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Ultrasound-guided *in utero* lentivirus transduction of the murine nervous system to investigate nervous system disorders

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Ultrasound-guided in utero lentivirus transduction of the murine nervous system to investigate nervous system disorders

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To my parents

ABSTRACT

Neurodevelopment is a highly dynamic process involving hundreds of molecular different cell types. Gain and loss of function studies provide valuable insight into the gene regulatory networks that orchestrate this morphological process. However, current methods to alter gene expression in mice either lack efficacy or consume a lot of time and resources.

In **Paper I** we developed NEPTUNE (**NE**ural **P**late **T**argeting by in **U**tero **N**anoinj**E**ction). This describes a technique, where lentivirus is injected into the amniotic cavity of embryonic day 7.5 mouse embryos in order to transduce the neural plate prior to the onset of neurulation. After determining optimal injection parameters, NEPTUNE achieves widespread infection of the brain and spinal cord, and transduction was stable for at least 6 months. Using cell type specific MiniPromoters, NEPTUNE could be adapted for conditional expression in neurons and glial cells. Finally, knock down of *Olig2* recapitulated the phenotype of Olig2$^{-/-}$ embryos, while knock down of *Sptbn2* induced neural tube defects, therefore suggesting a novel function of Sptbn2 during neurodevelopment.

In **Paper II** we prepared a detailed and through protocol to provide all the necessary information that enables reproducibility of the technique. In concordance with the 3Rs we have developed a staging protocol to assess amniotic cavities prior to surgery and determined maximum volumes that can be injected without compromising embryonic survival. We further highlight the flexible usage of NEPTUNE for either assessment of gene function using widespread transduction or for lineage tracing using clonal transduction. Finally, we address what other organs and tissues are targeted with non-conditional NEPTUNE, including the eye, neural crest cells and the peripheral nervous system.

In **Paper III** we use NEPTUNE to manipulate gene expression in the developing enteric nervous system. Single cell RNA sequencing of E9.5 embryos showed that NEPTUNE targets neural crest cells and neural crest derived tissues, for example cardiomyocytes, as well as enteric progenitors. Non-conditional NEPTUNE showed even transduction of both, neuronal and glial progenitors in the stomach, small and large intestine. By applying NEPTUNE in *Baf53b-Cre* transgenic mouse line, we achieved conditional targeting of neurons and assessed the role of transcription factor PBX3 during neuronal lineage specification in the gut. Overexpression of PBX3 lead to an increased number of CALB1+ excitatory neurons and reduction of NOS1+ inhibitory motor neurons.

Thus, the work presented in this thesis shows that NEPTUNE enables rapid and flexible assessment of gene function during nervous system development.

ZUSAMMENFASSUNG

Unser Nervensystem leistet täglich unglaubliches, ohne dass wir daran denken oder uns besonders anstrengen müssen. Unser Gehirn lässt und Ideen formulieren, Pläne schmieden und träumen. Dank der vielen Nervenstränge, die unseren gesamten Körper vernetzen, spüren wir Berührung und nehmen Änderungen in der Temperatur wahr. Unser Nervensystem reguliert außerdem unsere Verdauung, und steuert Bewegungsabläufe. Es ist daher nicht verwunderlich das Wissenschaftler seit Jahrhunderten versuchen herauszufinden, wie dieses Komplexe Netzwerk aus Zellen und Nervensträngen bilden kann. Die Antwort liegt in unserem genetischen code. Dank der technologischen Fortschritte der letzten 20 Jahren, ist es heute möglich die komplette DNS-Sequenz einer Person zu bestimmen. Das hat vor allem Vorteile für die klinische Forschung, denn viele Patienten mit neurologischen Entwicklungsstörungen haben keine richtige Diagnose. Das bedeutet, sie können zwar anhand ihrer Symptome einer bestimmten Erkrankung zugeordnet werden, allerdings ohne Befund was die Erkrankung ausgelöst hat. Das kann sowohl für den Patienten selbst aber auch für das Umfeld frustrierend sein. Eine komplette Sequenzierung kann deshalb besser Aufschluss darüber geben welche Teile der DNS betroffen sind und wie stark. Hier bahnt sich allerdings das nächste Problem an. Dank dieser schnellen Verfahren haben sich große Mengen an Daten angesammelt, die allerdings noch überprüft werden müssen.

Das Ziel dieser Doktorarbeit war deshalb eine neue Methode zu entwickeln, die die Validierung dieser Ergebnisse ermöglicht. Als Lösung präsentieren wir NEPTUNE, kurz für **NE**ural **P**late **T**argeting by in **U**tero **N**anoinj**E**ction. Dies beschreibt eine Technik, bei der Mäuseembryonen mit Virus infiziert werden können, noch bevor sich das Gehirn und Nervensystem entwickelt. Dadurch ist es möglich die Gen Expression zu manipulieren und deren Effekt auf die embryonale Neuroentwicklung zu untersuchen. Die Veröffentlichungen I und II beschreiben die genauen Optimierungsabläufe, in denen wir Virus verwendet haben, das alle infizierten Zellen mit einem grün fluoreszierenden Protein markiert. Unter optimalen Bedingungen ist es möglich mehr als 95% des gesamten Gehirns und mehr als 70% des Rückenmarks zu infizieren. Diese weitläufige Effizienz ermöglichte uns die Funktion eines Gens, dass in Patienten mit Erkrankungen des Bewegungsapparates betroffen ist, genauer zu untersuchen. Zusätzlich zeigen wir, wie das Virus spezialisiert werden kann, damit nur bestimmte Zelltypen, wie zum Beispiel Nervenzellen, infiziert werden. In Veröffentlichung III nutzen wir diese Technik, um das Nervensystem des Darms zu untersuchen. Dieses besteht aus einer Vielzahl unterschiedlicher Nervenzellen, die hauptsächlich die Peristaltik des Verdauungstraktes regulieren. Wie und wann genau sich diese Zellen aber spezialisieren ist noch unklar. Ein wichtiger Aspekt dieser Arbeit ist auch die Reduktion der Anzahl von Mäusen, die in der Wissenschaft angewendet werden. Mit unserer Methode ist es möglich mehrere Gene gleichzeitig zu untersuchen, da eine Maus mehrere Embryonen in sich trägt. Dadurch können Ergebnisse schneller erzielt werden und weniger Tiere müssen verwendet werden.

LIST OF SCIENTIFIC PAPERS

I. **Katrin Mangold**, Jan Mašek, Jingyan He, Urban Lendahl, Elaine Fuchs and Emma R. Andersson. Highly efficient manipulation of nervous system gene expression with NEPTUNE. *Cell Reports Methods* 1, 100043, 2021.

II. **Katrin Mangold**, Jingyan He, Sanne Stokman and Emma R Andersson. Murine Neural Plate Targeting by In Utero Nanoinjection (NEPTUNE) at Embryonic Day 7.5. *J. Vis. Exp.* (180), e63148, 2022.

III. **Katrin Mangold**, Ziwei Liu, Jingyan He, Lenka Belicova, Viktoria Knoflach, Sandra DeHaan, Ulrika Marklund and Emma R. Andersson. Lentiviral transduction of the peripheral nervous system via nerua crest using NEPTUNE. *Manuscript*

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LIST OF ABBREVIATIONS

AAV	Adeno associate virus
AC	Amniotic cavity
Arp1	Actin-related protein 1A, centractin alpha
BAF53b	Actin-like 6B
BMP	Bone morphogenetic protein
CALB1	Calbindin 1
CMV	Cytomegalovirus
CNS	Central nervous system
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DCX	Doublecortin
DLHP	Dorsolateral hinge point
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglia
DSB	DNA double strand breaks
E	Embryonic day
ENC	Enteric neuron class
ENS	Enteric nervous system
FGF	Fibroblast growth factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GI	Gastrointestinal
H2B	Histone 2B
HB9	Motor neuron and pancreas homeobox 1
HDR	Homology-directed repair
hPGK	Human Phosphoglycerate kinase
hPSC	Human pluripotent stem cell
HUC/D	ELAV like RNA binding protein 3
ISL1	ISL LIM Homeobox 1
LTR	Long terminal repeats
LV	Lentivirus

MHP	Median hinge point
NDD	Neurodevelopmental disorder
NEPTUNE	Neural Plate Targeting by in Utero Nanoinjections
NEUN	Neuronal nuclear protein
NHEJ	Non-homologous end joining
NMP	Neuromesodermal progenitors
NNC	Neural crest cell
NOS1	Nitric Oxide Synthase 1
NPC	Neural progenitor cell
NTD	Neural tube defect
OLIG1	Oligodendrocyte Transcription Factor 1
OLIG2	Oligodendrocyte Transcription Factor 2
PBX3	pre B cell leukemia homeobox 3
PCP	Planar cell polarity
PDGFRα	Platelet-derived growth factor alpha
PNS	Peripheral nervous system
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
SCA	Spinocerebellar ataxias
SCA5	Spinocerebellar ataxia 5
SCAR14	Spinocerebellar ataxia, autosomal recessive 14
SIN	Self-inactivating
SOX2	SRY-Box Transcription Factor 2
SOX10	SRY-Box Transcription Factor 10
SOX11	SRY-Box Transcription Factor 11
SPTBN2	Spectrin Beta, Non-Erythrocytic 2
VSVG	Vesicular stomatitis virus glycoprotein

1 INTRODUCTION

The beginning of the 21st century was marked with the release of the first sequence of a human genome (Craig Venter *et al.*, 2001; Lander *et al.*, 2001). Although it was an incomplete draft, focusing on the protein-coding part of the DNA (Abdellah *et al.*, 2004), it nonetheless marked an important date, as it enabled scientists around the globe to conduct more accurate studies on our genetic code and its influence on human health and disease. In addition, it fueled efforts to improve the current sequencing approaches to increase speed, accuracy as well as sample throughput (Margulies *et al.*, 2005; Shendure *et al.*, 2005; Thermes, 2014). Over the past twenty years, these next generation sequencing techniques have been used and adapted across multiple disciplines, which resulted in numerous, significant discoveries of how the genetic code is organized or the complexity of gene transcription in single cells and whole tissues (Ståhl *et al.*, 2016; Regev *et al.*, 2017; La Manno *et al.*, 2021).

Improvements in both technologies and protocols also led to rapid decrease of sequencing costs, thereby making it available for hospitals and in diagnostics (National Human Genome Research Institute, 2021). Previously, whole exome sequencing was the gold standard in clinical genetics. However, with growing evidence about regulatory elements outside the gene coding DNA sequence influencing gene expression, it became more and more relevant to used whole genome sequencing instead (Soden *et al.*, 2014; Taylor *et al.*, 2015; Clark *et al.*, 2018).

Neurodevelopmental disorders (NDDs) are a heterogenous group of physical and behavioral phenotypes with often complex genetic cause (Soden *et al.*, 2014). Several initiatives have been built to identify the mechanisms that lead to onset of NDDs. However, these initiatives rely on the use of online databases in order to assign a genomic mutation to a diagnosis. Unfortunately, for NDDs this is rarely possible and leaves between 45-75% of patients and families without diagnose or adequate treatment plans (Soden *et al.*, 2014; Turro *et al.*, 2020; Smedley *et al.*, 2021).

Therefore, there is a growing need for advanced tools that can be used to validate the vast amount of information that is acquired from proteome, transcriptome, and genome studies. The following introduction will focus on neurodevelopment and current approaches that are used to assess gene function *in vivo*.

1.1 NEURODEVELOPMENT IN MAMMALS

"What is perhaps the most intriguing question of all is whether the brain is powerful enough to solve the puzzle of its own creation." *Gregor Eichele, 1992*

The mystery of how an embryo unfolds from simple to complex has fascinated and perhaps haunted scientists, philosophers, and thinkers for centuries. Equally perplexing are the unseen forces that initiate and govern the development of one of the undoubtedly most complex organs in the human body. The brain is a remarkable tissue, built out of millions of cells that enable us to plan, structure, learn, remember, imagine, and think. Together with the spinal cord, the two organs comprise the central nervous system (CNS). However, the way we perceive and interact with our environment, how we feel pain or temperature is mediated by the peripheral nervous system (PNS). Therefore, PNS is a collective term that includes for example, the sensory receptors in the skin, the cranial nerves that enable facial expressions as well as the neurons that span the gastrointestinal tract.

Both CNS and PNS are derived from the embryonic ectoderm. Gastrulation is the process during embryogenesis that leads to the formation of three germ layers: endoderm, mesoderm, and ectoderm (Gilbert and Barresi, 2016). Essentially, these three layers of multipotent stem cells will give rise to every organ and tissue of the future embryo. As the name implies (*ektos* = Greek, "outside") the ectoderm is the outermost layer of the late-stage gastrula and will, aside from the CNS and PNS, also give rise to the surface ectoderm (for example skin, lens, and cornea epithelium).

Here we can observe another mysterious wonder of life. As just described, the CNS and PNS are derived from the outermost germ layer. However, in the fully developed embryo, the brain and spinal cord and all the nerves are clearly on the inside, surrounded by skin and bone. This is achieved by a process called neurulation, which describes the major confirmational change of the embryonic ectoderm from a flat epithelial sheet into a tube.

1.1.1 Primary neurulation

How does a sheet turn into a tube? Correct, by folding it. However, in the developing embryo, there are two different mechanisms that result in the same outcome. Primary neurulation describes how the rostral part of the neural tube is formed, by folding of the neural plate into a hollow tube. The caudal portion of the neural tube is formed by secondary neurulation. Here, a solid cord of aggregated mesenchymal cells, followed by subsequent cavitation, proceeds the tube formation. Both mechanisms will be discussed.

Like many other processes that occur during embryonic development, neurulation is one additional process, where proliferation, axis elongation and change in morphology happen in a beautifully synchronized manner. The first step of neurulation is neural induction (Colas and Schoenwolf, 2001). It describes the process of defining the neural plate, the area of the ectoderm that will become the future neural tube. This is achieved, by suppression of BMP (bone morphogenic protein) signaling via expression of Sox2 transcription factors, among

others (Ybot-Gonzalez *et al.*, 2007; Archer *et al.* 2011). Thereby, creating a defined gradient across the ectoderm, which will be crucial for further cell fate determination.

Neural induction is followed by cell proliferation and elongation of the neural plate along the rostral-caudal axis. The edges of the neural plate thicken and form the neural folds, which extend towards the dorsal midline. Elevation of the neural folds creates a U-shaped furrow, called the neural groove, which will become the lumen of the neural tube after neurulation is completed (Jacobson and Moury, 1995).

Bending of the neural plate is achieved by the formation of the hinge points. These describe three specific regions, where the neuroepithelial cells are anchored to the underlying tissues. The median hinge point (MHP) is overlaying the notochord and extends across the entire rostral-caudal axis. The two dorsolateral hinge points (DLHPs) are induced by the neighboring surface ectoderm (Nikolopoulou *et al.*, 2017). Cells at the hinge points undergo morphological changes along the apicobasal axis, where the apical side is narrowed, and the basal side expands. This is achieved via asymmetrical constriction of actin-myosin filaments and gives the cells a wedge-shaped morphology. Finally, a higher cell proliferation rate at the dorsal side of the neural plate compared to the ventral side, is adding additional force that pushes the neural folds closer towards the midline (McShane *et al.*, 2015).

When the two neural folds meet, neural tube closure is initiated. However, this does not occur simultaneously across the rostral-caudal axis but rather is initiated at specific regions (Juriloff *et al.*, 1991; Van Allen *et al.*, 1993). Starting from these initiation points, the neural tube is closed in a zipper-like manner . In addition, epidermal-neural attachments are separated, by cells at the border of the neural plate stop expressing E-Cadherin and instead begin to synthesize N-cadherin (McShane *et al.*, 2015).

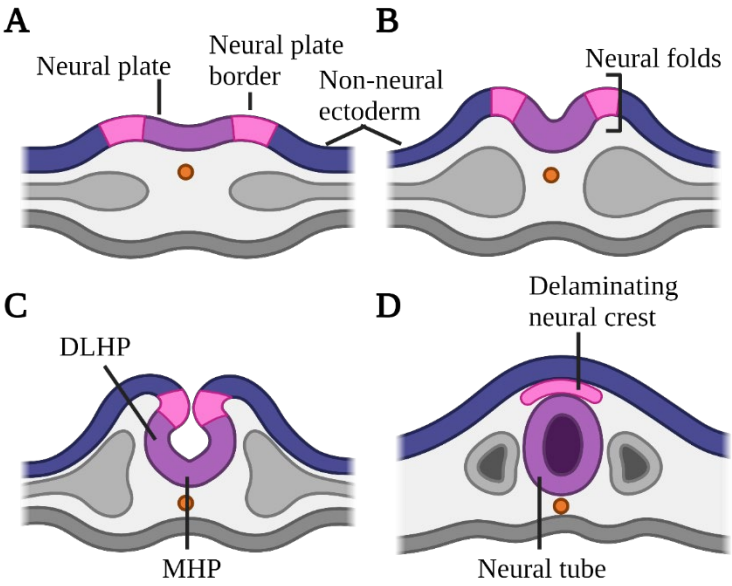

Figure 1. Stages of primary neurulation.
(A) Regionalization of the ectoderm into neural plate and surface ectoderm. (B) The edges of the plate move upwards, creating a U-shaped furrow. (C) Bending is achieved at the three hinge points. (D) Primary neurulation is completed upon fusion of the neural folds, separating neural from non-neural tissues.
DLHP = Dorsolateral hinge points; MHP = Median hinge point. Figure created with BioRender.com

1.1.2 Secondary Neurulation

The caudal part of the neural tube is formed via secondary neurulation and begins with the aggregation of tail bud cells into a solid epithelial cord called the medullary cord (Schoenwolf and Nichols, 1984). Simultaneously, these cells become polarized by the formation of lateral gap junctions (Schoenwolf and Kelley, 1980). This results in the development of small lumen between their apical surfaces and a remaining central cluster of mesenchymal cells. As secondary neurulation progresses, this cell clusters are removed by cell rearrangement and migration (Colas and Schoenwolf, 2001).

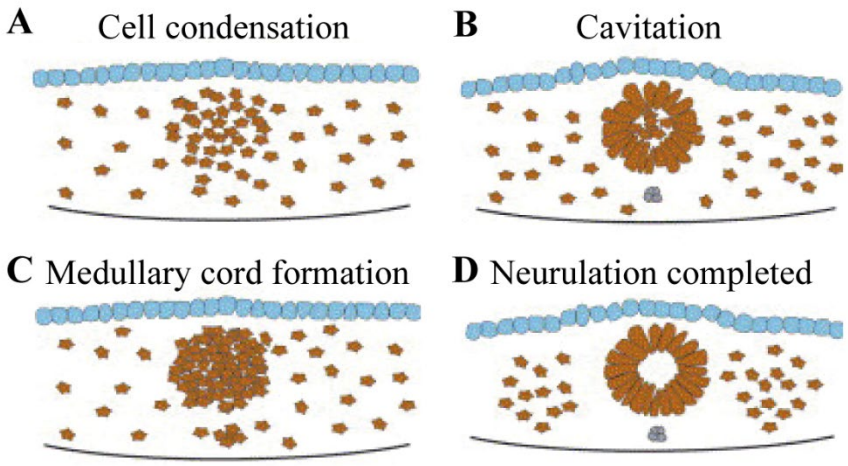

Figure 2. Stages of secondary neurulation. (A and B) Cells in the tail bud condense and form the medullary cord. (C) Cells become organized by formation of gap junctions. (D) Neural tube is cleared via cell rearrangement and cell migration. Modified from (Lowery and Sive, 2004).

Axial elongation and formation of the tail bud is driven by axial progenitor cells, which reside in the caudal progenitor zone (Wymeersch, 2021). Lineage tracing studies have shown that these cells have the potential to produce both, caudal neural and mesodermal tissue and are therefore termed neuromesodermal progenitors (NMP) (Tzouanacou *et al.*, 2009). However, their differentiation potential is affected by their spatial and temporal environment, as for example neural progenitors (NPCs) derived from NMPs at E8 were mostly localized to the ventral domain of the spinal cord, while NMP derived NPCs at E10 were preferentially detected at the dorsal domain (Shaker *et al.*, 2021). In addition, *in vitro* differentiation of human pluripotent stem cells (hPSCs) into neural crest cells (NCCs) suggests that axial progenitors give rise to trunk NCCs (Frith *et al.*, 2018).

1.2 NEURAL TUBE DEFECTS

Neural tube defects (NTDs) occur when neurulation fails and are among the most common structural birth defects in humans, with a prevalence ranging from 0.5-10 in 1,000 live births (Dolk *et al.,* 2010; Parker *et al.*, 2010). Spina bifida (latin for "split spine") occurs if the posterior neuropore fails to close and leaves parts of the spinal cord exposed. Severity is dependent on the size and placement of the lesion as well as to what extend spinal nerves and meninges are affected (Thompson, 2009). Anencephaly is a usually lethal condition, where the most anterior part of the neural tube remains open. This exposes the developing forebrain to the amniotic fluid which leads to subsequent degeneration. Finally, craniorachischisis occurs when neural tube closure fails along the entire length of the body axis and leads also to early lethality.

NTDs can result from genetic, epigenetic, or environmental causes (Wilde *et al.,* 2014). However, the majority of genes that have been associated to cause NTDs can be linked to the non-canonical or β-catenin independent Wnt pathway, that controls planar cell polarity (PCP) (Hamblet *et al.*, 2002; Curtin *et al.*, 2003; Murdoch *et al.*, 2003; Wang, Guo and Nathans, 2006; Kibar *et al.*, 2007, 2011). Binding of certain Wnt proteins (Wnt11, Wnt5a) to Frizzled-Ror receptor complex at the cell membrane induces signaling cascades that lead to the activation of Rho GTPases like Rac and RhoA. This results in reorganization of actin and microtubule cytoskeleton, to alter cell shape and often induces bipolar protrusive behaviors, necessary for cell migration. Non-canonical Wnt/PCP signaling is most notable for directing cells to divide or move within the same spatial plane within a tissue. During neurulation, the neural plate undergoes conformational changes as it lengthens along the anterior-posterior axis and narrows along the medial-lateral axis. Both is mediated via non-canonical Wnt/PCP pathway (Keller, 2002).

Multiple genetic mouse models have been generated to study NTDs. Disruption of PCP core regulators like Vangl1 and Vangl2, Scrib and Celsr1, Fzd3 and Fzd6 as well as Dvl1-3 results in craniorachischisis (Hamblet *et al.*, 2002; Curtin *et al.*, 2003; Murdoch *et al.*, 2003; Wang, Guo and Nathans, 2006; Kibar *et al.*, 2007, 2011). Heterozygous Vangl2$^{Lp/+}$ and Celsr1$^{Crsh/+}$ embryos are delayed in closure initiation but ultimately achieve closure and show a slightly wider and shorter midline. In contrast, Scrib$^{Crc/+}$ heterozygotes embryos initiated closure with a closely similar pattern to wild-type littermates (Murdoch *et al.*, 2014). This partially reflects the situation in humans, where heterozygous sequence variants detected in affected humans can often be found in unaffected individuals as well, suggesting. that there is a threshold for NTDs that requires several mutations (Murdoch *et al.*, 2014). Finally, mutations in Grhl2 and Grhl3 (expressed in the surface ectoderm), Rab23 (also named Openbrain) and Pax3 (regulators of Shh) or Noggin and Bmp7 also induce NTDs, further highlighting the genetic complexity (Eggenschwiler and Anderson, 2000; Stottmann *et al.*, 2006; Gustavsson *et al.*, 2007; Brouns *et al.*, 2011; Lin *et al.*, 2019).

1.3 SPINOCEREBELLAR ATAXIA

Ataxia is a collective term that describes medical conditions with poor or impaired muscle control that affects movement. Clinical features involve gait abnormalities, impaired coordination and fine motoric as well as change in speech. Spinocerebellar ataxias (SCAs) describe a large group of inheritable cerebellar degenerative disorders, which are caused by degeneration of the cerebellum and its afferent and efferent connections (Margolis, 2014). The genetic etiology of SCAs is quite complex since phenotypes can vary within SCA subtypes (Schelhaas *et al.*, 2000).

SCA5 and SCRA14 are both caused by mutations in the SPTBN2 gene (Spectrin Beta, Non-Erythrocytic 2) (Lise *et al.*, 2012; Cho and Fogel, 2013; Elsayed *et al.*, 2014; Wang *et al.*, 2014; Al-Muhaizea *et al.*, 2018; Nicita *et al.*, 2019). Sptbn2 is expressed throughout the CNS with highest levels in Purkinje cells (Sakaguchi *et al.*, 1998), which is the main neuronal type in the cerebellum. SPTBN2 interacts and stabilizes glutamate transporter EAAT4 at the cell membrane, which indicates a function in preventing excitotoxicity (Jackson *et al.*, 2001). In addition, it has been shown to interact with Ankyrin1 and mediate neuron excitability (Clarkson *et al.*, 2014) as well as Arp1 and Dynactin which suggest involvement in vesicle transport in the cytoplasm (Bennett and Healy, 2009). Mutations in humans often lead to missense or in-frame deletions, resulting in expression of a truncated or altered protein. $Sptbn2^{-/-}$ mice recapitulate the ataxic phenotype and survive until adulthood. Hover, these animals are considered hypomorphs, since a truncated version of SPTBN2 with exons 2-6 missing, is still expressed (Perkins *et al.*, 2010).

1.4 THE NEURAL CREST

Between the surface and the neuroectoderm resides the presumptive neural crest. Upon fusion of the neural folds and closure of the neural tube, these cells delaminate from the dorsal midline and migrate away. Depending on their anatomic position along the anterior-posterior axis at the neural tube, the NCC will give rise to a variety of tissues in the embryonic body. Cranial neural crest will give rise to the connective tissue in the face, such as cartilage and bones, odontoblasts of the tooth as well as craniofacial nerves and glia (Chai *et al.*, 2000; Golding *et al.*, 2000; Soo *et al.*, 2002). Cardiac neural crest, as the name implies, contribute to the muscular tissue wall of the large arteries (outflow tracts) as well as the septum, that separates the left and right heart chambers (Farrell *et al.*, 1999; Tang *et al.*, 2019). The dorsal root ganglia (DRG) are located in the spinal cord and belong to the PNS. It describes a cluster of sensory neurons and their supporting glia, that transmit somatosensory information to the brain. DRGs are derived from trunk neural crest cells (Kasemeir *et al.,*, 2005; Wiszniak *et al.*, 2019). Finally, vagal, and sacral neural crest give rise to the enteric nervous system (ENS) (Le Douarin and Teillet, 1973; Obermayr *et al.*, 2012). In addition, melanocytes (epidermal pigment cells) are derived from NCCs from all axial levels (Simões-Costa and Bronner, 2015).

NCC induction begins with formation of the neural plate in the embryonic ectoderm. This is meditated by a vast gene regulatory network that creates specific gradients of BMB, Wnt and FGF signaling pathways across the mediolateral axis (Groves and LaBonne, 2014). The emergence of NCCs from the lateral borders of the neural plate is then induced by expression of neural crest specifiers, for example FoxD3, Snail and Sox9/10. These sets of genes promote that NCCs undergo epithelial-to-mesenchymal transition, a crucial step to enables NCCs to delaminate from the neural tube and migrate into the periphery of the embryo (Groves and LaBonne, 2014; Simões-Costa and Bronner, 2015).

1.4.1 Molecular and cellular origin of the ENS

Vagal neural crest cells migrate and colonize the gastrointestinal (GI) tract in two ways. NCC adjacent to somites 1 and 2 migrate via the vagus nerve and colonize the esophagus and stomach, whereas vagal neural crest cells adjacent to somites 3-7 enter the foregut and colonize the entire GI tract from rostal to caudal (Espinosa-Medina *et al.*, 2017). In contrast, sacral NCCs enter the distal gut and colonize the colon in a caudal to rostral direction. The ENS regulates blood flow, peristaltic movements, and secretion. Therefore, it is not surprising that it consists of a wide range of different neuronal classes, for example inhibitory and excitatory motorneurons, sensory neurons and interneurons (Zeisel *et al.*, 2018; Morarach *et al.*, 2021a). Sox10 is a key regulator to maintain the pool of enteric progenitors throughout development, while Ascl1 and Hand2 as well as BMB and FGF signaling are involved in neuron and glial differentiation (Lei and Howard, 2011; Simões-Costa and Bronner, 2015; Memic *et al.*, 2016). However, the exact gene regulatory network that leads to generation of specific neuron subclasses of the ENS remains elusive.

1.5 STRATEGIES TO ASSESS GENE FUNCTION

Loss of function studies are commonly used to assess the normal function of a gene in a cell, tissue or within a larger organism and can be induced in different ways. Pharmacological inhibitors are small molecules or antibodies that can be used to block the usual function of a protein. Their application is widespread across different research fields, for example in cancer treatment studies and immunotherapy.(Arkin, Tang and Wells, 2014)

RNA interference (RNAi) is a widely conserved mechanism, where short, double stranded RNA molecules bind to their mRNA targets and mediate either degradation or block of translation. These short RNA species are usually 21-23nt long and interact with the RNA-induced silencing complex (RISC), where they mediate guidance to the target mRNA. If there is perfect sequence complementary between the two RNAs, RISC induces cleavage, however if there is only a partial matching, translation is only blocked (Kim and Rossi, 2007). Synthetic RNAi reagents like short hairpin RNAs (shRNA) have been used widely in the past for high throughput genetic screens to identify new genes, or gene networks that are involved in a wide variety of biological processes (Boutros *et al.*, 2004; Beronja *et al.*, 2010, 2013; Schramek *et al.*, 2014).

The CRISPR/Cas9 system is a form of RNA-mediated adaptive immunity in bacteria and has been widely used for targeted mutagenesis *in vitro* and *in vivo*. Thereby, the Cas9 nuclease is directed to a specific region of the DNA via a small guide RNA (sgRNA) where it introduces DNA double strand breaks (DSB). Mammalian cells have two main DSB repair machineries: Homology-directed repair (HDR) and nonhomologous end-joining (NHEJ). While the first mechanism requires a homologous DNA template to ensure precise reconstruction of the sequence, NHEJ is more error-prone and introduces insertion or deletion (indel) mutations at the repair site. That effect is used to cause a frameshift in the target sequence, resulting in a premature stop codon and non-functional protein (Cong *et al.*, 2013; Gilbert *et al.*, 2013).

1.6 IN VIVO DELIVERY SYSTEMS

1.6.1 Electroporation

To mediate long-term gene silencing, via RNAi or sgRNA/Cas9, DNA expression vectors are introduced into the host cell to facilitate integration into the genome. Electroporation is a physical transfection method, that relies on a short electric pulse to induce the formation of temporal pores in the cell membrane, momentarily allowing negatively charged nucleic acids to enter the cell (Chu *et al.*, 1987).

It has been widely used to transform cells that are resistant to chemical transfection as well as mouse and chick embryos to study neurodevelopment. Avian embryos are popular model systems to study neurodevelopment, because of their accessibility, ease of culture and planar topology. DNA can be injected into the neural tube as early as E1 (when the chick embryo contains ten somites) and has been extensively used to study neural patterning (Itasaki, Bel-Vialar and Krumlauf, 1999). In mice, it is mainly used for gain and loss of function studies in the cerebral cortex. DNA is injected into the ventricle and depending on position of the electrodes as well as duration of the current, different regions and cell types can be targeted (Saito and Nakatsuji, 2001). It is possible to target the neural tube as early as E9.5, however only thus far in cultured embryos (Akamatsu *et al.*, 1999). For *in vivo* studies, electroporation is only possible for embryonic stages post neurulation. Additional drawback is the high rate of cell mortality and the requirement of extensive optimization of several parameters, for example voltage and pulse length. Therefore, electroporation is not suitable for more complex studies like genetic screens or lineage tracing or targeting of early neural progenitors as well as NCCs.

1.6.2 Viral vectors

Viral vectors circumvent the problems of low targeting efficacy and toxicity. Retroviruses are of special use and interest, since the genes they transduce integrate into the genome of the target cell, causing stable and long-term expression of the transgene (Barquinero, Eixarch and Pérez-Melgosa, 2004). However, some of these viruses can only access the nucleus of the host cell during mitosis, when the nuclear envelope is temporarily disassembled, limiting their transduction efficacy to dividing cells only (Cohen, Au and Panté, 2011). Lentiviruses are a sub-class of retrovirus and are the most commonly used vectors, due to their ability to

infecting both dividing and non-dividing cells, since their genome is actively transported through the nuclear pore complex (Cohen, Au and Panté, 2011). In addition, they cause little to no immune reaction and have the capacity to carry large DNA fragments (Naldini *et al.*, 1996). Many of the currently used lentiviral vectors are based on an HIV-1 variant, which was modified so it can still infect and transduce target cells but is replication defective. An additional safety feature is the deletion of viral enhancer and promoter sequences to create self-inactivating (SIN) vectors. The transcriptional inactivation of the long terminal repeat (LTR) in the SIN provirus should enable the regulated expression of genes from internal promoters by eliminating any cis-acting effects of the LTR (Miyoshi *et al.*, 1998). Viral tropism is based on the glycoproteins on viral envelope. For lentivirus, the glycoprotein of vesicular stomatitis virus (VSV-G) has been the most popular envelope, allowing the virus to transduce a wide range of cell types and host species (Kim and Rossi, 2007).

Alternatively, Adeno associated viruses (AAV) are also able to infect dividing and quiescent cells of various tissues and cause long-term expression of the transgene. However, instead of random integration as it is the case for Lentiviruses, AAV either form stable episomal DNA forms or integrate site-specifically into the host cells genome (Naldini *et al.*, 1996). A major drawback, however, is the limited packaging capacity in AAV, which makes it problematic to deliver genes or inserts larger than 5kb (Duan, Yue and Engelhardt, 2001). Nevertheless, viral vectors have been widely used to either assess gene function or to correct genetic diseases. For AAVs, capsid and administration route are determining the cell types that can be targeted. If injected directly into the brain parenchyma, AAV serotypes 1, 9 and rh.10 show strong transduction of neurons (Burger *et al.*, 2004; Cearley and Wolfe, 2006), while AAV4 and rh.43 possesses strong tropism towards GFAP+ astrocytes (Liu *et al.*, 2005). When delivered into the ventricles AAV4 targets ependymal cells (Liu *et al.*, 2005), whereas AAV7 and AAV9 can bypass the ependymal layer and transduce neurons throughout the cortex, cerebellum, and spinal cord (Samaranch *et al.*, 2013).

1.6.3 Genetic mouse models

Creating a transgenic mouse can be done in several different ways, however the two most common approaches are: 1) direct injection of exogenous DNA into the pronuclei of fertilized one-cell mouse embryos and 2) targeted manipulation of mouse embryonic stem (ES) cells, derived from the inner cell mass of embryonic day (E) 3.5 mouse blastocyst.(Kumar *et al.*, 2009) In short, fertilized donor eggs are collected and microinjected with either DNA or ES cells, followed by implantation into a pseudo pregnant recipient female. The resulting offspring contains a subset of animals that harbours the insert, also called founder animals, which are used to generate a mouse line that stably expresses the transgene in every cell.

Conditional knockouts are used to assess the function of a gene in a specific cell type or during a certain developmental stage. This approach is adapted from the Cre/loxP system of bacteriophages, where the target gene (or a portion of it) is flanked by loxP sites and expression of the Cre-recombinase is under the control of a cell type specific promoter

(Lakso *et al.*, 1992). In this way, recombination and deletion of the target gene will only occur in cells or tissues that express Cre. To develop this further, Cre can be fused to a tamoxifen-responsive mutated estrogen receptor (CreERT2), which upon administration of the agent promotes translocation of Cre into the nucleus (inducible Cre) (Indra *et al.*, 1999).

1.7 ULTRASOUND GUIDED NANOINJECTIONS *IN UTERO*

Targeting and manipulating the developing mouse embryo at various time points with intra-amniotic injections of virus or cells has been used by several groups in the past (Liu *et al.*, 1998; Boyle *et al.*, 2001; Punzo *et al.*, 2008; Endo *et al.*, 2009). The technique of ultrasound guided nanoinjections *in utero* has been optimized and established to perform functional genetic studies in the mouse epithelium (Beronja *et al.*, 2010, 2013; Schramek *et al.*, 2014). Using high titer lentivirus and RNAi in wild type or transgenic mice, they have provided important insights into the gene regulatory network that governs skin morphogenesis and identified tumor suppressor genes involved in the onset and progression of skin cancer.

Gaiano et al first described injections targeting the developing brain as early as E8.5, using retrovirus to overexpress shh and notch components to study telencephalic development (Gaiano *et al.*, 1999, 2000). However, in these studies they mouse leukemia virus which only infects mitotic cells and reached an efficacy of 24%. Stitelman *et al.* (2010) used lentivirus encoding for *CMV-GFP* at E8.5 and described even transduction of neurons with stable expression of the transgene for up to two years. However, also in this study the maximum efficacy was less than 50%. Injections at E7.5 were described in Slevin *et al.* (2006). Fluorescent beads were used to assess injection precision to target the amniotic cavity (AC), exocoelomic cavity or ectoplacental cone at this stage. While they reported good survival following injections (68%) beads were detected in other tissues following injections into the AC. In addition, transduction efficacy was very low and only transient due to nature of the fluorescent beads. In conclusion, while there is strong evidence that injections can be performed prior to onset of neurulation in mice, at E7.5, previous studies have been lagging efficacy and have not revealed the techniques full potential.¨

2 RESEARCH AIMS

The overall goal of this PhD project was to develop a technique that could induce rapid, and permanent alteration of gene expression in the developing mouse nervous system. In addition, the technique should be powerful enough to model neurodevelopmental disorders, flexible and adaptable to allow conditional transduction or lineage tracing.

Paper I – To demonstrate the techniques versatility and to perform gene loss of function studies to investigate ataxic nervous system disorders.

Paper II – To describe targeting of the peripheral nervous system and other non-CNS tissues as well as to provide a detailed protocol of the method.

Paper III – To investigate gene regulatory networks that drive neuron specification in the developing ENS by conditional overexpression of Pbx3 transcription factor in neuronal progenitors.

3 ETHICAL CONSIDERATIONS

3R is a term that summarizes the words reduce, refine, and replace. The concept of the "Three Rs" has first been introduced during the late 1950s and is since 2010 legal requirement when using animals for scientific purposes in the European Union (Russell and Burch, 1959). Overall, 3R can be summarized as different strategies all aimed to achieve humane experimental techniques. The concept of reduction covers any approach that will result in fewer animals being used, without obtaining less precise results or information. This can include lowering the number of animals used for a study in general but also re-using animals for certain procedures if it does not result in poor animal welfare. Refinement means modification of all (experimental) procedures, to reduce pain or distress of the animal and at the same time enhance well-being. Replacement ultimately should lead to complete substitution of conscious, living animals for insentient material, for example cell culture or organoids.

Genetically modified mouse models are the gold standard for assessing a genes biological function because of their specificity. However, generation and maintenance of these mouse colonies usually requires multiple rounds of (in)breeding and therefore is a time and resource consuming procedure. In addition, the extent to which a transgene or mutation affects fertility or viability influences the number of homozygous offspring in one litter. Most importantly though it generates a large number of animals that is not used for scientific purposes.

The three studies described in this thesis focus on the optimization and use of the NEPTUNE technique to study development of the central and peripheral nervous system in mice. In this approach, mouse embryos are transduced with lentivirus to achieve permanent alteration of gene expression. Paper I and Paper II focus on identifying the optimal parameters to achieve widespread transduction without compromising survival. Therefore, it is technically feasible to inject between 75 to 100% of the litter. In addition, multiple viral constructs can be injected, either into the same amniotic cavity or into separate ones. This was especially important for Paper III, where NEPTUNE is used to study ENS development. Since the number of neurons in the embryonic gut can vary, littermates should always be compared. With NEPTUNE it was possible to inject two lentiviral constructs within the same female, resulting in non-injected controls, injected controls, and target construct injected animals in one litter.

Therefore, NEPTUNE contributes to the 3R initiative by reducing the number of animals used for scientific purpose as well as refining current techniques to assess gene function. However, the injections are technically difficult and required a lot of training and test injections. Therefore, this is a technique that probably should be carried out by only a view, trained people. Ultimately, NEPTUNE will also safe time since phenotypes can be assessed within hours or days following injections. This aspect can be particularly interesting for reverse genetic studies and screens, where mutations or transgenes introduce a genotype with unknown phenotype.

4 RESULTS AND DISCUSSION

4.1 PAPER I

Functional genetic studies of the developing CNS in mice have relied on either manipulation of embryonic stages post neurulation or the usage of transgenic animals. These approaches are not ideal for inducing changes of gene expression during development in a rapid or flexible manner. In **Paper I**, we present an approach that we termed NEPTUNE (Neural Plate Targeting by in Utero Nanoinjections). It describes an additional approach, where lentivirus is injected into the amniotic cavity of E7.5 mouse embryos, to transduce the neural plate and therefore the future brain and spinal cord (Figure).

In order to achieve maximum transduction efficacy together with best embryo survival rate, we started with the optimization of a number of different parameters concerning the ultrasound-guided injections technique. This included determination of the optimal embryonic state, suitable volumes that can be injected into the amniotic cavity as well as viral titers that are required to achieve maximum transduction. We found that injections at E7.5, an embryonic state where neurulation has not been initiated yet, resulted in more widespread transduction, compared to E8.0 injections. At E7.5, we found that there was great variation in size of the amniotic cavities even within the same litter. Therefore, we performed a number of control experiments, where we injected a range of different volumes (23-483nL) into E7.5 amniotic cavities. In addition to recording the survival rate following injections, we also acquired images of the cavities before and after injections and measured the relative volume increase for each cavity. We could show that an increase of up to 90% was tolerated well by the embryos and did not affect survival. We continued with injecting H2B-GFP encoding lentivirus (Beronja *et al.*, 2010) at varying titers and assessed the targeting efficacy in both brain and spinal cord of E13.5 embryos. Flow cytometer analysis of single cell suspensions showed a dose-dependent transduction, with viral titers of $2*10^{10}$ ifu/mL (infectious units per mL) resulting in >95% GFP+ cells. In addition, computational quantification of GFP+ nuclei on tissue sections with Cell Profiler, showed that transduction was even from anterior to posterior in the brain as well as rostral to caudal in the spinal cord.

GFP was expressed in both SOX2+ neural progenitors as well as in post-mitotic NEUN+ neurons. In addition, viral transduction was stable for at least 6 months, were GFP signal was detected in CALB1+ Purkinje neurons and SOX2+ glia in the cerebellum. As mentioned earlier in the introduction, formation of the future spinal cord occurs via primary and secondary neurulation. GFP+ cells were detected in the mesenchyme of lumbar and sacral spinal cord sections but not in thoracic levels, suggesting that injections also targeted NMPs. Furthermore, dorsal root ganglia and delaminating neural crest were also transduced.

Injections at E7.5 targeted not only the CNS and parts of the PNS. The skin, which also arises from the embryonic ectoderm, was well transduced, as well as epithelial cells lining the lumen of the lungs and stomach. In addition, scattered GFP+ cells were detected in the liver and the heart. Therefore, we continued to replace the *hPGK* promoter of our H2B-GFP

lentiviral construct with MiniPromoters (Portales-Casamar *et al.*, 2010) to achieve specific expression in neuronal or glial subtypes. Injections of *hPGK-H2B-GFP* lentivirus resulted in equal targeting of DXC+ neurons, GFAP+ astrocytes or OLIG1+ oligodendrocytes. In contrast, when *Dcx-H2B-GFP* lentivirus was injected, GFP signal was excluded from SOX2+ neural progenitors and furthermore restricted to only the CNS and spinal cord.

To show that NEPTUNE can be used as an alternative approach to gene manipulation during embryonic development, we cloned shRNA targeting *Olig2* into our *hPGK-H2B-GFP* viral vector. The transcription factor *Olig2* is an essential regulator of motorneuron and oligodendrocyte development and *Olig2*$^{-/-}$ leads to loss of both cell types (Takebayashi *et al.*, 2002). We could show that NEPTUNE and siRNA mediated knock down of *Olig2* was powerful enough to recapitulate the characteristics that can be found in *Olig2*$^{-/-}$ mice, which includes gross morphological abnormalities and strong reduction of ISL1+ and HB9+ motorneurons as well as PDGFRα+ oligodendrocytes in the spinal cord.

Finally, in order to investigate if NEPTUNE can be used to study neurodevelopmental disorders, we used a similar approach and injected H2B-GFP lentivirus that also encoded for shRNA against *Sptbn2*. Injected embryos at E9.5 displayed neural tube defects with varying severity (Class 1 = mildest phenotype, with a subtle straightening of the body axis; Class 2 = intermediate, with failure to undergo embryonic turning and a skewed body axis; Class 3 = strongest, embryos were developmentally delayed and had not undergone turning). SPTBN2 protein levels, correlated with the three phenotype classes, with the strongest reduction in class 3, compared to scrambled RNA injected controls. In situ hybridization for neural floor plate marker *Foxa2* and sections from thoracic to sacral spinal cord sections showed a wider floor plate region in *shSptbn2* embryos, which was more pronounced in lumbar levels. This recapitulates phenotypes similar to PCP mutants and therefore describes a novel function of *Sptbn2* during neural development.

Previous attempts to use ultrasound-guided *in utero* nanoinjections to target the developing nervous system have been lagging efficacy (Gaiano *et al.*, 1999; Slevin *et al.*, 2006; Stitelman *et al.*, 2010) or transduction stability. In this study we show that NEPTUNE can achieved widespread transduction of the neural plate with stable integration of the transgene for at least 6 months. In addition to determining the optimal embryonic stage and injection volumes, we also carefully assessed our virus production protocols and showed that not only the viral titer but also the quality of the virus affects embryo survival and transduction efficacy. One of the main advantages of NEPTUNE over conventional transgenic mouse models is its speed and flexibility. Conditional expression is usually achieved using Cre mice, however generation of these mouse lines can be time consuming and previous reports have been shown the Cre expression alone can produce multiple phenotypes (Cain-Hom *et al.*, 2017). Using MiniPromoters robustly removed GFP expression from SOX2+ neural progenitors and was specific to DCX+ neurons and GFAP+ astrocytes. However, GFP signal could be found in OLIG1 negative cells following injection of *OLIG1-H2B-GFP*. This is probably due to the transient *Olig1* expression during cell specification in combination with our H2B-GFP

reporter, which stabilizes the GFP in the cell nucleus and labels cells that might no longer express OLIG1. Thus, conditional NEPTUNE can be achieved via MiniPromoters, however every promoter construct should be carefully validated. In addition, modifications of the viral vector and using less stable reporter can further enhance specificity. Finally, we could show that NEPTUNE is a powerful enough to modulate gene expression during embryonic development. We describe a novel role of *Sptbn2* during neural development and embryonic turning. Mutations of *SPTBN2* in humans leads to ataxic syndromes SCA5 and SCAR14. However, most of these mutations don`t result in complete loss of function of the protein. This is also the case for current Sptbn2 mutant mouse models, which still express slightly shorter isoforms of SPTBN2. Sptbn2 is part of the cytoskeleton where it interacts with actin filaments to from a flexible network that stabilizes the cell membrane, cell contact and adhesion molecules. Phenotypes following shRNA mediated knock down mimic PCP mutants, however there were also differences as our embryos did not present an extensively open neural tube. Non-canonical Wnt/PCP is a dynamic process and requires an intact cytoskeleton (Nikolopoulou *et al.*, 2017). Therefore, this data suggests that Sptbn2 plays a mediating role during neural tube closure and embryo turning by stabilizing/supporting PCP. However, more studies will be necessary to support that theory.

4.2 PAPER II

Reproducibility is an important aspect of any scientific field. As described in Paper I, NEPTUNE holds great potential and will be a valuable addition to the genetic toolbox to study and unravel complex genetic networks. The NEPTUNE technique comprises a large number of individual steps and procedures, which contribute to a greater or lesser extend to successful transduction of the neural plate and minimal embryo loss. Therefore, in Paper II, we provide an in depth, step-by-step protocol with detailed information regarding surgery preparation, embryo staging and volume calculations.

Measurements have shown that at E7.5, amniotic cavities range between 0.2 to 1mm in diameter. Therefore, it is essential that the glass needles, used for injections, have a long and fine tip to circumvent that the amniotic sac is damaged during entering or retracting of the needle. Although glass needles can be purchased, we found it more cost effective and flexible to prepare ours in house. Needle tips can be sharpened by using a grinder. However, we found that it is important that the needle tip just touches the grinder, without the body of the needle bending. Otherwise, this creates a long and fragile needle tip that can easily break when entering the decidua and cause damage to the tissue or the embryo.

A big advantage of NEPTUNE is the reduction of animals used in research. However, as described in Paper I, we have observed that the size of the amniotic cavity at E7.5 varies greatly both between females but also within litters. To prevent that females undergo unnecessary surgery, we found that cavities can be assessed though the abdominal wall of the female during regular ultrasound checks. This way, it is possible to rank each female according to how many ideal-sized amniotic cavities (exocoelomic and amniotic cavity

clearly divided into two cavities) can be found and if injections can be performed immediately or should be postponed.

During surgery, uterine horns and deciduas should always be handled delicately, without any pulling or squeezing, as this can negatively influence embryo survival. In addition, only 3-4 deciduas should be exposed at the time to reduce the risk of drying out. Depending on if cavities of the left or right uterine horn will be injected, the orientation of the female or glass needle has to be adjusted. This is because of the anatomical structure of the implantation sites: the amniotic cavity is always the most distal cavity of the decidua, while the ectoplacental cone (containing blood vessels, appearing dark red) is always most proximal to the uterine horn. Implantation sites should always be arranged so the amniotic cavity is facing towards the needle tip. Even though cavity staging prior to surgery should result in most of the amniotic cavities being of similar size. However, to further reduce the risk of resorption due to injection of too large volumes, the diameter of every cavity can be measured prior to injections to calculate the maximum acceptable volume.

Another main advantage of NEPTUNE is the flexibility it offers. As indicated in Paper I, neural plate transduction efficacy is dose dependent and can be adjusted by the user via lowering viral titers or injection volumes. Here we compare E13.5 brains injected with either high or low titer HSB-GFP lentivirus, achieving high efficacy transduction of the entire brain or low efficacy transduction of single cell clones. In addition, NEPTUNE targets structures that have previously been difficult to target with electroporation such as the choroid plexus (Haddad *et al.*, 2013; Kaiser *et al.*, 2021).

The importance of performing injections prior to the onset of neurulation was demonstrated at the example of the developing eye. The retina is derived from the neuroectoderm, while the surface ectoderm gives rise to the lens and corneal epithelium. In addition, neural crest cells contribute to the corneal stoma as well as the corneal endothelium. All tissues were well transduced when injections were performed at E7.5. In contrast, injections at E9.5 result in GFP+ cells only in the lens and corneal epithelium. In addition, targeting of neuroectoderm-derived tissues was less efficient at E8.5.

Neural crest cells contribute to the development of multiple organs and tissues in the body, including the lingual epithelium (Liu *et al.*, 2012). This comprises the tongue mesenchyme and epithelium with papillae and taste buds. Following injections at E9.5, the lingual epithelium of the tongue is well transduced with GFP+ cells, while the underlying mesenchyme is negative. In addition, closer look at the tongue epithelium at E15.5 showed small crypts with GFP+ cells lining them. In comparison, injections at E7.5 led to widespread transduction of the tongue mesenchyme. In addition, neurons and progenitors in the DRG were well transduced. This data further suggests widespread targeting of neural crest cells via injections at E7.5.

4.3 PAPER III

While Paper I and Paper II are describing how NEPTUNE can be primarily used to study development of the CNS, in Paper III we are focusing on neural crest cells and the development of our second brain – the ENS.

While it was indicated in Paper I and Paper II that neural crest cells are targeted with NEPTUNE, we wanted to investigate this further. Therefore, we injected tdTomato-encoding lentivirus at E7.5 and collected at E9.5 for single cell RNA sequencing. We identified 10 clusters, of which 9 expressed pro-neuronal marker Sox11, showing how NEPTUNE predominantly targets cells of the neuroectoderm. Sox10 expressing neural crest cells were found as well as Ascl1, which has been previously linked to controlling neurogenesis and gliogenesis in the ENS (Memic *et al.*, 2016). Quantification of H2B-GFP+ and SOX2+ neural progenitors or NEUN+ neurons in the DRG from thoracic to sacral levels at E13.5 showed a transduction efficacy between 65 – 85%. This is similar to targeting efficacy of the spinal cord, suggesting that NEPTUNE equally well transduces neural crest cells and the PNS.

Indeed, when we stained E13.5 intestine with neuronal marker HUC/D and glial/progenitor marker SOX10, H2B-GFP was expressed in both cell types. Quantifications of HUC/D+ and GFP+ cells at E18.5 showed that NEPTUNE targets about 50% of the neuronal population in the stomach and intestine.

To specifically label neurons only, we used the *Baf53b-Cre* mouse line and injected a lentiviral vector expressing double floxed and inverted EGFP (DIO or FLEX, (Schnütgen *et al.*, 2003)). Sections at E18.5 confirmed conditional targeting of neurons, as EGFP expression was restricted to HUC/D+ cells only and was depleted from SOX10+ progenitors. Next, we used a similar lentiviral construct, to conditionally overexpress *Pbx3* transcription factor that has been shown to promote fate transition from enteric neuron class (ENCs) 8/9 to ENC 12 (Morarach *et al.*, 2021b). While injection of control virus (*DIO-EGFP*) did not interfere with the ratio of NOS1+ inhibitory motor neurons (ENC 8/9) to CALB1+ excitatory neurons (ENC 12), this became completely reversed upon overexpression of *Pbx3* (*DIO-Pbx3-EGFP*). In addition, at E18.5, EGFP was enriched in CALB1+ cells at the expense of NOS1+ cells.

In this Paper, we show that conditional NEPTUNE can be achieved, using *Baf53b-Cre* animals and that specific overexpression of *Pbx3* in neurons is powerful enough to enhance differentiation of Calbindin-expressing neurons in the ENS. However, one of the remaining challenges is NEPTUNEs targeting efficacy of ENS neurons. Further analysis of single cell sequencing data with focus on delaminating and migrating neural crest cells as well as the different neuronal classes of the ENS should reveal if some cell types are more susceptible to viral transduction than others. In addition, whole mounts of the intestine at different embryonic time points will further reveal when during development, the first GFP transduced cells are expressed in the ENS.

The *Baf53b-Cre* mouse line is specific to neurons (Zhan *et al.*, 2015), however not specific to neurons of the ENS. We have shown in Paper I, that by conditional expression can be achieved in wild type animals with MiniPromoters. With more data emerging that gives insights into the transcriptional profile during ENS development, it would be possible to tailor viral vectors and label specific cell lineages. Thus, NEPTUNE will be an essential tool to further resolve the complex trajectory of neuron and glia specification in the gut.

5 CONCLUDING REMARKS AND FUTURE PERSEPECTIVES

The technical advances of the past decade in genomics, transcriptomics and proteomics have immensely contributed to our understanding of the gene regulatory networks that govern nervous system development. This raises the need for advanced tools that allow functional assessment of these genes *in vivo*.

This thesis presents a method, that allows manipulation of the developing murine nervous system in a more rapid and flexible way, than classic genetic approaches. Using high titer lentivirus, we showed that NEPTUNE results in widespread transduction of the brain and spinal cord. This efficacy can be used, when one or several genes are assessed in their function during neural tube closure or pattering. In contrast, clonal NEPTUNE is possible by reducing the viral titers. Lineage tracing provides information on how particular cells have progressively acquired their final identities. Injections of lentivirus barcode libraries into wild type embryos have enabled trajectory analysis in the mouse telencephalon (Ratz *et al.*, 2022) or of neural crest derivates of the mouse adrenal gland region (Kameneva *et al.*, 2021). Although both studies circumvent the use of genetically modified animals, injections have been performed during or post neurulation. Nevertheless, viral barcode libraries hold great potential and together with NEPTUNE can be used to resolve lineage relationship from neural progenitors across the entire neural plate. In addition, it will be very useful to investigate which ENS populations are targeted and if there is a bias towards labelling different neural crest cell populations. Aside from lineage tracing, low transduction of only a few cells with virus is also desirable when performing genetic screens (Beronja *et al.*, 2010, 2013). Ultrasound-guided *in utero* nanoinjections of lentivirus RNAi libraries has allowed rapid and efficient identification of oncogenic lesions in skin cancer (Beronja *et al.*, 2013; Schramek *et al.*, 2014) and breast cancer (Ying and Beronja, 2020). A similar approach can be used with NEPTUNE to decipher complex neurodevelopmental disorders (De La Torre-Ubieta *et al.*, 2016; Fahey *et al.*, 2017); the genetic etiology of pediatric brain tumors (Simeonova and Huillard, 2014) or unravel gene redundancy in complex signaling cascades like Notch or Wnt (Kitamoto *et al.*, 2005; Fischer *et al.*, 2007). Finally, the combination of unique barcode identifiers with multiplex gene editing approaches will allow assessment of how lineage dynamics are affected upon gene knock down, knock out or overexpression (Rogers *et al.*, 2017; Ying and Beronja, 2020).

Knock down of *Sptbn2* resulted in a novel phenotype that partially resembled neural tube defects seen in PCP mutants (Murdoch *et al.*, 2014). Mutations of the SPTBN2 gene, where a truncated version of the protein is expressed, leads to onset of ataxia in both humans and mice (Perkins *et al.*, 2010; Lise *et al.*, 2012; Elsayed *et al.*, 2014; Wang *et al.*, 2014). To further investigate the function of Sptbn2 during development, it will be important to compare the knock down phenotype to a sgRNA mediated knock out. If depletion of Sptbn2 affects the cytoskeleton and therefore inhibits a cells' ability for conformational changes, *in vitro* motility and scratch assays can be performed to assess the functional outcome following

knock down or knock out. In addition, conditional knock down using either MiniPromoters or *Cre* specific mouse lines can further pinpoint if depletion in a specific cell type results in a similar phenotype.

Finally, expanding the "portfolio" of NEPTUNE will be an important aspect to increase its versatility. Recent advances in engineering rAAV capsids have enabled the generation of tailored viral vectors with cell specific tropism in the adult brain and spinal cord (Deverman *et al.*, 2016; Davidsson *et al.*, 2019). However, these vectors are usually injected directly into the brain ventricles or administrated into the blood system (Chen *et al.*, no date; Chan *et al.*, 2017). In addition, while rAAV robustly target neurons and glia in the CNS, the PNS and ENS remains difficult to transduce (Chan *et al.*, 2017). Therefore, it will be interesting to test how AAV injections compare to lentivirus transduction of the neural plate.

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