the aganglionic part becomes (Gariephy 2004). In most cases HSCR is sporadic but up to 20% of the cases are inherited (Mc Laughlin & Puri 2015).

One of the first genes linked to HSCR was RET (Lyonnet et al. 1993; Angrist et al. 1993). Between 15-35% of sporadic and 50% of familial cases present a mutation in the RET gene
Mutations in the RET ligand GDNF have also been identified (Angrist et al. 1996). Further studies revealed mutations in EDNRB (Puffenberger et al. 1994), EDN3 (Edery et al. 1996; Hofstra et al. 1996), SOX10 (Pingault et al. 1998) and PHOX2B (Benailly et al. 2003) in patients presented with HSCR.

Common treatment options include the resection of the aganglionic segment of the bowel and conjoining the normally innervated colon with the anus. Nevertheless, even after successful surgery, most children suffer from life-long fecal incontinence and constipation (Wester & Granström 2017). Therefore, novel treatment options are needed to increase the quality of life for these patients.

1.8.2 ENS disorders with selective loss of NOS1+ neurons

There are several ENS disorder with a selective loss of NOS1+ neurons including achalasia, Chaga’s Disease, hypertrophic pyloric stenosis and diabetic gastroparesis. The reason for the selective vulnerability of NOS1+ neurons might be that NOS is activated by Ca2+, which is increased upon cell stress. An increased production of NOS leads to an increase in NO, which can cause cell damage (Rivera et al. 2011). NOS1+ neurons are mainly inhibitory motor neurons responsible for the relaxation of the muscle during peristalsis and for relaxing the sphincters. Therefore, loss of NOS1+ neurons leads to an imbalance of excitatory and inhibitory signaling resulting in diminished relaxation.

Achalasia, Chaga’s Disease and diabetic gastroparesis manifest themselves primarily in adulthood and can result in enteric neurodegeneration and/or neuronal dysregulation. Pharmacological drugs, like Botulinum toxin A, can stop contractions and are used as a first line of treatment for Achalasia and Chaga’s Disease. Long-term treatments for achalasia involve surgery in which the sphincter gets manually dilated or the muscles in the sphincter are cut (Boeckxstaens et al. 2014). For surgical treatment of megacolon colorectal anastomosis or rectosigmoidectomy is performed (Pinazo et al. 2010). Prokinetics can help with improving gastric emptying in diabetic gastroparesis (Vannormelingen et al. 2013). Hypertrophic pyloric stenosis mainly occurs in infants, pointing towards an immaturity problem. Surgical opening of the pylorus is in most cases enough and result in spontaneous re-innervation with similar neuronal compositions compared to normal pylorus (Vanderwinden et al. 1996).

1.8.3 Parkinson’s Disease

Parkinson’s disease (PD) is a neurodegenerative disorder characterized by the loss of dopaminergic cells in the substantia nigra of the ventral midbrain and aggregated αSynuclein (αSYN) containing inclusion bodies (Lewy bodies) in the remaining neurons. Besides the typical symptoms of tremor, rigidity and slowness/difficulty of movement, there are a number of non-motor symptoms, amongst others impaired smell, constipation and gastric dysmotility. These non-motor symptoms manifest themselves several years before the onset of any motor symptoms (Hawkes et al. 2009).
As post-mortem studies showed Lewy bodies in the ENS, but not all patients displayed motor symptoms, Braak formulated a novel mode of pathogenesis for PD. He postulated that a potential pathogen is taken up by the nose or mouth and swallowed with the saliva. Once in the stomach it crosses the stomach wall and invades the ENS. From there it migrates in a retrograde fashion up the vagus nerve from where it spreads to further areas in the brain (Braak et al. 2003; 2006). Support for Braak’s hypothesis comes from in vivo experiments in mice in which rotenone, administered intragastrically, led to αSYN accumulation in the ENS, the dorsal motor nucleus of the vagus and substantia nigra (Pan-Montojo et al. 2010). A recent study in patients who underwent vagotomy showed that the subsequent risk for developing PD is lower, indicating that the vagus nerve plays a critical role in PD progression (Svensson et al. 2015). In line with the previous studies, Holmqvist et al. (2014) showed that human PD brain lysate injected into the gut wall of mice directed a retrograde transport of αSYN via the vagal nerve to the dorsal motor nucleus of the vagus in a time-dependent manner. Further research will be necessary to elucidate the exact mechanisms by which αSYN is spread from the gut to the CNS.

1.9 FUTURE TREATMENTS FOR ENTERIC NEUROPATHIES

Life-long complications even after successful surgeries call for an exploration of new treatment options for enteric neuropathies. The most discussed alternatives are cell replacement therapies, in which ENCCs or enteric neurons are transplanted into the gut to replenish lost neurons. Plausible cell sources range from ENCCs harvested from embryonic or postnatal gut, other neural crest derived stem cells or CNS stem cells to embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs).

In mouse ENCCs can easily be isolated via the use of genetic labeling of the progenitor population and consequent sorting of cells. Several studies have shown the transplantation and integration potential of these cells in mice (Hotta et al. 2013; Cooper et al. 2016; McKeown et al. 2017; McCann et al. 2017). No genetic labeling can be employed for the isolation of ENCCs of human origin. Thus, antibodies against the cell surface marker gene p75 are used for the isolation of fetal and postnatal human ENCCs. Recently their successful transplantation into the distal colon of immune-deficient mice was shown. Transplanted cells integrated into the existing ENS network, forming interconnections between existing cells and ganglia, and expressed mature markers for neurons and glia (Cooper et al. 2017). Rollo et al. (2016) isolated ENS cells from the ganglionic region of resected colon from HSCR patients. The aganglionic region of the same resected colon was cultured as an explant and consequently isolated ENS aggregates were cocultured with the explants. ENS cells spread over large parts of the aneuralonal colon and differentiated into mature neurons and glia. Even though, these initial experiments highlight the capacity of human fetal and postnatal isolated ENS cells for transplantation into aganglionic colon, the big hurdle of reaching appropriate numbers of cells that can colonize the entire aganglionic gut still remains.

In order to increase the number of starting cells that can be transplanted into the gut, researchers are deploying ESCs and iPSCs strategies to generate neural crest derivatives. Initial
experiments showed the differentiation of human pluripotent stem cells (PSCs) into neural crest precursor cells that can be further differentiated into peripheral neurons, smooth muscle cells and melanocytes (Lee et al. 2007; Chambers et al. 2013). In 2016 Studer’s group was able to generate enteric neurons from human PSCs. For the specific generation of ENS and no other peripheral neuron type, the differentiation has to be steered towards a more caudal neural crest fate via the use of retinoic acid. The generated cells differentiated into mature neurons expressing numerous neurotransmitters typically found in enteric neurons, but not other neural crest lineages. They went on to show that these cells can be transplanted into the mouse colon, where they migrated extensively to eventually repopulate the entire host colon (Fattahi et al. 2016). Recent adaptations to the protocol lead to a complete defined and xeno-free differentiation of human PSCs; an important step for later use in clinical trials (Barber et al. 2019). Other researchers also successfully generated enteric neurons from iPSCs and even engineered intestinal organoids with a functioning enteric innervation (Workman et al. 2016; Schlieve et al. 2017). So far the composition of the produced neurons is arbitrary. For regenerative purposes a balanced constitution of neurons is, however, necessary to regain functionality of the gut. Furthermore, no guiding cues have been identified that can steer PSCs into specific subclasses of enteric neurons.

Esophageal achalasia might provide a good first model for cell-based regenerative strategies as the target area is restricted to the lower esophageal sphincter. Generated cells can therefore be delivered endoscopically. Moreover, only one type of neuron is lost and has to be replaced (Burns et al. 2016).
2 RESULTS AND DISCUSSION

2.1 PAPER I

Even though the right cell composition of the ENS is crucial for its proper function, very little is known about the transcriptional network necessary to diversify ENCCs into functionally distinct neuron subtypes. The pro-neuronal transcription factor Ascl1 was previously detected in ENCCs and neurogenesis was found reduced in Ascl1-/- mutant animals (Lo et al. 1991; Guillemot et al. 1993). Furthermore, Ascl1 was implicated in the formation of 5-HT+ neurons in the ENS (Blaugrund et al. 1996). In this paper we sought to make a thorough study of the transcription factor Ascl1 during the development of the ENS.

ASCL1 was expressed in the majority of NCCs entering the foregut at E9.5 and throughout development ASCL1 was mainly detected in the SOX10+ progenitor population. As not all SOX10+ cells co-expressed ASCL1, we investigated if only certain neuronal subtypes are generated from ASCL1+ ENCCs by using the inducible transgenic mouse line Ascl1CreERT2/+ x R26ReYFP. All subtype markers, that are expressed at E18.5, showed co-expression with YFP, indicating that all enteric neuronal subtypes are generated from ASCL1+ progenitors. Also, enteric glia express ASCL1 and our tracing study showed YFP expression in S100+ and BLBP+ glial cells, demonstrating the generation of enteric glia from ASCL1+ progenitors.

Next, we analyzed the guts of Ascl1-/- mutant embryos. Loss of Ascl1 led to a delayed onset of HUC/D expression by 3 days and reduced numbers of neurons in the entire length of the GI tract, albeit most drastically in anterior parts of the gut. As Ret and SOX10 were readily detectable at early embryonic stages in the mutant embryo, migration of ENCCs does not seem to be impaired and there was no sign of increased cell death. The amount of progenitor cells going through the cell cycle were, however, reduced. Therefore, our study suggests that ENCCs do reach the gut and colonize it at normal rates, but are only slowly dividing or are temporarily stalled in the cell cycle. By counting the proportion of SOX10+ cells that also co-express the mature glial marker S100, we saw a reduction of enteric glia in the distal part of the intestine at E18.5 in Ascl1-/- mutant embryos compared to wildtype (wt) controls.

To evaluate if the generation of specific neuronal subtypes is affected in Ascl1-/- mutants, we counted the percentage of neurons expressing a certain subtype marker. The ratios of CALB1+ (stomach and small intestine), TH+ (stomach) and VIP+ (stomach) neurons were drastically reduced in the mutant compared to wt control. NOS1+ neurons were increased in numbers in the small intestine. Interestingly, the ratio of neurons expressing 5-HT was not differentially expressed in the mutant.

As neurogenesis was impaired in Ascl1-/- mutants, the reduction of neuronal subtype markers could be attributed to the delay in marker expression. To exclude this potential confounding factor, we analyzed Ascl1KINgn2 mutant animals, in which the coding region of Ascl1 is replaced by another proneuronal gene Ngn2. In these animals neurogenesis was rescued in the CNS, but
subtype differentiation was still impaired (Parras et al. 2002). $\text{Ascl1}^{\text{KINgn2}}$ mutant embryos showed similar numbers of HUC/D$^+$ enteric neurons at E12.5 as $\text{wt}$ controls and at E18.5 the same number of neurons could be found along the entire length of the gut in mutant and $\text{wt}$ control embryos. Proliferation was rescued as well. Therefore, $\text{Ngn2}$ seems to counteract the loss of $\text{Ascl1}$ and restores normal neurogenesis. The percentage of enteric glial cells, however, was still impaired in the posterior part of the GI tract. Similarly, the same subtype markers as in the $\text{Ascl1}^{-/-}$ mutant (CALB1, TH and VIP) were reduced in the $\text{Ascl1}^{\text{KINgn2}}$ mutants compared to $\text{wt}$ controls. This indicates the necessity of $\text{Ascl1}$ for the specification of certain neuronal subtypes.

2.2 PAPER II

The goal of paper II was to identify transcription factors and signaling pathways that regulate the diversification of neurons during embryonic development of the ENS. Previous studies indicated that enteric neurons expressing different subtype markers are born sequentially during embryonic and early postnatal development (Pham et al. 1991; Chalazonitis et al. 2008; Bergner et al. 2014). We therefore hypothesized that the transcriptome of ENS cells at distinct developmental time points should reflect this differentiation potential.

We subjected progenitor cells, the whole ENS population and non-ENS gut cells from developmental time points E11.5 and E15.5 to a microarray analysis to determine their RNA expression. To this end we conducted pairwise comparisons between the 6 cell populations to identify genes that are differentially expressed. After thorough investigation of the RNA expression in online in situ hybridization resources, we selected 31 transcription factors for an in depth IHC analysis in mouse and human embryonic gut tissue. We were able to categorize the transcription factors into four groups based on their spatiotemporal expression patterns. Group I included transcription factors that were expressed early and abundant, indicating roles in neurogenesis. Transcription factors belonging to group II and III were mainly confined to HUC/D expressing neurons and showed early and late onset, respectively. These genes have probably roles in neuronal differentiation. Transcription factors with highly selective expression dynamics were categorized into group IV and those are most likely responsible for neuronal subtype differentiation. Staining of human embryonic tissue revealed similar expression patterns of the transcription factors compared to mouse tissue, demonstrating their conservation across species. We subsequently co-stained our identified transcription factors with known neurotransmitters/peptides. Most transcription factors were co-localized with all tested marker proteins, but 10 transcription factors showed selective expression patterns with only a subset of marker proteins.

One identified transcription factor with highly selective expression dynamics was SOX6. SOX6$^+$ cells could only be found in the stomach and were co-localized in neurons expressing either TH, CALB1 or NPY. Double staining of TH, NPY and CALB1 revealed that NPY and CALB1 are expressed in the same cells and are therefore most likely labeling one gastric subtype and TH$^+$ neurons define another gastric subtype. We next analyzed $\text{Wnt1Cre x Sox6}^{\text{fl/fl}}$
embryos, in which Sox6 is specifically deleted in neural crest derivatives. The expression of CALB1 and NPY was not affected in the mutant compared to wt littermates, whereas TH+ neurons were drastically reduced in mutant embryos and this decrease in TH+ neurons remained in adult Sox6 mutant mice. Functional studies in Sox6 mutant animals revealed the importance of TH+ neurons for gastric motility.

Similar to the transcription factors, we analyzed the expression of signaling factors and receptors. Our screen identified various new signaling components of already known signaling pathways including BMP and WNT signaling. Furthermore, we detected components of 9 signaling pathways not previously described. Some of the signaling factors were found to be produced by ENS cells, indicating autocrine/paracrine signaling mechanisms.

2.3 PAPER III

For the past two decades researchers have categorized enteric neurons into around 16 different subtypes according to their neurotransmitter/peptide expression, morphology and function (Sang & Young 1996; Sang et al. 1997; Qu et al. 2008; Mongardi Fantaguzzi et al. 2009). However, overlapping and species-specific expression of marker proteins prevented their unequivocal use to distinguish functionally distinct neurons.

In this study we used single cell transcriptomics to define enteric neuron subclasses in the myenteric plexus of juvenile mice according to their molecular architecture. 727 neurons were clustered into 6 molecular distinct classes, denoted Enteric Neuron Class (ENC)1-6. ENC1 and 4 correspond to excitatory motor neurons and ENC2 relates to inhibitory motor neurons. ENC3, 5 and 6 express genes traditionally linked to IPANs.

To better understand the emergence of ENC1-6 during embryonic development we performed single cell RNA sequencing (scRNAseq) of the small intestine ENS at E15.5 and E18.5. Our data clearly showed progenitor cells at different stages in the cell cycle, cells undergoing neurogenesis and lastly immature neurons that were branching off into two main trajectories (ENC1 and 2) and that were differentiating further into their final cell types (ENC3-6). This analysis revealed, that instead of emerging from 6 distinct stem cell pools, progenitor cells only take on either one of two identities once neurogenesis is completed and further mature in that identity or convert into the remaining enteric neuron lineages.

Interrogation of the scRNAseq data revealed that the expression of the transcription factor Pbx3 coincided with the conversion of ENC2 to ENC6. We therefore hypothesized that Pbx3 is involved in the lineage specification of this putative IPAN subclass. IHC analysis showed expression of PBX3 at all developmental time points in HUC/D+ cells. Double staining of PBX3 with known neurotransmitters/peptides revealed the absence of PBX3+NOS1+ neurons at E18.5, whereas double positive cells were clearly visible at E15.5. As Pbx genes are known to work in concert with other transcription factors from the same gene family we verified the expression of MEIS1. MEIS1 was similarly expressed in the developing ENS and was co-expressed in PBX3+ neurons, as well as it was excluded from NOS1+ neurons at late embryonic
stages. This made us speculate whether expression of PBX3 together with MEIS1 leads to the
downregulation of NOS1 and consequent conversion of neurons to class ENC6.

To test this, we investigated the effects of loss of Pbx3 in the developing ENS. In the guts of
Pbx3−/− mutant embryos the ratio of neurons expressing NOS1 was increased compared to wt
controls. GAL+ and VIP+ neurons, labeling the same class of neurons as NOS1, were also
upregulated. CALB1+ neurons (marker for ENC6) on the other hand were reduced in numbers
in the mutant compared to wt control. Our data signifies the necessity of Pbx3 for the
differentiation of ENC2 cells into the ENC6 phenotype.
3 CONCLUSION AND PERSPECTIVE

In the presented thesis we investigated the specification of enteric neurons during ENS development.

In paper I we showed that neurogenesis and gliogenesis are dependent on Ascl1 expression. Our study recognized that even though all enteric neurons and glia are generated from Ascl1+ progenitors, only specific subtypes of neurons expressing CALB1, TH and VIP require Ascl1 for their specification. Notably, our study contradicts the earlier notion that generation of 5-HT+ neurons depends on Ascl1 expression.

In paper II we identified a plethora of new transcription and signaling factors with spatial and temporal expression during mouse and human ENS development with potential roles in stem cell maintenance/neurogenesis, neural specification/differentiation or circuit assembly. Interestingly, the detection of signaling ligands in the ENS itself suggests a self-regulatory mechanism in which early-born neurons regulate the diversification of later-born neurons.

Our study revealed the first transcription factor linked to the generation of one specific enteric neuron subtype. The generation of dopaminergic neurons in the stomach depends on the expression of Sox6. This indicates that our atlas is a rich resource of genes with regulatory functions in ENS development.

In paper III we propose a new classification of enteric neuron subtypes in the myenteric plexus. Analysis of scRNAseq data from juvenile mice established the presence of 6 mature neuron subtypes, denoted ENC1-6. Subsequent scRNAseq analysis of embryonic gut tissue elucidated the developmental trajectories from which those 6 subtypes emerge.

In our search for transcription regulators controlling the diversification of neurons we discovered Pbx3 as a potential candidate. Our study indicates that expression of Pbx3 in a subset of cells in class ENC2 is initiating their differentiation into neurons belonging to ENC6; probably by repressing Nos1.

Besides expression of Pbx3 in ENC6, our scRNAseq analysis unveiled expression of Pbx3 in another putative IPAN subclass, ENC5. Due to staining difficulties and antibody incompatibility no assessment could be made on whether Pbx3 is similarly regulating the conversion of subsets of neurons in ENC1 to neurons belong to ENC5.

Our classification of the juvenile myenteric plexus is based on only 727 neurons. Analysis of scRNAseq datasets with higher cell numbers will likely reveal subclasses of enteric neurons within the 6 major ENCs identified in this study. This will hopefully also uncover the presence of different interneuron subtypes, something that is lacking in this classification of neuronal subtypes in the ENS probably due to the low proportion of these neurons compared to the entire ENS.
We limited our scRNAseq analysis of the juvenile ENS to neurons in the myenteric plexus as those cells are more easily dissociated. It will be interesting to see how many more neuronal classes can be found in the submucosal plexus. This is especially noteworthy in relation to the embryonic data sets, in which no discrimination between the two plexuses during dissociation was possible.

We are currently establishing an *in utero* over-expression system in mouse to see if ectopic expression of *Pbx3* is sufficient to re-program ENS cells into ENC6 neurons. Almost certainly the effect will be dependent though on the expression of co-factors, like *Meis1*.

In paper I we speculated that lack of *Ascl1* could lead to a fate switch of CALB1*+* neurons to NOS1*+* neurons. In light of the new scRNAseq data, it could be that, similarly to *Pbx3*, *Ascl1* is necessary for the conversion of ENC2 neurons to ENC6 and, in the absence of *Ascl1*, neurons in ENC2 are halted.

Together, paper II and III disclosed a huge array of possible determinant genes with potential roles in specification of enteric neuron subtypes. iPSC technologies could be deployed to test if these regulators can steer iPSCs towards a specific enteric neuronal lineage. Signaling factors could simply be added to the differentiation medium. Transcription factors would need to be conditionally introduced into iPSCs to assure their expression at the correct time during the differentiation protocol.
4 ACKNOWLEDGEMENTS

Ulrika thank you for giving me the opportunity to explore the unknowns of ENS development. From day one you granted me a lot of trust to develop freely and become the researcher I am today. Thank you for always having an open door to discuss really anything.

Ernest thank you for our fruitful discussions about PD in the gut, even though the project failed in the end. Thank you for all the nostalgic and hilarious stories at lunch/fika about the old days of MolNeuro.

Fatima what would I do without you?! You guided me from day one and showed me all the ropes around here. Thank you for your patience, I can’t stress that enough!!! Thank you for being an amazing travel companion (I will never forget our gluttony in Kuala Lumpur) and of course for feeding me your mind-blowing cakes.

Josefin and Katarina, for being the best animal care takers one can wish for, for doing things without me even asking and being kind to me if I send impossible last-minute requests; due to your dedication this thesis was possible.

My old office mates; Carolina, for all the entertainment that comes from sitting next to you and for always motivating me in the gym, Daniel, for always criticizing my illustrator skills (this kappa is a testament to your absence while preparing my figures) and for all the times you fixed my bike, Martin, for our ski trips in Trysil, the ice cream chats in the summer and for leaving parties as early as me, Sam, for your patience with the loud chats going on in the office, Jose, for your absentmindedness and being completely startled, when we start talking to you.

My new office mates; Tony, for cheering me up when I struggled in the lab, all the below the belt jokes and let’s not forget your beauty, Emilia, for our endless conversations in the office, that drive everybody else crazy, and starting the Pub quiz tradition, Puneet, for enduring us gossip girls and joining in every now and then.

My flat mates; I don’t know how I can ever thank you for sharing apartments with me; you’ve seen me at my worst, you endured me bossing you around and you tolerated my weirdness, and somehow you all still like to spend time with me (well, I guess feeding you with sweet treats helps). Karol, there are not enough pages in this thesis to thank you. It all started with you agreeing to share an apartment with me and Willy and from then on it was just one big blast. Thank you for being an amazing office mate, talented squash teacher, uncertified psychotherapist, most grateful dinner guest and chirpy movie watching companion. Willy, for your remarkable cooking skills and giving me a hard time baking something for you without bananas and pears and other random things in it (but who am I to talk), Lars, for our insect-documentary-watching evenings, your exclusive knife-sharpening service and fixing my bike endless times, Dennis, for your guitar sessions and being there for me when my aunt died, David, for all the hour-long talks starting the second before I want to go to bed, your talent for blocking the shower just before I come home sweaty from the gym and for fixing my bike,
Markus, for sharing Mohnstrudel with me, keeping my Austrian dialect in a somewhat acceptable state and fixing my bike, Anne, for going to Gröna Lund and Parkteatern with me, Ka Wai, for your delicious baked goods and your amusing reactions while watching movies.

Rest of MolNeuro; Dagmara, for joining me in the craziness of catching free breakfast/water bottles/towels in SATS, couldn’t say it any better than: sooo awesome, you are the best, Hermany, for our gym times and all your tries getting me into clubbing (and being the only one that succeeded once or twice), Camiel & Alejandro, for the chaotic cooking and game evenings, Mandy, for lunches at 12 sharp and bringing the best board games to MolNeuro games night, Simone, for the 5am chats, Jana, for never getting annoyed when I ask for antibodies, Ivar, for the amusing lunches and apple supply, Lauri, for the provoking lunch conversations and joining the baking club, Emelie, for the fun times in Trysil, Ana F. & Anneke, for the lunches and theater visits, Elisa, for the lunches, Enrique, for helping me with my laptop issues, Alca, for worrying with me about Karol, Alessandro, for your help in the lab and your sarcastic comments about everything, and all the other people, past and present, that make MolNeuro so delightful to work in.

Katrin M., for being my favorite collaborator, Silke, Paula & Lora, for our struggles finding a time when everybody can meet for dinner, Theresa & Tom, for the Christmas and Easter dinners and the Christmas cookie baking lunacy, Moritz & Katrin K., for understanding my cravings for sweet main dishes and preparing them for me, and fixing my bike (yes I am repeating myself, but if you are as unskilled as me, you need to have a handy man in every corner of the city), Giuseppe, for letting me sometimes win at squash, Mateusz, for preferring me over your wife as work-out buddy in MA Circuit, Dieke, for trusting me with a cactus, even though you have seen so many other plants die in our apartment (it is still alive!).

Anna-Maria, for being my first Swedish friend (showing me it is possible), for all the travels and New Year’s Eves, Magdalena, for our swimming sessions, Birgit, for starting the whole Sweden adventure with me all those years ago, Claudia, for our endless walks through Stockholm while trying to have a conversation in Swedish.

And most importantly my family; Matthias, dass du mich daran erinnerst, dass ich mir das letzte bisschen Hausverstand auch noch heraus studiert habe und mich darauf hinweist Tirolerisch zu sprechen, Christoph, für all die Besuche hier in Schweden, unser Schi fahren daheim und unser gemeinsames Kochen. Du warst zwar schneller mit deinem Bachelor und Master Abschluss, aber der Doktor gehört mir, Mama & Papa, dass ihr mich von Anfang an unterstützt habt und eure erste Reaktion immer war: dorthin kommen wir dich doch gerne besuchen. Danke, dass ihr mir immer zuhört und mich aufmuntert, wenn ich mir meine Zehe verstaue, meine Kontaktlinse ruiniere oder einfach nur mit der Gesamtsituation unzufrieden bin, und dass ihr mir nur manchmal zu verstehen gebt, dass ich doch noch nicht so ganz lebensfähig bin.
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