Heinrich-Heine-Universität Düsseldorf

# Heterogeneous fate choice of neural stem cells in the damaged and intact central nervous system

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*Für meine Eltern*

# Abstract

The vertebrate central nervous system (CNS) primarily consists of the brain and spinal cord. In both structures, so-called white and gray matter regions (WM and GM, respectively) can be discriminated by their cell composition and hence functionality. While highly efficient, the CNS shows a low regenerative capacity following an acute injury or while suffering from other neuropathological diseases during which neural cells are lost. Adult neural stem cells (NSCs) harbor the potential to replace lost cells but are locally restricted to only two discrete niches in the brain. However, the transplantation of multipotent NSCs holds great promises in terms of cell replacement strategies, e.g. when myelinating oligodendrocytes are lost. In this scenario, fate choice of exogenously applied NSCs must be directed towards the oligodendroglial lineage in order to achieve sufficient cell replacement. The p57kip2 gene has been described as a negative regulator of oligodendroglial fate choice in rat NSCs. However, it was unknown whether p57kip2 suppressed NSCs show a similar increase in oligodendrogenesis following transplantation into either healthy or injured GM- and WM tissue. Moreover, the impact on fate choice of transplanted NSCs in different neuropathological diseases has been abundantly described but rarely compared from a disease-instructing perspective.

To address these issues, I first analyzed the publicly available literature describing the transplantation of NSCs into different unharmed CNS regions and various neuropathological models, respectively. It became evident, that different brain and spinal cord regions exert different fate directing instructions on transplanted NSCs. Surprisingly, transplantation into the same regions under pathological paradigms led to dissenting results depending on the kind of pathology or acute injury. Since evidence for a differential impact of GM versus WM on NSC fate was rare, I next transplanted p57kip2 suppressed and control NSCs into the GM and WM of both, brain and spinal cord. White matter structures in both CNS compartments promoted an oligodendroglial fate compared to gray matter. Moreover, besides an additional increase in oligodendroglial descendants of p57kip2 suppressed NSCs in WM, this intrinsic modulation overruled the inhibitory nature of GM areas with regard to oligodendrocyte generation. Finally, I showed that the transplantation of p57kip2 suppressed NSCs in an acutely injured spinal cord also led to the promotion of an oligodendroglial fate choice but resulted in a net cell loss, probably due to a higher vulnerability of this lineage in a hostile environment.

This study reveals the high degree in adaptability of transplanted NSCs as the fate choice was almost exclusively driven by the cellular need of the respective injury. Furthermore, it discloses the necessity for more comparability within and between NSC transplantation studies owing to the high degree in heterogeneity between different CNS regions and between healthy and pathological tissue. Since an intrinsic modulation could partially overrule microenvironmental-driven lineage instructions in unharmed and injured CNS tissue, this study provides evidence for a hierarchy of intrinsic versus extrinsic fate directing signals, which should be considered in future cell replacement studies.

# Zusammenfassung

Das Zentralnervensystem (CNS) der Wirbeltiere besteht hauptsächlich aus Gehirn und Rückenmark. In beiden Strukturen können weiße und graue Substanz (WM bzw. GM) durch ihre Zellzusammen-setzung und damit Funktionalität unterschieden werden. Obwohl das ZNS hocheffizient ist, zeigt es nach einer akuten Verletzung oder anderen neuropathologischen Erkrankungen, bei denen Nervenzellen verloren gehen, eine geringe Regenerationsfähigkeit. Adulte neurale Stammzellen (NSCs) können möglicherweise verlorene Zellen ersetzen. Jedoch sind sie lokal auf nur zwei diskrete Nischen im Gehirn beschränkt. Die Transplantation multipotenter NSCs birgt jedoch ein großes Potenzial in Bezug auf Zellersatzstrategien, z.B. wenn myelinisierende Oligodendrozyten verloren gehen. In diesem Szenario muss die Schicksalsentscheidung von exogen applizierten NSCs in Richtung der oligodendrogliale Entwicklung gerichtet sein, um einen ausreichenden Zellersatz zu erzielen. Das p57kip2-Gen wurde als negativer Regulator der oligodendroglialen Schicksalsentscheidung bei Ratten-NSCs beschrieben. Es war jedoch nicht bekannt, ob p57kip2-supprimierte NSCs nach einer Transplantation in gesundes oder verletztes GM- und WM-Gewebe einen ähnlichen Anstieg der Oligodendrogenese zeigen. Darüber hinaus wurde das Ergebnis der Schicksalswahl transplantierter NSCs bei verschiedenen neuropathologischen Erkrankungen zwar ausführlich beschrieben, jedoch selten aus einem krankheitsinstruierenden Blickwinkel verglichen.

Um diese Fragestellungen anzugehen, analysierte ich zunächst die öffentlich verfügbare Literatur, welche die Transplantation von NSCs in verschiedene unversehrte CNS-Regionen bzw. verschiedene neuropathologische Modelle beschreibt. Es wurde deutlich, dass verschiedene Gehirn- und Rückenmarksregionen einen unterschiedlichen Einfluss auf die Schicksalssteuerung transplantierter NSCs ausüben. Überraschenderweise führte die Transplantation in dieselben Regionen unter pathologischen Paradigmen zu anderen Ergebnissen, abhängig von der Art der Pathologie bzw. der akuten Verletzung. Da Hinweise auf einen unterschiedlichen Einfluss von GM gegenüber WM auf das Schicksal von NSCs selten waren, transplantierte ich als nächstes p57kip2-supprimierte sowie Kontroll-NSCs in die GM und WM von Gehirn und Rückenmark. Strukturen der weißen Substanz in beiden CNS-Kompartimenten förderten ein oligodendrogliales Schicksal im Vergleich zur grauen Substanz. Neben einem zusätzlichen Anstieg von Oligodendroglia nach p57kip2-Suppression in NSCs in der WM setzte diese intrinsische Modulation zusätzlich die Unterdrückung von GM-Bereichen hinsichtlich der Oligodendrozytenerzeugung außer Kraft. Schließlich zeigte ich, dass die Transplantation von p57kip2-supprimierten NSCs in ein akut verletztes Rückenmark zwar ebenfalls zur Förderung eines oligodendroglialen Schicksals führte, jedoch einen Nettozellverlust bedeutete, was wahrscheinlich auf eine höhere Anfälligkeit dieser Zellen gegenüber einer lebensfeindlichen Umgebung zurückzuführen ist.

Diese Studie zeigt den hohen Grad an Anpassungsfähigkeit transplantierter NSCs, da die Wahl des Schicksals fast ausschließlich vom zellulären Bedarf der jeweiligen Verletzung abhing. Darüber hinaus wird die Notwendigkeit einer besseren Vergleichbarkeit innerhalb und zwischen NSC-Transplantationsstudien aufgrund des hohen Grads an Heterogenität zwischen verschiedenen CNS-Regionen sowie zwischen gesundem und pathologischem Gewebe offenbart. Da eine intrinsische Modulation die schicksalinstruierenden Signale von Mikroumgebungen in unversehrtem und verletztem CNS-Gewebe teilweise außer Kraft setzen konnte, liefert diese Studie Hinweise auf eine Hierarchie zwischen intrinsischen und extrinsischen Schicksalssignalen, welche in zukünftigen Zellersatzstudien berücksichtigt werden sollten.

# Abbreviations

| | |
|---|---|
| ALS | Amyotrophic Lateral Sclerosis |
| aNSC | adult Neural Stem Cell |
| Aqp4 | Aquaporin-4 |
| BBB | Blood-Brain-Barrier |
| BDNF | Brain-Derived Neurotrophic Factor |
| BMP | Bone-Morphogenic Protein |
| Cdkn1c | Cyclin-Dependent Kinase Inhibitor 1C |
| CNS | Central Nervous System |
| Cspg4 | Chondroitin Sulfate Proteoglycan 4 |
| Dcx | Doublecortin |
| DG | Dentate Gyrus |
| EAE | Experimental Autoimmune Encephalomyelitis |
| ESC | Embryonic Stem Cell |
| GE | Ganglionic Eminence |
| GFAP | Glial Fibrillary Acidic protein |
| GM | Gray Matter |
| GSTπ | Glutathione S-Transferase pi |
| GZ | Granule Zone |
| Hes | Hairy and Enhancer of Split |
| iPSC | Induced Pluripotent Stem Cell |
| LGE | Lateral Ganglionic Eminence |
| LIF | Leukemia Inhibitory Factor |
| MCAO | Middle Cerebral Artery Occlusion |
| MGE | Medial Ganglionic Eminence |
| mOL | Myelinating Oligodendrocyte |
| MS | Multiple Sclerosis |

| | |
|---|---|
| MSC-CM | Mesenchymal Stem Cell Conditioned Medium |
| NB | Neuroblast |
| NEC | Neuroepithelial Cell |
| NeuN | Hexaribonucleotide binding Protein-3 |
| Neurog2 | Neurogenin 2 |
| NG2 | Neuron-Glia antigen 2 |
| OB | Olfactory Bulb |
| Olig1 | Oligodendrocyte transcription factor 1 |
| Olig2 | Oligodendrocyte transcription factor 2 |
| OPC | Oligodendroglial Precursor Cell |
| PDGFRα | Platelet Derived Growth Factor Receptor, alpha |
| Plp1 | Proteolipid protein 1 |
| PMD | Pelizaeus-Merzbacher Disease |
| PNS | Peripheral Nervous System |
| RGC | Radial Glial Cell |
| RMS | Rostral Migratory Stream |
| SCI | Spinal Cord Injury |
| SGZ | Subgranular Zone |
| Shh | Sonic hedgehog |
| Shi | shiverer |
| Sox10 | SRY-box 10 |
| Sox9 | SRY-box 9 |
| SVZ | Subventricular Zone |
| TAP | Transient Amplifying Cell |
| TBI | Traumatic Brain Injury |
| VZ | Ventricular Zone |
| WM | White Matter |
| β-III-Tub | Class III β-tubulin |

# Contents

# 1. Introduction

## 1.1 Cellular composition of the central nervous system

The nervous system of all mammalian species, including human, is composed of a peripheral and a central nervous system (PNS and CNS, respectively). The PNS serves to sense environmental stimuli and forward this information via afferent nerve fibers to the CNS. Here, the information is processed and efferent signals propagate back into the periphery, e.g. a motoric signal along a peripheral nerve fiber ending at a neuromuscular junction causing this muscle to contract. The CNS is composed of brain and spinal cord. The brain can be further classified into a multitude of sub regions such as the cerebellum, hippocampal formation, stratum radiatum, motor- and sensory cortex. Simplistically, brain- and spinal cord tissue can be broadly categorized into two distinct types of tissue: gray and white matter (GM and WM, respectively). On a macroscopic level, these two classifications arise either from the more gray (non-myelinated) or from white (myelinated, lipid-rich structures) appearance of the respective tissue. Further differences of the two tissue classes will be described in more detail later during the introduction. However, it is noteworthy that the two main cell types, neurons and macroglia, reside in both matters. In general, neurons reflect the electro-chemically active functional subunit in the CNS. Typically, a neuron consists of a neuronal cell body harboring the nucleus and other fundamental organelles, a dendritic tree serving as input region to receive information, and a single axon that serves to propagate the signal to other neurons or muscles. The ends of both, dendrites and the axon, show membrane protuberances called synapses, which enable chemical signal transduction via the release of neurotransmitters. Eventually, neurotransmitters released by pre-synapses on the axon side diffuse via the synaptic cleft to reach receptors on the dendritic post-synapse. Here, binding of the neurotransmitter leads to a change in ion homeostasis in the neuron which, when reaching a certain threshold, evokes an action potential at the axon hillock leading to the depolarization of the membrane. This electrical signal then propagates along the axon to activate neurotransmitter release at the next pre-synapse. In order for an action potential to propagate faster along an axon, the axon diameter has to be increased as described for giant squids (Zalc B et al., 2008). However, limited spatial properties in higher (land-)animals has impacted evolution in a way to keep smaller sized axons with yet fast signal conduction by arranging the electrical current to „jump“ along an axon. This so-called saltatory signal transduction is warranted by insulating axons with myelin sheaths that show regularly occurring gaps, at which the action potential is newly generated (Cohen CCH et al., 2020). Myelin consists of lipid and myelin proteins and is wrapped around axonal structures by myelinating oligodendrocytes (mOLs) in the CNS. One mature mOL can insulate multiple axons (in contrast to their peripheral counterparts: Schwann cells) and constitutes one of the main macroglial cell types. Very recent evidence attributes even supporting metabolic functions to these mOLs (Funfschilling U et al., 2012;Lee Y et al., 2012;Saab AS et al.,

2016;Snaidero N et al., 2017). The other macroglial cell mainly provides structural and metabolic support to neurons, ensheathes the synaptic cleft in a structure called tripartite synapse to avoid neurotransmitter spillover and consequently excitotoxicity, and serves as a filter cell between blood vessels and neural cells as part of the blood-brain-barrier (BBB): the astrocyte (Sofroniew MV and Vinters HV, 2010). Besides these two macroglial cells, neuroectoderm-derived ependymal cells reside in the brain and build up a barrier between the cerebral-spinal fluid filled ventricles and neural tissue. In contrast to neurons, macroglia and ependymal cells, microglia are mesoderm-derived cells in the CNS. These cells reside in all parts of the CNS and play a critical role as the immune response system of the CNS (Prinz M et al., 2019). The generation of new neural cells, referred to as neurogenesis or gliogenesis, respectively, decreases dramatically from early postnatal development to adulthood (Encinas JM et al., 2011;Katsimpardi L and Lledo PM, 2018;Kuhn HG et al., 1996;Sorrells SF et al., 2018). In the CNS, both processes are limited by the number of precursors (e.g. oligodendroglial precursor cells, OPCs) of each individual cell type at post-developmental time points. Moreover, two niches in the adult mammal (and bird) harbor adult neural stem cells (aNSCs) which potentially give rise to neurons and macroglia: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG) within the hippocampal formation (Gotz M et al., 2016;Kriegstein A and Alvarez-Buylla A, 2009).

## 1.2 Central nervous system development

Following the formation of the blastula from a fertilized egg, three germ layers form: endoderm, mesoderm, and ectoderm. Ectoderm in proximity to the noto chord thickens and develops into the neuroectoderm: the neural plate. As lateral edges of this cell layer fuse, the neural plate folds and eventually forms the neural tube. While these former lateral edges consist of neural crest cells, which will generate the PNS, cells of the neural tube will give rise to neurons and glial cells of the CNS. The formation of three primary vesicles at the anterior part of the tube particularly mark the beginning of brain development. These vesicles will subdivide into five secondary vesicles and already define the major structures of the adult brain (e.g.: cortex, midbrain, or cerebellum). The single cell layered wall of the neural tube mainly contains NSC, the neuroepithelium. By numerous symmetric cell divisions these cells drive enlargement of the brain until asymmetric cell divisions (division of an NSC into a self-renewing NSC and a daughter cell primed to differentiate) eventually produces neuronal and glial precursor cells. Increased neuronal cell migration hallmarks the next phase of brain development, while proliferation starts to decline. Once neuronal precursors and neurons have reached their final position, they differentiate and mature in order to form functional neuronal networks. While developmental neurogenesis is declining, gliogenesis begins (Taverna E et al., 2014).

### 1.2.1 Developmental and adult neurogenesis in the subventricular zone

The generation of neurons from stem cells is referred to as neurogenesis. While its existence in the adult human is still under debate (Boldrini M et al., 2018;Moreno-Jimenez EP et al., 2019;Sorrells SF, et al., 2018), adult neurogenesis has been well studied in rodents and allowed insight into key similarities and differences compared to developmental neurogenesis (Gotz M, et al., 2016). In rodents, developmental neurogenesis starts with the formation of the neural tube. The first stem cells of the CNS, neuroepithelial cells (NECs), form the germinal zone along the ventricle called the ventricular zone (VZ) of the neural tube. During this early phase (starting at E10.5 in mice), NECs transform into radial glial cells (RGCs), which show an elongated radial morphology and proliferate symmetrically to increase the pool of these primary NSCs (Kriegstein A and Alvarez-Buylla A, 2009;Taverna E, et al., 2014). Subsequently, proliferation changes from symmetric to asymmetric cell division in which one RGC gives rise to a daughter RGC and an intermediate progenitor cell, which either directly differentiates into a neuron or produces two neurons by asymmetric cell division. By this mechanism, the pool of primary NSCs remains constant while the number of progenitors and subsequently neuronal cells increases. During development, RGCs serve as "highways" for newly produced progenitors to migrate in radial direction along radial fibers in order to reach cortical structures. Following proliferation and migration phases, neural progenitors start to mature by becoming post-mitotic and forming synaptic networks. Remaining RGCs in the adult SVZ are considered adult NSCs also called type B cells (Doetsch F et al., 1999;Gotz M, et al., 2016). Although adult NSCs still express the astrocyte marker glial fibrillary acidic protein (GFAP, as do their embryonic counterparts, the RGCs), they can be well discriminated from astrocytes by their expression of the cytoskeletal protein Nestin. Upon activation, these slowly dividing stem cells give rise to type C cells (transient amplifying progenitors, TAPs), which are highly proliferative. In the SVZ, TAPs give rise to type A cells, also termed neuroblasts, which are less proliferative and mainly migrate along the rostral migratory stream (RMS) towards the olfactory bulb (OB). Here, neurogenesis ends with the differentiation and maturation of neuroblasts (NBs) into glomerular- and granule neurons. With the transition into migrating NBs, these cells become positive of the protein Doublecortin (Dcx), indicating their commitment towards the neuronal lineage. Once mature, neuronal cells lose Dcx-positivity and can hence be identified by the expression of Hexaribonucleotide binding Protein-3 (also termed neuronal nuclear antigen, NeuN).

### 1.2.2 Gliogenesis

Astrocytes and oligodendrocytes are summarized as macroglial cells and are believed to outnumber neurons in an approximately 2:1 ratio in the CNS (von Bartheld CS et al., 2016). In general, neurogenesis precedes gliogenesis and the conversion from one process to the other is referred to as neuron-glial switch. The emergence of both cell types, neurons and macroglia, is equally dependent on the expression of pro-neural versus pro-glial factors as well as inhibitory signals for both lineages. During late embryogenesis and early postnatal stages, macroglial cells develop from RGCs which involves a variety

of extrinsic signaling molecules and intrinsic transcription factors (Kriegstein A and Alvarez-Buylla A, 2009). These molecules orchestrate the fate specification of neuronal versus glial progenitors and subsequently that of oligodendroglial versus astroglial cells. Components of the sonic hedgehog- (Shh) and bone morphogenic protein- (BMP) pathway pattern the neural tube (Liem KF, Jr. et al., 2000;Timmer JR et al., 2002;Zagorski M et al., 2017). While cells residing in the ventral neural tube secrete Shh, the origin of BMPs lays in the dorsal region and both molecules show antagonistic function. Astrocytes are the first macroglial cells that are generated during development. Here, BMP signaling induces the expression of hairy and enhancer of split (Hes) leading to the repression of downstream pro-neuronal genes. Simultaneously, activated component of the Notch signaling pathway further drive gliogenesis (Ge W et al., 2002). The expression of the basic-helix-loop-helix (bHLH) transcription factor Sox9 marks glial progenitors primed to differentiate into astrocytes (Stolt CC et al., 2003;Sun W et al., 2017). As astrocyte mature, their volume increases and they become highly ramified or elongated, depending on their final destination (astrocyte subtypes will be introduced in a later chapter). In addition, mature astrocytes express distinct marker proteins that can help to identify them such as the water channel protein aquaporin 4 (Aqp4), SRY-box 9 (Sox9), and GFAP.

In the developing telencephalon, OPC fate specification takes place in the ventral lateral ganglionic eminence (LGE) as well as the medial ganglionic eminence (MGE) shortly after astroglial specification. Oligodendroglial precursor cells are marked by their expression of platelet derived growth factor receptor, alpha (PDGFRα) as well as the bHLH transcription factors Olig1, Olig2 (oligodendrocyte transcription factor 1 and 2, respectively), and SRY-box 10 (Sox10) and initially emerge from the ventral pMN domain to migrate throughout the whole developing spinal cord and telencephalon (El Waly B et al., 2014). These transcription factors actively promote the generation of OPCs. The maturation into oligodendrocytes is accompanied by a change in marker protein expression. While Olig2 and Sox10 serve as pan-oligodendroglial markers and are involved in oligodendroglial fate specification as well as the initiation of myelination, the expression of glutathione S-transferase pi (GSTπ) has been used to identify mature OLs (Bunk EC et al., 2016;Kremer D et al., 2009). Once activated to insulate an axon with myelin, myelin protein (e.g. 2',3'-cyclic nucleotide 3' phosphodiesterase (CNPase), myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG)) expression starts (Bercury KK and Macklin WB, 2015).

### 1.2.3 Oligodendrogenesis

Oligodendrocytes are mature glial cells that differentiate from OPCs to myelinate axons, thereby facilitating saltatory signal propagation throughout the CNS. Developmental oligodendrogenesis in the brain is characterized by three consecutive waves of OPC generation. The first wave of OPCs arises at E12.5 in the mouse telencephalon from NK2 homeobox 1 (Nkx2.1) expressing precursors in the medial ganglionic eminence (MGE) and ventral parts of the lateral ganglionic eminence (LGE) (Spassky N et al., 2001). These early OPCs are replaced by a second and third wave of OPCs from the lateral and

caudal GE at E15.5 and the dorsal VZ at early postnatal time points, respectively (Kessaris N et al., 2006). Similarly, in the neural tube/developing spinal cord two prenatal waves of OPCs from the ventral and dorsal region, respectively, are followed by a third postnatal wave (Spassky N, et al., 2001). The exact oligodendroglial patterning of the neural tube progenitor domains (denoted p0, p1 p2, p3 and pMN) is yet not fully understood but involves a combination of extrinsic and intrinsic neural repressors as well as the expression of pro-oligodendroglial factors (El Waly B, et al., 2014). While extrinsic Shh signaling and intrinsic expression of Olig2 and Sox10 serve as pro-oligodendroglial determinants, downregulation of pro-neuronal neurogenin 2 (Neurog2) is equally important. Some of the OPCs do not differentiate and can be identified by their expression of chondroitin sulfate proteoglycan 4 (Cspg4; referred to as neuron-glia antigen 2, NG2). These cells remain proliferative into adulthood and potentially serve as a backup pool for lost mOLs (Vigano F et al., 2016).

### 1.2.4 Adult oligodendrogenesis in health and disease

During development, not all OPCs differentiate into mOLs but remain as slowly proliferating or quiescent NG2-cells throughout the whole CNS (El Waly B, et al., 2014). While OPCs in the GM are almost exclusively activated to proliferate and differentiate following an injury, WM-OPCs slowly divide and give rise to new OLs during adulthood, both during health and disease state (Spitzer SO et al., 2019). However, the production of new OPCs and subsequently mOLs rapidly declines with age to reach a low generation rate in rodents (Psachoulia K et al., 2009). Besides OPCs, NSCs from the SVZ contribute to adult oligodendrogenesis. Here, stem cells from the SVZ (foremost the dorsal SVZ adjacent to the corpus callosum) first give rise to rapidly dividing transient amplifying cells (TAPs) that in turn can give rise to OPCs, which migrate radially into the WM or GM (Menn B et al., 2006). Of note, to this point it is unknown whether adult oligodendrogenesis is also a feature of the human CNS (exceeding the age of 19) (Giedd JN and Rapoport JL, 2010).

Different stimuli such as voluntary exercise increase the rate of adult OPC/OL generation (Alvarez-Saavedra M et al., 2016) in the healthy state. Furthermore, the strongest stimuli for OPC and OL generation is damage to the CNS by e.g. traumatic injury, autoimmune diseases or infections leading to demyelination – the breakdown of myelin. In general, demyelination is accompanied by the death of mOLs even though two studies using RNA-Sequencing- and $C^{14}$-analysis of Multiple sclerosis (a demyelinating autoimmune disease) post mortem brain tissue showed that in these patients mOLs only degenerated without being lost (Jakel S et al., 2019;Yeung MSY et al., 2019). However, the general features seen after demyelination are proliferation of adult OPCs, their migration towards the lesion and subsequent differentiation leading to an increased number of mOLs at the expense of the OPC pool (Franklin RJ et al., 1997;Levine JM and Reynolds R, 1999). Besides resident OPCs, aNSCs from the SVZ also contribute to the generation of new oligodendroglial cells upon demyelinating events (Nait-Oumesmar B et al., 1999;Picard-Riera N et al., 2002). While these stem cells primarily give rise to NBs, which migrate along the RMS to eventually differentiate into neurons of the OB, their potential to also

generate mature OLs was increased four-fold following demyelination. Furthermore, Nait-Oumesmar and colleagues could show a similar expression of NSC- and OPC-markers in cells migrating from the human SVZ of MS patients (Nait-Oumesmar B et al., 2007).

## 1.3 Neural stem cells and their niches

Every stem cell needs its niche. In the CNS, NSCs reside either in the neural tube during development or in the SGZ of the DG and in the SVZ in the postnatal and adult organism, respectively. Here, contact with the surrounding cells as well as secreted signaling molecules affect the state (e.g. either quiescent or activated) of these stem cells. In principle, aNSCs are multipotent stem cells with the potential to give rise to neurons, astrocytes and oligodendrocytes directly or via the generation of intermediate cell states such as TAPs and respective progenitor cell types (Kriegstein A and Alvarez-Buylla A, 2009). However, depending on the niche, the regional position of one cell in this niche, and the current state of the organism (e.g. healthy versus diseased, young versus old) the ratio of aNSC-derived cells is not overall equal (Encinas JM, et al., 2011;Katsimpardi L and Lledo PM, 2018;Moreno-Jimenez EP, et al., 2019;Zweifel S et al., 2018). This plasticity of aNSCs to generate different proportions of descendants at different rates depending on environmental cues has promised great potential to manipulate the fate of these cells. Therefore, isolated and *in vitro* cultured NSCs are kept in „niches“ (culture media) which are carefully designed by researchers to i) provide necessary factors ensuring cell survival and ii) manipulate cell fate decisions and behavior of either wild type or further genetically engineered NSCs.

### 1.3.1 Adult neural stem cells

During development, the majority of RGC descendants eventually give rise to post-mitotic cells of the CNS. A small proportion of RGC-derived adult radial glial-like cells remain as aNSCs in two niches of the adult brain: the SGZ and the SVZ (Altman J and Das GD, 1965;Doetsch F, et al., 1999;Fuentealba LC et al., 2012;Kriegstein A and Alvarez-Buylla A, 2009).

The DG of the hippocampal formation is compartmentalized into molecular layer, granule zone (GZ), hilus and a one cell layer thin SGZ between hilus and GZ (Eriksson PS et al., 1998). Radial glial-like cells can be identified molecularly by their expression of Nestin and GFAP as well as their distinct morphology of a cell body residing in the SGZ and a radial process projecting thru the GZ towards the molecular layer. Acting as NSCs in the SGZ, these cells spontaneously give rise to neurons and some astrocytes (Boldrini M, et al., 2018;Eriksson PS, et al., 1998;Ming GL and Song H, 2011;Moreno-Jimenez EP, et al., 2019;Spalding KL et al., 2013). While it is not clear whether astrocytic generation is direct or follows the generation of a glial precursor, different intermediate cells are generated before final differentiation into a neuron. Two-photon *in vivo* live cell imaging showed several symmetric and asymmetric cell divisions of radial glia-like cells and their non-radial glia-like descendant before these neural progenitors terminally differentiated into neurons of the GZ (Pilz GA et al., 2018). The generation of oligodendroglial cells by SGZ-NSCs does not occur without further ectopic manipulation of these

cells such as the inhibition of negative regulators of oligodendrogenesis (e.g. p57kip2, Drosha, Prox1) (Akkermann R et al., 2017). Of note, the number of OLs is very low compared to neurons and astrocytes in the DG.

The second niche that harbors adult NSCs, the SVZ, lays adjacent to the ventricular wall of the lateral ventricles. Here, multi-cilia ependymal cells line the inner wall of the lateral ventricles. Adult neural stem and progenitor cells are located adjacent to and partially intermingled between these ependymal cells. This pool of stem and progenitor cells consists of three main cell types: type A-, B, and C-cells (Doetsch F and Alvarez-Buylla A, 1996;Doetsch F, et al., 1999). Type B-cells represent the pool of quiescent and slowly dividing aNSCs, which give rise to fast proliferating type C-cells (also called TAPs). These TAPs are the major source of neuronal and glial cells from the SVZ. In the case of neuronal primed cells, TAPs convert into type A-cells (referred to as NBs) which migrate along the RMS towards the OB to eventually differentiate into granule- or glomerular neurons. While the majority of SVZ-derived cells adapt a neuronal identity, SVZ-NSCs harbor the potential to spontaneously give rise to glial cells as well. Besides the generation of astrocytes (Faiz M et al., 2015), pro-oligodendroglial cues such as the expression of the transcription factors Olig2 or Sox10 drive NSCs to generate OPCs (Menn B, et al., 2006;Pozniak CD et al., 2010).

### 1.3.2 The role of p57kip2 in directing neural stem cell fate

While many factors have been explored to be involved in the regulation of cell fate decisions in aNSCs, my thesis focuses on the cyclin-dependent kinase inhibitor 1C (Cdkn1c) p57kip2. p57kip2 is involved in several processes throughout the nervous system (CNS and PNS) during development and in the adult state. Classically (from a cancer research perspective) p57kip2 is regarded as a tumor suppressor gene and therefore a negative regulator of proliferation by binding to G1 cyclin-CDK complexes. Furthermore, p57kip2 is associated with Beckwith-Wiedemann syndrome, which leads to an increased risk of tumor formation in children. However, studies of myelinating cells of the PNS, Schwann cells, could attribute an additional role to p57kip2 as a negative regulator of Schwann cell differentiation (Heinen A et al., 2008). Small hairpin RNA (shRNA) interference mediated knock down of p57kip2 in cultured Schwann cells led to an increased differentiation rate and myelin protein expression (Heinen A, et al., 2008). Similarly, several studies proved a direct implication of p57kip2 in the control of proliferation versus differentiation in OPCs (Dugas JC et al., 2007;Gottle P et al., 2015;Kremer D, et al., 2009). Our own laboratory could not only show that p57kip2 knock down but also its specific subcellular localization (out of the nucleus into the cytoplasm) promoted OPC differentiation and OL-mediated myelination (Gottle P, et al., 2015). In neural crest cells of the developing zebrafish, p57kip2 promotes both neuronal development in cells with high p57kip2 expression and oligodendroglial fate in cells with low p57kip2 levels (Park HC et al., 2005). In contrast to Parks results, a study of SGZ-NSCs suggests that p57kip2 is important for the maintenance of NSC quiescence in the SGZ and that reduction of p57kip2 leads to NSC activation and increased neurogenesis *in vivo* (Furutachi S et al., 2013).

Moreover, the emergence of slowly dividing, aNSCs of the SVZ depends on the expression of p57kip2 in embryonic neural progenitor cells as its deletion led to an impaired emergence of SVZ-NSCs (Furutachi S et al., 2015). Experiments of our own group further revealed involvement of p57kip2 in adult NSC fate modulation for both, SGZ- and SVZ-NSCs (Jadasz JJ et al., 2012;Jadasz JJ et al., 2018). Stem cells from both niches were subjected to shRNA-mediated suppression of p57kip2 by nucleofection *in vitro*. Positively transfected aNSCs showed decreased expression of neuronal and astroglial marker genes such as Class III β-tubulin (β-III-Tub) and GFAP, respectively. At the same time, the expression of proteins highlighting different stages of oligodendroglial development (for OPCs: ceramide galactosyltransferase (CGT), pre-mature OLs: Adenomatous Polyposis Coli (APC), mature OLs: GSTπ) was increased. Additionally, transplantation of SGZ-NSCs into healthy rat spinal cords led to an increased differentiation into GSTπ-positive OLs at the expense of GFAP-positive astrocytes upon p57kip2 knock down, demonstrating initial relevance of p57kip2 modulated aNSCs for the treatment of demyelinating injuries or diseases.

## 1.4 Heterogeneity in the central nervous system

Heterogeneity occurs in many different facets, especially in the CNS. Although hippocampus, cerebellum, and spinal cord are all part of the CNS, they are very different in their functionality and cell composition. More than 100 years ago, Ramon y Cajal already showed how nerve cells (neurons) appear in different morphologies. In the time since, it became clear that also other cells found throughout the entire CNS, such as OPCs and astrocytes, appear in many different morphologies and molecular compositions potentially leading to functional differences (Cadwell CR et al., 2016;Fuzik J et al., 2016;Petitpre C et al., 2018;Spitzer SO, et al., 2019). The level of functional heterogeneity further increases if the CNS is subjected to damage, as for example astrocytes become reactive (Liddelow SA et al., 2017) or cells from the systemic milieu can enter the CNS upon disruption of the BBB. The most important levels of heterogeneity concerning the subject of this thesis will be introduced in the following chapters in more detail.

### 1.4.1 Glial heterogeneity

„*Understanding how myriad different cell types in the brain communicate to give rise to cognition and perception is the central challenge of neurobiology today*.“ (Emery B and Barres BA, 2008). Understanding of the multitude of neurons in the CNS has been the focus of neuroscientific research in the past decade. However, more and more evidence accumulates hinting towards a comparable level of heterogeneity among glial cells. Its deeper understanding is the basis for future clinical approaches tackling diseases in which glial cells are affected.

Among astrocytes, two broad morphologies can be distinguished: i) protoplasmic astrocytes populating the GM and ii) fibrous astrocytes residing in WM structures (Andriezen WL, 1893;Matias I et al., 2019). Protoplasmic astrocytes are heavily ramified, which hints towards their contribution to neuromodulatory

functions in the tripartite synapse as the majority of synapses are found in GM structures (Bushong EA et al., 2002;Matias I, et al., 2019;Oberheim NA et al., 2012). In contrast, fibrous astrocytes of the WM have a less complex structure and are probably functionally restricted to nutritious and homeostatic support (Lundgaard I et al., 2014). Furthermore, astrocytes differ in their expression profile as revealed by RNA-sequencing- and proteome analyses (Bachoo RM et al., 2004;Chai H et al., 2017) depending on their localization in the CNS and the age of the organism. Molecularly, the identification of astrocytes throughout the CNS has been challenging due to their inhomogeneous expression of a specific astrocyte marker. While GFAP has been widely accepted and used to identify astrocytes *in vitro* and *in vivo* (Bonaguidi MA et al., 2005;Bushong EA, et al., 2002;Jadasz JJ, et al., 2012;Jadasz JJ, et al., 2018;Zhang Z et al., 2019), its expression does vary among astrocytes (Zhang Z, et al., 2019). Other markers such as aldehyde dehydrogenase 1 family member L1 (Aldh1l1), S100 protein beta polypeptide neural (s100b) or solute carrier family 1 member 3 (Slc1A3) have been equally challenged by the heterogeneous nature of astrocytes. Here, Aldh1l1 mainly labels cortical and hippocampal astrocytes and s100b additionally labels subsets of oligodendroglia (Hachem S et al., 2005).

Mature mOLs ensure saltatory signal propagation and axonal integrity by insulating axons with myelin sheaths. This function is mainly attributed to WM structures, since mOLs reside in the GM in much fewer numbers. Recent data suggests that mOLs also provide energy metabolites to neurons via so-called cytoplasmic „myelinic“ channels (Philips T and Rothstein JD, 2017). Knock out studies of different myelin proteins (compact and uncompact) elegantly showed that axon degeneration does not occur in knock out animals, where uncompact myelin was still present as opposed to knock outs with only compact myelin (Snaidero N, et al., 2017). Whether this newly discovered function is a feature shared by all mOLs (e.g. GM vs WM and independent of the myelinated axon diameter) is still under debate. Single cell RNA-Sequencing analysis of mature and myelinating oligodendroglial cells, respectively, along different brain regions identified six molecularly similar (expression of mature OL genes), yet unequal OL populations which furthermore differed from two distinguishable myelinating OL states (Marques S et al., 2016). Oligodendroglial progenitor cells serve as a reservoir pool for mOLs during OL turnover and during re-myelination phases following demyelinating events (Vigano F, et al., 2016). Despite being morphologically homogenous, at least three OPC sub clusters can be distinguished by their unique combinatorial expression of canonical OPC marker genes (Marques S, et al., 2016). Canonical OPC marker genes (Olig1, Olig2, NG2, PDGFRα) are expressed by all OPCs, however, the expression level between each cluster differs and further led to the identification of cell surface proteins, unique for each subtype (Spitzer SO, et al., 2019). Here, GO term analysis (as well as other studies on functional OPC heterogeneity) (Fernandez-Castaneda A et al., 2020;Marisca R et al., 2020;Vigano F et al., 2013;Vigano F, et al., 2016) highlighted the role of varying OPC subpopulations in functionally divers brain functions ranging from reserve pool for mOLs to integrating neuronal activity. With regard to GM versus WM differences, Vigano and colleagues elegantly showed by hetero- and homotopically

transplanting OPCs from GM into WM tissue of the mouse cerebral cortex (and vice versa) a higher intrinsic determination of WM-derived OPCs to differentiate into mOLs (Vigano F, et al., 2013). Furthermore, a WM tissue environment led to a myelination phenotype by transplanted GM-derived OPCs more similar to WM-derived OPCs, revealing environmental cues specific for GM versus WM cerebral tissue.

### 1.4.2 Heterogeneity between the diseased/injured versus healthy central nervous system

Gray matter and white matter structures differ in their cell composition, e.g. WM being mostly devoid of neuronal cell bodies. Furthermore, the differences in relative number of neuronal- and glial cells between these two tissues lead to a heterogeneous environment. Normal homeostasis and basic CNS function across the entire CNS must be warranted by all (glial) cells in the healthy state using comparable biochemical pathways. However, transplantation experiments of multipotent NSCs have revealed: i) remarkable adaptability of NSCs to sense and react to their environment (which will be subjected in more detail in a later paragraph), and ii) undisputed proof about the heterogeneous effects different brain areas exert on NSCs fate (Gage FH et al., 1995;Herrera DG et al., 1999;Seidenfaden R et al., 2006). Interestingly, these host evoked effects on differentiation seem to be conserved across species as transplantation of human embryonic neural precursor cells into murine CNS tissue showed a comparable outcome (Fricker RA et al., 1999). Additionally, these studies revealed a limited migratory feasibility within the CNS except for specialized structures since significant migration only occurred when NSC populations were grafted near the RMS (Fricker RA, et al., 1999). This heterogeneity within the CNS further increases by introduction of neuropathological paradigms (e.g. traumatic brain- or spinal cord injury, demyelination, stroke) (Beyer F et al., 2019). The increase is based on three single events or a combination of them, depending on the pathological model: i) (local) breakdown of the BBB as for example in spinal cord injury models leads to the infiltration of immune cells (further increasing cell heterogeneity) and cytokines, which under healthy conditions would not be able to pass the BBB, further increasing heterogeneity in local signaling molecules; ii) the composition of cells in lesion areas changes (e.g. demyelinated, mOL-devoid lesions in MS models; recruitment of microglia and NG2-cells to the side of traumatic brain injury) and leads to cell-cell contacts and paracrine signaling cocktails differing from that of the heathy state; and iii) cell debris and inflammatory signals similarly activate microglial cells to change their state from „M2“(quiescent/immunosuppressive) to„M1“ (pro-inflammatory) (Tang Y and Le W, 2016) and astrocytes from homeostatic “A2” to a reactive type “A1” (Clarke LE et al., 2018;Liddelow SA, et al., 2017). Here, changes in morphology, proliferation capacity, mobility, transcriptome, and functionality separate these cells clearly from their „homeostatic cell origin“, leading to increased local diversification in neuropathological affected areas.

## 1.5 Central nervous system diseases and injuries affecting oligodendrocytes

Due to its multiplicity in functions essential for survival as well as in defining the human self, assault to the CNS has dramatic effects. This chapter will serve to give a broad overview of the different kinds of CNS pathologies (including acute injuries) relevant to both publications in this thesis and to introduce corresponding animal models before highlighting different scientific efforts which aimed to ameliorate the negative effects of CNS damage.

Deficiency in neurological function can be based on either focal (e.g. traumatic injury to the brain or spinal cord) or global (e.g. autoimmune responses and hereditary diseases) insults to the CNS. A focal insult by direct, forced impact to the brain or spinal cord results in a primary- and secondary injury (Ditunno JF et al., 2004). The primary, immediate consequence is destruction of the tissue architecture and homeostasis while the secondary injury is the result of cell death in and around the lesion area as well as the infiltration of immune cells, which results in an inflammatory response. Moreover, the generation of a glial scar by reactive GFAP-positive astrocytes around the lesion border constitutes a major challenge for re-growing axons following spinal cord injuries (SCIs) (Adams KL and Gallo V, 2018). The rodent models for traumatic brain injury (TBI) to the brain (stab wound model) and SCI (compression and hemi- or completely transected spinal cord) can be modified according to the region and tissue depth of interest and closely resemble the clinical situation in terms of wound infliction as well as primary and secondary effects. Middle cerebral artery occlusion (MCAO) serves as a model for stroke – another focal injury to the CNS (Carmichael ST, 2005). Here, focal ischemia is induced by blocking the blood flow thru the middle cerebral artery (by intraluminal suture, inserting a suture until the tip occludes the middle artery, or by injection of blood cloths) leading to a lack of oxygen supply in certain brain areas. Despite resembling the impact of an actual stroke on multiple different cells in the oxygen deprived area, MCAO in rodents usually affects areas relatively larger than stroke-affected areas in the human brain (Carmichael ST, 2005).

In contrast to defined areas of injury, neuropathology with negative effects on a global CNS scale harbor different challenges. Leukodystrophies like the Pelizaeus-Merzbacher disease (PMD) often derive from mutations in myelin genes (Koeppen AH and Robitaille Y, 2002). In PMD, multiple mutations in the proteolipid-protein 1 gene (a major component of CNS myelin sheaths) lead to disturbed formation or complete lack of myelin sheaths around axons. The so-called shiverer (shi) mouse has been developed to study such undirected failure in myelin sheath formation (Molineaux SM et al., 1986). A homozygous mutation in the gene encoding for MBP (a major component of compact myelin) results in the formation of abnormal myelin in which only uncompact myelin is formed. Although different in the (mutational) origin and unable to resemble the multitude of dysmyelinating neuropathology, shi mice show global failure in myelination and have allowed the study of reinforcing myelination by transplanted stem cell derived mOLs (Uchida N et al., 2012).

Apart from clearly hereditary/mutation-driven diseases negatively affecting myelin integrity, autoimmune diseases can lead to demyelination and consequently degeneration of axons. Multiple sclerosis (MS) patients suffer from multiple focal lesions on a global CNS scale showing demyelination, microglial activation, failure in OPC differentiation, and subsequently neuronal death (Goldenberg MM, 2012;Weinshenker BG, 1996). Consequently, this autoimmune-driven attack of myelin and mOLs results in sensory- and motor-dysfunction as well as cognitive decline (Thompson AJ et al., 2018). Experimental autoimmune encephalomyelitis (EAE) serves as an animal model to study especially the inflammatory aspects of MS. Here, in general an injection of a peptide of the myelin oligodendrocyte glycoprotein ($MOG_{35-55}$) into healthy adult mice results in a T-cell driven autoimmune response against the body's mOLs. Although direct evidence for the cause of MS in human is still unknown, the initial EAE etiopathology is comparable to the (inflammatory) processes documented in human MS tissue (Miller SD and Karpus WJ, 2007;Rivers TM and Schwentker FF, 1935).

### 1.5.1 Treatment strategies

In line with the plurality of neuropathology, their cause, etiopathology, affected cell types and areas, strategies for supporting CNS regeneration are diverse. These strategies range from pharmacological approaches to support the survival and differentiation of affected cell types to computational devices implanted in Parkinson´s disease (PD) patients for deep brain stimulation (Groiss SJ et al., 2009) or in SCI models in monkeys to alleviate gait deficits (Capogrosso M et al., 2016). Moreover, stem cell based therapies either transplanted into the CNS tissue or applied systemically aim to replace lost cells or support remaining cells via the secretion of paracrine factors (Beyer F, et al., 2019;Han F et al., 2015;Tang Y et al., 2017). In MS models, myelin breakdown and OL death is followed by perish of demyelinated axons. As symptoms in MS usually occur after the death of neurons, the search for treatment strategies has aimed at supporting the resident OPC pool to differentiate and make up for lost myelin (Gruchot J et al., 2019;Kremer D et al., 2019). Although the suppression or nuclear exclusion of the p57kip2 protein o supports OPC differentiation (Gottle P, et al., 2015;Kremer D, et al., 2009) *in vitro*, the translation into an *in vivo* MS model is pending. Here, genetic engineering of endogenous OPCs in several demyelinated areas constitutes a major challenge. In light of the tight regulation of the BBB and the problem to target only a specific cell type, the use of nanoparticles coated with OPC-specific antibodies has offered a new promising approach. Using NG2-coated nanoparticles, leukemia inhibitory factor (LIF), a cytokine that promotes developmental myelination (Ishibashi T et al., 2009) as well as remyelination (Deverman BE and Patterson PH, 2012;Laterza C et al., 2013;Slaets H et al., 2010), was successfully delivered to OPCs *in vivo* and led to an increase in myelin repair (Rittchen S et al., 2015).

A similar pro-oligodendroglial role of p57kip2 has been attributed to NSCs. Its suppression led to an increased oligodendroglial marker expression while astroglial identity declined (Jadasz JJ, et al., 2012). As these cells reside in only two discrete niches deep in the brain tissue (e.g. the SVZ), targeting of this

cell pool has been equally challenging. The use of implanted minipumps into the ventricle has been shown to successfully target endogenous NSCs (Shen Q et al., 2008). However, in the context of MS, it seems unlikely that endogenous stem cells are able to reach sides of demyelination in the brain and spinal cord. Instead of endogenously activating oligodendroglial differentiation (e.g. via genetic engineering), the use of mesenchymal stem cell conditioned medium (MSC-CM) resulted in a fate switch of cultured NSCs favoring an oligodendroglial phenotype (Jadasz JJ, et al., 2018;Rivera FJ et al., 2006). The transplantation of MSCs in neurodegenerative animal models supports the survival of these animals (Jin HK et al., 2002). Elucidation of the factor(s) responsible for the pro-oligodendroglial effect on NSCs and subsequent activation of endogenous MSCs (residing in the bone marrow) constitutes yet another promising approach in tackling global neurodegenerative diseases.

### 1.5.2 Additional stem cell based therapies

Since this thesis contains a detailed review article describing the use of bona fide NSCs, this chapter serves to introduce therapeutic strategies using alternative stem cells. In contrast to multipotent NSCs, pluripotent stem cells, including embryonic stem cells (ESCs), bear the potential to produce cells of all three germ layers. While the use of ESCs raise several ethical issues and does not circumvent possible immune repulsion, the discovery of induced pluripotent stem cells (iPSCs) has promised great potential for transplantation strategies in neurodegenerative diseases. Using an overexpression cocktail of transcription factor (also known as the Yamanaka factors), the Yamanaka laboratory was able to induce pluripotency in somatic cells (Takahashi K and Yamanaka S, 2006). Subsequent research led to the generation of protocols that either promoted the transition of iPSCs towards the NSC lineage or the direct conversion of somatic cells into induced neural stem cells (iNSCs) (Kim J et al., 2011;Meneghini V et al., 2017;Thier M et al., 2012;Wen Y and Jin S, 2014). Here, mouse and human fibroblasts were successfully transdifferentiated into iNSCs using only one transcription factor (either zinc finger protein 521 or SRY-box2) (Ring KL et al., 2012;Shahbazi E et al., 2016). While the introduction of viral components for the overexpression of such genes may be accompanied by unwanted off target effects as well as the necessity for silencing these constructs post-lineage reprogramming, the use of chemical compounds led to the generation of chemically induced NSCs (ciNSCs) (Tang Y, et al., 2017). Meanwhile, multiple transplantation studies have used iNSCs in neurodegenerative models, such as in amyotrophic lateral sclerosis (ALS), PD and Huntington´s disease (HD) or Alzheimer´s disease (AD) (Beyer F, et al., 2019;Tang Y, et al., 2017). Here, transplanted iPSC-derived NSCs either led to the replacement of lost neuronal and glial cells (McBride JL et al., 2004;Wu J et al., 2015) in PD and HD models or supported endogenous repair mechanisms by the secretion of beneficial growth factors such as brain-derived neurotrophic factor (BDNF) (Xu L et al., 2006;Yasuhara T et al., 2006;Zuo FX et al., 2015). It is not always clear whether mere cell replacement or the secretion of paracrine signals supporting endogenous repair mechanisms lead to functional improvements after stem cell transplantation (Tang Y, et al., 2017). However, the continuous development of new protocols for the

fast and robust generation of iNSCs and iPSC-derived NSCs promises to raise further knowledge about the mechanisms that mediate functional improvement following stem cell transplantation into patients suffering from a neurodegenerative disease.

## 1.6 Aims of this thesis

A low regenerative capacity marks the CNS. Once an injury or neurological pathology leads to the loss of neurons or glial cells, only a limited cell pool of progenitors and intrinsic aNSCs is available to repopulate these areas. Although multiple transplantation studies using exogenously propagated and partially modulated NSCs with the aim to replace lost cells have been conducted, no successful clinical translation has been achieved so far. Therefore, the aim of this thesis was to closely evaluate and compare NSC transplantation studies of the past and to conduct own transplantation studies in order to answer the following questions:

i) Does tissues heterogeneity between GM and WM affect the fate choice of transplanted aNSCs?

ii) How and to what degree does an injury environment influence these fate choices?

iii) Do we find evidence for a hierarchy of extrinsic versus intrinsic fate modulating signals? More specifically, does the suppression of the negative regulator of oligodendroglial differentiation p57kip2 overrule exogenous fate directing effects of different microenvironments?

# 2. Results (Publications)

*The publications are displayed in chapter 5 (Publications).*

## 2.1 Heterogeneous fate choice of genetically modulated adult neural stem cells in gray and white matter of the central nervous system

Felix Beyer*, Janusz Jadasz*, Iria Samper Agrelo, Jessica Schira-Heinen, Janos Groh, Anastasia Manousi, Christine Bütermann, Veronica Estrada, Laura Reiche, Martina Cantone, Julio Vera, Fancesca Viganò, Leda Dimou, Hans Werner Müller, Hans-Peter Hartung, Patrick Küry

*Abstract*

Apart from dedicated oligodendroglial progenitor cells, adult neural stem cells (aNSCs) can also give rise to new oligodendrocytes in the adult central nervous system (CNS). This process mainly confers myelinating glial cell replacement in pathological situations and can hence contribute to glial heterogeneity. Our previous studies demonstrated that the p57kip2 gene encodes an intrinsic regulator of glial fate acquisition and we here investigated to what degree its modulation can affect stem cell-dependent oligodendrogenesis in different CNS environments. We therefore transplanted p57kip2 knockdown aNSCs into white and gray matter (WM and GM) regions of the mouse brain, into uninjured spinal cords as well as in the vicinity of spinal cord injuries and evaluated integration and differentiation in vivo. Our experiments revealed that under healthy conditions intrinsic suppression of p57kip2 as well as WM localization promote differentiation toward myelinating oligodendrocytes at the expense of astrocyte generation. Moreover, p57kip2 knockdown conferred a strong benefit on cell survival augmenting net oligodendrocyte generation. In the vicinity of hemisectioned spinal cords, the gene knockdown led to a similar induction of oligodendroglial features; however, newly generated oligodendrocytes appeared to suffer more from the hostile environment. This study contributes to our understanding of mechanisms of adult oligodendrogenesis and glial heterogeneity and further reveals critical factors when considering aNSC mediated cell replacement in injury and disease.

*these authors contributed equally to this work

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*Contribution on experimental design, experimental procedure and publication*

Approximated share of contribution: 70%

Minor contribution to the design of the study by Felix Beyer; Major contribution to the experimental realization (adult mouse neural stem cell culture, transfection, qRT-PCR, mouse transplantations and handling, tissue processing, immunocyto- and immunohistochemistry, tissue pre-processing for immunoelectron microscopy, data analysis and figure preparation) by Felix Beyer; medium contribution to manuscript preparation and subsequently finalization following revision by Felix Beyer.

*Link to the publication*

https://onlinelibrary.wiley.com/doi/abs/10.1002/glia.23724

## 2.2 Do neural stem cells have a choice? Heterogenic outcome of cell fate acquisition in different injury models

Felix Beyer*, Iria Samper Agrelo*, Patrick Küry

*Abstract*

The adult mammalian central nervous system (CNS) is generally considered as repair restricted organ with limited capacities to regenerate lost cells and to successfully integrate them into damaged nerve tracts. Despite the presence of endogenous immature cell types that can be activated upon injury or in disease cell replacement generally remains insufficient, undirected, or lost cell types are not properly generated. This limitation also accounts for the myelin repair capacity that still constitutes the default regenerative activity at least in inflammatory demyelinating conditions. Ever since the discovery of endogenous neural stem cells (NSCs) residing within specific niches of the adult brain, as well as the description of procedures to either isolate and propagate or artificially induce NSCs from various origins ex vivo, the field has been rejuvenated. Various sources of NSCs have been investigated and applied in current neuropathological paradigms aiming at the replacement of lost cells and the restoration of functionality based on successful integration. Whereas directing and supporting stem cells residing in brain niches constitutes one possible approach many investigations addressed their potential upon transplantation. Given the heterogeneity of these studies related to the nature of grafted cells, the local CNS environment, and applied implantation procedures we here set out to review and compare their applied protocols in order to evaluate rate-limiting parameters. Based on our compilation, we conclude that in healthy CNS tissue region specific cues dominate cell fate decisions. However, although increasing evidence points to the capacity of transplanted NSCs to reflect the regenerative need of an injury environment, a still heterogenic picture emerges when analyzing transplantation outcomes in injury or disease models. These are likely due to methodological differences despite preserved injury environments. Based on this meta-analysis, we suggest future NSC transplantation experiments to be conducted in a more comparable way to previous studies and that subsequent analyses must emphasize regional heterogeneity such as accounting for differences in gray versus white matter.

*these authors contributed equally to this work

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*Contribution on experimental design, experimental procedure and publication*

Approximated share of contribution: 35%

Co-conceptualization and manuscript writing was performed by Felix Beyer.

*Link to the publication*

https://www.mdpi.com/1422-0067/20/2/455

# 3. Discussion

The high degree in complexity and heterogeneity of the adult CNS are two sides of the same coin. On the one hand, it enables it to simultaneously fulfill a multitude of complex, diverse tasks, to learn and adapt to environmental changes, and to being creative and inventive. On the other hand, damage to the CNS has devastating consequences reaching from failures in sensory and motor function, consequently impeding the interaction with its environment, to changes in personality and potentially death. In Addition, the high degree in heterogeneity makes the CNS less redundant, meaning that, in cases where cells are lost, only a small amount of progenitors with equal determination can serve as back-up pool. Multiple sclerosis is a bitter example of how challenging a comprehensive understanding and the development of therapeutic strategies for neuropathology are. As an autoimmune disease, regardless of whether the trigger lies in the CNS or the immune system, cells from the immune system outside the CNS attack myelinating OLs, which ensheathe axons from the eye, brain and spinal cord. Consequently, demyelinated axons degrade, neurons die and subsequently activate surrounding glial cells leaving multiple „hotspots“ of demyelination, inflammation, and cell death. Therefore, MS is a disease, which affects the CNS on a global scale in contrast to a focal injury (e.g. SCI or TBI). Due to the lower degree in heterogeneity in focal lesions, the investigation of treatment options seems less challenging. Although this might be true and promising on the way to find strategies to replace lost neurons and regain functional recovery following SCI, no successful therapy has been clinically applied, yet. Since excessive neurodevelopmental research and *in vitro* studies on („healthy“) adult CNS cells have brought detailed insight into the molecular hallmarks of neuronal and glial cell development, the question arises: Why haven´t these discoveries led to successful treatment strategies in a neuropathological background? Here it might be helpful to take a step back and look at i) the distinct differences between purified cells kept under defined conditions *in vitro* versus the chemical and physical mixture every cell is exposed to in CNS tissue, ii) the variety of tissue microenvironments present in the CNS in healthy organisms, and iii) the change in these microenvironments depending on a specific disease or injury.

Slowly dividing progenitor cells like OPCs reside throughout the CNS, which theoretically hold the potential to expand their pool via an accelerated cell cycle and mature into myelinating OLs (Vigano F, et al., 2016). Furthermore, the discovery of aNSCs have rejuvenated the clinical research on cell replacement strategies in neuropathology where cell replacement is required for functional recovery. Nevertheless, the regenerative capacity of the CNS especially with regard to cell replacement is overall poor compared to other organs such as the liver. This limited regeneration is due to inhibitory signals released during CNS insults as well as physical barriers such as the formation of a glial scar (Adams KL and Gallo V, 2018;Huebner EA and Strittmatter SM, 2009;Silver J et al., 2014) and the fact that aNSCs only reside in two discrete niches in the adult CNS. Consequently, using multipotent aNSCs for cell replacement strategies requires *in vitro* cultivation and expansion of aNSCs, fate modulation towards

specific cell types lost during the course of a disease, and detailed knowledge about the feasibility of translating *in vitro* findings into *in vivo* situations. Given the heterogeneity in CNS tissue- and cell composition, analysis of how different neuro-microenvironments affect fate, survival, and the migratory potential of transplanted aNSCs is a necessary step towards successful clinical translation.

This thesis describes the successful pro-oligodendroglial fate modulation by intrinsically modulating the expression of p57kip2 in mouse aNSCs *in vitro*. Consequently, albeit culture conditions promoting astroglial differentiation, modulated aNSCs showed decreased differentiation along this glial lineage. Transplantation of unmodified aNSCs into cortical tissue of young adult mice revealed that in contrast to GM tissue a WM environment exerted beneficial impact on long-term survival and oligodendroglial fate. The suppression of p57kip2 synergistically corroborated these effects. Additionally, p57kip2 suppressed aNSCs-derived mOLs expressed myelin proteins and ensheathed corpus callosum axons with myelin *in vivo*. Similar observations were made upon transplantation of rat aNSCs into intact rat spinal cord GM and WM, respectively. Despite the introduction of a spinal cord injury by means of a hemi section prior to transplantation of aNSCs close to the lesion side, p57kip2 suppressed aNSCs showed advanced oligodendroglial marker expression in WM tissue. However, increased oligodendroglial identity led to a higher vulnerability and consequently an overall diminished survival rate in the injury paradigm. The experimentally acquired data is mostly in line with published data on NSC transplantation studies into different CNS tissues. However, to my knowledge no NSC transplantation study has consequently compared the impact of i) GM versus WM as well as of ii) the microenvironmental changes of a given tissue upon acute injury on transplanted aNSCs.

## 3.1 Intrinsic versus extrinsic signals directing the fate of adult neural stem cells

Ever since the discovery of aNSCs in higher organisms including human (Altman J and Das GD, 1965;Moreno-Jimenez EP, et al., 2019;Spalding KL, et al., 2013) gene expression silencing or overexpression of intrinsic fate regulators has been in the focus of clinical stem cell research in order to direct lineage commitment of these cells (Braccioli L et al., 2018;Garcia-Leon JA et al., 2018;Jadasz JJ, et al., 2012). Additionally, exogenous application of signaling molecules such MSC-CM modulates fate decisions of cultured aNSCs towards an oligodendroglial phenotype (Jadasz JJ, et al., 2018;Rivera FJ, et al., 2006). Transplantation of NSCs into intact brain tissue revealed a surprising degree of host tissue driven adaptability resulting in comparable lineage commitment, independent of the donor cell origin (SVZ versus SGZ), animal age or prior *in vitro* propagation (Gage FH, et al., 1995;Seidenfaden R, et al., 2006). It is likely, that upon intrinsic ablation of certain receptors and/or downstream pathways, extrinsic cues will not be able to drive a distinct fate directing expression program. An activation or

disinhibition might reinforce effects by exogenously applied fate directing molecules. However, a hierarchical classification between intrinsic and extrinsic fate directing signaling cues is missing.
Consequently, we asked how the pro-oligodendroglial paradigm of p57kip2 suppressed *in vitro* cultivated aNSCs changes due to exposure to different tissue compositions *in vivo*. Vigano and colleagues elegantly revealed a differential impact of gray- versus white matter tissue on OPCs (Vigano F, et al., 2013) *in vivo*. In line with these findings, our analysis of transplanted aNSCs confirmed that a WM microenvironment in both the cortex and the spinal cord had a pro-oligodendroglial effect leading to higher numbers in mature and myelinating OLs. In addition, we could reveal that prior p57kip2 suppression either enhanced WM driven lineage commitment or counteracted inhibitory cues from GM tissue. Transplanted, p57kip2 suppressed cells progressed even further than under *in vitro* culture conditions in a way, that myelin sheaths by aNSC-derived OLs were formed *in situ*. Myelination might also have been supported by accessibility to corpus callosum spanning axons expressing and presenting Neuregulin-1 Type III (NRG1 type III) (Taveggia C et al., 2005) – a trigger signal for myelination.
Understanding the interaction of how intrinsic and extrinsic signals shape a stem cells fate commitment will be key to clinical approaches aiming at NSC-driven cell replacement. For example, Koutsoudaki and colleagues overexpressed insulin-like growth factor 1 (IGF1) in transplanted aNSCs prior to transplantation in a model of TBI (Koutsoudaki PN et al., 2016). *In vitro*, IGF1 overexpression led to an enhanced neuronal differentiation. However, upon transplantation close to the lesion side, these stem cells did not show any preferential lineage commitment compared to control NSCs. This underlines the need for critical determination of factors accumulating during inflammatory phases of disease (such as in MS patients or after infiltration of immune cells following a SCI or TBI) and its interplay with endogenously modulated aNSCs. Here, the suppression p57kip2 supported aNSCs to overcome inhibitory GM cues with regard to oligodendroglial differentiation. Albeit leading to similar effects when transplanted into the vicinity of a hemisected spinal cord, the net outcome of surviving cells was diminished. Here, additional application of pro-survival cues will warrant a prolonged beneficial remyelination outcome initiated by intrinsic aNSC fate modulation.

## 3.2 p57kip2´s potential to direct oligodendroglial fate acquisition

Cdkn1c (p57kip2) is a negative regulator of myelinating cell differentiation both of PNS and CNS origin (Gottle P, et al., 2015;Heinen A, et al., 2008;Kremer D, et al., 2009). Moreover, p57kip2 suppression leads to a higher rate in oligodendroglial lineage commitment in cultured rat aNSCs (Jadasz JJ, et al., 2012). Comparable results were achieved using mouse NSCs from the adult SVZ even under pro-astroglial culturing conditions (Beyer F et al., 2020). Whether p57kip2 also exerts a negative role on oligodendroglial fate choice in NSCs, residing in their *in vivo* niches in the adult awaits analysis. In general, *in vitro* cultured aNSCs have the potential to differentiate along the neuronal, astroglial, or oligodendroglial lineage (Kriegstein A and Alvarez-Buylla A, 2009). However, in our *in vitro*

experiments no spontaneous neuronal differentiation occurred. Nevertheless, cultured aNSCs showed commitment towards the neuronal lineage upon transplantation into GM and WM, respectively. Here, neither p57kip2 modulation nor the varying tissue microenvironment resulted in changes in the number of cells expressing the NB marker protein Dcx. Therefore we hypothesize that p57kip2 plays a more significant role at the astrocyte-to-oligodendroglial decision axis in aNSCs. Does p57kip2 suppression lead to the active inhibition of astroglial identities or does it initiate a pro-oligodendroglial expression program? Upon suppression of p57kip2, early marker expression of oligodendroglial identity (e.g. NG2) increase and negative regulators of oligodendrogenesis decrease (e.g. Hes5). At the same time, genes representative of an astroglial and stem cell identity decrease. An earlier study on p57kip2 and rat aNSCs could comprehensively show that upon suppression, the expression of negative regulators of BMP-signaling (driving astrocyte generation, (Bonaguidi MA, et al., 2005)) is increased leading to the assumption that astrogenesis is actively inhibited. This is in line with our *in vivo* finding documenting that the highest degree of astrocyte generation in GM transplants of unmodulated aNSCs – astroglial differentiation decreased upon p57kip2 suppression. On the other hand, p57kip2 suppression enhanced WM tissue-driven oligodendroglial differentiation of transplanted aNSCs, which leads to the assumption that reduced p57kip2 levels additionally lead to a higher susceptibility of aNSCs for oligodendroglial cues. Analysis of the exact mechanism by which p57kip2 suppression leads to fate modulation in aNSCs requires deeper insight into the molecular changes following transplantation. Here, the analysis of early time windows via single cell RNA-Sequencing of sorted cells post-transplantation could reveal (pseudo time-dependent) changes in their transcriptome. Moreover, additional axes of modulation would be revealed and help to pave the way to a successful clinical translation of aNSC-dependent cell replacement.

## 3.3 Neural stem cell heterogeneity and it´s consequence on transplantation outcomes

In light of the idea to use NSCs for cell replacement strategies in neuropathological diseases or following an acute injury, the following question arises: Does the NSC origin and donor age influence transplantation outcome in terms of cell survival and differentiation? Therefore, the analysis of injury-free transplantation studies is warranted in order to define donor cell properties, which affect the before mentioned parameters most. Neural stem cells are heterogeneous along temporal and spatial axes. While sharing principle stem cell properties such as self-renewal and multipotency, NSCs from developmental and adult origin differ in cell cycle length and mode of progeny generation (Gotz M, et al., 2016). Moreover, the primary NSC pool of the developing neural tube vanishes, while adult NSCs reside in two discrete niches lifelong in adult vertebrates (Altman J and Das GD, 1965;Doetsch F and Alvarez-Buylla A, 1996;Doetsch F, et al., 1999;Lois C and Alvarez-Buylla A, 1993;Menn B, et al., 2006). Primary NSCs from the neuroepithelium first undergo rapid symmetric cell divisions to expand the stem

cell pool. This phase is followed by asymmetric cell divisions generating RGCs, which give rise to neurons and subsequently glial cells. In the adult, radial glial-like cells of the SVZ and DG are slowly dividing NSCs. Depending on the niche and their location within this niche, these cells can give rise to neurons and glia alike upon activation (Akkermann R, et al., 2017;Zweifel S, et al., 2018). For transplantations conducted during this doctoral thesis, I exclusively used SVZ stem cells of mice with comparable adult age. Successful identification of specific markers to separate dorsal, medial and lateral SVZ stem cells would allow for a more detailed analysis of differentially primed NSCs in this niche. Here, Zweifel and colleagues recently showed that an increased expression gradient of Hopx along the medio-lateral axis of the dorsal SVZ defines the priming towards an astrocytic fate (Zweifel S, et al., 2018). However, as reviewed in this thesis (Beyer F, et al., 2019), even the enrichment in PSA-NCAM expressing (more neuronal primed) NSC from this niche did not change the outcome in cell identities compared to mixed NSC transplants into the mouse striatum and motor cortex. Surprisingly, even neuronal primed NSCs adapted, in line with my findings, predominantly a glial fate (Beyer F, et al., 2020;Seidenfaden R, et al., 2006). The same study showed that NSCs extracted from P75 and P5 (time of gliogenesis during development) mice did also not differ in differentiation outcome (Seidenfaden R, et al., 2006). *In vivo*, NSCs residing in the DG of the hippocampus almost exclusively give rise to neuronal progeny before final differentiation into post-mitotic astrocytes (Pilz GA, et al., 2018). When these cells were isolated and propagated in culture, proofing stem cell self-renewing properties, transplantation of this pool into the hippocampus and adjacent corpus callosum led to glial differentiation (Gage FH, et al., 1995) – a result, dominating the picture of injury-free transplantation studies using NSCs from different niches, donor species and age (Beyer F, et al., 2019). Consequently, the answer to the question whether NSC donor age and origin influence transplantation outcome is no. However, as heterogeneity is also a feature among all NSC-derived cells and because detailed analysis in this regard (e.g. by single cell RNA-Sequencing of NSC progeny following NCS transplantation) has not been conducted yet, it remains to be shown whether different NSC populations will give rise to either more or less heterogeneous neurons and glial cells. As for transplantation studies conducted in our laboratory, an intrinsic fate modulation by suppression of p57kip2 did change both, survival and fate outcome (Beyer F, et al., 2020;Jadasz JJ, et al., 2012). Here, analysis of how susceptible differing NSC sub-populations are in regard to the described changes following p57kip2 suppression would lead to a more detailed analysis and be of interest for future clinical approaches.

## 3.4 How does an injury or disease change the outcome of transplanted neural stem cells?

Neurodegenerative disease or acute injury to the CNS changes multiple parameters possibly affecting the fate of transplanted NSCs. While in contact with the systemic milieu via the tightly controlled BBB, the CNS still represents a rather closed system, which leads to the accumulation of cell debris whenever

cells die. Consequently, microglia and astrocytes change to a reactive state, which leads to changes in their phagocytosis capacity, morphology, and cytokine expression and secretion (Clarke LE, et al., 2018;Liddelow SA, et al., 2017;Tang Y and Le W, 2016). Moreover, following TBI using a stab wound model, a subset of NG2 glia positive for Gpr17 migrates towards the wound to undergo maturation, thereby changing the cell composition in the affected area (Boda E et al., 2011;Vigano F, et al., 2016). Results of several injury-free transplantation studies suggested, that transplanted NSCs could sense their environment and acquire fates that often closely represent the naive state of the individual areas (Beyer F, et al., 2019). Interestingly, fate choice analysis of transplanted NSCs in different injury/neuropathological models reinforced the idea of a highly adaptable nature of NSCs. When SVZ-derived NSCs were transplanted in wild type and shiverer (dysmyelinated) mouse brains, shi brains showed an increase in oligodendroglial differentiation by the transplanted NSCs (Yandava BD et al., 1999). In line with these findings, comparison of different transplantation studies sheds light on the impact of a changed microenvironment on NSC fate. Intra-hippocampal transplantation in healthy mice led to primarily neuronal and astroglial differentiation (Gage FH, et al., 1995;Raedt R et al., 2009). However, in a model of TBI in which a stab wound to the hippocampus causes a local injury, a higher number of transplanted NSCs differentiated into OLs (Koutsoudaki PN, et al., 2016). Surprisingly, neuronal cell death in a model of TLE induced by kainic acid injection into the hippocampus instructed NSCs to differentiate primarily into neurons (Miltiadous P et al., 2013). These findings lead to the assumption that a hierarchy for NSC-driven cell replacement exists. Under healthy conditions, exogenously applied NSCs seem to merely adapt to the existing network (Beyer F, et al., 2019). In case when primarily neurons are affected (kainic acid induced neuronal stress/death), NSCs are primed to refill the neuronal pool. However, in a focal stab wound to the hippocampus in which also cortical areas are injured leading to an equal damage of glial and neuronal cells, NSCs favor glial fate choice (Koutsoudaki PN, et al., 2016). This is in line with my own findings of NSC transplantation into both, hemisected and unharmed spinal cord (Beyer F, et al., 2020). Here, the ratio of NSC descendants with oligodendroglial identity increased upon transplantation into the injury model. Moreover, p57kip2 suppression increased the proportion of OL generation but simultaneously resulted in a net cell loss probably owing to a higher vulnerability of oligodendroglial cells towards injury inflicted hostile cues. Taking into account that no neurons were generated, time windows with the aim to replace lost cells such as neurons by applied NSCs need to be well defined in order to achieve functional improvement for patients suffering from SCI and other neurodegenerative diseases. As chronic temporal lobe epilepsy (TLE) models have shown, that the injury microenvironment changes compared to an acute TLE in mice with respect to NSC fate choice (Miltiadous P, et al., 2013;Waldau B et al., 2010), close analysis of the temporal changes in the injury side would extend our knowledge on clinically relevant time windows for exogenous cell replacement.

## 3.5 Alternatives to extrinsic neural stem cells for cell replacement strategies

The use of (neural) stem cells for cell replacement strategies in patients where cells of the CNS are lost constitutes just one of many approaches neuroscientists and neurologists follow. Neural stem cells can be propagated in culture (Reynolds BA and Weiss S, 1992;Weiss S et al., 1996) to reach a sufficient number of cells needed, depending on the lesion extend. Moreover, NSCs are multipotent and can therefore differentiate into all neuroectoderm-derived cells, which they seem to do in a self-organized, hierarchic way as discussed in the chapter before. Finally, at least using model systems in mice and rats, neither embryonic and adult rodent NSCs nor human derived NSCs showed any tumor formation upon transplantation (Beyer F, et al., 2019). However, as no NSC based approach has been successfully translated into clinical application, alternative strategies need to be analyzed in parallel. Another extrinsic cell replacement approach could be facilitated by the use of multiple progenitor cells. For OPCs, a certain degree of adaptability was shown when progenitors from WM (corpus callosum) and GM (neocortex) were transplanted into the other region (GM and WM, respectively) (Vigano F, et al., 2013). Here, GM-derived OPC differentiated into myelinating OLs in a fashion characteristic for resident WM OPCs. Since usually both cell classes (neurons and glia) are affected in a disease or injury over time (Ditunno JF, et al., 2004;Goldenberg MM, 2012) a "multiple progenitor approach" would ensure the provision of potentially generated neurons as well as glial cells. However, for the prevention of an early disease progression such as in MS patients, where oligodendroglial cells die first prior to neuronal death (Thompson AJ, et al., 2018;Weinshenker BG, 1996) an overload of unnecessary cells could lead to a harmful immune response. Furthermore, the addition of astroglial progenitors to an acute spinal cord injury harbors the risk of enforcing the formation of a glial scar, which is considered a major obstacle for regrowing axons (Adams KL and Gallo V, 2018;Yuan YM and He C, 2013). All extrinsic cell replacement strategies not only face the obstacle of immune comparability but also a debate over cell origin accompanied by an ethical concern. The discovery of iPSC technology made both immune reaction and ethical questioning mostly dispensable. By inducing a transcription factor cocktail in somatic, post-mitotic cells (e.g. fibroblasts), these cells are reprogrammed and convert into pluripotent stem cells (Takahashi K and Yamanaka S, 2006). Further research led to protocols that drive either the direct generation of neurons and glia or the generation of NSCs from iPSCs (Tang Y, et al., 2017) paving the way for autologous neural cell replacement without ethical concerns or the fear of an immune rejection. Here, the beneficial use of (human) iPSCs and iPSC-derived NSCs in several neurodegenerative disease models is frequently reported (Lee-Kubli CA and Lu P, 2015;Meneghini V, et al., 2017). Furthermore, research in the field is currently focusing on improving protocols to minimize mutations and tumorigenicity, identify optimal treatment windows and select the most promising cell type and dosage for individual diseases and injuries (Knoepfler PS, 2009;Kooreman NG and Wu JC, 2010).

Whether multipotent NSCs with proliferating capacity exist in the adult human is currently under debate (Moreno-Jimenez EP, et al., 2019;Sorrells SF, et al., 2018). Targeting endogenous NSCs, if available in sufficient number, would redundantise the use of immunosuppressive drugs following an allotransplantation, as these NSCs would be genetically identical to the hosts' immune system. However, since human NSCs are also restricted to their niches it is unlikely that a sufficient number of cells would migrate towards multiple focal lesions, such as in MS patients, or along the spinal cord. Moreover, genetic engineering to promote a certain cell type (e.g. oligodendroglial differentiation by suppression of p57kip2) harbors several obstacles such as cell specific delivery of genetic engineering elements (e.g. shRNA) without any spill over. Finally, adult NSCs might not be suited for every possible neuropathology as Rowland and colleagues showed differences in oligodendroglial fate choice between primitive and adult NSCs in the shi mouse (Rowland JW et al., 2011). Circumventing immune rejection and avoiding the risks of neurological surgeries for transplantation of cells also holds great advantage when considering to target endogenous progenitor cells. However, neuronal progenitors have so far only been identified in close proximity to stem cell niches in the adult human CNS (Moreno-Jimenez EP, et al., 2019) while proliferative astrocyte progenitors were not detected in the adult human brain but only at fetal stages (Zhang Y et al., 2016). Adult OPCs reside in the adult human white matter (Roy NS et al., 1999;Windrem MS et al., 2004) and thus provide a targeted for cell replacement of lost OLs via application of stimulating cytokines. Here, theoretically proliferative signals should precede pro-differentiation cues. However, analysis of integrated nuclear bomb test-derived $C^{14}$ isotope in human post mortem brain tissue revealed little to no turnover of the OL pool (Yeung MS et al., 2014), raising the question of how potential resident adult OPCs are to differentiate. Moreover, in MS patients recent evidence suggests that mature OLs retract myelin fibers but survive in an inhibited, damaged state (Jakel S, et al., 2019) potentially being able to re-myelinate without the need of cell replacement by OPCs. Whether and how adult OPCs contribute to re-myelination in human neurological diseases such as MS needs further investigation prior to human-specific studies aiming at boosting the potential of endogenous (oligodendroglial) progenitor cells.

## 3.6 The importance of my results for future cell replacement strategies

Both, my experimental work and the review summarizing the heterogeneous impact of different injuries and neurological disease model systems on transplanted NSCs contribute to a more detailed knowledge about stem cell based cell replacement strategies. Especially in light of the heterogeneous effects of individual injury microenvironments on NSCs, which show remarkable adaptability, more attention needs to be raised to the course of a disease/injury. Finding the right time window and region for cell transplantations seems crucial as an injury/neurological disease is dynamic in terms of an altered microenvironment as well as cell composition concomitantly changing cell-cell contact mediated and

paracrine signals (Ditunno JF, et al., 2004;Goldenberg MM, 2012;Han F, et al., 2015;Liddelow SA, et al., 2017;Weinshenker BG, 1996). My transplantation experiments revealed that differential cell composition not only affects survival (with WM showing a beneficial effect on long-term survival) but also a differing impact on fate choice of transplanted aNSCs. Moreover, chronic TLE showed a different impact on fate choice compared to transplanted NSCs in an acute TLE mouse model (Miltiadous P et al., 2011;Waldau B, et al., 2010) underlining the dynamics in neuropathology lesions. So far, detailed knowledge about changes in the microenvironment is missing. Therefore, changes in the injury environment need to be considered more carefully especially in light of different cell kinetics in patients of different age. For example, in humans developmental oligodendrogenesis is ongoing until the age of 20 and declines rapidly after that (Giedd JN and Rapoport JL, 2010).

Several NSC transplantation studies have used embryonic or fetal, primitive NSCs in neurological disease models aiming at either cell replacement or trophic support (Beyer F, et al., 2019;Tang Y, et al., 2017) possibly not foreseeing ethical issues when translating results and application into the human. I successfully used adult NSCs and summarized published work from other aNSC transplantation studies, thereby revealing the high degree in adaptability of these adult derived stem cells. While my analysis proofed a differential impact of an unharmed versus damaged CNS tissue and could reveal gray versus white matter differences when comparing NSC fate choice, the majority of other NSC transplantation studies focused on injury model and transplantation side only (Beyer F, et al., 2019). Nevertheless, my transplantation studies lack information about a differential impact of a hemi-sectioned spinal cord on exogenously applied NSCs over time, which should be addressed in a comparable manner in future experiments.

Finally, easy cell access for genetic modulations constitutes just one advantage of using *in vitro* cultured NSCs for disease amelioration upon transplantation into or close to lesion sides. For example, overexpression of growth factors in NSCs prior to transplantation aims at both, cell replacement as well as disease amelioration by providing paracrine signals with neuroprotective properties such as IGF1 (Koutsoudaki PN, et al., 2016;Miltiadous P, et al., 2011). I used a shRNA-mediated approach to suppress the negative regulator of oligodendrogenesis p57kip2, which, from a pro-oligodendroglial perspective, antagonized inhibitory signals from GM and further increased NSC-derived OL yield in a WM environment. However, transplantation of p57kip2 suppressed NSCs near a hemisected spinal cord led to a net cell loss, probably due to a higher vulnerability of generated oligodendroglial cells. Therefore, carefully evaluating the precise timing of genetic modulation as well as the time point of transplantation in future experiments will help to reach a maximum efficiency of cell replacement and consequently functional improvements.

## 3.7 Conclusion

In conclusion, this thesis describes the differential impact of healthy versus damaged central nervous system tissue on the fate decision of transplanted neural stem cells (NSCs). Moreover, even in healthy tissue the fate choice of transplanted NSCs was dependent on the transplantation side with white matter structures supporting oligodendroglial fate. While NSCs show a remarkable adaptability in terms of cell replacement upon transplantation into varying tissues, I could show that an intrinsic suppression of p57kip2 in NSCs partially overruled microenvironmental driven effects. A more detailed knowledge about the heterogeneity of both the NSC pool as well as spatio-temporal changes in the microenvironment of neuropathological affected tissues will potentially lead to treatment strategies that are more effective using exogenously applied NSCs.

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# 5. Publications

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RESEARCH ARTICLE

# Heterogeneous fate choice of genetically modulated adult neural stem cells in gray and white matter of the central nervous system

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## Abstract

Apart from dedicated oligodendroglial progenitor cells, adult neural stem cells (aNSCs) can also give rise to new oligodendrocytes in the adult central nervous system (CNS). This process mainly confers myelinating glial cell replacement in pathological situations and can hence contribute to glial heterogeneity. Our previous studies demonstrated that the p57kip2 gene encodes an intrinsic regulator of glial fate acquisition and we here investigated to what degree its modulation can affect stem cell-dependent oligodendrogenesis in different CNS environments. We therefore transplanted p57kip2 knockdown aNSCs into white and gray matter (WM and GM) regions of the mouse brain, into uninjured spinal cords as well as in the vicinity of spinal cord injuries and evaluated integration and differentiation in vivo. Our experiments revealed that under healthy conditions intrinsic suppression of p57kip2 as well as WM localization promote differentiation toward myelinating oligodendrocytes at the expense of astrocyte generation. Moreover, p57kip2 knockdown conferred a strong benefit on cell survival augmenting net oligodendrocyte generation. In the vicinity of hemisectioned spinal cords, the gene knockdown led to a similar induction of oligodendroglial features; however, newly generated oligodendrocytes appeared to suffer more from the hostile environment. This study contributes to our understanding of mechanisms of adult oligodendrogenesis and glial heterogeneity and further reveals critical factors when considering aNSC mediated cell replacement in injury and disease.

KEYWORDS

glial fate modulation, myelin, neural stem cell, p57kip2, regional heterogeneity, spinal cord injury, transplantation

Felix Beyer and Janusz Jadasz contributed equally to this study.

## 1 | BACKGROUND

Heterogeneity among oligodendroglial progenitor cells (OPCs) has previously been related to their gray (GM) or white matter (WM) localization in the central nervous system (CNS; (Lentferink, Jongsma, Werkman, & Baron, 2018; Vigano, Möbius, Götz, & Dimou, 2013)). Based on single cell sequencing data, this information has recently been expanded describing regional differences among oligodendroglial cells (Marques et al., 2016) as well toward disease-specific lineages upon demyelination (Falcao et al., 2018; Jäkel et al., 2019). Moreover, new reports point to an additional contribution to myelin repair in the adult CNS from partially lesioned oligodendrocytes (Duncan et al., 2018; Yeung et al., 2019). Besides GM and WM structures, the subventricular zone (SVZ), one of the stem cell niches of the adult brain, represents yet another source for myelinating cells, thus possibly contributing to glial heterogeneity. Adult self-renewing, multipotent neural stem cells (adult neural stem cells [aNSCs]) mainly generate neuroblasts which eventually differentiate into mature neurons of the olfactory bulb or the striatum (Bond, Ming, & Song, 2015; Lim & Alvarez-Buylla, 2016) but were also shown to give rise to new oligodendroglial cells in the corpus callosum following demyelinating events (Brousse, Magalon, Durbec, & Cayre, 2015; Menn et al., 2006; Nait-Oumesmar et al., 1999; Nait-Oumesmar et al., 2007; Picard-Riera et al., 2002; Xing et al., 2014). Signals responsible for neuronal versus glial progeny decisions remain yet to be identified but a number of factors were shown to either inhibit or promote stem cell-dependent oligodendrogenesis (Akkermann, Beyer, & Küry, 2017). In addition, bioinformatics analysis predicts the existence of a number of unexploited signaling pathways acting on niche cells (Azim et al., 2018). Nevertheless, a detailed knowledge about intrinsic regulators and extrinsic influences is needed in order to understand whether and how neural stem cells can be exploited for robust oligodendrocyte replacement, as warranted by WM loss observed in many neuropathologies. It also remains to be shown to what degree activation of endogenous stem/progenitor cells versus supply of exogenous cells (transplantation) is applicable and whether generated cell derivatives correspond to fully myelinating oligodendrocytes or are alike cells only. Likewise, little is known about the influence of regional differences (GM and WM) as well as of an injury environment on transplanted aNSCs. As previous stem cell transplantation studies revealed poor fate directing properties when NSCs were, for example, instructed to differentiate into neurons prior to transplantation (Koutsoudaki et al., 2016) or when cells overexpressed IGF-1 (Miltiadous et al., 2013), this supports a potential dominant effect of the host tissue.

The p57kip2/cdkn1c gene encodes one of the intrinsic regulators involved in oligodendroglial cell fate acquisition. We previously demonstrated that short-hairpin RNA (shRNA) mediated suppression of this gene in rat aNSCs, derived from the subgranular zone and SVZ stem cell niches, induces oligodendroglial features at the expense of astrocyte generation, notably despite the presence of strong astrogenic cues (Jadasz et al., 2012). In addition, there is evidence for a differential expression of p57kip2 along SVZ subregions correlating with variations in the extent of oligodendrocyte progeny generation (Akkermann et al., 2017). In the current study, we investigated whether this intrinsic fate modulation leads to the same degree of oligodendrocyte formation following aNSC transplantation into different healthy brain and spinal cord regions and whether it can also dominate over factors emanating from a hostile, pathophysiological environment. To this end, genetically modulated aNSCs were transplanted into cortex (GM) and corpus callosum (WM), GM and WM of the spinal cord as well as in the vicinity of acutely lesioned spinal cords in order to investigate differential instructive signals influencing survival, tissue integration, and oligodendroglial differentiation. While we show that also in mouse, SVZ-derived aNSCs p57kip2 knockdown generally induces an oligodendroglial fate we nevertheless detected remarkable differences between WM and GM grafted cells as well as a differential impact from the injury environment on grafted cell survival. Taken together, our results uncover heterogeneous oligodendroglial fate determining and maturation effects mediated by extrinsic and intrinsic regulators.

## 2 | MATERIAL AND METHODS

### 2.1 | Animals

Female mice (C57Bl/6) and rats (RjHan:W) were used as host animals in order to avoid possible gender dependent heterogeneity. All rodents were housed in a pathogen-free facility with 12 hr light/dark cycle and supplied with food/water ad libitum. Transplantation experiments into wildtype mice and rats were all approved by the LANUV (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen; Az.: 84-02.04.2015.A239; Az.: 84-02.04.2015.A525) and carried out in accordance with ethical care. For the preparation of adult mouse NSCs from wildtype C57Bl/6 or transgenic (NesCreERT2::tdTomato) mice 3 month old female animals were used. Please note that NesCreERT2::tdTomato mice were received by introducing an inducible tdTomato cassette (Gt(ROSA)26Sor locus; for further detail, please see Jackson Laboratory stock No. 007914) into NesCreERT2 mice (Imayoshi, Ohtsuka, Metzger, Chambon, & Kageyama, 2006). Rat aNSCs were prepared from 8 to 10 week old female Wistar rats.

### 2.2 | Adult mouse and rat neural stem cell culture and transfection

Preparation of aNSCs was conducted using adult mouse and rat SVZs as previously described (Jadasz et al., 2012; Jadasz et al., 2018). Briefly, isoflurane anesthetized animals were killed by decapitation and after removal of the brains SVZ of both hemispheres were prepared, mechanically digested and transferred to 4°C phosphate buffered saline (PBS; D8537; Sigma-Aldrich, Taufkirchen, Germany). After washing in PBS, cells were enzymatically digested in PDD solution containing papain (0.01%, Worthington Biochemicals, Lakewood, CA), Dispase II (0.1%, Boehringer, Ingelheim, Germany), DNase I (0.01%, Worthington Biochemicals), and 12.4 mM MgSO4, dissolved in HBSS (PAA Laboratories) for 30 min at 37°C with trituration steps every 10 minutes. After additional washing steps in neurobasal (NB) medium

(Gibco BRL, Karlsruhe, Germany) supplemented with B27 (Gibco), 2 mM L-glutamine (Gibco), 100 U/mL penicillin/0.1 mg/L streptomycin (Gibco) and centrifugation at 140 rcf for 5 min, cells were resuspended in NB medium supplemented with 2 mg/mL heparin (Sigma-Aldrich), 20 ng/mL FGF-2 (R&D Systems, Wiesbaden-Nordenstadt, Germany), and 20 ng/mL EGF (R&D Systems) from here on referred to as NBall medium. Cells were seeded at $1 \times 10^6$ cells in one T75 culture flask in 10 mL NBall medium and cultured for 7 days at 37°C in a humidified incubator with 5% $CO_2$. The medium was exchanged twice a week and on Day 7 cells were passaged using accutase (PAA Laboratories) for separation (10 min at 37°C). Mouse and rat aNSCs were dispersed using an accutase step and subjected to nucleofection using a Lonza nucleofection device and the adult mouse NSC nucleofector kit (Lonza, Basel, Switzerland) as described before (Jadasz et al., 2018). Briefly, $4 \times 10^6$ cells were transfected using the program A-033 (high efficiency) resuspended in 100 μL nucleofection solution and 10 μg plasmid DNA. Nucleofections were performed using constructs pSUPER ("ctrl"; empty vector control) and pSUPER-p57kip2 ("p57-KD"; suppression of p57kip2) and for visualization of transfected cells a citrine expression vector was cotransfected at a ratio of 5:1 all as published previously (Heinen et al., 2008; Jadasz et al., 2012; Kremer et al., 2009). Transfected cells were plated on poly-L-ornithine/laminin (100 and 5 μg/mL; Sigma-Aldrich) coated and acid pretreated 13 mm glass cover slips ($8 \times 10^4$ cells/coverslip) for 24 hr in NBall medium before changing to astrocyte fate stimulating medium (Minimum Essential Medium Alpha (α-MEM) supplemented with 10% fetal calf serum (FCS)). For in vitro quantitative real-time reverse transcription PCR (qRT-PCR) analysis, transfected cells were first cultured in 10 mL NBall medium for 24 hr in T75 flasks. Prior to fluorescent activated cell sorting (Jadasz et al., 2012), cells were gently washed from the flasks, treated for 5 min with accutase, washed with PBS containing 2 mM ethylenediaminetetraacetic acid, and subsequently sorted by means of citrine expression. Sorted cells were plated on 13 mm glass cover slips and cultured in α-MEM + 10% FCS medium. For in vivo transplantation experiments, cells (derived from transgenic tdTomato-positive mice or from wildtype rats) were transfected as described above 1 hour prior to grafting.

## 2.3 | Immunocytochemistry

α-MEM + 10% FCS cultured mouse aNSCs were fixed after 4 or 7 days using 4% paraformaldehyde (PFA) and stained for the detection of marker proteins as previously described (Jadasz et al., 2018) using the following antibodies: rabbit anti-neural/glial antigen 2 (NG2; 1:100, AB5320, Millipore, RRID: AB_11213678), rabbit anti-glutathione-S-transferase-π (GSTπ; 1:500, ADI-MSA-101, ENZO, RRID: AB_10615079) and rabbit anti-glial fibrillary acidic protein (GFAP; 1:4000, Z0334, DAKO, RRID: AB_10013382). Primary antibodies were incubated at 4°C overnight, followed by three washing steps (1x PBS) and incubation with the secondary antibody (goat anti-rabbit Alexa 594; 1:500; A-11037, Thermo Fisher Scientific, RRID: AB_2534095) in PBS (supplemented with 4′,6-diamidin-2-phenylindol [DAPI]) at room temperature (RT) for 30 min. Images were taken using a Zeiss Axioplan2 microscope and analyzed using the ImageJ BioVoxxel software.

## 2.4 | Gene expression analysis

Transfected and sorted mouse aNSCs were lysed after 7 days using 350 μL RLT lysis buffer (Qiagen) supplemented with β-mercaptoethanol (1:100, Sigma). Total RNA purification, cDNA synthesis, and qRT-PCR were performed as previously described (Jadasz et al., 2012). For sequence detection, the following forward (fwd) and reverse (rev) primers were used, with TBP and ODC serving as reference genes:

TBP_fwd: AGAATAAGAGAGCCACGGACAACT, TBP_rev: TGGCT CCTGTGCACACCAT; ODC_fwd: GGTTCCAGAGGCCAAACATC, ODC_rev: GTTGCCACATTGACCGTGAC; p57kip2_fwd: CCGAC TGAGAGCAAGCGAAC, p57kip2_rev: ATTGGTGATGGACGGCTCCT; NG2_fwd: ACGATCCACCTCGCATCATC, NG2_rev: GTTCCACA GGGACACCAGAG; GSTπ_fwd: CATGCCACCATACACCATTGTC, GSTπ_rev: CATTCGCATGGCCTCCA; nestin_fwd: AGCCATTGTGGT CTACGGAAGT, nestin _rev: TCCACACACCCCAGTGGTT; Hes5_fwd: TGCAGGAGGCGGTACAGTTG, Hes5_rev: GCTGGAAGTGGTAAG CAGCTT; GFAP_fwd: CCAGCTTCGAGCCAAGAA, GFAP_rev: GAAGCTCCGCCTGGTAGACA; AQP4_fwd: TCCTGATGTGGAGCTCA AACG, AQP4_rev: GCTGCGCGGCTT TGC; Sox2_fwd: CCAGCGCAT GGACAGCTA, Sox2_rev: GCTGCTCCTGCATCATGCT.

## 2.5 | Stereotactic transplantation into GM and WM of the mouse brain

Then, 1 hr prior to transplantation, mouse aNSCs were transfected as described above and kept at RT in PBS. For transplantation into the mouse brain, cells were centrifuged for 5 min at 140 rcf and resuspended in PBS to a density of $1 \times 10^5$ cells/μL. Recipient C57Bl/6J mice were deeply anesthetized using isoflurane inhalation. Approximately 0.75 μL of the cell suspension was injected in either the WM or GM of the somatosensory cortex of (13–14 week old) mice according to Vigano et al. (2013). Transplantations were performed with a Hamilton syringe (10 μL Neuros Model 1701 RN, ga 33, *L* 0–20 mm) into the WM at 0.7 mm (anterior–posterior), ±1 mm (medial–lateral), 2.1–1.8 mm (dorsal–ventral) relative to the bregma and into the GM at 0.7 mm (anterioposterior), ±1 mm (medial–lateral), 1.5–1.3 mm (dorsal–ventral) relative to the bregma using a motorized robot stereotaxic instrument and StereoDrive software (Neurostar). Postoperative care comprised an analgesic treatment (RIMADYL, Pfizer; 5 mg/kg) for 3 days starting on the day of operation. For tissue removal, mice were deeply anesthetized with isoflurane and transcardially perfused with 20 mL ice-cold PBS followed by 20 mL 4% PFA. Mouse brains were harvested and postfixed overnight in 4% PFA at 4°C, followed by 24–48 hr cryoprotection in 30% sucrose (in PBS) at 4°C. Brains were embedded in Tissue-Tek OCT (Sakura Finetek Europe, Netherlands), frozen, and stored at −80°C until preparation of 10 μm sections using a cryostat (Leica CM3050S). Sections were stored at −80°C.

## 2.6 | Stereotactic transplantation into the intact and hemisected rat spinal cord

Adult female rats were operated as previously described (Schira et al., 2012) with slight modifications. Briefly, after dorsal hemisection at thoracic level eight (Th8) using a Scouten wire knife (Bilaney), the dura was sutured and then rat aNSC transplantation was performed using a glass capillary, attached to a Small Animal Stereotaxic Instrument (David Kopf Instruments), at 2 mm rostral and 2 mm caudal to the lesion, 0.1 mm lateral to the midline and 1.1 mm (for GM) or 0.7 mm (for WM) dorsal–ventral from the dural surface. For the transplantation of aNSCs into the intact spinal cord, no hemisection was performed but cells were implanted at the same coordinates as previously mentioned. At each transplantation site 2 μL containing $1 \times 10^5$ either control transfected (ctrl; empty vector) or p57kip2-suppressed (p57-KD) cells in PBS were injected slowly within 4 min. Cells were transfected 60 min prior to grafting and for cell detection a citrine expression vector was cotransfected as indicated above. Please note that for both conditions, control and p57-KD, transfection efficiency was comparably high (Supplementary Figure 2). Postoperative care included prophylactic oral antibiotic treatment (Baytril, Bayer Health Care; 0.4 mL/kg) and manual bladder expression for 1 week. Further to this, rats received an analgesic treatment (RIMADYL, Pfizer; 5 mg/kg) for 3 days starting on the day of operation. Seven days postoperation, animals were transcardially perfused. Rats were deeply anesthetized using a mixed solution containing Ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) and transcardially perfused with 200 mL 4°C PBS followed by 400 mL 4% PFA. Spinal cords were harvested and postfixed overnight in 4% PFA at 4°C, followed by 24–48 hr cryoprotection in 30% sucrose (in PBS) at 4°C. Spinal cords were then embedded in Tissue-Tek OCT (Sakura Finetek Europe), frozen, and stored at −30°C until preparation of 10 μm sections using a cryostat (Leica, CM3050S). Sections were stored at −30°C.

## 2.7 | Immunohistochemistry

Brain and spinal cord sections were thawed and left to dry for at least 15 min at RT. Before blocking, sections were rehydrated for 5 min in distilled water, transferred to −20°C acetone (5 min), and washed in 1x TBS (pH 7.6) and 1x TBS-T (TBS containing 0.02% Triton) for 5 min each. Blocking was performed with 5–10% biotin-free bovine serum albumin (BSA; in TBS-T) for 30 min at RT, followed by application of the following antibodies (in 5–10% BSA in TBS) and incubation overnight: rabbit anti-NG2 (1:100; MAB5320; Millipore, RRID: AB_11213678; RT), rabbit anti-sex determining region Y-Box 10 (Sox10; 1:100, S1058C002, DCS Immunoline, RRID: AB_2313583; RT), rabbit anti-GFAP (1:10,000; Z0334, DAKO, RRID: AB_10013382; 4°C), goat anti-doublecortin (Dcx; 1:100, sc-8066, Santa Cruz, RRID: AB_2088494; 4°C), rabbit anti-GSTπ (1:4,000; ADI-MSA-101, ENZO, RRID: AB_10615079; 4°C), mouse anti-2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNPase; 1:5,000, 836,402, Biolegend, RRID: AB_2565362; 4°C), rat anti-myelin basic protein (MBP; 1:500, MCA409S, BioRad, RRID: AB_325004; 4°C), rabbit anti-NeuN (1:500; ab177487, Abcam, RRID: AB_2532109; 4°C), mouse anti-myelin oligodendrocytes glycoprotein (MOG; 1:500, MAB5680, Millipore, RRID: AB_1587278; 4°C), and chicken anti-green fluorescent protein/citrine (1:500–2,000; GFP-1020, Aves, RRID: AB_10000240; 4°C or RT). Sections were washed two times for 7.5 min in TBS and incubated with the species-appropriate fluorochrome-conjugated secondary antibody (1:500 in PBS) for 30 min at RT: donkey anti-chicken Alexa 488 (703-545-155, Jackson Immuno Research Labs, RRID: AB_2340375), donkey anti-goat Alexa 647 (A-21447, Thermo Fisher Scientific, RRID: AB_2535864), goat anti-chicken Alexa 488 (A-11039, Thermo Fisher Scientific, RRID: AB_2534096), goat anti-rabbit Alexa 405 (A-31556, Thermo Fisher Scientific, RRID: AB_221605), goat anti-mouse Alexa 647 (A-32728, Thermo Fisher Scientific, RRID: AB_2633277), goat anti-rat Alexa 647 (A-21247, Thermo Fisher Scientific, RRID: AB_141778), goat anti-rabbit Alexa 594 (A-11037, Thermo Fisher Scientific, RRID: AB_2534095), and DAPI or RedDot 2 (Biotium, Cat.#: 40061). Images were taken using a Zeiss CLSM microscope 510 (CLSM 510, Zeiss, Jena, Germany) and analyzed using the ImageJ BioVoxxel software.

## 2.8 | Histological cell quantification

Immunohistochemical staining was done on brain sections of the corresponding centers of transplantation (on average, 25 sections per marker per time point were analyzed). Fluorescently marked cells were counted on each picture/section leading to an average value for each animal. Observed cell numbers are represented by the area of an individual circle (Figure 3). For quantification of cell numbers in spinal cord, transplantation experiments corresponding centers of transplantation were used and 14–24 sections encompassing the transplantation zone per animal were analyzed. After calculating the mean number of citrine-positive cells per section, this number was then multiplied with the total number of sections containing citrine-positive cells (between 40 and 65 sections per animal) to indicate total numbers of surviving cells in the spinal cord.

## 2.9 | Immunoelectron microscopy

For immunoelectron microscopy, mice were perfused with 4% PFA in cacodylate buffer, brains were dissected and postfixed overnight. Brains were embedded in 6% agarose in cacodylate buffer, and 50-μm-thick coronal sections were cut in PBS using a microtome (Microm HM 650 V, Thermo Fisher Scientific). Free-floating sections were blocked with 1% BSA in PBS and incubated with 0.1 M $NaIO_3$ in PBS and subsequently in 5% dimethyl sulfoxide in PBS. Sections were incubated with rabbit anti-GFP antibody (1:100; AB3080, Millipore, RRID:AB_91337) in 1% BSA in PBS overnight at 4°C and immune reactions were subsequently visualized using a biotinylated secondary antibody (biotinylated goat anti-rabbit IgG; 1:50; BA-1000, Vector, RRID: AB_2313606) and streptavidin-biotin-peroxidase (PK-6100, Vector Laboratories, RRID:AB_2336819) complex using diaminobenzidine–HCl (SK-4105, Vector Laboratories, RRID:AB_2336520) and $H_2O_2$. After diaminobenzidine staining, appropriate regions of the corpus callosum were cut, the sections were osmicated and processed for light and electron microscopy by dehydration and embedding in Spurr's medium.

Ultrathin sections (70 nm) were mounted to copper grids, counterstained with lead citrate, and investigated using a ProScan Slow Scan CCD camera mounted to a Leo 906 E electron microscope (Zeiss) and corresponding software iTEM (Soft Imaging System).

### 2.10 | Statistical analysis

Statistical analyses and graphs were done using Excel and Graph-Pad Prism 5.0 software. To determine statistical significance in graphs with more than two conditions, ordinary (not repeated measures) two-way analysis of variance with Bonferroni posttest was applied. For datasets with two conditions, Student's two-sided, unpaired *t* test was applied. Statistical significance thresholds were set as follows: $*p < .05$; $**p < .01$; $***p < .001$. All data are shown as mean values ± *SEM* and "*n*" represents the number of independent experiments performed.

## 3 | RESULTS

### 3.1 | p57kip2 gene suppression leads to oligodendroglial fate acquisition in cultured aNSCs

The p57kip2/cdkn1c gene has previously been shown to encode a negative regulator of Schwann cell and oligodendroglial precursor cell differentiation (Heinen et al., 2008; Kremer et al., 2009). Moreover, we demonstrated that enforced downregulation of p57kip2 expression leads to accumulation of oligodendroglial features and markers in rat hippocampus-derived aNSCs (Jadasz et al., 2012). Please note that in this previous publication, we demonstrated that p57kip2 suppression induces a moderate survival benefit in aNSCs and that it also slightly promoted their proliferation. In order to prepare in vivo stem cell maturation studies, we conducted a series of experiments using cultured adult mouse SVZ-derived NSCs. To assess whether p57kip2 gene suppression also instructs an oligodendroglial fate in mouse aNSCs, we transfected them with a p57kip2 specific shRNA generating vector (p57-KD; Heinen et al., 2008; Jadasz et al., 2012; Kremer et al., 2009). Control cells were transfected with an empty expression vector and for identification of transfected cells, a citrine-encoding construct was cotransfected. Transfected cells were fluorescence-activated cell sorted by means of their citrine expression (Jadasz et al., 2012) 1 day following transfection and subsequently cultured in an astroglial fate promoting medium as described previously (Jadasz et al., 2012; Jadasz et al., 2018). Seven days following transfection, transcript levels were measured and suppression of p57kip2 was confirmed (Figure 1a). This was accompanied by an increased gene expression of NG2 (oligodendroglial precursor marker; Figure 1b) and of the mature oligodendrocyte marker GSTπ (Figure 1c). At the same time, the expression of the negative regulator of oligodendroglial differentiation Hes5, of astroglial GFAP and aquaporin-4 (AQP4) and of stem cell markers nestin and Sox2 was decreased (Figure 1d-h). For validation of RNA expression, immunocytochemical staining using antibodies directed against NG2 (4d), GSTπ

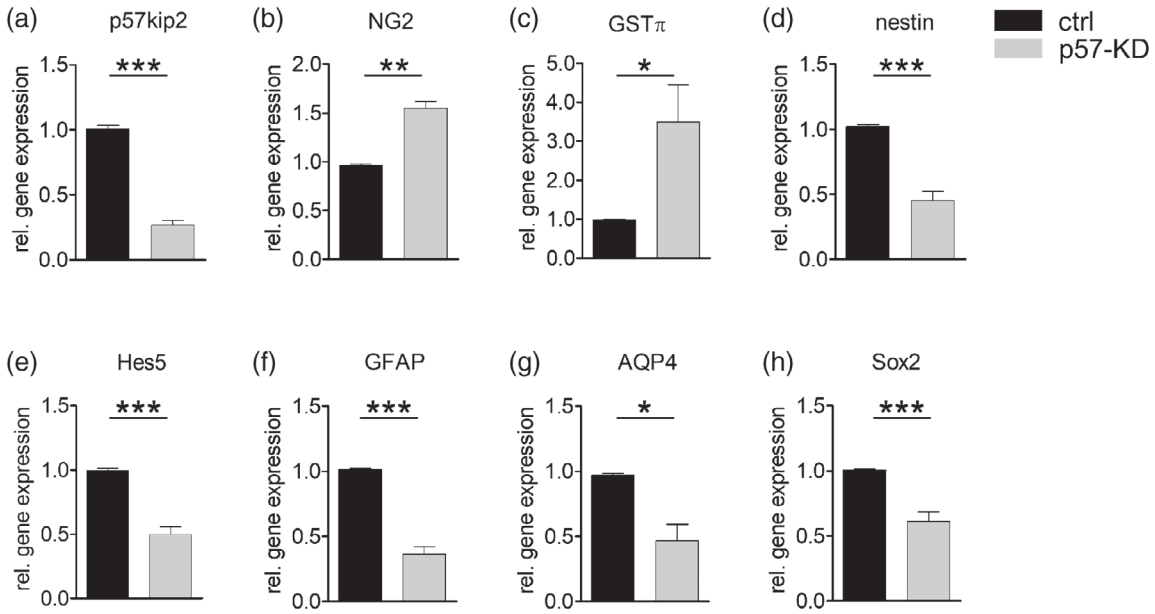
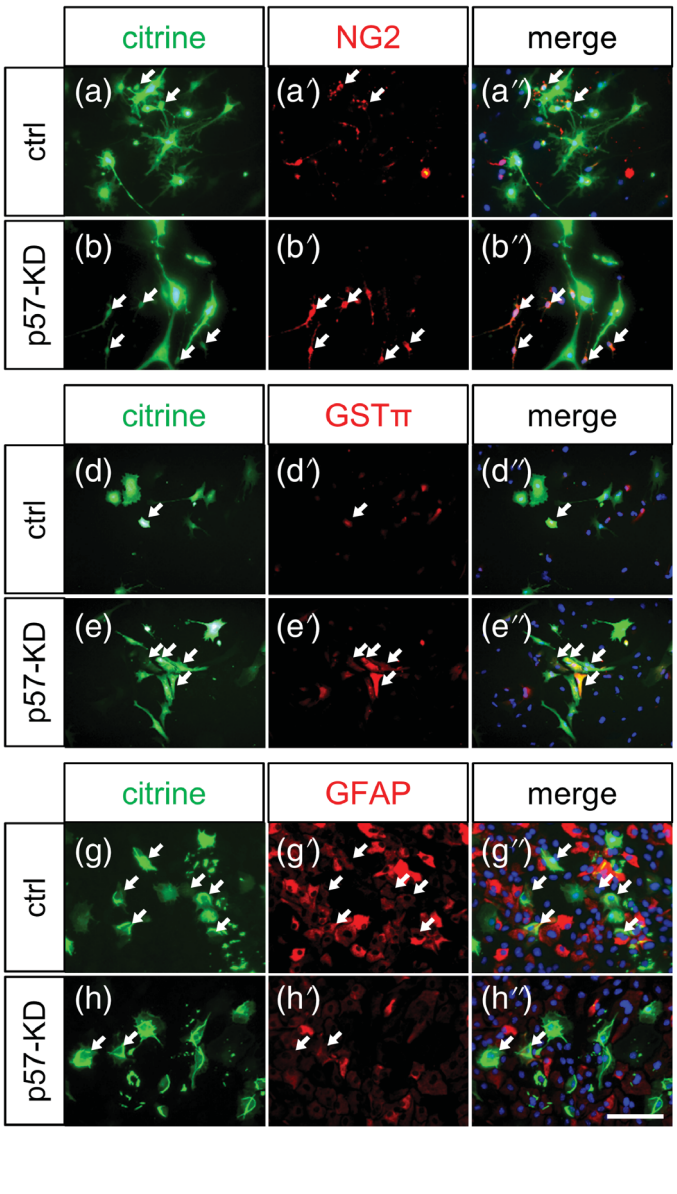



**FIGURE 1** Increased oligodendroglial gene expression in adult NSCs following suppression of p57kip2. qRT-PCR of FACS-sorted aNSCs 7 days following shRNA-mediated suppression of p57kip2 (p57-KD; gray bars) revealed p57kip2 transcript level reduction (a) compared to control transfected cells (ctrl; black bars). This led to an increased expression of early (NG2; b) as well as of mature oligodendroglial marker genes (GSTπ; c). The increase in oligodendroglial gene expression was accompanied by a decreased expression of stem cell genes nestin (d) and Sox2 (h), of the oligodendroglial differentiation inhibitor Hes5 (e), and of the astrocytic marker genes GFAP (f) and AQP4 (g). Data are shown as mean values, error bars represent *SEM*. Number of independent experiments: $n = 3$ for (b,g), $n = 4$ for (d), $n = 5$ for (c,h,f), and $n = 6$ for (a,e). Statistical significance was calculated using Student's two-sided, unpaired *t* test: $*p < .05$; $**p < .01$; $***p < .001$. aNSCs, adult neural stem cells; FACS, fluorescent activated cell sorting; GFAP, glial fibrillary acidic protein; GSTπ, glutathione-S-transferase-π; qRT-PCR, quantitative real-time reverse transcription PCR; shRNA, short-hairpin RNA



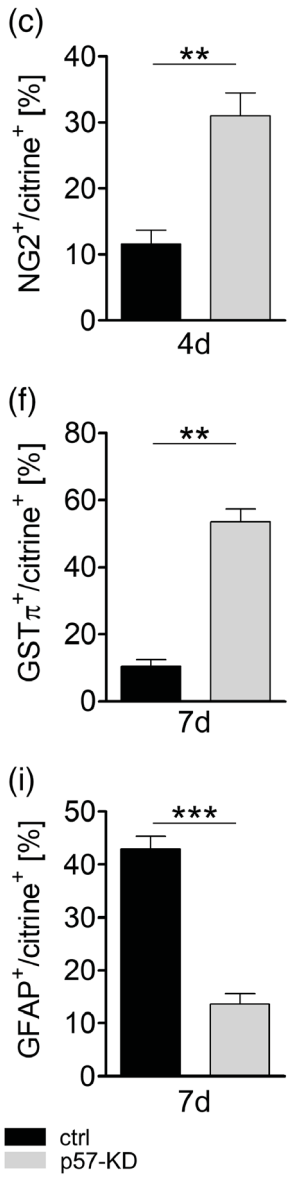




**FIGURE 2** Suppression of p57kip2 in adult NSCs induces oligodendroglial protein expression. Immunocytochemical analysis of p57kip2 suppressed (p57-KD; gray bars) and control transfected aNSCs (ctrl; black bars) for the oligodendroglial precursor marker NG2 after 4 days (a–c), for the mature oligodendrocyte protein GSTπ after 7 days (d–f) and for GFAP after 7 days (g–i). Note that only transfected cells (citrine-positive) were analyzed. Arrows in representative images point toward double positive (citrine/corresponding marker protein) cells. Scale bar in (h): 200 μm. Data are shown as mean values, error bars represent *SEM*. Number of independent experiments: $n = 3$. Statistical significance was calculated using Student's two-sided, unpaired *t* test: *$p < .05$; **$p < .01$; ***$p < .001$. aNSCs, adult neural stem cells; CNS, central nervous system; GFAP, glial fibrillary acidic protein; GSTπ, glutathione-S-transferase-π; shRNA, short-hairpin RNA [Color figure can be viewed at wileyonlinelibrary.com]

(7d), and GFAP (7d) were performed. Only transfected cells (marked by citrine expression) were scored and quantification confirmed increased oligodendroglial identities at the expense of GFAP expression in p57kip2 suppressed aNSCs (Figure 2).

Having established that transfection of the p57kip2 suppression vector confers an oligodendroglial fate on mouse SVZ-derived aNSCs as compared to control transfected cells (ctrl), we next investigated how to accomplish in vivo transplantation experiments with the lowest degree of technical variance among different host animals and surgeries. We therefore examined whether SVZ-derived aNSCs from transgenic tdTomato expressing mice (driven by nestin promoter (Imayoshi et al., 2006)) showed similar (p57kip2 dependent) properties in terms of oligodendroglial fate choice as nontransgenic transfected cells. For this purpose, we used a transgenic reporter mouse line in which the tdTomato expression was observed in approximately 50% of the cultured aNSCs (data not shown) as additional source for the preparation of SVZ aNSCs. Transfection of the p57kip2 suppression vector/citrine vector combination reproducibly resulted in a third of cells being citrine-positive (of which 50% were also tdTomato-positive), hence with knocked down p57kip2 levels, whereas another third of cells was tdTomato-positive only. In our in vivo approaches, we then strictly compared green to red-only cells in order to have a site-by-site comparison of p57kip2 suppressed compared to control aNSCs. Note that cultured nontransfected/wildtype aNSCs, control-transfected/wildtype aNSCs and nontransfected/tdTom-positive (transgenic) aNSCs showed the same astroglial and oligodendroglial protein marker expression dynamics (Supplementary Figure 1). Immunocytochemistry revealed that the degree of nestin positivity was the same (100%) in both, wildtype and tdTom-positive, stem cell populations. In addition, GFAP, GSTπ, and Hes5 gene expression analysis revealed the same p57-KD-dependent expression dynamics as shown for transfected wildtype aNSCs (Supplementary Figure 3). We also transplanted this cell population onto myelinating cocultures (Göttle et al., 2015; Göttle et al., 2018) in order to provide an as much as possible physiological ex vivo

environment. These experiments revealed that suppression of p57kip2-induced Sox10 expression, a marker expressed in the whole oligodendrocytic lineage, in transgenic (tdTomato-positive) aNSCs from 14.63% ± 0.84% to 30.37% ± 2.17% which was to a similar degree as when control transfected wildtype aNSCs were compared to p57-KD transfected wildtype aNSCs (data not shown). This provided strong evidence that a direct assessment of citrine-positive (p57-KD) versus tdTomato-only-positive (ctrl) cells perfectly reflects the differences as previously seen when p57-KD transfected cells were compared to control transfected cells. This constitutes an important aspect as we aimed at comparing cell fates upon modulation of p57kip2 expression in each individual host animal in situ, hence with a minimum of variation arising from the use of different mice or injection sites.

## 3.2 | p57kip2 suppressed aNSCs show survival advantage in both, GM and WM

As a next step, transgenic (tdTomato-positive) aNSCs transfected with the p57-KD/citrine vector combination were transplanted into 13–14 week old mouse brains. Coordinates were chosen according to a previous study challenging the fate of grafted OPCs (Vigano et al., 2013) and modulated aNSCs were either implanted into WM or GM regions (corpus callosum and neocortex, respectively). Note that every graft was composed of tdTomato-only-positive control cells and of cells with downregulated p57kip2 expression (citrine-positive). Counting the number of fluorescent cells at 4, 14, and 42 days post-transplantation (dpt) revealed differences in cell survival rates depending on the genetic modulation and site of transplantation (as presented as pie charts in Figure 3). Overall, p57kip2 suppressed aNSCs showed greater survival rates as compared to control cells in both GM and WM, and at all time points examined. Such an increased survival upon p57kip2 knockdown, despite the fact that these cells underwent nucleofection, suggests that gene suppression or the subsequently induced oligodendroglial fate acquisition confer a survival benefit. Over time, a modest decline in surviving cell numbers was observed which was less prominent in WM as compared to GM (compare Figure 3c to b). Furthermore, the survival benefit of p57-KD cells appeared to increase over time. Importantly, because cell counts were not generated by means of a longitudinal analysis but derived by examining tissues from different cohorts of grafted mice, a direct comparison in numbers is only possible between GM and WM at a single time point but not within a given tissue between different time points. Nevertheless, using the here presented grafting procedure, we were able to reproducibly generate mice with implanted, genetically modified cells over a period of up to 6 weeks, thus allowing detailed analysis of maturation in vivo and tissue integration.

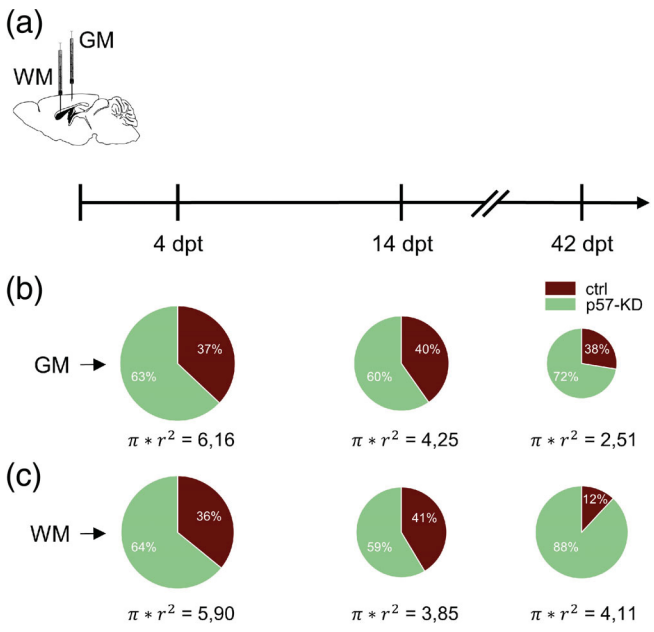



**FIGURE 3** WM tissue and suppression of p57kip2 support survival of transplanted adult NSCs. (a) aNSC transplantation set up into GM or WM (GM, WM) and time points (ctrl: tdTomato-only-positive nontransfected cells; p57-KD: citrine-positive p57kip2 suppressed cells). Grafting into GM (b) and WM (c) of young adult mouse brains. Brains were analyzed 4, 14, or 42 days posttransplantation (dpt). (b,c) Area ($A = \pi r^2$) of pies represent the average cell count per field of view following all immunohistochemical analyses for each time point and tissue (GM vs. WM). Average survival rates of p57-KD cells (green) and ctrl cells (red) are given in percent within each pie. aNSCs, adult neural stem cells; GM, gray matter; WM, white matter [Color figure can be viewed at wileyonlinelibrary.com]

## 3.3 | WM signals and suppression of p57kip2 promote oligodendroglial fate choice

In order to determine the impact of a modulated p57kip2 expression in addition to GM versus WM environmental cues on fate acquisition of adult NSCs, brain sections (4, 14, and 42 dpt) were subjected to immunofluorescent staining (Figures 4 and 5). Quantitative analysis revealed that at 4 dpt Sox10 protein could only be detected in WM implanted cells or in GM implanted cells when they were p57kip2 suppressed (Figure 4b–d‴,l). In the WM, the number of Sox10-positive cells was further increased by p57kip2 knockdown. A similar induction of NG2 positivity was observed in response to p57kip2 suppression at 4 dpt in both tissues (Figure 4e–g‴,m). Note that at this stage, both tissues featured comparable numbers of implanted cells as represented by the pie charts in Figure 3. Concomitantly, the expression of GFAP was significantly decreased due to the p57kip2 knockdown at 4 dpt, whereas levels were generally lower in the WM (Figure 4h–i‴,n). To test for a possible early neuronal fate, brain sections were stained using an antibody directed against the neuroblast marker Dcx. Neither suppression of p57kip2 nor tissue specific effects led to significant differences in the ratio of transplanted aNSC-derived neuroblasts (Figure 4j–k‴,o).

We next examined expression of the mature oligodendrocyte marker GSTπ among transplanted aNSCs at 14 dpt (Figure 5b–e). Generally, GSTπ was found to be expressed in more cells transplanted into the WM, we nevertheless observed a robust and significant induction in response to the p57kip2 knockdown. On the other hand,

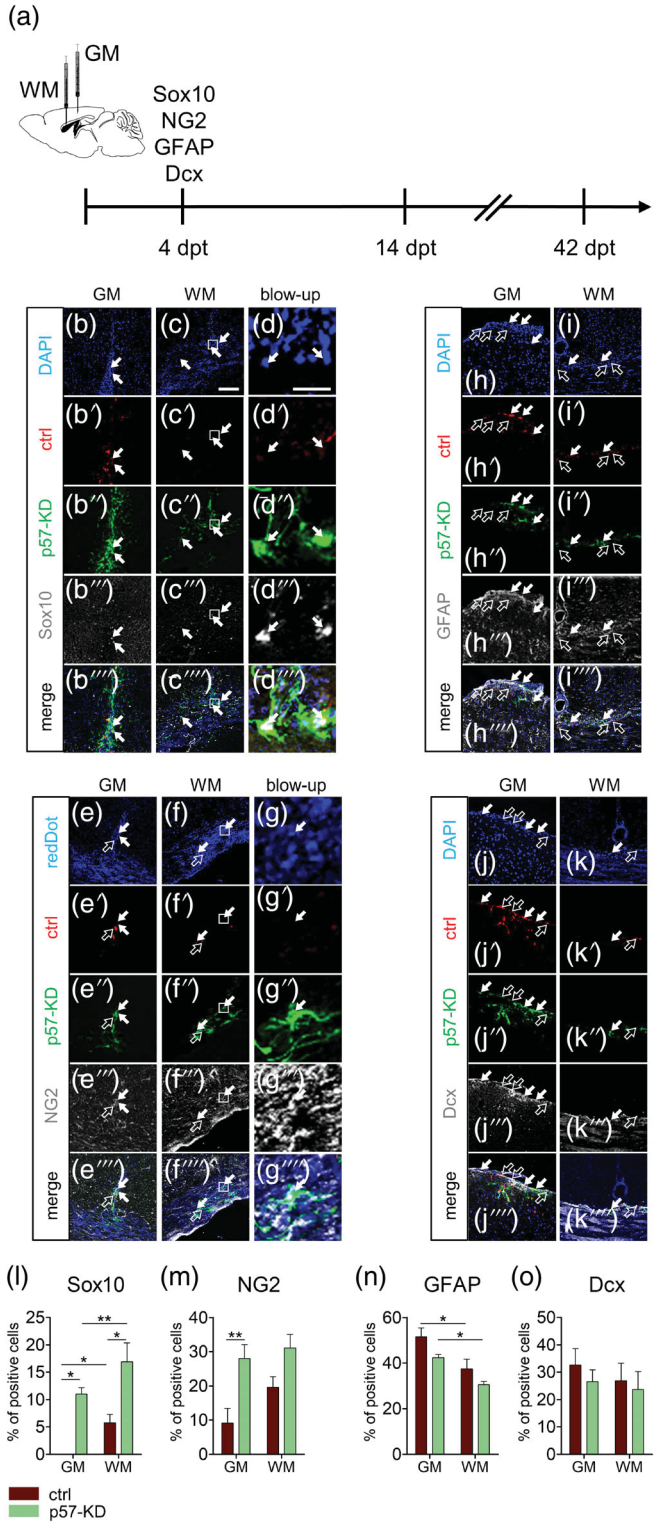



**FIGURE 4** Conferring an oligodendroglial fate to adult NSCs in vivo following suppression of p57kip2 and transplantation into WM tissue. (a) aNSC transplantation set up into GM or WM, time point, and examined marker proteins (ctrl: tdTomato-only-positive nontransfected cells; p57-KD: citrine-positive p57kip2 suppressed cells). (b–o) Immunohistochemical analysis for Sox10, NG2, GFAP, and Dcx was performed 4 days posttransplantation to reveal early fate choices of transplanted aNSCs. While oligodendroglial and astroglial marker protein expression correlate inversely, expression of Dcx showed neither tissue nor p57kip2-dependent changes. Inserts in (c–c‴) and (f–f‴) correspond to blow-ups in (d–d‴) and (g–g‴), respectively. White arrows point toward citrine- and Sox10-/NG2-/GFAP-/Dcx- double-positive cells, respectively, while open arrows point toward tdTomato- and NG2-/GFAP-/Dcx- double-positive cells, respectively. Scale bar in (c) for all nonblow up pictures: 100 μm. Scale bar in (d) for all blow-ups: 20 μm. Data are shown as mean values, error bars represent *SEM*. Number of independent experiments: $n = 5$ animals per graft site. Statistical significance was calculated using two-way ANOVA with Bonferroni posttest: $^{*}p < .05$; $^{**}p < .01$. ANOVA, analysis of variance; aNSCs, adult neural stem cells; GFAP, glial fibrillary acidic protein; GM, gray matter; WM, white matter [Color figure can be viewed at wileyonlinelibrary.com]

at this time point, reduction in GFAP-positivity was more pronounced among WM implanted cells (Figure 5f). Finally, in order to test for functional integration including stem cell-derived myelin protein expression and the establishment of myelin sheaths, transplanted cells were analyzed at 42 dpt, mainly focusing on WM. We examined to what degree p57kip2 knockdown also confers a benefit in myelination when cells were exposed to axons in vivo. Moreover, we also wanted to address the question whether aNSCs after being cultured for several passages in vitro still have the potential to fully differentiate into myelinating oligodendrocytes following transplantation. Brain sections were subjected to immunofluorescent staining using antibodies against myelin proteins CNPase, MBP, and MOG. In WM, a strong increase in the degree of myelin protein expressing cells was detected in response to the long-term p57kip2 knockdown (Figure 5g–j). Note that a comparison between GM and WM, which was conducted using MOG stained cells, revealed that p57kip2 suppression boosts myelin expression in both tissues (Figure 5j). Furthermore, overall induction levels were higher in WM and together with the fact that at this time point significantly more p57kip2-suppressed cells survived in WM as compared to GM (see Figure 3), the overall yield in mature myelinating oligodendrocytes was severely elevated in this condition. These cells also displayed multiple myelin positive cell extensions morphologically resembling mature oligodendrocytes (Figure 5g–g‴; i–iv). An initial qualitative electron microscopic analysis further confirmed that processes of transplanted p57kip2-suppressed cells, identified by means of electron dense phenazine precipitates derived from anti-citrine immunohistochemical detection, were indeed found in direct proximity to myelin sheaths around corpus callosum axons. These myelinating extensions are therefore likely to derive from transplanted aNSC-derived oligodendrocytes (Figure 5k–m). The degree of actively myelinating cells out of the pool of p57kip2 suppressed cells, over time as well as in relation to their integration sites, should be determined in upcoming studies.

## 3.4 | Stem cell-derived oligodendrogenesis in the spinal cord

In order to test whether the observed pro-oligodendroglial behavior of modulated stem cells is maintained under hostile conditions, aNSCs were transplanted into the lesioned spinal cord. For practical reasons, rat spinal cord hemisection characterized by a profound local lesion

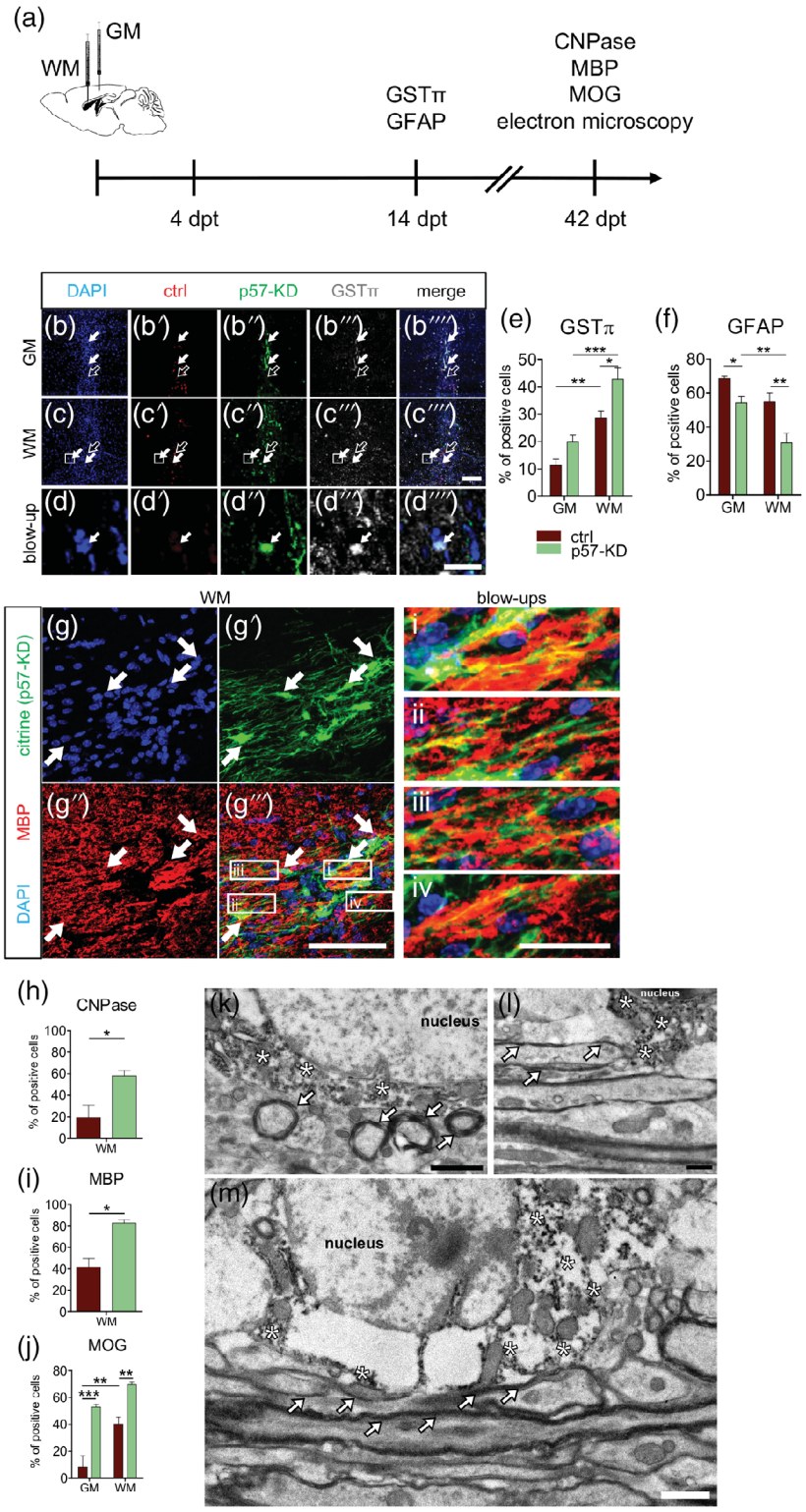



**FIGURE 5** Suppression of p57kip2 leads to an increased yield of adult NSC-derived myelinating oligodendrocytes. (a) aNSC transplantation set up into GM or WM, time points and examined marker proteins (ctrl: tdTomato-only-positive nontransfected cells; p57-KD: citrine-positive p57kip2 suppressed cells). (b–e) Immunohistochemical analysis 14 days posttransplantation revealed that the ratio of GSTπ expressing cells was increased in WM and upon p57kip2 suppression. (f) At the same time, less transplanted aNSCs differentiated into GFAP expressing astrocytes. Immunohistochemical assessment of myelin protein expressing cells in the corpus callosum 42 days after transplantation revealed significant increases in CNPase- (h), MBP- (g–g‴), and MOG-positive cells (j) following p57kip2 suppression (green bars). Inserts in (c–c⁗) and (g‴) correspond to blow-ups in (d–d⁗) and (i–iv), respectively. White rectangles in (g‴) surround MBP-positive processes of transplanted p57-KD aNSC in the corpus callosum. White arrows point toward citrine- and GSTπ- (b-d⁗) or MBP- (g–g‴) double-positive cells. (k–m) Electron microscopy images show that p57-KD cells after 42 days posttransplantation exhibit anti-GFP staining-derived electron dense precipitates in the cytoplasm of myelinating extension (asterisks) and are in direct proximity to myelinated axons (arrows) in the corpus callosum. Data are shown as mean values, error bars represent *SEM*. Number of independent experiments: *n* = four animals per graft site for (e,f,j) and *n* = three animals for (h,i). Statistical significance was calculated using Student's two-sided, unpaired *t* test (h,i) and using two-way ANOVA with Bonferroni posttest (e,f,j): **p* < .05; ***p* < .01; ****p* < .001. Scale bar in (c⁗,g‴): 100 μm, scale bar in (d⁗): 20 μm, scale bar in (iv): 25 μm, scale bar in (k,l): 1 μm, scale bar in (m): 500 nm. ANOVA, analysis of variance; aNSCs, adult neural stem cells; GFAP, glial fibrillary acidic protein; GM, gray matter; GSTπ, glutathione-S-transferase-π; MBP, myelin basic protein; WM, white matter [Color figure can be viewed at wileyonlinelibrary.com]

(an overview of such a lesion and citrine-positive transplanted cells [green] is shown in Supplementary Figure 4) was chosen and SVZ-derived rat aNSCs were used according to our previous study (Jadasz et al., 2012). Here, either empty vector control transfected- (ctrl) or p57kip2-suppressed (p57-KD) aNSCs were transplanted into GM and WM rostral and caudal to the lesion site, immediately after spinal cords were hemisectioned (Figure 6a). In contrast to our observations on mouse brain implanted stem cells, the average number of transplanted and surviving cells in the lesioned spinal cord significantly dropped in response to the p57kip2 knockdown at 7 dpt (Figure 6b). Immunofluorescent staining revealed no difference in the extent of Sox10-positivity in control cells between GM and WM and a significantly increased degree of Sox10-positivity in p57-KD cells transplanted into WM tracts (Figure 6c–h″,i). The number of GSTπ

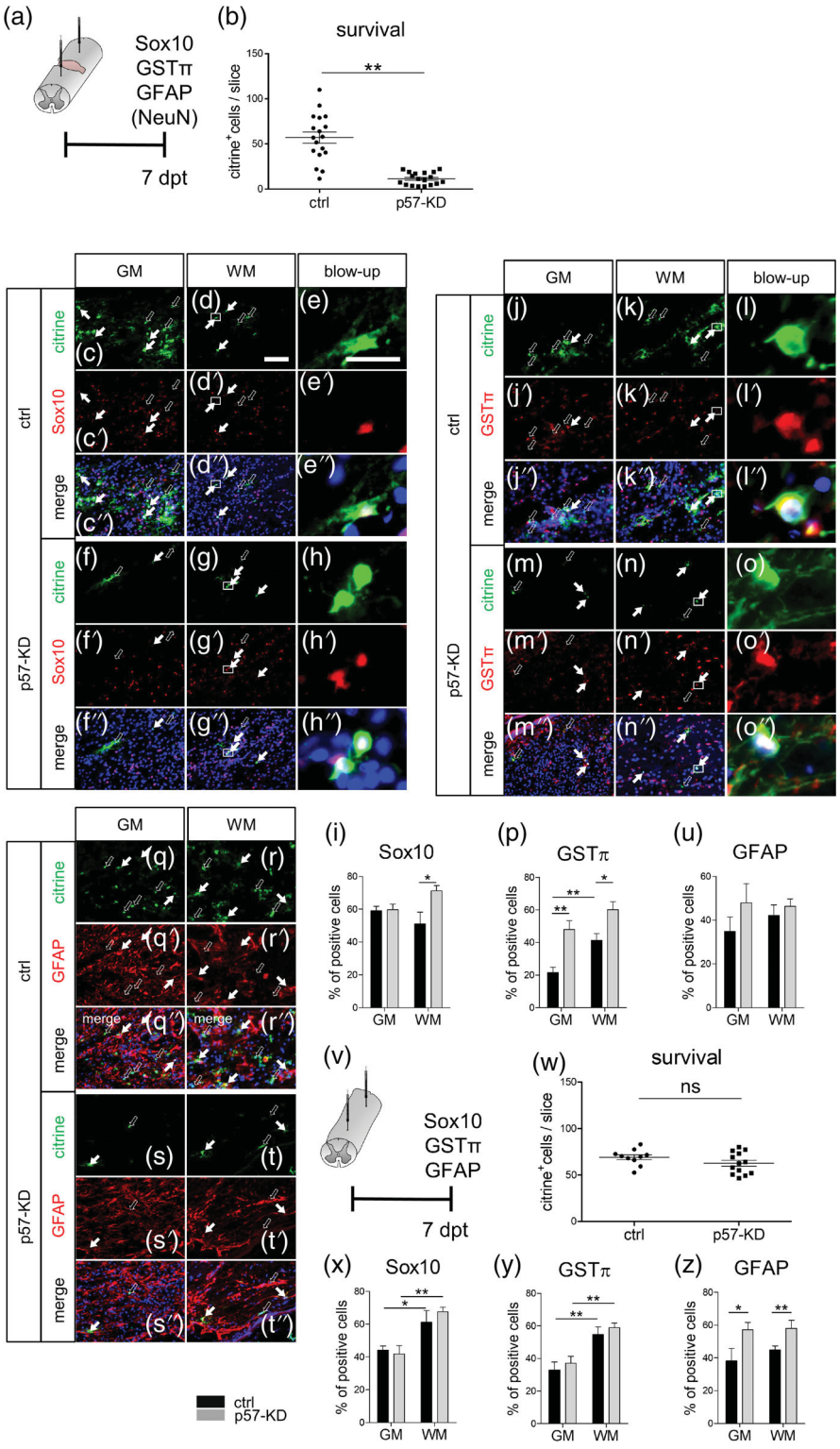



**FIGURE 6** High vulnerability of aNSC-derived oligodendroglial cells in the spinal cord injury microenvironment. (a) Hemisected rat spinal cord transplantation set up, time point, and examined marker proteins (ctrl: control transfected cells [black bars]; p57-KD: p57kip2 suppressed cells [gray bars]; both populations marked by means of citrine cotransfection). (b) Cell survival was negatively affected in the p57-KD aNSC population. (c–u) Immunohistochemical analyses for Sox10, GSTπ, and GFAP 7 days posttransplantation. Inserts in (d–d″), (g–g″), (k–k″), and (n–n″) correspond to blow-ups shown in (e–e″), (h–h″), (l–l″), and (o–o″), respectively. White arrows point toward citrine- and Sox10-/GSTπ-/GFAP- double-positive cells, respectively, while open arrows point toward citrine-positive cells only. Scale bar in (d) for all nonmagnified pictures: 100 μm. Scale bar in (e) for all blow-up pictures: 20 μm. (v) Transplantation set up into the noninjured rat spinal cord, time point, and examined marker proteins (ctrl: control transfected cells [black bars]; p57-KD: p57kip2 suppressed cells [gray bars]; both populations marked by means of citrine cotransfection). (w) Cell survival of p57-KD and ctrl-transfected cells was not affected in the lesion-free transplantation paradigm. (x–z) In the intact spinal cord transplantation set up, the p57-KD cell population showed low (z) or no (x,y) pro-glial differentiation compared to the control aNSCs. However, transplantation into WM resulted in a significantly increased number of oligodendroglial cells compared to GM grafts (x,y). Data are shown as mean values, error bars represent *SEM*. Number of independent experiments: $n = 6$ control- versus 5 p57-KD animals for hemisected animals and 4 control- versus 5 p57-KD animals for transplantation into the noninjured spinal cord. Statistical significance was calculated using Student's two-sided, unpaired *t* test (b,w) and using two-way ANOVA with Bonferroni posttest (i,p,u,x–z): $*p < .05$; $**p < .01$. aNSCs, adult neural stem cells; GFAP, glial fibrillary acidic protein; GM, gray matter; GSTπ, glutathione-S-transferase-π; WM, white matter [Color figure can be viewed at wileyonlinelibrary.com]

protein-positive transplanted stem cells was increased by the p57kip2 knockdown in both, GM as well as WM (Figure 6j–o″,p) with the highest ratio of GSTπ-positive aNSC-derived cells to be found upon gene knockdown in the WM area. While we showed that in the intact (or mildly traumatically affected) mouse brain the impact of WM as well as of the p57kip2-knockdown leads to a reduced astrocyte generation, staining for GFAP expression in spinal cord implanted stem cells revealed almost no change in the extent of this marker expression (Figure 6q–t″,u). In line with the observation that none of the transplanted aNSCs were found expressing the neuronal marker NeuN (data not shown), it can be concluded that almost exclusively glial derivates arose from grafted stem cells, probably due to the astrogenic environment (Beyer, Samper Agrelo, & Küry, 2019 and references therein). Note that since in the lesion paradigm we decided to

perform our analyses at 7 dpt, so considerably later than in the brain (4 dpt), neuron detection was carried out using NeuN as marker. This goes along with the finding that for control- as well as for p57-KD cells, respective proportions of GFAP- and Sox10-positive cells added up close to 100% in both GM and WM. In contrast to our findings of reduced numbers of citrine-positive/p57kip2 suppressed cells in the vicinity of hemisectioned spinal cords (Figure 6b), survival rates between transplanted control- and p57-KD aNSCs did not differ in the nonlesioned spinal cord (Figure 6w). Here, we found that a total number of 686.9 ± 27.3 p57-KD cells and 662.4 ± 29.9 ctrl cells survived and integrated into the host tissue at 7 dpt. Moreover, upon transplantation into the intact spinal cord no profound pro-oligodendroglial effect in response to suppression of p57kip2 was observed as assessed using markers such as Sox10 and GSTπ (Figure 6x,y). The extent of GFAP-positive cells was comparable in lesioned and nonlesioned tissues (Figure 6u,z) with a differentiation effect upon knockdown of p57kip2 resulting in a slight increase in GFAP-positive astrocytes after transplantation in the lesion-free spinal cord. However, aNSCs transplanted into the WM still showed increased oligodendroglial marker expression as compared to GM transplants (Figure 6x,y).

## 4 | DISCUSSION

An assessment of glial heterogeneity within the CNS is an important yet unsolved aspect of cell lineage analysis and for myelinating cells, it includes the description of different oligodendroglial cell populations within different brain regions (Vigano et al., 2013), during development (Spitzer et al., 2019) or in demyelinating diseases (Jäkel et al., 2019). Vigano et al., for example, reported an impaired differentiation of GM-derived OPCs compared to WM-derived OPCs upon heterotopic transplantation into GM and WM suggesting intrinsic differences between adult OPC subpopulations to occur (Vigano et al., 2013). Furthermore, they demonstrated that the WM environment can help to overcome intrinsic inhibitory cues acting on GM OPCs providing evidence for a role of differentially expressed extracellular matrix and/or diffusible ligands. Additional impact results from the contribution of aNSCs being heterogeneous between not only different niches but also revealing variations within well-defined niches such as the SVZ (Mizrak et al., 2019). For OPCs and aNSCs, overlapping intrinsic regulators of oligodendroglial differentiation have been described, such as, for example, Sox10, Olig2, and p57kip2 (Copray et al., 2006; Jadasz et al., 2012; Pozniak et al., 2010). Yet, it remains to be shown whether overall differentiation and maturation processes are really identical and to what degree progenitor or stem cell derivatives depend on environmental cues. The latter fact is also important considering the observation that internodes in remyelinated animals differ in terms of length and thickness depending on the origin of the myelinating cells (Xing et al., 2014). In order to achieve a better understanding of how aNSCs can contribute to glial cell heterogeneity either in different brain regions or under hostile conditions and to see whether extrinsic signals can be overcome by intrinsic manipulation of the stem cell fate choice, we transplanted genetically modulated aNSCs in different CNS regions of mice and rats. By using less committed neural stem cells (as compared to committed OPCs), we aimed to reveal the full potential of healthy and lesioned GM- versus WM-derived heterogeneous signals on cell fate acquisition and integration.

Our data clearly demonstrated that mouse and rat neural stem cells equally respond to lowered p57kip2 expression levels in that this manipulation increased the establishment of oligodendroglial features, improved and accelerated the generation of myelinating oligodendroglial cells at the expense of astroglial marker expression. Such directed fate acquisition and differentiation appeared to dominate over tissue specific cues such as in GM areas of the brain, where oligodendroglial fate directing cues seem to be either limited or of inhibitory nature. Moreover, increased oligodendroglial differentiation upon transplantation into WM tracts was further enhanced by the intrinsic p57kip2 modulation, which additionally granted a survival benefit resulting in a much higher yield of myelinating aNSC-derived oligodendrocytes. Of note, neuronal differentiation was not observed in cultured cells and following transplantation, no differences in the extent of neurogenesis were observed. Whereas in stem cell culture this is likely to result from the astroglial promoting medium hence conferring a restriction to glial decisions it can be concluded that in vivo p57kip2 does not influence neurogenesis in this context.

Experimental transplantation of NSCs into CNS tissue aiming either at directed cell replacement or the local generation of trophic signals has so far almost exclusively been conducted under pathophysiological conditions (Assinck, Duncan, Hilton, Plemel, & Tetzlaff, 2017; Beyer et al., 2019). Few studies only engaged into regional differences (Fricker et al., 1999; Gage et al., 1995; Herrera, Garcia-Verdugo, & Alvarez-Buylla, 1999; Seidenfaden, Desoeuvre, Bosio, Virard, & Cremer, 2006) and none of them considered a potential impact of GM versus WM on implanted stem cells. Overall, the currently published injury- and pathology-free CNS transplantation studies imply that transplanted NSCs do sense their ectopic environment and to some degree adapt environment-specific migratory features and fate acquisition (summarized in Beyer et al. (2019)). However, direct assessment of GM versus WM differences on NCS fate and a detailed overview of oligodendroglial differentiation over time has so far been missing.

The here described differences observed between GM and WM of healthy and lesioned CNS point to dominant roles of an intrinsic fate modulation (here by suppression of p57kip2) and WM signals regarding oligodendroglial fate acquisition and successful execution of differentiation programs in the healthy CNS and of injury environmental cues related to cell survival. Based on these observations, particularly taking into account lowered long-term survival rates among GM grafts, it can be concluded that therapeutic cell replacement strategies either should be limited to diseases with a clear WM impact or must consider additional survival promoting manipulations. The absent pro-oligodendroglial effect in response to p57kip2 knockdown in the healthy spinal cord differed from our observations in the healthy brain. Several reasons might account for these differences such as, for

example, myelination dynamics and myelin as well as oligodendrocyte turnover not being identical over all CNS regions (Foran & Peterson, 1992; Snaidero & Simons, 2014; Williamson & Lyons, 2018). Further, we have to acknowledge that the composition of both tissues and their signaling cues affecting NSC fate and differentiation might differ. Finally, we cannot rule out species differences between our two rodent transplantation models. This can be interpreted in that p57kip2 suppression driven pro-oligodendroglial differentiation and cell integration can only be seen in tissues with a certain need for (re) myelination. Another question to be solved in the future relates to the heterogeneity among SVZ aNSCs (Azim et al., 2018) which might have an impact on how receptive these cells respond to an intrinsic p57kip2 modulation or extrinsic cues upon transplantation. Whether this includes for example the expression of a specific subset of gap junction proteins such as connexins would be of interest to address. Dynamic connexin expression following transplantation into striatal slice cultures and the generation of connexin-dependent networks were reported to affect adult neural precursor survival (Jaderstad, Jaderstad, & Herlenius, 2011; Ravella, Ringstedt, Brion, Pandolfo, & Herlenius, 2015). To what degree observed dependencies also apply to endogenous stem cells naturally giving rise to new oligodendrocytic cells, hence in a noninvasive experimental paradigm, thus most likely limited to myelin repair in demyelinating conditions, needs to be addressed in future experiments.

Taking other transplantation studies into account, it appears that the number of surviving NSCs seems to be overall rather low (as exemplified in Herrera et al. (1999), Raedt et al. (2009), and Seidenfaden et al. (2006)); summarized in Beyer et al. (2019)). It is therefore of considerable interest to see that suppression of the intrinsic regulator p57kip2 imparts a survival benefit. This might indeed be promising in light of improving potential myelinating cell replacement strategies. However, we also found that the number of p57kip2 suppressed cells was severely reduced upon grafting into lesioned CNS tissue suggesting that a promoted oligodendroglial fate acquisition on the one hand facilitates successful tissue integration but on the other hand might come with an increased sensitivity toward hostile cues as described before (Casha, Yu, & Fehlings, 2001; Crowe, Bresnahan, Shuman, Masters, & Beattie, 1997; Pfeifer et al., 2006; Rowland, Hawryluk, Kwon, & Fehlings, 2008; Vroemen, Aigner, Winkler, & Weidner, 2003). An influence of spinal cord infiltrating peripheral immune cells (Anderson, 2002; Margul et al., 2016) can also not be excluded yet. Such an impact could only be addressed by future transplantation experiments into subacute spinal cord injuries at later time points featuring reduced inflammation and beyond secondary cell death. Changes toward less hostile lesion conditions have in fact already been described for transplanted NSCs in temporal lobe epilepsy models (Raedt et al., 2009). However, it remains to be seen to what degree such a delayed aNSC application makes sense as grafting into lesion regions primarily aims at protection of demyelinated/endangered axons. In this regard, cotransplantation of NSCs with other cells that can exert an overall benefit to the whole lesion environment such as mediated by unrestricted somatic stem cells (Schira et al., 2012) or mesenchymal stem cells (Jadasz et al., 2012; Jadasz et al., 2018) might therefore constitute a promising alternative approach. Of note, cotransplantations of NSCs with other cells revealed indeed to be beneficial in terms of cell survival and functional improvements in ischemic stroke animal models (Cai et al., 2015; Luo et al., 2017). Moreover, coapplication of blockers of spinal cord injury-evoked oligodendroglia hostile cues such as chondroitin sulfate proteoglycans (Dyck, Kataria, Akbari-Kelachayeh, Silver, & Karimi-Abdolrezaee, 2019) might further increase the yield in p57-KD aNSC-derived oligodendrocytes.

In conclusion, our work uncovered regional heterogeneity between rodent CNS GM and WM affecting survival and fate of transplanted aNSCs. Here, WM tissue substantially promoted both, survival as well as differentiation into myelinating oligodendrocytes. In addition, we showed that suppression of the p57kip2 gene further increased WM effects and in part antagonized lower oligodendroglial yield in GM grafts. Gene knockdown-promoted oligodendrogenesis, however, suffers from a negative impact on cell survival mediated by the injured CNS hostile microenvironment, which must therefore be addressed prior to future cell replacement therapies.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

## ETHICS STATEMENT

Rodent primary stem cell preparation was approved by the ZETT (Zentrale Einrichtung für Tierforschung und wissenschaftliche Tierschutzaufgaben; O90/15, O118/11). Cell transplantation experiments presented were approved by the authorities LANUV (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen; Az.: 84-02.04.2015.A239; Az.: 84-02.04.2015.A525).

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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# Do Neural Stem Cells Have a Choice? Heterogenic Outcome of Cell Fate Acquisition in Different Injury Models

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**Abstract:** The adult mammalian central nervous system (CNS) is generally considered as repair restricted organ with limited capacities to regenerate lost cells and to successfully integrate them into damaged nerve tracts. Despite the presence of endogenous immature cell types that can be activated upon injury or in disease cell replacement generally remains insufficient, undirected, or lost cell types are not properly generated. This limitation also accounts for the myelin repair capacity that still constitutes the default regenerative activity at least in inflammatory demyelinating conditions. Ever since the discovery of endogenous neural stem cells (NSCs) residing within specific niches of the adult brain, as well as the description of procedures to either isolate and propagate or artificially induce NSCs from various origins ex vivo, the field has been rejuvenated. Various sources of NSCs have been investigated and applied in current neuropathological paradigms aiming at the replacement of lost cells and the restoration of functionality based on successful integration. Whereas directing and supporting stem cells residing in brain niches constitutes one possible approach many investigations addressed their potential upon transplantation. Given the heterogeneity of these studies related to the nature of grafted cells, the local CNS environment, and applied implantation procedures we here set out to review and compare their applied protocols in order to evaluate rate-limiting parameters. Based on our compilation, we conclude that in healthy CNS tissue region specific cues dominate cell fate decisions. However, although increasing evidence points to the capacity of transplanted NSCs to reflect the regenerative need of an injury environment, a still heterogenic picture emerges when analyzing transplantation outcomes in injury or disease models. These are likely due to methodological differences despite preserved injury environments. Based on this meta-analysis, we suggest future NSC transplantation experiments to be conducted in a more comparable way to previous studies and that subsequent analyses must emphasize regional heterogeneity such as accounting for differences in gray versus white matter.

**Keywords:** neural stem cell; subventricular zone; subgranular zone; CNS injury; disease; regeneration; transplantation; therapy; injury environment; regional heterogeneity

## 1. Introduction

Ever since the discovery of naturally occurring neural stem cells (NSCs) residing in discrete niches of the adult mammalian central nervous system (CNS) [1–5], these cryptic cell populations received considerable interest in terms of their contribution to brain plasticity, learning, and repair. In this regard, most work addressed structure, function, and maintenance on stem cell niches located in the subventricular zone (SVZ) of the lateral brain ventricles as well as in the subgranular zone

(SGZ) of the dentate gyrus. Whereas cells with stem-like properties contained within the ependymal cell population of the adult spinal cord [6,7] received less attention. Years of research have brought advances in NSC mediated regeneration and also pointed particularly to NSC grafting into affected CNS tissues and tracts as a potential therapeutic choice for a variety of neuropathologies. Yet, no clinical trial has been able to successfully translate these approaches into clinical treatments. While the large degree of heterogeneity of applied NSCs, even when isolated from defined stem cell niches [8,9], is likely to affect reproducibility, standardization, and clinical translation, different brain regions and injury types additionally contribute to the number of parameters affecting cell fate acquisition. Most NSC mediated regeneration studies focus on stem cell modulation, induced lineage heterogeneity, and their impact on the treated injury. However, an inverse view has rarely been considered so far and is therefore the main scope of this review. In order to interpret the power of an injury microenvironment on grafted cells, one has to elucidate the effects mediated by different CNS regions on introduced cell survival, proliferation, migration, and fate acquisition. We will therefore first discuss injury-free NSC engraftment studies in order to compare different outcomes on the above-mentioned parameters. In the second part, additional impact arising from host tissue injuries and lesion inflicted reactions will be addressed.

While screening the publicly available literature, it became evident that there is a large degree of heterogeneity when it comes to the NSC transplantation procedure itself, related for example to age and species of donor- as well as host tissues, the question whether sorted/enriched cell populations versus mixed cell grafts were applied or concerning time-points at which host tissue and grafted cells were analyzed. Likewise, the localization and type of an injury prior to engraftment of stem cells, as well as their positioning within lesion zones additionally influence cellular integration and differentiation. It would therefore be important to define rate limiting and dominating parameters to ensure a larger degree of comparability across different investigations and to promote the development of protocols that will eventually lead to a successful clinical translation.

## 2. Injury-Free Neural Stem Cell Transplantation Studies

Clinical research depends on animal models, which mimic human disease or injury. For neuropathological studies of the CNS various animal models such as acute and chronic spinal cord injury (SCI); traumatic brain injury (TBI); inflammatory-, genetically-, or chemically induced demyelination/neurodegeneration have been used to assess the impact of NSC transplantation on either lesion amelioration or tissue regeneration. The outcome of these studies shows a surprising degree of variability in terms of cell fate acquisition, migration within the host tissue as well as the implanted cell's potential to fully mature (relevant studies discussed in detail below and summarized in Table 1). To better compare and interpret these differential outcomes an initial assessment of cellular reactions in the healthy CNS is warranted. Whether transplanted NSCs strictly recapitulate a developmental-like program within a non-hostile environment, as described for NSCs and their progenitor descendants in the healthy adult SGZ [10], or whether regional CNS heterogeneity decides on donor cell parameters is therefore a key question.

**Table 1.** Summary of injury-free NSC transplantation studies.

| Reference | Donor NSC Origin | Host Animal | CNS Region | Time Post-Transplantation | Outcome |
|---|---|---|---|---|---|
| Seidenfaden et al. 2006 | SVZ<br>Mice<br>P5 or P75<br>- | Mice (C57BL/6)<br>Six to ten weeks old | Striatum (mainly)<br>Motor cortex<br>Lateral posterior thalamic nucleus | 3 wpt and 6 mpt | Survival: independent of the donor animal age<br>Migration: no preference for GM or WM<br>Cell fate: glial |
| Gage et al. 1995 | Hpc<br>Adult female Fischer 344 rats<br>>3 months old<br>33 passages | Adult female Fischer 344 rats<br>>3 months old | Hippocampus | 1, 4, 8, 12 wpt | Survival: yes<br>Migration: some up to 3 mm<br>Cell fate: glial in Cc and neuronal in Hpc |
| Raedt et al. 2009 | SVZ<br>Male mice (C57BL/6×DBA2/J)<br>-<br>10 passages | Sprague Dawley rats<br>175–200 g | Hippocampus | 3 and 6 wpt | Survival: yes<br>Migration: low degree<br>Cell fate: astroglia (38.6%) and neuronal (5.8%) |
| Herrera et al. 1999 | SVZ<br>Mice (NSE-LacZ)<br>2–3 months old<br>Directly isolated and transplanted | Male mice (CD-1)<br>2–3 months old | Cortex<br>Striatum<br>Hippocampus<br>Olfactory bulb | 2, 4, 6, 8 wpt | Survival: comparable between Str, Cx and OB, less survival in Hpc<br>Migration: only in the OB<br>Cell fate: non-neuronal type-C (or astrocyte) and type-A (neuronal precursor) phenotypes in Cx and Str, neurons in OB |
| Lois and Alvarez-Buylla 1994 | SVZ<br>Mice (NSE-LacZ)<br>Adult<br>- | Mice<br>Adult | Lateral ventricle | 30 dpt | Survival: -<br>Migration: along RMS up to OB<br>Cell fate: - |
| Fricker et al. 1999 | Forebrain<br>Human<br>6.5 to 9 weeks old<br>9 to 21 passages | Female Sprague Dawley rats<br>Adult (250 g)<br>Immunosuppressed | Dentate gyrus<br>RMS<br>Striatum<br>Subventricular zone | 2 and 6 wpt | Survival: yes<br>Migration: low (DG, Str), high (SVZ, RMS)<br>Cell fate: neuroblast features in SVZ and RMS neuronal in OB and Hpc, glial and neuronal in Str |
| Brock et al. 1998 | SVZ<br>Sprague Dawley rats<br>P0, P1, or P2<br>Transplanted 24 h after isolation<br>---<br>VZ<br>Rat embryos<br>E16 to E17<br>Transplanted 24 h after isolation | Rats<br>P0–P1 | Subventricular zone | 1 to 4 wpt | Survival: yes<br>Migration: along RMS up to OB (SVZ-NSCs) cells remain at the injection site (VZ-NSCs)<br>Cell fate: neurons |

The table provides (from left-to-right) information on the original literature, donor cell origin (tissue, species, age, and passage number in culture if applicable), host animals (species, weight/age), region of transplantation within the CNS, time points of analysis, and features of transplanted cells (survival, migration, cell fate). A "-" indicates that no information on this feature was given in the original publication. Abbreviations: SVZ—subventricular zone; P—postnatal day; dpt, wpt, mpt—days, weeks, months post-transplantation, respectively; GM/WM—gray- and white matter, respectively; Hpc—hippocampus; Cc—corpus callosum; Str—striatum; Cx—cortex; OB—olfactory bulb; RMS—rostral migratory stream; DG—dentate gyrus; VZ—ventricular zone.

Using NSCs isolated from the SVZ of either postnatal day (P)5 or P75 old mice for transplantation into the striatum, motor cortex, and lateral posterior thalamic nucleus, Seidenfaden and colleagues showed that transplanted cell survival is independent of the donor animal age—at least when comparing immature postnatal- to young adult brains [11]. Furthermore, they observed that when these cells were implanted into the healthy striatum of adult mice, they did not show any migratory preference for either gray matter (GM) or white matter (WM) structures. In terms of fate choice, these SVZ-derived NSCs that are rather primed to take on a neuronal fate in their in vivo niche [12], primarily adopted glial phenotypes equally split between astroglia and oligodendroglia. Similar cell fates were observed in the additional transplantation regions such as cerebral motor cortex and lateral posterior thalamic nucleus. Surprisingly, even when grafted cells were enriched for neuronal precursors by means of the neuronal marker polysialylated neuronal cell adhesion molecule (PSA-NCAM) and subsequently transplanted into the healthy striatum, the fate outcome in vivo was unchanged compared to non-sorted cells, indicating that striatal cues dominantly suppress neuronal lineage and favor glial descendants.

A related transplantation study used rats instead of mice for both donor cells as well as host animals [13]. However, "cells capable of proliferation and neurogenesis" [13] from the adult rat hippocampus were used as donor cells and most likely represent the NSC pool of the SGZ, which are even more neuronal primed as compared to SVZ-derived cells. Additionally, prior to transplantation these cells were passaged for over 1.5 years. Despite these numerous differences, fate acquisition was again mainly described as being glial (analyzed by morphology) upon transplantation back into the hippocampus and subsequent analysis in hippocampus and the adjacent corpus callosum. Furthermore, even though some degree of cell migration was described (adjacent to striatum and corpus callosum), it was considered as minor and the majority of cells remained close to the injection site. Of note, transplanted cells found in the corpus callosum, a region absent of neuronal cell bodies and primarily characterized by myelinating oligodendrocytes (OLs) and oligodendroglial precursor cells (OPCs), adopted an oligodendroglial morphology. Only transplanted cells directly located in the granule cell layer of the hippocampus, indeed acquired a neuronal cell fate, which, even back then, evoked the author's statement that "the in vivo fate of these cells is clearly influenced by exogenous factors" [13]. In contrast to these two allotransplantations (transplantation in which the donor material is derived from a different donor of the same species), Raedt and colleagues xenografted (donor and host differ in species) SVZ-derived NSCs from young mice, which additionally were propagated over 10 passages prior to transplantation, into the adult rat hippocampus [14]. Similar to Gage´s observations, transplanted cells did not show high degrees of migration except for a few cells entering the granule cell layer of the hippocampus. However, in contrast to SGZ-derived NSCs, some of these SVZ-NSCs were detected close to the SVZ of the host rat, indicating SVZ niche specific cues, which might have attracted intra-hippocampal SVZ-NSC grafts. Even though oligodendroglial differentiation was not accessed, the majority of surviving NSC descendants were positive for glial markers such as astroglial glial fibrillary acidic protein (GFAP) (38.6%) and only a few cells (5.8%) showed mature neuronal marker RNA binding protein, fox-1 homolog (C. elegans) 3 (Rbfox3 or NeuN) expression, which is in agreement with earlier observations [11,13].

Further support for the assumption that exogenous factors influence the fate of transplanted NSCs [13] resulted from an extensive transplantation study published four years later [15]. While different injection sites within young adult, non-injured mice were chosen, a defined single donor cell condition was maintained for all transplantations, allowing for a direct comparison of fate acquisition and other cell properties within different brain regions. Neural stem cell survival rates were largely comparable between striatum, cortex, and olfactory bulb indicating that no preferential survival cues are expressed and secreted in these brain regions. Hippocampus implanted cells, however, survived less well for non-disclosed reasons. Moreover, it was also stated that proliferation (or even tumorigenesis resulting from aberrant cell expansion) was not observed among transplanted NSCs—an observation that was confirmed by many follow-up studies in the field. Whether this implies

the existence of mechanisms that actively restrict NSC division outside of their niches or whether this is rather a consequence of host-initiated cell differentiation remains to be shown but is promising in light of preventing the generation of brain tumors such as for example glioblastoma. Given that donor cells were acutely isolated from the adult SVZ and were therefore more primed towards neuronal differentiation subsequent analysis of fate choice in this study must be interpreted carefully, as even mentioned by the authors. Cortex- and striatum-transplanted cells attained primarily non-neuronal type-C (or astrocyte) and type-A (neuronal precursor) phenotypes. On the other hand, when these cells were transplanted into the olfactory bulb, a region receiving neuronal progenitors via the rostral migratory stream (RMS) more mature granule neurons were found to descend from transplanted NSCs [15]. Moreover, significant cell migration was observed in this situation. Of note, donor SVZ cells were isolated from a transgenic mouse line in which LacZ was expressed under the control of the neuron-specific enolase (NSE) promotor, which limits a proper analysis of fate choice due to reporter restriction to neuronal descendants. Still, some degree of astrocytic phenotype acquisition was observed indicating that the transgenic NSE promotor activity was not too specific. Oligodendroglial differentiation was not assessed in this study.

For a successful treatment of neurological conditions such as multiple sclerosis (MS) or adrenoleukodystrophies transplanted NSCs would have to distribute well within diseased brains in order to access multiple and irregularly dispersed lesions. Active migration within the brain parenchyma appears indeed to be a rare feature of implanted NSCs as for example transplantation of SVZ-derived NSCs into neighboring SVZ regions of adult healthy mice did not result in any observable migration activities [16]. Transplantation into the lateral ventricle, however, resulted in migration along the RMS up to the olfactory bulb, indicating that also artificially implemented cells are restricted to naturally occurring migration routes and cues.

Supporting evidence that brain region specific cues act across different species arose from a subsequent study in which embryonic human NSCs (huNSCs) were applied to injury-free models [17]. Here, donor cells extracted from 6.5 to 9-week-old embryonic human forebrains and subsequently expanded over 9 to 21 passages were transplanted into either the dentate gyrus, the RMS, the striatum, or the SVZ of adult immunosuppressed rats. These multipotent NSCs showed no tumor formation within the first six weeks post transplantation. In line with the previous reports on rodent-to-rodent transplantations, only minor NSC migration was observed into dentate gyrus or striatum. Moreover, such low migration rates were questioned in terms of whether they result from true (host evoked) cell movement or rather from random dispersion as consequence of pressure implemented during the implantation procedure. Similar to rodent-to-rodent NSC engraftments, larger migration activities were only observed when huNSCs were transplanted into the SVZ or RMS. In there, these embryonic cells adopted features similar to those of the surrounding endogenous neuroblasts. Interestingly, despite the multipotency and the rather high proliferative capacity of these huNSCs of embryonic origin, they adopted exclusively a neuronal fate when reaching the olfactory bulb or within the neuronal SGZ of the hippocampus. On the other hand, within the striatum, both glial- and neuronal NSC derivatives were described. Since NeuN expression was still absent these were most likely immature neuronal cells—a notion which supports the above-mentioned type-A cell (neuronal precursor) generation by rodent NSCs in the striatum [15]. Absent NeuN expression was also reported for the majority of host neurons in the striatum [17]. In this regard, the use of additional neuronal markers in order to achieve a more detailed description of the acquired cell fate resulted in the observation that the acquired neuronal phenotypes of engrafted NSC derivatives matched the neuronal population of the host striatum. Here, the authors describe that the transplanted NSCs differentiated into either glutamic acid decarboxylase (GAD) 67-, calbindin-, or dopamine- and cAMP-regulated neuronal phosphoprotein-(DARPP-) 32-positive neurons, three neuronal types located in the host tissue [17].

The astonishing impact especially the RMS exerts on transplanted NSCs was corroborated by yet another study [18]. This investigation provides additional support for the SVZ/RMS environment acting as key guidance structure for transplanted cells since neonatal SVZ-derived NSCs transplanted

into the neonatal SVZ migrated with the same properties (route, speed, morphology) along the RMS as the endogenous cells arising from the SVZ. Even at their final destination in the olfactory bulb, the ratio of granule- versus glomerular-cell layer infiltration was maintained at 3:1, independent of whether these were transplanted NSCs or endogenous NSC-derived neuroblasts. However, when embryonic ventricular zone-derived cells were used they found that these NSCs never entered the RMS pathway but strictly remained at the injection sites within the neonatal SVZ. The authors thus suggested that this might be due to their inability to recognize specific guidance cues along postnatal migratory routes, which could be attributed to naturally different migratory properties and their role in populating the developing neocortex [18,19]. Nevertheless, this described inability to migrate along the RMS is contradictory to results by Fricker and colleagues, who also used embryonic NSCs (although from human embryonic tissue) and reported migration along the host RMS. Apart from species-specific migratory properties, observed differences in the migratory behavior could also derive from the fact that Brock and colleagues analyzed host brains only up to 15 days post-transplantation whereas the other team analyzed host brains six weeks post-transplantation into the SVZ. Moreover, human embryonic NSCs were passaged between 9 and 12 times prior to transplantation [17], potentially impacting a number of cellular parameters (for comparison see Table 1).

## 3. Brain Pathology Models and Their Heterogenic Impact on NSC Fate

Comparing outcomes of NSC transplantation in different neuropathological or injury inflicted models is important in order to understand the adaptability NSCs are capable of and also for the development of CNS repair strategies. Nevertheless, due to the high degree of variation and heterogeneity across the different model systems this section will focus on selected models representing both common and rare as well as global and focal pathologies. Working out the details between different injury models, and taking into account heterogenic responses of different brain regions depends on comparable starting conditions. This includes information on donor age, on the particular donor stem cell niche, on isolated cell types and whether they were propagated in culture or whether genetic manipulation was applied prior to transplantation. Moreover, variations deriving from different model systems will also be considered.

### *3.1. Dysmyelinating Neuropathologies*

Treatment of hereditary white matter disorders characterized by abnormal or complete absence of myelin due to mutations in genes encoding for myelin proteins (such as in Pelizaeus-Merzbacher disease) will most likely depend on engraftment of healthy cells giving rise to functionally unimpaired myelin forming oligodendrocytes. In shiverer mice (shi) homozygous mutations in the myelin basic protein (MBP) gene lead to the absence of MBP expression and consequently to low levels of compact and functional myelin [20,21] and it therefore serves as an animal model for dysmyelinating neuropathologies. Intracerebroventricular transplantation of C17.2 NSCs (an immortalized cell line derived from neonatal mouse cerebellum) into newborn (P0) shi mice resulted in excessive cell migration within the brain parenchyma, both into GM and WM. The degree of oligodendroglial differentiation was significantly higher when engrafted into these myelination deficient mice as compared to wildtype hosts. While in healthy mice 16% of the transplanted cells differentiated into the oligodendroglial lineage, up to 28% of them generated oligodendroglia in the shi brain [22]. This might therefore reflect the need for myelinating glial cells in the shiverer CNS but also the capacity of NSCs to sense and react to such a deficient background. This is a promising hint that NSCs can indeed compensate according to missing/impaired cell types and, as stated by the authors: "Such behavior might reflect a fundamental developmental strategy with therapeutic utility".

However, intracerebroventricular transplantation of the same NSC cell line (C17.2) into shi mice of comparable age (P1-P3) resulted in mainly neuronal differentiation [23]. While this was primarily a feasibility study and the primary focus of this study was not on cell fate acquisition, the authors still state that no oligodendroglial differentiation, and hence no myelin production, was observed.

Transplanted NSCs did accumulate into the surrounding parenchyma and expressed the neuronal marker TuJ-1 [23]. Interestingly, C17.2 cells were a courtesy of the former-mentioned study´s senior author (Prof. Evan Snyder) and in both studies homozygous mouse mutants (*shi/shi*) were used as host animals, which in terms of methodology makes these studies highly comparable. Therefore, the reason for such differential fate outcome remains obscure.

Studies in which transplantation experiments were performed using different (neural) stem cell types but exclusively applying a single neuropathological model are of special value since methodological differences can be ruled out. When adult forebrain SVZ neural precursor cells (aNPCs) were grafted into the dysmyelinated spinal cord of shiverer rats, the majority differentiated into oligodendroglial cells and even into mature myelinating OLs [24]. In contrast, transplantation of embryonic stem cell-derived primitive neural stem cells (pNSCs) into the same model did not result in successful integration and even led to heterotoma formation. However, definitive NSCs (dNSCs) which were also investigated in this study and which derived from pNSCs by leukemia inhibitory factor (LIF) and fibroblast growth factor 2 (FGF2) application revealed oligodendroglial fate acquisition and generation of mature myelinating cells comparable to aNPC descendants. For dNSCs and aNPCs differentiation into oligodendroglia was in the range of 48–58% whereas only 3–4% astrocytes and 3–4% neurons were observed. Thus, a natural restriction point for (neural) stem cells appears to exist deciding on whether and when they are able to properly react to neuropathological dysfunctions and deficits, at least in a dysmyelinated environment such as the shiverer rodent.

### *3.2. Traumatic Brain Injury*

Traumatic brain injury (TBI) results from forced impact to the skull and brain, which leads to a primary- (direct negative influence on tissue architecture and homeostasis) as well as a secondary (cell death, inflammation) injury [25]. Due to their multipotent nature, NSC transplantation into TBI lesions is thus considered as a promising approach for broad cell replacement and functional improvement.

The study by Koutsoudaki and colleagues elegantly demonstrated the non-necessity of NSC modulation prior to transplantation into injured adult mouse brains. Upon TBI to the hippocampus by stabbing multiple times 2 mm deep through the cortex, corpus callosum, and hippocampus, non-modified (reporter-gene expression only) and insulin like growth factor 1 (IGF1) overexpressing NSCs (mouse P5, SVZ-derived) were transplanted close to the lesion site. Considering fate outcome as well as functional improvement (as assessed by the Morris Water Maze test, MWM), no differences between IGF1 producing and reporter gene only expressing NSCs were observed. Both cell populations ameliorated injury-induced spatial learning deficits and in both cases transplanted NSCs mainly differentiated into oligodendroglial cells [26]. As in some hippocampal injury procedures, also white matter structures of the corpus callosum are disrupted resulting in focal oligodendrocyte death [27], subsequent migration of NSCs into the corpus callosum and oligodendroglial differentiation (both in the corpus callosum and hippocampus) are probably driven by the need to repair WM structures. However, this observation is somehow in contrast to what has been described in injury-free hippocampus transplantations, where mainly astroglial and neuronal differentiation was described [13,14]. Since in both experimental set-ups (studies by Gage et al. and Raedt et al.) fate acquisition was largely comparable despite different donor cell origin, differences in terms of a higher OL differentiation in Koutsoudaki´s TBI model might be best explained by an injury-dependent change in the tissue microenvironment.

In contrast to the TBI wound induced by a blunt-end needle, which also disrupts WM structures [26], the modified Feeney method was used to injure the cortical motor area in rats [28]. Unfortunately, from the data presented in this study it is not clear whether this modified method also results in hemorrhage in the underlying WM leading to the formation of a necrotic cavity within the corpus callosum as originally described [29]. According to the authors, transplantation of mouse embryonic hippocampus-derived NSCs 3 mm from the lesion (rostral, caudal, left, and right) at the day of injury resulted in migration of these NSCs towards the injured region. Analysis of fate

choice revealed that a few transplanted NSCs gave rise to TuJ-1-positive neurons and GFAP-positive astrocytes [28]. Differentiation into oligodendroglial cells was not reported so it can only be assumed that this modified procedure did not harm WM structures.

In a comparable experimental set up (modified Feeney method) neonatal mouse hippocampus-derived NSCs were transplanted 24 h after injury into the pericontusional region [30]. Similar to the above-mentioned investigation transplanted NSCs survived, migrated towards the lesion site differentiating into few TuJ-1-positive neurons and GFAP-positive astrocytes [30]. Although this study focused on BDNF expression-mediated effects of NSCs on the extent of injury and subsequent functional improvements, a proper description of all three cell types (neuronal, astroglial, as well as oligodendroglial cells) was not provided.

### 3.3. Temporal Lobe Epilepsy

Temporal lobe epilepsy (TLE) is a common form seen in about 30% of epileptic patients. By injecting kainic acid (KA) into the rodent hippocampus or the SVZ, neurodegeneration is induced and neuronal cell death and functional impairments similar to TLE patients can be mimicked. While NSC transplantation has also been investigated in terms of trophic support these NSCs can confer to the damaged environment, this section will focus on the aspect of NSC mediated cell replacement and fate choice.

Since kainate induced hippocampal degeneration represents a focal CNS injury, transplantation of P5 mouse SVZ-derived NSCs into the hippocampus close to the injection site resulted in only minor migration towards the lesion area [31]. Stem cell marker (nestin) expression as well as transplanted NSC proliferation were rarely or not all observed after longer time-points (up to 60 days) post-transplantation [31]. Interestingly, transplantation of NSCs directly into such kainate-treated tissues led to significant differences in fate choice compared to non-injured or TBI-injured hippocampi. Basically no glial cells as assessed by GFAP- and O4 staining for astrocytes and oligodendroglia, respectively, derived from the transplanted NSCs within the kainate injured hippocampus. Instead, approximately 36% of the engrafted cells were immunopositive for the neuronal marker NeuN and the majority of cells expressed the neuronal progenitor marker doublecortin (Dcx) [31]. Further highlighting the major impact an injury microenvironment can exert on engrafted NSCs, the authors also demonstrated that only few or even no beneficial effects could be attributed to IGF1 overexpressing NSCs when looking at later time-points. In fact they observed, that the naïve NSCs did not express IGF1 in culture but rather adapted this beneficial feature upon transplantation into the kainate-treated hippocampus. The exact reason for the cognitive improvement (MWM test) upon NSC transplantation needs further investigation since several beneficial effects of the NSC transplants were described such as neuronal cell replacement, IGF1 expression, decreased astroglial activation as well as normalized proliferation rates in the dentate gyrus. In terms of clinical translation, these results might also be promising for the development of potential treatment options for patients with TLE.

While transplantation of NSCs into the kainate-induced neurodegenerative hippocampus resulted in mostly neuronal differentiation and ameliorated cognitive function [31], another TLE study using NSCs derived from the embryonic rat medial ganglionic eminence (mgeNSCs) showed substantial different cell fates and behavioral outcome. Hippocampal neurodegeneration was induced by injecting kainate intraperitoneally in rats to generate a chronic injury and NSCs were therefore transplanted several months later [32]. In this situation, the majority of transplanted mgeNSCs differentiated into astrocytes (57%). Only a minor fraction differentiated into mature NeuN expressing neurons (13%), GABAergic interneurons (10%) and into few OPCs (3%). This finding seems contradictory to the hypothesis that the same kind of injury microenvironment exerts a dominant impact on the fate of (different) engrafted NSCs, indicating that timing is another critical factor, not only in terms of survival and integration as revealed for spinal cord injuries but also for acquired cell fates. It can therefore be assumed that chronic neurodegeneration has changed the injury microenvironment so that it mainly contains astrogenic cues (perhaps in a more rapid manner than the healthy hippocampus

as it becomes more astrogenic with age anyways [33]). This is also supported by the observation that the majority of cultured mgeNSCs differentiated into neurons and OPCs in vitro [32]. Consequently, while in acute TLE lesions neuronal fate acquisition and cognitive improvement were observed [31], NSC transplantation in chronic TLE lesions resulted in astroglial differentiation, subsequent GDNF expression, a restored GDNF expression in host astrocytes, no cognitive improvement (MWM) but beneficial effects on spontaneous recurrent motor seizures (SRMS) [32]. How engrafted NSCs sense such subtle environmental changes remains to be investigated. Of note, no or only minor migration of transplanted cells within the hippocampal tissue and no tumor formation was observed in both acute and chronic TLE models.

A further TLE study also reported primarily astroglial fate choices although NSCs were transplanted into an acute TLE model [14]. A change in the injury microenvironment over time was supported by the observation that transplantation of mouse SVZ neurospheres at three weeks post-kainate lesion significantly improved the survival of transplanted cells compared to implantation after only three days. The striking differences in fate outcome despite similar starting conditions might indeed be dependent on subtle variations of transplanted cells. Raedt and colleagues used NSCs derived from young adult mouse SVZ, which were passaged at least ten times in vitro prior to transplantation, whereas Miltiadous' team used NSCs derived from P5 mice, which were propagated for "at least 3–4 passages". Moreover, while Raedt and colleagues xenografted mouse cells into the rat TLE model, the other group induced TLE in mouse hippocampi and grafted mouse cells. In addition, while Raedt's team transplanted SVZ NSC-derived neurospheres into kainate injection sites, Miltiadous' group transplanted dissociated SVZ-derived NSCs 600 µm away from the initial kainate injection site into the more rostral part of the injured hippocampus. Finally, the xenografting procedure was accompanied by cyclosporine application for immunosuppression, which can exert additional influences on the injury microenvironment due to a diminished immune response. Of note, beneficial effects of cyclosporine have been described for different injury models [34–36].

Another interesting finding is that SVZ-derived NSCs transplanted into healthy non-injured hippocampi were also located close to the SVZ several weeks after transplantation [14]—a distribution that was, however, not seen in TLE animals. This suggests that SVZ-derived NSCs can sense cues from the SVZ, which can be overridden by signals from the injured hippocampal tissue. Such a potential signal hierarchy with injury-derived signals dominating over naïve cues could be promising when it comes to NSC transplants in neuropathologies featuring dispersed lesions such as in MS.

### 3.4. Sly Disease

Sly disease is a rare hereditary lysosomal storage disorder, characterized by the deficiency in β-glucuronidase (GUSB) and subsequent accumulation of glycosaminglycans in many organs including the brain leading to mental retardation. Applying NSCs as early as technically feasible might provide a potential therapeutic approach in order to restore global dysfunction of the developing brain. The mucopolysaccharidosis VII (MPS VII) mouse strain mimics human sly disease pathological features. Upon transplantation of GUSB-expressing C17.2 NSCs into lateral ventricles of neonatal MPS VII mice, cells distributed and integrated in the whole brain and no tumorigenesis was observed [37]. Widespread integration into most brain regions (from olfactory bulb back to the hippocampal area) is most likely attributed to the global impact of the developing brain since a similar distribution was described in healthy animals [37]. Interestingly, donor cells did not spread completely throughout the whole brain, since in regions to which cells from the host SVZ do not contribute (e.g., retina) GUSB activity from donor cells was absent. Integration into the host brain tissue was maintained for up to 12 months post-transplantation indicating that even in a less beneficial environment (MPS VII brain) NSCs might be capable of surviving and contributing to normal CNS homeostasis. Morphological analysis three weeks post-transplantation revealed "normal neural morphologies" [37]. However, a detailed immunohistochemical characterization was missing. In light of the observations from this study it will be of interest to see whether also in mouse injury models with distinct focal brain region

impairment (e.g., stab wound to the cortical tissue) NSC transplantation into the neonatal developing brain would result into more widespread cell integration as opposed to the generally observed focal NSC occurrence around adult lesion sites.

### 3.5. Stroke

The lack of oxygen supply in the brain results in ischemic stroke, leading to irreversible neuronal damage [38]. Ischemia can be induced in the rodent brain via middle cerebral artery occlusion (MCAO) resulting in a similar striatal injury as in stroke patients. Upon intrastriatal transplantation of embryonic cortical mouse NSCs, which in vitro primarily express nestin and immature neuronal markers (Dcx, β-III-Tub) and incorporate BrdU (labeling proliferative cells), into non-MCAO rat brains, these cells did not migrate and the majority died within a few days [39]. However, in MCAO rats where the ischemic epicenter lies in the striatum, transplanted cells survived and migrated throughout the ischemic striatum [39]. In terms of fate acquisition, sham and MCAO striatal tissue both allowed differentiation of engrafted NSCs into neuronal cells, astrocytes and oligodendrocytes. The majority of NSCs in fact differentiated into GFAP-positive astrocytes and into Dcx-positive neuronal precursors—a pattern that was also described upon grafting of embryonic human NSCs into the healthy striatum [17]. Of note, the number of generated mature NeuN-positive neurons increased in the MCAO group compared to sham animals six days post-transplantation [39]. Interestingly, upon transplantation into the lateral ventricle of the adult rat (MCAO or sham), robust migration into the striatum was only observed in the MCAO group [39]. Here, assessment of fate choice in the ischemic striatum revealed an increase in Hu-positive neuronal cells while astroglial differentiation was decreased compared to sham operated animals six days post-intracerebroventricular transplantation. The high proportion in astroglial descendants of the intrastriatal—compared to intracerebroventricular—transplantations could thus reflect the acute necessity for functional astrocytes following MCAO. Furthermore, while transplantation of neonatal mouse SVZ-derived NSCs into the rat striatum 48 h following MCAO also resulted in neuronal differentiation (22%) accompanied by cells with astroglial- and oligodendroglial fates, co-transplantation of astrocytes and NSCs resulted in an increase in generated neurons (37%) [40]. Such co-transplantation also led to a higher ratio of proliferating and surviving NSCs seven days after transplantation.

Transplantation of neurosphere-derived cells from neonatal mice hence containing NSCs, various progenitors and more differentiated cells [41] into the ventricle of 12-week-old mice four hours following MCAO resulted in migration into striatal- and cortical tissue. Sham operated animals receiving the same grafts were devoid of donor cells in the brain parenchyma [41]. Even though neuronal fate acquisition was not assessed, early time-point analyses (one and seven days post-transplantation) revealed nestin-, GFAP-, as well as chondroitin sulfate proteoglycan 4- (Cspg4 or NG2) positive NSC descendants which, however, had disappeared after 14 days. Assessment of mRNA expression in the cortex revealed an increase in various cytokine- and trophic factor messages (such as CXCL12, TGF-β1, VEGF-A, IGF1, and BDNF) in the transplantation-free MCAO-group compared to sham operated animals, which points towards a rapid host initiated regeneration reaction. Interestingly, the authors report further increased transcript levels in the ischemic group, which received NSC transplants [41]. Even though mRNA origin (host or donor) could not be discriminated, transplanted NSCs thus appear to also modulate endogenous repair mechanisms.

Regarding differences in the microenvironment of ischemic brain regions over time, Darsalia and colleagues compared the differential outcome of intrastriatally transplanted human fetal striatal NSCs 48 h and six weeks after stroke. Transplantation after 48 h following stroke resulted in a higher NSC survival rate as compared to transplantation at the later time-point. However, different time-points did not affect the extent of migration, differentiation and proliferation. Interestingly, analysis of neuronal fate acquisition revealed no change in the percentage of Dcx-positive neuroblasts but for both transplantation time-points the percentage was lower as compared to injury-free animals [42]. Such observations are indeed relevant in light of the necessity to conduct autologous transplantation

procedures at rather late stages in stroke patients. Moreover, looking at the currently available studies it becomes clear that fate acquisition of transplanted NSCs in the MCAO stroke model must be analyzed more carefully in future studies taking into account all neural lineages NSCs can give rise to, their maturation kinetics as well as subregional differences. This is even more important in light of the current assumption that transplanted NSCs can reflect, to some degree, the regenerative need of an injury environment.

### 3.6. Multiple Sclerosis

Multiple sclerosis (MS) is an autoimmune disease characterized by oligodendrocyte and myelin loss, which ultimately leads to axonal degeneration and subsequent sensory, motor, and cognitive dysfunction [43]. The most common animal model used for recapitulation of mainly inflammatory aspects of this disease is experimental autoimmune encephalomyelitis (EAE). The majority of the here considered studies used myelin oligodendrocyte glycoprotein ($MOG_{35–55}$) peptide induced EAE, which leads to a T-cell mediated autoimmune response towards oligodendrocytes with the first symptoms appearing ten days after initial immunization.

Intracerebroventricular transplantation of human embryonic stem cell-derived early multipotent neural precursor cells (hESC-NPCs) demonstrated robust migration into the host WM, which led to significantly reduced clinical sings of EAE in host mice [44]. The beneficial effects presumably resulted from diminution of inflammatory processes and subsequent reduced demyelination, as well as axonal damage [44].

Further evidence for a high migratory activity of implanted cells in the EAE brain derived from a report on human induced pluripotent stem cell-derived NSCs (iPSC-NSCs) having migrated into the dentate gyrus one month following intraventricular transplantation in EAE mice [45]. Co-localization of reporter protein-positive iPSC-NSCs and neuronal protein TuJ-1 indicated integration of graft-derived neurons into injured areas of the dentate gyrus. Two months following transplantation endogenous remyelination in the marginal zone of the WM was detectable. Thus, iPSC-NSCs dramatically reduced T-cell infiltration and ameliorated EAE dependent demyelination resulting in functional recovery [45]. It seems striking that in contrast to dysmyelinating pathologies [22,24] no substantial oligodendroglial differentiation of transplanted NSCs was described. Since endogenous remyelination was in fact detected, such effects either resulted from an immunomodulatory action of the stem cells, along with some of them giving rise to neurons, eventually tuning down the T-cell response. Alternatively, transplanted NSCs might also have activated host OPCs to differentiate and myelinate.

Minor regeneration promoting effects using allogenic NSCs were also reported for a chronic EAE model, into which transplantation was performed 40 days post-immunization. Here, treatment with non-modified bone marrow-derived (BM) NSCs (derived from six- to eight-week-old mice) [46] blocked the demyelination process but did not favor remyelination in the diseased spinal cord. On the other hand, BM-NSCs molecularly engineered to produce LINGO-1-Fc, a soluble LINGO-1 (leucine rich repeat and IG domain containing 1) antagonist (LINGO-1´s role in neuropathologies is reviewed in [47]), significantly promoted remyelination in the chronic stage of EAE and reduced further demyelination processes as compared to control animals [48]. Additionally, LINGO-1-Fc expressing BM-NSCs significantly promoted neurological recovery. Histological assessment revealed improvements in axonal integrity, enhancement of oligodendrocyte maturation, and neuronal repopulation of the degenerated areas.

While the two previous studies report minor regeneration promoting effects using allogenic or naïve NSCs in EAE mice, Einstein and colleagues could show that intraventricularly transplanted striatal newborn rat multipotential NSCs migrated into inflamed WM tracts and subsequently differentiated into glial cells in EAE rats. This transplantation strategy resulted in a reduced inflammation of the host brain and ameliorated the disease course. Whether beneficial effects were due to additional processes attributed to particularly the rat system remains open.

Intraventricular transplantation of both, glial-derived neurotrophic factor (GDNF) overexpressing-(GDNF-NSCs) or naïve newborn rat cerebrum-derived NSCs ten days following EAE induction led to a delayed disease onset and significant reduction of clinical EAE signs [49]. More specifically, GDNF-NSC receiving rats recovered five days earlier to basal gait as opposed to rats that received non-modified NSCs. This observation might be attributed to the significant reduction in the number of inflammatory infiltrates in the striatum and the lower number of cells within each infiltrate in animals receiving naive NSCs or even to a greater extent upon application of GDNF-NSCs. Interestingly, the majority of NSCs migrated specifically towards inflamed areas in the corpus callosum and striatum. In addition, assessment of GDNF-NSC differentiation in the striatum revealed a significantly higher neuronal and oligodendroglial fate acquisition as compared to naïve NSCs. The latter of which preferentially generated GFAP-positive astrocytes.

### 3.7. Alzheimer´s Disease

Neurodegeneration in Alzheimer´s disease (AD) results in learning deficits and dementia. In a murine AD model (APP/PS1 mice), neuron-specific Thy1 promotor driving the co-expression of KM670/671NL mutated amyloid precursor protein (APP) and of L166P mutated presenilin-1 (PS1) leads to human amyloid depositions and local neuronal loss in the dentate gyrus [49,50]. The majority of hippocampus transplanted neonatal mouse SVZ-derived NSCs (primed towards the neuronal lineage by retinoic acid application in vitro) differentiated into neurons [51]. However, despite neuronal priming 24% of the total NSC population still differentiated into glial cells, implying the additional necessity to replace glial cells as well. In total, 8% NSC-derived oligodendroglia were reported—a surprising finding considering that upon NSC transplantation into the healthy hippocampus or hippocampal TLE models almost no oligodendroglial differentiation was described [14,31,32]. Unfortunately, efforts aiming at the identification of additional progenitor populations were not undertaken. A variety of AD mouse models have been generated, such as for example APP/PS1, B6C3-Tg, 5xFAD, 3xTg, APPSw-NSE (www.alzforum.org) [50,52–55] of which the APP/PS1 model is most widely used featuring amyloid plaque generation, mild to robust neuronal cell loss and cognitive impairments [51,56–62] and reporting on cognitive improvement after NSC transplantation [56,57,61,62]. However, Marsh and colleagues showed that fetal-derived human NSCs engrafted into the hippocampus migrated up to 1.7 mm and were detected in the lateral ventricle five months after transplantation, where they failed to differentiate and formed ectopic human cell clusters. Probably due to the lack of differentiation, the authors found no evidence for cognitive improvement [63]. Using an APPSw-NSE transgenic mouse line, lateral ventricle transplanted fetal human telencephalon NSCs (13 weeks of gestation) showed extensive migratory activity as indicated by broad distribution of these cells in SVZ, WM tracts, striatum, thalamus, hypothalamus and cortex. Some of the transplanted NSCs differentiated into neuronal- (5.8%) and glial cells (oligodendroglia 2.3% and astroglia 11.7%) but most of them remained nestin-positive (82.4%). Despite the low differentiation rate, transplantation of these NSCs resulted in improved spatial memory, decreased tau phosphorylation, lowered Aβ42 levels, and attenuated microgliosis and astrogliosis [58]. The observed contribution to regeneration was therefore unlikely a consequence of direct cell replacement but rather due to trophic, modulatory effects.

Interestingly, most of the compiled studies report that transplanted NSCs showed high migratory behavior leaving injection sites [56–65]. In addition, transplanted cells that remained mostly undifferentiated were mainly reported in cases where human NSCs were used [57,58,63]. Whether this reflects a natural restriction of human cells to adapt to an AD related environment remains to be shown. On the other hand, murine NSCs preferentially acquired either astrocytic [59,60,62,64] or neuronal fates [51]. While some studies report increased synaptic densities following NSC transplantation with a concomitant reduction in Aß concentration [56,58] others declare positive effects of transplanted NSCs without Aß alterations [51,57,62].

Using a chemical model to mimic AD related neuropathological features by okadaic acid injection into the lateral ventricles [65] only transplantation of rat NSCs (both derived from the SVZ or SGZ

of embryonic rats) overexpressing human nerve growth factor (NGF-NSCs) led to robust survival, migration, and integration while non-overexpressing NSCs did not. Besides, NGF expressing cells also enhanced cognitive performance. Whether this is due to an inhibited differentiation process, as reported for other AD studies, remains to be shown as no detailed assessment of fate choice was performed [65].

### 3.8. Huntington´s Disease

Huntington´s disease (HD) is an inherited neurodegenerative disease caused by the progressive loss of GABAergic medium spiny neurons (MSNs) in the striatum. Injection of 3-nitropropionic acid (3-NP) into the striatum serves as an animal model to mimic HD symptoms and related neurodegenerative aspects. Transplantation of human NSCs (v-myc immortalized and derived from fetal telencephalic tissue) into the injured striatum one week prior to the injury resulted in decreased loss of striatal neurons as well as the appearance of calbindin-expressing (marker for medium spiny striatal projection neurons) donor cell-derived neurons [66]. Upon immunohistochemical analysis of the grafting site, the authors stated that the transplanted NSCs were able to read signals operating in the damaged striata and to appropriately differentiate into GABAergic neurons. However, transplantation of the same NSC pool 12 h after the 3-NP injection did not confer such beneficial effects. As only a single time-point at one week post-3-NP injection was immunohistochemically analyzed, it is therefore possible, that the observation window was too small in order to detect a similar cell replacement role of these NSCs in this second set-up. A further study using YAC128 mice as yet another HD animal model confirmed that transplanted mouse iPSC-derived NSCs are capable of replacing lost neurons in the striatum [67]. YAC128 mice carry a full-length human mutant huntingtin gene (mHTT), which leads to selective, age-dependent progressive impairments in motor- and cognitive functions due to neuronal loss in the striatum [68–70]. Upon transplantation of mouse iPSC-derived NSCs into YAC128 mice, these mice showed better motor function (rotarod test), which was accompanied by neuronal differentiation of transplanted NSCs [67]. These mature NeuN-positive neurons were also positive for DARPP-32 (medium-sized spiny striatal projection neurons)—a feature that was also observed in injury-free striatal transplantation studies [17]. Moreover, it was of interest to see that NSC transplantation into control (wildtype) mice resulted in decreased survival of the engrafted cell population as opposed to the YAC128 striatum indicating a beneficial role of the diseased host environment. This observation finds further support by a transplantation study in which another chemical HD animal model was investigated. Here, injection of quinolinic acid into the striatum leads to regional excitotoxicity and subsequent degeneration of DARPP-32-positive, medium spiny projection neurons [71–73]. Even though the authors referred to the implanted cells as neural precursors, corresponding in vitro analyses revealed that the isolated cells were proliferating and could give rise to both, neurons and astroglia, which suggests that they were still NSCs. Finally, transplantation of human fetal, striatal eminence-derived NSCs six hours after striatal injury induction led to broad migration rostral and caudal to the injection site and to robust tissue integration [73]. Analysis after 12 weeks post-transplantation revealed that the majority of NSCs indeed differentiated into neurons of which some also displayed DARPP-32 expression. Glial descendants were not investigated.

## 4. Heterogeneity among Spinal Cord Injury Models and Donor Cell Origin

Spinal cord injury (SCI) is a devastating neurological condition, which is caused by a traumatic impact to the spinal cord. It results in permanent impairment of motor- and sensory functions due to the interruption of descending and ascending nerve fiber tracts. Local cell loss at sites of injury is followed by glial scar formation and accompanied by inflammation which prevent regeneration of transected axons and exert an additional negative impact on functionality and survival of remote neurons [74,75]. Stem cell transplantation seems to be a beneficial therapeutic approach in order to promote spinal cord regeneration either via secretion of neurotrophic factors or in that engrafted stem cells adopt neuronal and glial identities and functionally integrate into damaged neuronal

circuits [76,77]. However, the question to what extent regeneration can take place and how the lesion environment affects fate and differentiation of transplanted NSCs is still under investigation addressing different spinal cord injury models as well as NSC populations of different origin.

In this regard, we compared data on fate acquisition of transplanted NSCs from two methodologically different SCI models. Compression or contusion used to induce broader and probably medically more relevant spinal cord lesions is compared to more defined injuries resulting from spinal cord hemisection or complete transection. Both models affect spinal cord integrity and lead to motor and sensory dysfunction while differing in terms of lesion volume and extent of secondary damage. Transplantation of various NSCs indeed led to the generation of different cell fates correlating with the type of evoked injury.

Interestingly, rodent and human NSCs transplanted into a compression or a contusion lesion preferentially differentiated into oligodendrocytes [78–91] while cells transplanted into hemisected or transected lesions predominantly differentiated into astrocytes [92–97]. This effect seems to be independent of host species (mouse, rat), donor cell tissue and species (SGZ, SVZ, spinal cord, iPSCs from fibroblasts; mouse, rat, human) and age (adult, embryonic, fetal) of donor animals [81,89,94,98]—a relevant aspect for clinical translation. In the majority of the described cases, where these NSCs have been transplanted into a compression lesion, cells differentiated into oligodendrocytes (41–51%), followed by astrocytes (5–31.2%) and neurons (0–21%) [79,85,87,99]. Engrafted cells, which showed neither neuronal nor glial marker protein expression apparently remained as non-differentiated nestin-positive cells [82]; however, without developing signs of tumorigenesis. In contrast to this pro-oligodendroglial fate acquisition in compression lesions, NSCs transplanted in a hemisected or transected spinal cord model mostly differentiate into astrocytes, a few into oligodendroglial cells (except for up to 44% in one study) [97] and rarely into neurons as most studies state [95,96]. Therefore, the choice of the SCI model apparently exerts a great impact on the fate outcome, which could indeed influence the degree of cellular and functional regeneration. Nevertheless, there are always exceptions to the rule where environmental influences are not dominating [80,82,100,101]. Transplantation of fetal rat spinal cord NSCs into an adult rat SCI compression model resulted in a majority of GFAP-positive astrocytes (32.6%), a few 2′,3′-cyclic nucleotide 3′ phosphodiesterase- (CNP) positive oligodendrocytes (4.4%) and low degree of neuronal cells (5.9%) whereas precursor marker expression (such as neuronal Dcx or oligodendroglial NG2) was not investigated [80].

While most NSC transplantation based SCI studies focused on rodent models, Iwanami and colleagues used a primate contusion model. Upon cervical contusion in marmosets and subsequent transplantation of human NSCs, assessment of fate acquisition of the engrafted NSCs showed the following distribution: 46% GFAP-positive astrocytes, 25% nestin-positive stem cells, 21% β-III-Tub-positive neurons, and 5% Olig2-positive oligodendroglial cells [82]. Since in rodents a contused tissue environment generated overall higher oligodendroglial cell numbers as shown by the expression of myelin basic protein, remyelination events or axonal ensheathment by myelinating descendants of transplanted NSCs [79,84,85,90,102], these observations thus question how well rodent models can recapitulate the spinal cord pathology in higher mammals such as primates and consequently also in human.

Migration of transplanted NSCs towards an injury site, into lesion zones or close to demyelinated axonal fibers is key for a potential beneficial role of transplanted NSCs in terms of local neurotrophic factor secretion or remyelination. Considering the migratory potential of transplanted NSCs, it was found that migration exclusively occurs in spinal cords with a contusion or compression injury [83–87,89,91,99,103]. For hemisected and transected tissues the majority of studies did either not describe or discuss cell distribution or provided no explanations why migration was not observed [93–97,101,104]. Moreover, in most studies cells were grafted to positions rostral and/or caudal from the lesion sites and not directly into lesion zones thereby ensuring maximal survival rates of implanted cells [81,84,105]. Nevertheless, a single study directly assessed different positions of NSC injection and reported that a transplantation rostral and caudal of the lesion site leads to an increase in NSC survival when compared to injection into the lesion epicentre [86,87]. Such observations must

therefore be considered in terms of clinical translation dealing with NSC-based regeneration therapies balancing survival of transplants vs. a limited degree of distribution which owning to the tissue sizes in higher mammals might indeed critically restrict a positive impact on cell replacement and functional restoration.

Despite the well described self-renewing potential of (neural) stem cells in vitro as well as in their discrete niches in vivo, transplanted stem cells showed little or no proliferation activities within host tissues in all SCI studies considered here [86,91,95,98]. Besides the described absence of tumorigenesis by NSCs in different SCI models, studies where NSCs were applied to an injury-free spinal cord also report no tumor formation [106,107]. The observed high differentiation rate and low proliferation potential of NSCs post-transplantation indicates that NSCs most likely sense and react to injury-specific cues which constitutes an important safety aspect. This is of even greater interest as indeed nearly all studies reported a certain degree of motor function recovery upon transplantation of diverse NSCs into the injured spinal cord independent of the chosen SCI model [82,102,103,105]. In this regard one has to take into account that studies with no observed functional benefit might remain unpublished, given that in SCI research recovery of lost functions constitutes a primary endpoint. However, reports on NSC populations failing to support neuroregeneration would still contribute to our still limited understanding of NSC biology under traumatic conditions.

The aforementioned studies revealing differences and similarities in different SCI models all used subacute or acute application conditions, in that NSC transplantation was performed immediately or shortly after injury. However, this does not necessarily reflect a patient's situation who will first receive emergency surgery in order to stabilize general conditions and to avoid secondary damage preceding possible regeneration directed therapies. Moreover, for autologous stem cell transplantation a timely application of appropriate cell numbers is not feasible. Investigating chronic SCI models in which cells are transplanted several weeks after SCI might therefore be more relevant in terms of clinical translation and feasibility. In this regard, not only the type of induced injury (pressure or cut) appears to be important but also the disease process seems to account for the NSC fate. This particular question was addressed by several groups comparing chronic (several weeks after injury) to a subacute (days after injury) transplantation approaches. In two studies, a lower survival rate of NSCs transplanted after four and eight weeks into contusion lesions compared to a subacute transplantation process was observed [85,86]. While Karimi-Abdolrezaee and colleagues could not find any cells after one to two weeks following transplantation even with positive stimulating growth factors infused for seven days after transplantation, subsequent investigations [87,89,97] were able to detect engrafted cells several weeks later independent of the lesion model used (contusion, hemisection). Interestingly, even though NSCs were transplanted into chronic lesion conditions fate assessment analysis revealed that engrafted cells still differentiated mainly into glial cells and that only a few neurons were generated [86,97] bearing a high degree of similarity to the observations made in acute models. Thus, despite a lowered survival rate, the chronic lesion environment appears to still exert a beneficial influence on the differentiation of human NSCs and of iPSC-NPCs as two groups reported a more mature oligodendrocyte phenotype in stem cell derivatives upon transplantation into early chronic- or chronic lesions [98,99].

Of note, given that at least for transplanted OPCs heterogenic responses in gray vs. white matter have been described [108], a systematic investigation to reveal potential heterogeneity between gray/white matter instructed fates of transplanted NSCs has not been conducted yet. To our knowledge, a single study from 2008 reported that transplanted NSCs mainly accumulated in GM at the lesion site and with only a small percentage of cells found within WM regions [105]. As gray or white matter specific cues might indeed be relevant for survival or migration of transplanted NSCs such differences should be addressed more carefully when planning and analysing NSC transplantation studies in both, injury-free spinal cords and SCI models—also in light of the desired cellular outcome (neurons vs. oligodendroglia).

## 5. Conclusions

The high complexity of adult mammalian central nervous systems and the low degree of intrinsic repair capacity results in devastating functional impairments and persisting disabilities in most neuropathologies. The discovery of neural stem cells and their potential to give rise to all cell lineages of the CNS has revived the field in terms of functional cell replacement. Initial concerns related to exogenously applied NSCs and possible adverse effects were refuted as the majority of NSC transplantation studies revealed no tumor formation. Interestingly, studies investigating the outcome of NSC transplantations into healthy CNS tissue revealed dominant region-specific cues instructing the grafts. Despite a great heterogeneity among different CNS lesion models, transplanted NSC migration towards lesion sites and subsequent differentiation into to be replenished cell types are common observations in the majority of neuropathological models. These observations raise hope for the development of NSC-mediated CNS regeneration therapies as most neural stem cell types seem to be sensible to injury environments and their requirements and since even delayed NSC applications can confer functional benefits.

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# Eidesstattliche Erklärung

Ich, Felix Beyer, versichere an Eides Statt, dass die vorliegende Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist. Textstellen, die wörtlich oder abgewandelt aus anderen Arbeiten stammen, habe ich mit einer Quellenangabe versehen. Diese Arbeit wurde weder vollständig noch in Teilen einem anderen Prüfungsamt zur Erlangung eines akademischen Grades vorgelegt.

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Felix Beyer, 05.10.2020