From DEPARTMENT OF CLINICAL NEUROSCIENCE

INFLAMMATORY EVENTS AND THEIR EFFECT ON NEURAL STEM CELL DIFFERENTIATION

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Inflammatory events and their effect on neural stem cell differentiation

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Für meine Mama, die immer, trotz meiner Unwilligkeit zu erzählen, fragt was ich so mache.

ABSTRACT

Inflammation plays a dual role in the central nervous system (CNS), serving as a defence mechanism to protect and restore neural tissue following injuries or infections, but also driving degeneration and aggravating damage. This study examines the intricate relationship between inflammation and neural stem cells (NSCs) within the CNS. NSCs are highly versatile cells capable of self-renewal and differentiation into various brain cell types, such as neurons, oligodendrocytes, and astrocytes, which are crucial for maintaining brain homeostasis. In **Paper I** we investigated the impact of NSC transplantation into an inflammatory environment following a spinal cord injury (SCI). The transplanted NSCs differentiated into oligodendrocytes and modulated the inflammatory environment, resulting in accelerated functional recovery after SCI. In **Paper II** we focused on the effect of irradiation on NSCs in young mice and their subsequent response to brain injuries. Irradiation poses an inflammatory challenge to the irradiated areas initiating for example microglial activation. Irradiated mice demonstrated a reduction in new neuron production post-stroke and a decrease in microglia cell numbers, indicating the influence of radiation on NSC behaviour during inflammation. In **Paper III** we delved into the role of secreted factors during an inflammatory reaction. We created a region-specific model by generating brain-stem specific astrocytes from embryonic stem cells (ESC) which, when exposed to inflammatory cues, exert neurotoxic effects on motor neurons. These findings present possibilities to recapitulate inflammatory scenarios using ESC. Finally, the **Manuscript** examines the impact of hydrogen peroxide (H_2O_2), a free radical released during inflammation, on the proliferation and differentiation of NSCs *in vitro* and *in vivo*. The results show that H_2O_2 increased NSC division and prompted a higher proportion of these cells to differentiate into oligodendrocytes. Moreover, this NSC behaviour was accompanied by transcriptional changes as seen in bulk RNA sequencing.

Collectively, this doctoral thesis provided new cell-molecular insights into NSC biology in disease models of inflammatory responses involved in stroke, spinal cord injury or to inflammatory mediators. This is essential knowledge when developing therapeutic strategies aimed at mitigating harmful outcomes and promoting neurological health. Such insights may pave the way for future advancements in treating neurological disorders and injuries by leveraging the interaction between inflammation and NSC.

POPULAR SCIENTIFIC ABSTRACT

Inflammation, the body's natural response to injuries or infections, can have a profound impact on neural stem cells (NSCs). NSC are cells in our brain that can self-renew and develop into various cells in the central nervous system (CNS) such as neurons - cells that generate electrical signals, oligodendrocytes - cells that support neuron signalling, and astrocytes - cells that regulate brain metabolism and modulate signal transmission. NSCs can also modulate their environment, and thereby influence the inflammatory and other processes in the brain. Inflammatory processes and programmed cell death in the CNS are necessary during development to optimize brain composition, as well as to serve as a complex defence mechanism aimed at protecting and restoring neural tissue in response to injuries, infections, or diseases, ultimately restoring brain homeostasis. However, the effect depends on the context and duration. Uncontrolled or prolonged inflammation can also contribute to neurological disorders and damage.

In this thesis, I examined the influence of inflammation on NSC and *vice versa*. In **Paper I**, we transplanted NSCs into the inflammation centre following of the spinal cord following injury. We observed that transplanted NSC tend to become oligodendrocytes, that the transplantation changed the inflammatory environment in which these cells resided and that treated animals regained hind limb function faster. In **Paper II**, we investigated how radiation affects NSC in young mice. We observed that radiation affected how NSC could respond to brain injuries such as strokes. Irradiated mice produced fewer new neurons after a stroke and had fewer microglia cells. In **Paper III**, we generated brain stem region-specific astrocytes from embryonic stem cells and showed that factors secreted during an inflammatory reaction can cause these astrocytes to become toxic to neurons. Lastly, in my final **Manuscript**, we focused on hydrogen peroxide, a free radical released during inflammation, and its effects on the proliferation and differentiation of NSCs. Here, we found that hydrogen peroxide increased the division of NSC and led to a higher percentage of these cells differentiating into oligodendrocytes.

The balance of the inflammatory response in the CNS is crucial to prevent excessive damage and promote healing and tissue regeneration Understanding the underlying mechanisms of inflammation in the CNS is critical for developing therapeutic strategies to mitigate harmful effects and promote neurological health.

POPULÄRWISSENSCHAFTLICHE ZUSAMMENFASSUNG

Entzündung, die natürliche Reaktion des Körpers auf Verletzungen oder Infektionen, kann einen tiefgreifenden Einfluss auf neuronale Stammzellen (NSZ) haben. Neuronale Stammzellen sind die Zellen in unserem Gehirn die sich selbst erneuern und sich in verschiedene Arten von Gehirnzellen wie Neuronen – Zellen die elektrischen Signale generieren, Oligodendrozyten – Zellen, die die Neuronen bei der Signalübertragung unterstützen und Astrozyten – Zellen die den Gehirnstoffwechsel regulieren und die Signalübertragung modulieren, umwandeln können. NSZ können auch ihre Umgebung modulieren und damit mögliche Entzündungsvorgänge im Gehirn. Entzündungen im zentralen Nervensystem (ZNS) sind während der Entwicklung erforderlich, um die Zusammensetzung des Gehirns zu optimieren, sowie als komplexer Abwehrmechanismus, der darauf abzielt, das neuronale Gewebe als Reaktion auf Verletzungen, Infektionen oder Krankheiten zu schützen und wiederherzustellen und letztendlich eine Homöostase im Gehirn wiederherzustellen. Die Wirkung hängt jedoch vom Kontext und der Dauer ab. Bei unkontrollierter oder langanhaltender Entzündung kann sie auch zu neurologischen Störungen und Schäden beitragen.

In dieser Thesis habe ich den Einfluss von Entzündung auf neuronale Stammzellen und vice versa untersucht. In Studie I haben wir NSZ nach einer Rückenmarksverletzung (SCI) in das Entzündungszentrum transplantiert. Transplantierte NSZ entwickeln sich vermehrt zu Oligodendrozyten, sie veränderten die entzündlichen Umgebung in der sie sich befanden und die transplantation beschleinigte die Rückgewinnung der Funktion in den gelähmten hinteren Extremitäten der Ratten. In Studie II haben wir untersucht, wie Bestrahlung in jungen Mäusen NSZ beeinflusst. Bestrahlung zeigt einen Effekt darauf wie NSZ in erwachsenen Tieren auf Schlaganfälle reagierten. Bestrahlte Mäuse produzieren weniger neue Neuronen nach einem Schlaganfall und haben weniger Mikroglia Zellen. In Studie III betrachten wir, wie Faktoren die während einer Entzündungsreaktion sekretiert werden dazu führen können, dass aus Stammzellen entstandene Astrozyten für Neuronen toxisch werden. Schließlich betrachten wir in meinem letzten Manuskript Wasserstoffperoxid (H_2O_2), ein freies Radikal, das während Entzündungsreaktionen freigesetzt wird, und seine Auswirkungen auf die Proliferation und Differenzierung von NSZ. H_2O_2 führt zu einer erhöhten Teilungsrate sowie zu einem höheren Prozentsatz dieser Zellen, die zu Oligodendrozyten werden. Das Gleichgewicht der entzündlichen Reaktion im ZNS ist entscheidend, um übermäßige Schäden zu verhindern und Heilung und Geweberegeneration zu fördern. Das Verständnis der zugrunde liegenden Mechanismen von Entzündungen im ZNS ist entscheidend für die Entwicklung therapeutischer Strategien zur Minderung der schädlichen Auswirkungen und Förderung der neurologischen Gesundheit.

LIST OF SCIENTIFIC PAPERS AND MANUSCRIPTS INCLUDED IN THE THESIS

Paper I: Sankavaram S R, Hakim R, Covacu R, Frostell A, **Neumann S**, Svensson M, Brundin L, *Adult Neural Progenitor Cells Transplanted into Spinal Cord Injury Differentiate into Oligodendrocytes, Enhance Myelination, and Contribute to Recovery.*
Stem Cell Reports. 12(5):950-966. (2019)

Paper II: Neumann S, Porritt M J, Osman A M, Kuhn H G, *Cranial irradiation at early postnatal age impairs stroke-induced neural stem/progenitor cell response in the adult brain.*
Scientific Reports, 2020 Volume 10:12369

Paper III: Lindblad C, **Neumann S**, Kolbeinsdóttir S, Zachariadis V, Thelin EP, Enge M, Thams S, Brundin L, Svensson M. *Stem cell-derived brainstem mouse astrocytes obtain a neurotoxic phenotype in vitro upon neuroinflammation.*
Journal of Inflammation (2023) 20:22

Manuscript: Neumann S, Ewing E, Fonseca L, Covacu R, Brundin L, *H_2O_2 exposure increases Neural Stem and Progenitor Cell proliferation and Oligodendrocyte lineage expansion.*
Manuscript

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

I Osman A. M., **Neumann S.**, Kuhn H. G., Blomgren K. *Caspase inhibition impaired the neural stem/progenitor cell response after cortical ischemia in mice*

Oncotarget. | 7 (3), 2239-48 (2016)

DOI: 10.18632/oncotarget.6803

II Keiner S., Niv F., **Neumann S.**, Steinbach T., Schmeer C, Hornung K., Schlenker Y., Förster M., Witte O. W. and Redecker C. *Effect of skilled reaching training and enriched environment on generation of oligodendrocytes in the adult sensorimotor cortex and corpus callosum.*

BMC Neuroscience. | 18(1) (2017)

DOI: 10.1186/s12868-017-0347-2

III Granberg T., Moridi T., Brand J. S., **Neumann S.**, Hlavica M., Piehl F., Ineichen, B V. *Enlarged perivascular spaces in multiple sclerosis on magnetic resonance imaging: a systematic review and meta-analysis*

Journal of Neurology | (2020)

DOI:10.1007/s00415-020-09971-5

IV Ineichen BV, Di Palma S, Laczko E, Liddelow SA, **Neumann S**, Schwab ME, Mosberger AC, *Regional Differences in Penetration of the Protein Stabilizer Trimethoprim (TMP) in the Rat Central Nervous System.*

Frontiers in Molecular Neuroscience. | 2020;13(167)

DOI: 10.3389/fnmol.2020.00167

CONTENTS

LIST OF ABBREVATIONS

BBB	Blood-Brain-Barrier
BED	Biological Effective Dose
bFGF	basic Fibroblast Growth Factor
BrdU	Bromodeoxyuridine
CNS	Central Nervous system
CSF	Cerebro-spinal fluid
DNA	Deoxyribonucleic Acid
DT	Diphtheria Toxin
E (11.5)	Embryonic Day (11.5)
ECM	Extra Cellular Matrix
EGF	Epidermal Growth Factor
ER	Endoplasmatic Reticulum
GCS	Glasgow Coma Scale
gd	Gestation Day
GOS	Glasgow Outcome Scale
GSH	Glutathione
Gy	Gray
HIF	Hypoxia Induced Factor
HMG	High Mobility Group
ICC	Immunocytochemistry
IHC	Immunohistochemistry
MS	Multiple Sclerosis
NEC	Neural Epithelia Cells
NSC	Neural Stem Cells
OB	Olfactory Bulb
OPC	Oligodendrocyte Progenitor Cells
OS	Oxidative Stress
PNS	Peripheral Nervous System
PSA-NCAM	Polysialylated Neural Cell Adhesion Molecule
RGC	Radial Glia Cell
RMS	Rostral Migratory Stream

ROS	Reactive Oxygen Species
RT	Room Temperature (22° C)
SC	Spinal Cord
SCI	Spinal Cord Injury
SGZ	Subgranular Zone
SVZ	Subventricular Zone
TBI	Traumatic Brain Injury
TF	Transcription Factor
VZ	Ventricular Zone

1 LITERATURE REVIEW

The central nervous system (CNS) serves as the body's control centre but is susceptible to various insults, making CNS pathologies a significant global health concern [6] and having an impact on society and families. While the causes of CNS disorders are wide-ranging and include e.g. autoimmune reactions, genetic factors, morphological changes, and mechanical injuries [7], the extent of disability and recovery heavily depends on correct functionality of CNS cell types: neurons, astrocytes, and oligodendrocytes. Unlike lower vertebrates which may have remarkable regenerative abilities [8], humans, rodents, and other mammals exhibit limited regeneration, particularly in the mature CNS where plasticity is scarce. Adult neurogenesis was discovered in mammals in 1962 by Josef Altman [9] and gained attention in the early 1980s when observed in various regions of the avian brain [10-13]. Since then, researchers and clinicians have investigated the regenerative potential of Neural Stem Cells (NSCs) due to the pressing need for new therapeutic interventions. However, stem cell therapy remains complex, and approaches such as integrating new-born cells into existing circuits pose challenges. Inflammation, commonly accompanying CNS pathologies and insults, plays a crucial role in removing foreign substances and damaged cells, actively orchestrating repair processes. Such insults also trigger a response in dormant NSCs, leading to their proliferation and migration. For instance, a positive correlation between focal cerebral ischemia extent and stem cell activity has been observed [14]. Inflammatory cues, such as cytokines, chemokines, and free radicals, play versatile roles such as contributing to the response to damage [15], influencing the aging process and cellular senescence [16, 17]. This thesis focuses on studying the interaction between transplanted NSCs and CNS injury (**Paper I**). It also explores the impact of irradiation [18] on the stem cell niche during brain development and its effects on regeneration and potential recovery (**Paper II**). The inflammatory environment's influence on CNS region specific neuronal survival, particularly the ramifications of astrocyte activity, has been examined in **Paper III**. Finally, the effect of inflammatory reactive oxygen species (H_2O_2) on NSC proliferation and differentiation was investigated in **Manuscript**.

1.1 NEUROINFLAMMATION

Injuries to the CNS, such as SCI, stroke/ischemia, and traumatic brain injury (TBI), are among the leading causes of death and disability worldwide [19]. They can create inflammatory conditions similarly to some autoimmune diseases such as Multiple Sclerosis (MS) and overall disturbances in homeostasis (e.g., irradiation). Regeneration of damaged tissue depends on the extent of injury, area of damage and importantly the inflammatory processes during the aftermath of the incident [20]. Inflammatory processes assist in healing and repair, immune-mediated clearance of damage and debris contributing significantly to the resolution of inflammation. Neuroinflammation is also part of, and necessary for, normal neural development [288, 289], as well as driving pathological neurodevelopmental mechanisms [290]. The brain has its own immune cell type called the microglia, but the view of the brain as an immune privileged organ due to the presence of the blood-brain barrier has changed over the years. The CNS is susceptible to peripheral immune factors and cells can infiltrate. The blood-brain barrier is more permeable than initially thought, with inflammation creating additional entrance points in the blood-brain barrier (BBB) [21, 22]. Furthermore, greater BBB permeability in areas adjacent to NSCs provides a platform for communication with the periphery [23, 24]. Acute neuroinflammation is often self-limiting and resolves naturally through debris clearance [25]. This process is aberrant in chronic inflammation, as evident in neurodegenerative diseases, in which the intended cycle of inflammation repair and resolution is broken [26]. Chemokines, cytokines, and the complement cascade play crucial roles in neuroinflammation. Cytokines are small proteins produced by immune cells, but also other cells such as oligodendrocytes and astrocytes, and are released in response to a plethora of stimuli and cell death. They can be either proinflammatory, e.g. TNF, IL-1, IL-6 interleukins, and IFNs, or anti-inflammatory, such as IL-4 and IL-10. They recruit leukocytes and stimulate their adhesion by inducting expression of adhesion molecules in the vascular endothelium [27] and extravasation [28]. Both chemokines and cytokines are upregulated following CNS injury and initiate inflammatory cascades, as well as participate in neural development and neuroprotection [29]. Tumor Necrosis Factor 1α (TNF) and interleukin 1α (IL-1α), discussed in **Paper I** and **Paper III**, belong to this category. TNF induces glutamate release, leading to neurotoxicity [30]. Several studies have shown that inactivating TNF reduces neural death and neurodegeneration in Sandhoff's disease and West Nile virus models [31, 32]. However, TNF receptor-1 deficient mice exhibit a more severe course of disease in a model of

experimental autoimmune neuritis [33]. This bilateral role of TNF can be credited to cell-specific receptor-related signalling pathways [34, 35]. Blockage of TNF has been unsuccessfully tried in MS treatment approaches, most likely failing due to the role of TNF in oligodendrocyte maturation [36] and the impact of TNF as a signal to regulate NSC activity during inflammation [37]. IL-1α, another pro-inflammatory cytokine, is present in all cells and is released upon cell damage or by myeloid cells. It is particularly abundant in cells with a barrier function, such as vascular endothelial cells and brain astrocytes[38]. IL-1α binds to IL-receptor family 1 [39] and activates transcription factors, for example nuclear factor-kB (NF-kB), activator protein-1 (AP-1) and c-Jun N-terminal kinase (JNK) ultimately initiating an immune response through the synthesis of additional cytokines, supporting vasodilation and extravasation and adaptive immunity [40]. The complement cascade, consisting of approximately 30 proteins, facilitates and amplifies immune responses. Under physiological conditions, complement cascade components cannot cross the BBB [41], further highlighting the role of resident cells of the CNS in neuroinflammation. Complement factors, especially C3, within the CNS can be produced by glial cells and neurons in response to inflammation [42, 43]. These factors are implicated in neurodegenerative diseases [44-47]. A CNS resident cell type surveilling the homeostasis are astrocytes. They support neural functions, while also influencing CNS immunity through cytokine receptor expression and recruitment of regulatory T cells (Tregs) via TGF-β and CXCL12 [48]. Oligodendrocytes were traditionally viewed as bystanders in CNS immune reactions and are often the target of the immunoinflammatory response in the CNS. But more recently oligodendrocytes are recognized as actively partaking in CNS immunity. They produce immune-mediators modulating microglia fate, express MHCII in MS [49] and receptors to IL-4, IL-6, IL-10, IL-12 [50]. Chemoattractants CXCL10, CCL2, CXCR2, and CCL3 amplify migration and proliferation of oligodendroglia [51]. Inflammatory processes assist healing and repair as the immune-mediated clearance of damaged tissue and debris significantly contributes to the resolution of inflammation. Neuroinflammation is required for normal neural development [52, 53] and it can drive pathological neurodevelopmental mechanisms [54]. Inflammation also exerts complex effects on NSCs within distinct niches of the adult CNS. Inflammatory factors such as IL-6 and NO disrupt neurogenesis in the subventricular zone (SVZ) and subgranular zone (SGZ), inducing gliogenesis via NRSF/REST upregulation. NSCs possess non-canonical anti-inflammatory roles, releasing trophic factors and modulating immune cells [55]. While the destructive features of inflammation have been studied with great interest, the field of reparative inflammation (Fig. 1) is still in its early stages. Our group studies how inflammation

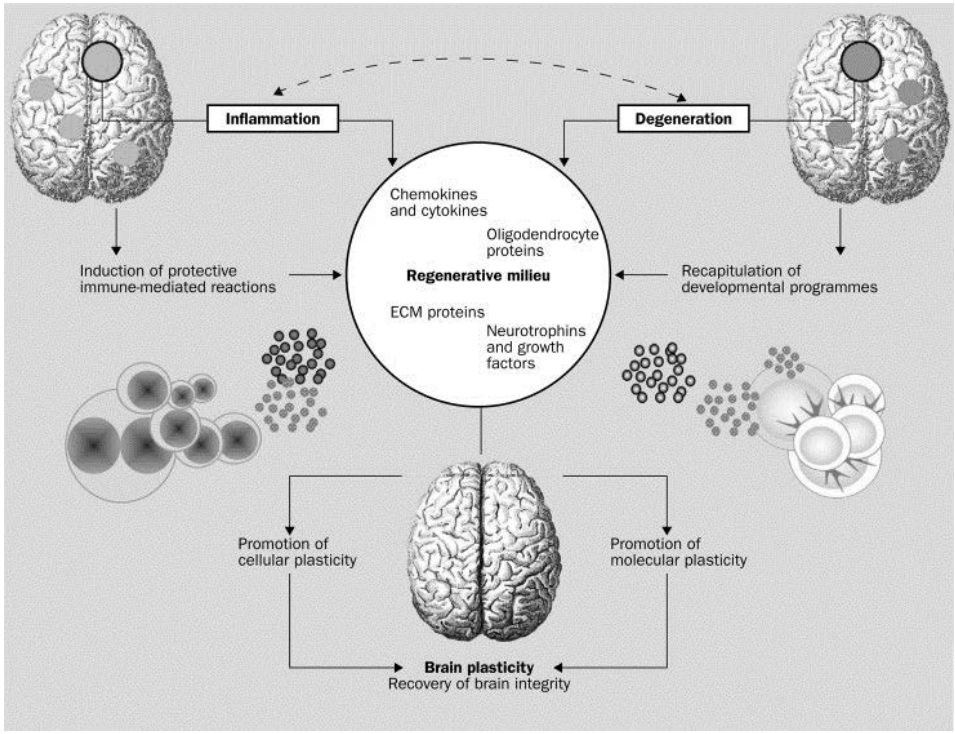


Figure 1 Molecules produced at the site of brain injury and during CNS degeneration have several cellular targets and mode of action. They act in a synergistic or antagonistic way. Inflammation can lead to degeneration and *vice versa*. In reparative inflammatory stages plasticity can be initiated and damaged tissue can recover, this can happen via the recapitulation of developmental programs or due to protective immune-mediate mechanisms. [4] License number: 5581390003574

affects the neural stem cells. We are interested in these processes in the context of CNS injury such as TBI and SCI as well as in autoimmune diseases such as Multiple sclerosis. We have previously assessed how NSC fate is impacted by oxidative stressors. This PhD study aims to contribute to the progression of this understudied field with our research on the impact of H_2O_2 on stem cell proliferation and differentiation in the **Manuscript** [26] as well as the various approaches used to examine different aspects of inflammation in **Paper I-III**. In the following sections I will discuss the approaches we used in this thesis to study the impact of neuroinflammation on NSC.

1.1.1 Microglia

Microglia, the CNS-resident immune cells, maintain CNS homeostasis [56] and play a role in development during which microglia express high levels of complement receptor and participate in inflammation through cytokine production. As such, they tag synapses releasing

complement proteins, by which they contribute to synaptic pruning during neurodevelopment [57]. As previously mentioned, when faced with tissue damage or inflammation, microglia undergo phenotypical and functional changes and express various surface receptors such as pattern recognition receptors (PRRs), toll-like receptors (TLR), phagocytic receptors (CR3 and CR4), and triggering receptor expressed on myeloid cells (TREM) [48]. Microglia can shift between pro-inflammatory (M1) and anti-inflammatory (M2) profiles, aiding in restricting neuroinflammation [58, 59]. Microglia, originally noted by Franz Nissl, were officially identified as a distinct cell type responding to brain injury by Pio Del Rio Hortega in 1919 [60]. They constitute 10-15% of the cells in the brain and spinal cord parenchyma [61]. Derived from hematopoietic mesoderm, microglia migrate towards the CNS during early development, before BBB closure (before embryonic day 8 in mice) [62-65]. They retain self-renewal capacities to replenish the microglia population. However, recent research indicates that peripheral monocytes can also repopulate depleted microglia, adopting microglia-specific DNA methylation signatures and upregulating microglia gene expression [39]. Microglia continuously interact with neurons, astrocytes, and the vascular system, contributing to brain development [66], hippocampal neurogenesis [67] and CNS network establishment [68]. Functioning as CNS macrophages, resting or "ramified" microglia constantly survey the CNS through dynamic cytoplasmic extensions that sense changes in homeostasis such as plaque formation, apoptotic or necrotic cells, and pathogens [69, 70] [71-73]. Special potassium channels in microglia enhance their sensitivity to minute differences in extracellular potassium levels [74]. Upon detecting disturbances in homeostasis, microglia become activated, assume a pro-inflammatory state, adopt an amoeboid morphology, and attempt to restore physiological conditions through phagocytosis [75]. Depending on the context, microglia can also assume anti-inflammatory roles. They are the main producers of extracellular reactive oxygen species (ROS) in the CNS [76] and present an important component of the innate immune response in the CNS. Their activation is a common feature of CNS diseases modulating the immune response by secretion of essential mediators.

1.1.2 Spinal Cord Injury

SCI is globally prevalent in young adults (20-29 years) and leads to severe disabilities [77]. It is defined as an insult to the spinal cord or cauda equina causing permanent changes in body functions below the site of injury. Statistics vary among countries. In Sweden, 19 per 1 million individuals experience SCI annually, with 60% being male and a mean incidence age of 55 years

old. The USA has the highest incidence at 40-50 cases per million individuals [19, 78]. Approximately 80% of SCI patients worldwide are males under the age of 30 [79]. Statistics from developmental countries are lacking. The leading cause of SCI is falling, followed by

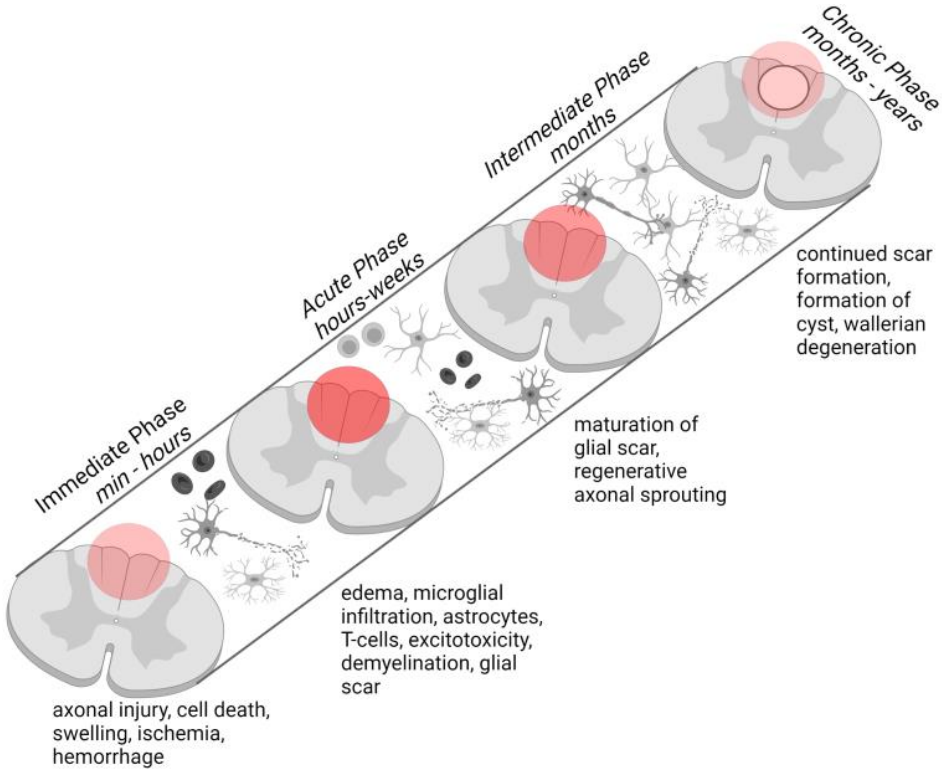


Figure 2 Time-course of a spinal cord injury depicting the different phases and ongoing changes, adapted from [309] License Number 5604180032972

transport related injuries [80].

The disruption/compromise of tissue due to mechanical impact is called the '*primary injury*' and the affected area will usually form the SCI epicentre. This is followed by inflammation, release of reactive species, glial scarring, and cyst formation, collectively termed the `*secondary injury*`. This usually represents a more detrimental period of SCI. The process of SCI can be categorized into four phases: immediate, acute, intermediate, and chronic (Fig. 2). The immediate phase comprises the direct effects of the primary injury. Within minutes after impact, the often macroscopically normal-appearing SC experiences microglia activation, increased pro-inflammatory cytokine levels, cellular disruption, and necrosis, often

accompanied by oedema and haemorrhage leading to spinal shock[1]. The **acute phase** typically lasts between 2h up to 2 weeks after insult, and it involves further immune cell infiltration due to a disrupted blood-spinal cord barrier [82, 83]. Destroyed cell bodies cause Ca^{2+} dysregulation leading to neuronal loss [84, 85]. Moreover, increased ROS levels might potentially cause damage to lipids, DNA and proteins, thus contributing to injury aggravation. ROS levels can be increased for up to two weeks after injury [86-88]. Inflammatory cues initiate astrocyte differentiation and activation from ependymal stem cells, and formation of reactive astrocytes from pericytes located in blood vessels. Morphological alterations indicating reactivity in astrocytes are upregulation of GFAP [395, 396] and other intermediary filament proteins [397], as well as cell soma/process hypertrophy [395-398]. Similarly to astrocytes in the healthy state, reactive astrocytes exhibit heterogeneity based on the severity and location of injury and are influenced by the type of neuroinflammatory stimuli [395, 399-401]. Depending on CNS location, reactive astrocytes may become neuroprotective or neurotoxic [402-404]. The role of reactive astrocytes also changes longitudinally during injury progression. Astrocytes eventually contribute to glial scar formation, separating healthy from injured tissue, which aids ionic homeostasis and angiogenesis, and hence decreases oedema and BBB porosity [89]. Over time this scar becomes compact and impermeable [405], hampering axonal regrowth and thereby preventing recovery [395, 406]. Ablation of astrocytic activity in the injury area negatively impacts recovery, increasing peripheral immune cell infiltration and impairing BBB repair [407] [408]. The **intermediate phase** of SCI encompasses the manifestation and maturation of the glial scar through activity of astrocytes and pericytes and lasts up to 6 months after injury. Restoration processes such as axonal sprouting can be observed from 3 weeks after injury. These are insufficient to regenerate the full functionality of the injured areas. Affected axons degenerate, and the cell death and inflammation due to the secondary injury often leads to the formation of a CSF-filled cyst at the injury site. These processes also persist into the **chronic phase** with receding inflammatory activity. Although there is no cure for SCI yet, some compensating mechanisms have been described. The partial recovery observed in animal and human models is established through compensatory mechanisms and plasticity in the form of reorganization of circuits or by neurogenesis [90]. For example, the formation of corticospinal circuits below the injury site has been demonstrated in humans and monkeys in

[1] Loss of sensation, paralysis with gradual recovery following SCI described in 1750 by Whytt 81. Ditunno, J.F., et al., *Spinal shock revisited: a four-phase model.* Spinal Cord, 2004. **42**(7): p. 383-395.

lateralized SCI, contributing to functional recovery, whereas this concept is less present in rats [91]. In contrast, symmetrical SCI in rats show greater recovery than in humans and primates. This reorganization and recovery are very variable and can be enhanced by physical training [92] and the contribution of transplanted NSCs [93, 94].

1.1.2.1 SCI scoring

Historically a vast array of mammals such as rats, cats, dogs and even monkeys have been used to study SCI. Rats are commonly used for studying SCI due to their similarity to human pathology, exhibiting cyst formation and poor motoric and sensory recovery [89, 92-95] Mechanistically, various methods have been employed to study SCI. Our group used a contusion model [96], with different impactor systems and parameters like weight and drop-height allowing for modulation of injury severity [94]. Other techniques include dislocation, transection, dissection, and chemical-induced SCI, yielding different tissue lesions with varied responses [97]. To assess SCI severity and recovery, we utilized the Basso, Beattie, and Bresnahan-locomotor rating scale (BBB-scale) [98], a well-established tool for hindlimb motility scoring. The BBB-scale is widely used to compare SCI severity across studies, with high reproducibility in mild to moderate SCI [99]. Additionally, we implemented a novel kinematic evaluation approach to assess hindlimb functionality during locomotion. This method is more sensitive in detecting functional changes, and hence allowed us to reduce the number of animals used.

1.1.3 Stroke

Stroke is a leading cause of adult disability and the second leading cause of death globally Stroke patients not only experience high mortality rates but also significant disability, affecting physical and mental health, quality of life and daily activities [100]. Stroke can be categorized into ischemic and haemorrhagic types. Ischemic stroke represents approximately 87% of all stroke cases [101] and occurs when blood vessels supplying oxygen-rich blood to the brain are blocked. Haemorrhagic stroke, on the other hand, results from a ruptured blood vessel in the brain causing bleeding. Both forms lead to insufficient oxygen and glucose supply, disrupting metabolic processes and causing cell death. This cascade of events involves excitotoxicity, ion imbalance, oxidative and nitric stress, and inflammation, exacerbating the injury [102]. Microglial activation, a key component of this nonspecific innate immune response is characterized by the release of reactive oxygen species, cytokines, and proteases, potentially

worsening the damage [103, 104]. The damaged area forms two distinct regions: the ischemic core and the penumbra. The ischemic core has experienced irreparable cellular damage and lacks electrical activity, making it impossible to be repaired through therapeutic interventions. The penumbra is the hypoperfused area surrounding the infarct core, where the tissue is at risk but can be salvaged through therapeutic interventions [105]. After stroke, NSCs located in the SVZ and SGZ exhibit increased proliferation, typically reaching a peak around 1-2 weeks after the initial injury [106, 107, 108] Subsequent NSC migration towards the site of injury, caused by inflammatory chemokines such as chemokine (C-C motif) ligand 2 (CCL2) and stromal cell-derived factor 1 α (SDF-1α) can be observed [109, 110]. Although the migratory response can last up to a year [111], the majority of these migrating progenitors will not functionally integrate into e.g. the striatum, and will subsequently die [106, 107, 112].

1.1.3.1 Models and applicability

Studying stroke in mice helps to understand the underlying mechanisms and to develop potential therapies. Several model systems are utilized in stroke research, each offering unique advantages and disadvantages. Commonly used model systems include transient middle cerebral artery occlusion (tMCAO) and photothrombotic stroke. tMCAO involves temporarily blocking the middle cerebral artery through a surgical procedure, mimicking the reperfusion seen in stroke patients, and allowing investigation of therapeutic interventions during this critical period. However, variability in extent and position of the induced stroke and additional damage and inflammation during reperfusion are limitations to this [113]. In **Paper II**, we

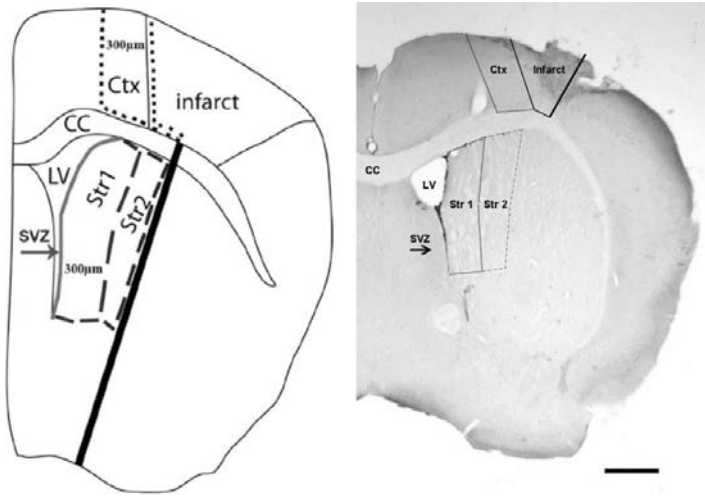


Figure 3 Infarct Area **A** Illustration depicts areas of histological quantification. Ctx: Periinfarct cortex: Str1: Striatum 1 (closest to the SVZ); Str2: Striatum 2 (in 300 µm distance from the SVZ); CC: Corpus callosum; LV: lateral ventricle. B translation to a section of tissue representing the accuracy of infarct size.

applied the photothrombotic stroke model that uses injection of a photosensitive dye systemically and using focal illumination to create blood clots and localized ischemia. This technique enables precise control over the location and size of the induced stroke, allowing for standardized experiments (Fig. 3). Of note, the stroke is limited to the illuminated area, making it less suitable for studying global brain ischemia [114]. In addition, while animal models provide valuable insights into stroke mechanisms, they have limitations in replicating the full complexity of human stroke. Therefore, findings from these models need to be carefully interpreted and validated in clinical studies [115].

1.1.4 Irradiation treatment

Radiotherapy is a primary treatment for tumours in challenging-to-reach areas or specific cancer types[2] [116-118]. The cytotoxic effects of radiation on tumour cells are attributed to double-strand DNA breaks or the formation of free radicals. In proliferating cells, frequent DNA integrity checks, and DNA damage triggers apoptotic pathways if not repaired during replication [119-121]. Photons exciting atoms generate free radicals, leading to the formation of ROS in the presence of water, causing secondary DNA, lipid and protein damage, ER stress which compromise cellular integrity [122, 123], [124, 125]. Importantly, radiation-induced damage affects neurogenic niches due to the presence of mitotically active cells. Microglial activation and autophagic activation accompany tissue repair and inflammation during irradiation [126, 127]. Beside the actual cell loss, stem cell and progenitor cell survival is also affected by irradiation, leading to complications in terms of regeneration [128]. This is often attributed to the neuroinflammatory aspects of irradiation [129]. Brain and spinal cord cancers are the second most common cancer in children and young adolescents (26%), with a rising survival rate due to irradiation treatment [130-133]. However, cognitive deficits and structural changes are observed in irradiated children [134, 135]. Various debilitating effects are already known such as impaired neural progenitor differentiation, cognitive late effects, and social impairments [136, 137]. This is caused by a plethora of irradiation-induced structural changes[3] [138-140], as well as changes in the contribution of new-born neurons during learning and memory formation [141]. Ionizing radiation is measured in grays (Gy), representing the absorbed dose of one joule of energy per kilogram of matter. It has three defined stages of

[2] Considered the appropriate treatment in more than 50% of cases.
[3] Such as abnormalities in vasculature, demyelination, and white matter necrosis.

effects: the *physical phase (I)*, where charged particles interact with tissue; the *chemical phase (II)*, leading to the formation of free radicals from damaged molecules; and the *biological phase*, which involves enzyme reactions, repair processes, cell death, and inflammation, ultimately leading to late radiation effects [107]. Fukuda et al. 2005 reported that a dose of 8 Gy leads to growth retardation within the stem cell niches of the dentate gyrus (DG) and the SVZ [142]. While the DG does not seem to recover with time, the SVZ appears to recover to some extent [143]. Hence, ablation of neurogenesis by irradiation as well as changes in the niche microenvironment due to irradiation-induced neuroinflammation could contribute to the cognitive changes after treatment [144-148]. To evaluate the effect of irradiation on cell survival in living tissue, the biological effective dose (BED) is used. To illustrate BED: a single physical dose of 10 Gy is a biological effective dose (BED) of approximately 47 Gy. To put this in context: a dose of 8 Gy causes growth retardation of DG and SVZ and exacerbates hypoxia-induced injury in mice [143, 149]. Malignant brain tumors are treated with up to 55 Gy, and children with leukemia may receive 18 Gy through whole-brain irradiation [150]. Modern radiation protocols employ multiple smaller doses, significantly reducing neurodegeneration. However, progressive memory and learning deficits may still occur in patients when specific brain regions, such as the temporal lobe and hippocampus, are irradiated [151, 152]. It is not clear in which way the response to CNS insults is affected post-irradiation and which cell types are afflicted. In this thesis we tried to further characterize the impact of irradiation in the developing brain on potential adult regeneration mechanisms and could show that formation of DCX^+ neural precursor cells are decreased in irradiated animals [153]. The same is true for microglia cells.

1.1.5 Traumatic brain injury (TBI) and traumatic axonal injury (TAI)

TBI is characterized as an alteration in brain function caused by an external force. TBI encompasses a heterogeneous group of injuries with varying severity and outcomes depending on the societal possibilities to manage and treat the condition [154]. The initial impact on the brain results from the primary injury, followed by secondary pathological processes, often leading to secondary injury [155-157]. These processes induce oedema, vascular injury, mitochondrial dysfunction, excitotoxicity, and the formation of free radical species. The involvement of TAI lesions is a particularly negative prognostic marker, especially in brainstem areas [158, 159]. The Glasgow Coma Scale (GCS) is the gold standard for scoring TBI in clinical practice, assessing the consciousness level as well as eye, motor and verbal responses on a

scale from 3 (worst) to 15 (best). A GCS score ≤ 8 is classified as severe TBI [160]. Further evaluation of the injury during the acute phase is also facilitated by neuroradiology with computerized tomography being the gold standard [161].

1.1.5.1 Neuroinflammation in TBI

Inflammation accompanies TBI, with both detrimental and beneficial effects on recovery [162]. Neuroinflammation following TBI is elicited by the damaged tissue [162] and BBB disruption after trauma [157], allowing molecules of the periphery, such as albumin, fibrinogen [163] and complement factors [164] from the periphery to enter the CNS. Conversely, factors released from the damaged tissue, commonly known as Damage associated molecular patterns (DAMPs), enter the bloodstream [162, 165]. This initiates a cascade of early immune system responses. DAMPs additionally activate microglia and astrocytes [162]. Astrocytes can be further activated by microglia. Astrocytes release IL-6 and matrix metalloproteinase (MMP-) 9 which increases BBB permeability [166, 167]. Furthermore, they produce additional cytokines and chemokines, which attract peripheral immune cells, with neutrophils arriving first followed by monocytes [168, 169]. The released immune mediators can have neurotoxic effects, stimulate ROS production, and support microglial-mediated MMP production [170-173]. At the same time, infiltrating macrophages are reported to be neuroprotective [162].

Astrocytes possess great heterogeneity. More than 10 subtypes have been described in the healthy CNS, based on their function, expression profile, location, and morphology. They are involved in synapse formation and maintenance, neurotransmitter homeostasis, and contribute to BBB functionality. Astrocytes can also contribute to BBB disruption [174] and their activation is clear in e.g. TAI [175] even without the presence of peripheral immune cells. After TBI or other CNS insults astrocytes change function as described previously in the section on SCI. The brain stem is a region especially vulnerable to TBI/TAI, nevertheless the effect of region-specific astrocyte reactivity on neural survival has not been assessed. Moreover, most of the secondary pathological processes can be targets for prognosis, treatment, and injury management. Having access to a suitable model system is important to improve these approaches. In **Paper III** we assess the response of brainstem and spinal cord motor neurons to astrocytes activated by inflammatory stimuli trying to discern a potential secondary mechanism aggravating secondary axotomy in TAI. There we aimed to model region-specific effects of TBI-associated neuroinflammation astrocyte reactivity and neuronal survival.

1.1.6 Oxidative Stress

Oxidative stress (OS) is a term to describe a flawed mechanistic interplay of pro- and antioxidants and their signalling roles in biological systems. OS is the result of excessive ROS and/or a failure in ROS scavenging systems. ROS scavenging is tightly controlled and mostly mediated by various combinations of reduction and oxidation reactions, so-called "redox" reactions, creating a "intracellular redox equilibrium". Imbalances in this tightly regulated process result in disturbed cellular homeostasis and damage or structural changes of proteins, nucleic acids, and lipids in a biological system, leading to fragmentation of these macromolecules [176]. OS is traditionally used to describe detrimental effects in a system, such as DNA damage and lipid oxidisation. This has also led to a negative view of chemical compounds mediating OS: reactive oxygen species. This view of ROS has changed in recent years, with studies reporting that a redox imbalance in favour of oxidants can have positive effects on biological systems. For example, in *Caenorhabditis elegans*, where the deletion of mitochondrial superoxide dismutase (sod-2, antioxidant) leads to an increased lifespan [177].

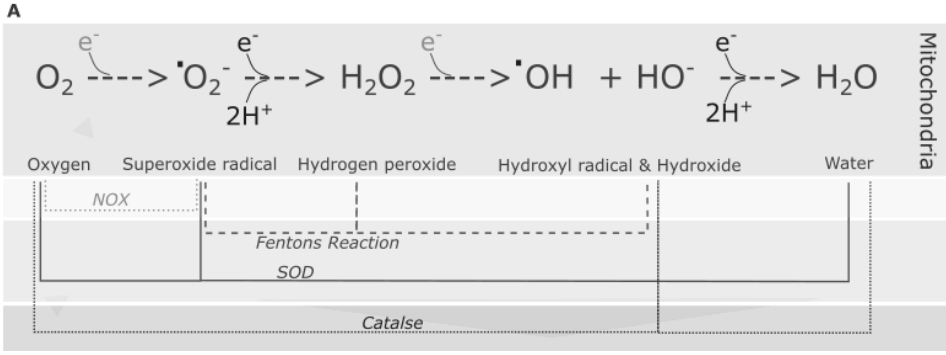


Figure 4 depiction of ROS formation from oxygen. Horizontal layers indicate sub location of process: top layer: mitochondria, second layer: cell membranes, third layer: present in ubiquitous fashion, fourth layer: peroxisome.

1.1.6.1 Reactive Oxygen Species

Reactive Oxygen Species (ROS) are highly reactive molecules derived from molecular oxygen (O_2) reduction/partial reduction, and they are central to oxidative stress. While oxygen is vital for aerobic life, its derivatives, including unstable free radicals with unpaired electrons in their outer molecular orbitals and potent oxidizing agents like hydrogen peroxide (H_2O_2) (Table 2), have a destructive potential. Initially, ROS were believed to only originate from the mitochondrial electron transport chain, releasing superoxide anions (O^{-2}) as an unwanted

byproduct [178]. Superoxide dismutase subsequently converts major parts of O_2 to H_2O_2 that will be further processed to other ROS [179], for example superoxide anions, hydrogen peroxide and hydroxyl (OH^-) (Fig. 4, Table 1). ROS readily engage in reversible or irreversible redox modifications when interacting with other molecules [180] in the form of reversible or irreversible redox modifications. The oxidant is thus reduced, and the reductant is oxidized, thereby compromising its identity. The redox reaction is not just the chemical activity of ROS that determines their impact; their biological response also plays a critical role. For instance, cell-cycle exit and entry into G0 or cell-cycle arrest is a typical response to ROS exposure. While high ROS levels can induce apoptosis, phagocytic cells (e.g. macrophages and microglia) produce ROS as part of the "oxidative burst" to eliminate invading microorganisms. Oxidoreductase enzymes, metal-catalysed oxidation, Fenton Reaction [181] and several other processes also lead to ROS production in a biological system (Fig 4). ROS play a role as intra- and intercellular signalling molecules and regulate the activity of transcription factors like p53, AP1, Nrf2, and NF-κB [182, 183].

Table 1 Reactive species and their degradation products.

REACTIVE SPECIES		ACTIVITY
Superoxide anion	$\cdot O_2^-$	*free radical, $t_{1/2}$= 1 x 10^-6 sec, generates H_2O_2*
Hydrogen Peroxide	H_2O_2	*oxidative agent, generates HO·, $t_{1/2}$= h, depending on environment [109]*
Hydroxyl Radical	HO·	*high reactivity, $t_{1/2}$= 1 x 10^-9 sec*
Peroxynitrite	$ONOO^-$	*can generate HO·*
Ferrous iron	Fe^{2+}	*Reacts with H_2O_2, generates $\cdot O_2^-$*

1.1.7 Hydrogen peroxide

H_2O_2 is a chemical compound consisting of two hydrogen and two oxygen molecules that is weakly acidic (pH 4.2-5.1) and unstable, as it readily decomposes to oxygen and water under the liberation of heat. It can be stabilized by addition of acetanilide or similar organic materials [184]. H_2O_2 is used to clean wounds and as bleaching agent. Several reports indicate its protective and signalling functions within the cell – providing for example axon pathfinding cues [185-189]. Hydrogen Peroxide is a so-called 'unfree radical' and in chemical terms is poorly reactive due the lack of unpaired electrons. However, it is the main source of hydroxyl radicals when it decomposes in the presence of transition metal ions that are available in

biological systems (in the human body mostly iron in the Fenton Reaction). The hydroxyl radicals are in turn one of the most reactive chemical species known [181] and have the potential to compromise the integrity of other molecules (Table 2). Their half-life is dependent on several factors such as temperature and composition of the solution [190]. Transmission distances of 1μm-10μm of signals via H_2O_2 in cells have been reported [191]. H_2O_2can diffuse up to 1.5 mm within tissue since it crosses cell membranes [192]. It is produced in every cell as a waste product during mitochondrial respiration, by the Nox1/2 complex, mediating redox reactions in the endothelium [193] or in phagocytes as a host defence mechanism [194].

Table 2 Reactive species and their relative oxidation number, indicating the tendency to gain electrons from another source [195].

REACTIVE SPECIES	RELATIVE OXIDATION NUMBER
Fluorine	*2.23*
Hydroxyl radical	*2.06*
Atomic oxygen (singlet)	*1.78*
Hydrogen peroxide	*1.31*
Perhydroxyl radical	*1.25*
Permanganate	*1.24*
Hypobromous acid	*1.17*
Chlorine dioxide	*1.15*

1.1.7.1.1 ROS in the central nervous system

The brain, despite being a small fraction of the body's weight, consumes a significant amount of oxygen in order to generate ATP (20% total body O_2 consumption on 2% of the total body's weight) [196], leading to increased production of free radicals [197]. Importantly, the brain is sensitive to oxidative stress due to low catalase levels, high iron content, and limited antioxidants, of which some being unable to cross the BBB [198, 199], among other factors [200-202]. High glutamate levels in the CNS can affect the glutamate/cysteine antiporter Xc- transporter, interrupting cysteine transport into the cell and as such the *de novo* synthesis of glutathione (GSH), leading to increased oxidative stress [201, 202]. Another source of ROS is the NADPH (nicotinamide adenine dinucleotide phosphate) oxidase 2 (NOX2) with various isoforms (e.g. NOX4 in the Endoplasmic Reticulum (ER)), which generates H_2O_2 during protein folding [203]. While those ROS sources are common to multiple cells/organs, the CNS also possesses some additional and unique ROS derived from excitatory amino acids and

neurotransmitters. For instance, ROS are formed following the auto-oxidation of e.g., norepinephrine and dopamine [204]. Calcium is needed for signal transduction but can also activate neuronal nitric oxide synthase (nNOS) and increase nitric oxide (NO) levels. The latter can form peroxynitrite ($ONOO^-$; Table 2). It is well known that especially the developing brain, which is rich in unsaturated fatty acids and redox-active iron and poor in antioxidants, is in great need of oxygen and thus vulnerable for OS [205]. Physiological levels of ROS affect the maintenance and viability of cells. Nrf2-Keap1 pathway plays a fundamental role in these processes by promoting an antioxidant transcription program [206]. Damage in microglia leads to elevated ROS production, which has been proposed as a driver of multiple sclerosis pathology, exerting OS and injury to tissue surrounding the MS lesions [207, 208]. OS can furthermore affect the endoplasmatic reticulum (ER) and increase cellular stress levels [209, 210]. ROS-induced functional and mitochondrial loss as well as apoptosis contribute to ageing and neurodegenerative pathologies including MS, Parkinson's disease, Alzheimer's disease [211] and amyotrophic lateral sclerosis. The aged brain especially accumulates redox metals, and an abnormal metal metabolism accounts for a large portion of the generated ROS accredited to these pathologies [212]. Therefore, ROS research used to primarily focus on ROS in ageing and disease [17, 213-215], however, ROS levels can change in a biological context without causing oxidative damage per se. Chang et al. reported that PI3K/Akt signalling involved in hippocampal progenitor cell growth is governed by NOX2-derived H_2O_2 and O_2 [216]. The PI3K/Akt pathway is generally involved selecting growth and proliferation over differentiation in adult neural stem cells [217] this has shown to be affected by endogenous ROS levels [554]. Indeed, hippocampal long-term potentiation (LTP) and axonal outgrowth and regeneration is regulated by NOX2, while H_2O_2 seems to act as an endogenous chemoattractant (e.g., for microglia) [185, 188]. H_2O_2 in specific has been shown to affect neural differentiation in an embryonic stem cell line through AKT and p38 pathways [218]. Cognitive impairments have been reported in mice lacking H_2O_2, as H_2O_2 from the electron transport chain (ETC) is involved in the regulation of dopamine release [219]. H_2O_2 also affects cell migration by modulating actin and cytoskeleton organization through cofilin [220]. Additionally, when transplanted, H_2O_2-treated mesenchymal stem cells increase BDNF and enhance therapeutic efficacy in SCI [186]. Furthermore, exposure to specific ROS types can lead to increased proliferation of NSCs [221]. One can conclude that ROS in the CNS can have contradictory effects. It is highly important to consider context, duration, and concentration,

but also the state (developmental, healthy, inflammatory and more) of the tissue and cells in contact with ROS.

1.1.7.2 Defence line of antioxidants – Redox regulation

With the appearance of aerobic metabolism, the need for antioxidants regulating oxidative radicals emerged (Table 3) [180]. The body produces and takes up antioxidants, which can be enzymatic or non-enzymatic. Non-enzymatic antioxidants include direct-acting molecules such as carotenoids, lipoic acid, and ascorbic acid, as well as indirect-acting molecules that bind

Table 3 Antioxidants/oxidant scavengers and their mechanisms of action. Taken from [222] under the Creative Commons Attribution-Non-commercial-No Derivative Works 3.0 Unported License

ENZYMATIC ANTIOXIDANT	CELLULAR LOCATION	SUBSTRATE	REACTION
Superoxide dismutase (Mn/Cu/ZnSOD)	Mitochondrial matrix (MnSOD) Cytosol (Cu/ZnSOD)	Superoxide ($O_2 \cdot^-$)	$O_2 \cdot^- \rightarrow H_2O_2$
Catalase	Peroxisomes Cytosol	Hydrogen peroxide (H_2O_2)	$2H_2O_2 \rightarrow O_2 + H_2O$
Glutathione peroxidase (GPX)	Cytosol	Hydrogen peroxide (H_2O_2)	$H_2O_2 + GSH \rightarrow GSSG + H_2O$
Peroxiredoxin I → VI (Prx)	Cytosol	Hydrogen peroxide (H_2O_2)	$H_2O_2 + TrxS_2 \rightarrow Trx(SH)_2 + H_2O$

metals to inhibit ROS generation. Transcription factors such as Nrf2 are also involved in OS defence. Nrf2 physiologically resides in the cytoplasm and is bound to the Kelch-like ECH-associated protein 1 (Keap1)-Cul3 E3 ligase complex. In the absence of OS, Nrf2 is continuously recycled by the proteasome. Keap1 contains thiol (-SH) which makes it prone to oxidization. Once Keap1 is oxidized, Nrf2 is liberated and can translocate into the nucleus where it dimerizes with proteins from the MAF family [223, 224]. The Nrf2-MAF heterodimer regulates the expression of approximately 200 different transcripts of antioxidant response elements (ARE). The Nrf2-MAP complex plays an important role in sensing the redox balance in concert with thioredoxin (thioredoxin and thioredoxin reductase) and the glutathione system (thiol glutathione (GSH)) to contribute to the restoration of the redox homeostasis [225, 226]. Both pathways overlap and compensate for each other during OS defence. The key molecule in many redox reactions is cysteine, specifically its sulfuric atom which converts

peroxides and free radicals into less destructive compounds such as water. There are several cells within the CNS that facilitate the OS defence: glial cells (especially astrocytes) provide a source of GSH, by releasing it into the extracellular matrix, and subsequently supporting neurons that have a high metabolic activity but a low antioxidant defence system [227, 228]. The cell type with the highest GSH content in the brain is microglia, which are the major source of ROS [229, 230].

Our group has reported that H_2O_2 exposure can led to downregulation of Gpx2,Gpx4, Sod1 and peroxiredoxin 1,2,5 expression [221]. Furthermore, OS-mediated modulation of occludin affects the function of tight junctions in the BBB [231]. ROS can thus also compromise the integrity of the BBB, making it more permeable for inflammatory components but also for antioxidants.

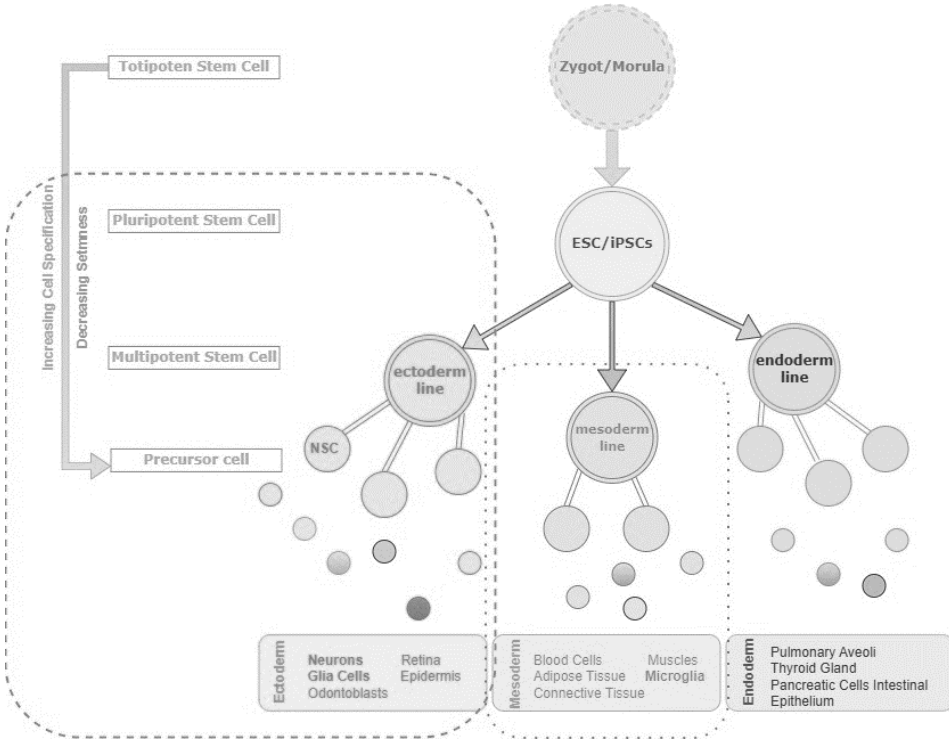


Figure 5 Stem Cell Differentiation - hierarchical presentation of stem cells in respect to their differentiation potential. Ectodermal tissue gives rise to multipotent NSCs which generate cells from the neural lineage, such as neurons, astrocytes, and oligodendrocytes. The mesodermal lineage gives rise to e.g., microglia. All these cells contribute to proper CNS function. With increased cellular specification, the potential stemness decreases. ESC = embryonic stem cells, IPSC = inducible pluripotent stem cells.

1.2 STEM CELLS

tem cells play a vital role in development, cellular differentiation, and tissue repair within an organism. The term 'stem cell' encompasses a diverse group of cells capable of self-renewal and differentiation [232, 233]. These contribute to development, cellular differentiation and repair processes within an organism. The archetypical stem cell, known as totipotent stem cell, originates from the fusion of a spermatocyte and an oocyte during early embryonic divisions (morula) [234], while all derived stem cells possess a restricted lineage (Fig. 5). NSCs arise from the ectoderm and are located along the ventricular neuroaxis, which serves as the starting point for brain development during embryogenesis [235]. NSCs demonstrate stem cell-like properties, including self-renewal and differentiation into specialized cell types such as neurons, oligodendrocytes, and astrocytes. While NSCs have been considered mostly dormant after the age of 25 [236], adult neurogenesis is nowadays well-established in mice, where the addition of e.g. newborn cells in the olfactory bulb through the rostral migratory stream (RMS) is most prominent [237]. In humans, adult neurogenesis is mostly associated with learning and memory, and occurs in the subgranular zone (SGZ) of the hippocampus. A few studies documented that these newborn neurons survive, integrate, and function in SGZ. However, whether and how these processes take place in other regions of the human CNS, for example the subventricular zone, remains to be proven [238, 239]. Reports on physiological adult neurogenesis in the human SVZ are limited, but NSCs from the SVZ can be reactivated in response to CNS injury, potentially replenishing lost cells [240]. However, the overall capacity

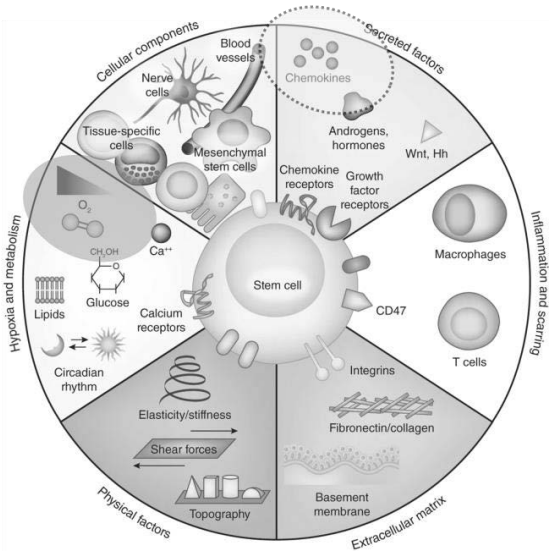


Figure 6 *Overview of intrinsic and extrinsic factors within the stem cell niche.* We are interested in the interplay of inflammation (ellipse dotted line) and redox balance (grey ellipse) with neural stem cells. [Adapted from Y. Reinwald, J. Bratt and A. El Haj [1] under the creative commons license attribution 3.0, we rendered the image black and white and added the ellipses]

of NSCs to produce new cells declines with age [241, 242]. This has been proposed to be due to factors such as impaired lysozyme function leading to protein accumulation [243, 244], which can also be compromised by reactive oxygen species (ROS) [245].

1.2.1 Stem Cell Niche

The mammalian brain arises from several stem cell niches[4]: In this thesis, the main focus is on the SVZ, a cell layer adjacent to the lateral ventricles, known as the largest stem cell niche in the rodent brain [246, 247]. The stem cell niche refers to a specialized microenvironment surrounding stem cells, consisting of the stem cells themselves, the extracellular matrix (ECM), the vascular system, and supportive cells [248, 249]. Within the niche, various cell types mutually influence one another to regulate self-renewal and differentiation. Intrinsic processes, mechanical cues, and paracrine/autocrine signals (e.g., ROS, ions, Notch signalling) in combination with signal gradients play critical roles in keeping stem cells quiescent or proliferating, inducing migration, and ultimately govern differentiation or self-renewal (stemness) [250-253] (Fig. 6). High levels of canonical developmental signalling (Wnt, Notch,

Table 4 Pathways/Factors and their effects on Stem Cells vary depending on the niche/tissue they are active in. Wnt/β-catenin can lead to self-renewal in certain cells while it promotes differentiation in others.

CANONICAL SIGNALING PATHWAYS IN ADULT STEM CELLS AND THEIR NICHES				
Factor	**Tissue**	**Stem cell**	**Effect**	**Reference**
Wnt/β-catenin	Hematopoietic System		Self-renewal/ proliferation	[255]
JAK-STAT &TGF-β	Drosophila, support cells	Cells surrounding support cells	Self-renewal/ proliferation	[256]
Wnt/β-catenin	Skin	Hair follicle precursor	Differentiation	[257]
Wnt/β-catenin	Mammalian Brain	Neural Stem Cell	Expansion of population	[258]
BMPRIA signalling	Haematopoietic System	Haematopoietic stem cell	Regulation of niche size	[259]

[4] subgranular zone, the central canal in the spinal cord and the filum terminale

BMP) are commonly found in stem cell niches, underscoring their significance in maintaining the niche homeostasis [254]. Proper niche function is also essential for balancing proliferation and facilitating tissue regeneration. The importance of surrounding cells and released factors [260] was demonstrated in hematopoietic stem cells by e.g. Zhang et al., who identified spindle-shaped N-cadherin^{+}CD45^{-} osteoblastic cells and BMPR1A (Bone Morphogenetic Protein Receptor Type 1A) signalling as crucial in maintaining niche size and promoting stem cell features [259]. Although all stem cell niches share fundamental characteristics, the signalling pathways and factors that establish the niche environment vary between different niches (Table 4)[261]. In the CNS the SVZ is one of the main sites containing NSCs and lines the lateral ventricle wall. Additionally, reports indicate the existence of tissue-resident cells unrelated to any specific niche that exhibit stem cell-like behaviour. For instance, specialized tissue-resident astrocytes have been described as acting like NSCs, being capable of generating neurons under pathological conditions [3, 262].

1.2.1.1 Neural stem cell niches: The subventricular zone

The SVZ comprises four cell types: ependymal cells (E-type), stem cells (B1 type), transient amplifying intermediate progenitor cells (C type), and neuroblasts (A type cells) (Fig. 7). Ependymal cells exist in two morphologically distinct forms: Type-E1 cells are multiciliated,

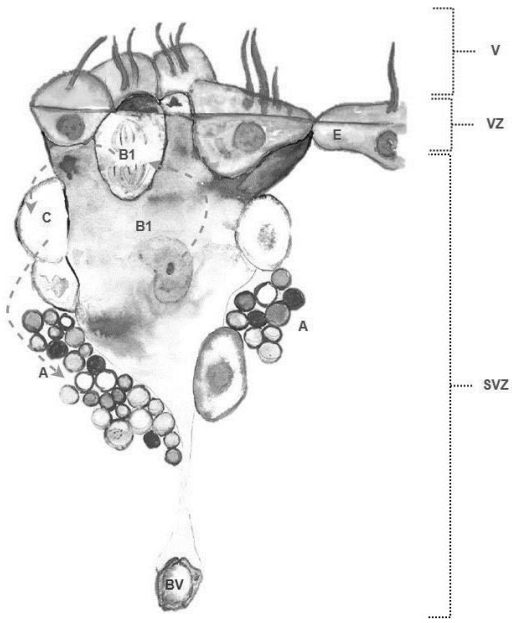


Figure 7 NSC niche composition in the SVZ. Schematic overview on the SVZ in the murine brain. E = ependymal cells in direct contact with the CSF; Type-B cells in the subventricular zone (SVZ) are recognized as NSCs, and they give rise to intermediate transit-amplifying progenitors known as Type-C cells. Subsequently, Type-C cells generate migrating neuroblasts referred to as Type-A cells, which ultimately differentiate into mature interneurons within the olfactory bulb. Dashed arrows indicate the differentiation steps stem cells undergo starting from B Type Cells; V = Ventricle filled with cerebrospinal fluid (CSF), VZ = Ventricular zone, SVZ = Subventricular zone. [adapted after Bernstock [2] under the Creative Commons Attribution License (CC BY)]

while Type-E2 cells are bi-ciliated. Both express S100B and CD24c and are post-mitotic. They are in direct contact with cerebrospinal

fluid (CSF) [263], a crucial factor preserving stemness [264], and are connected to B-type cells through gap junctions [265], allowing them to modulate B-type cell proliferation. B-type cells give rise to B1-type cells which are also in contact with the CSF and can directly generate oligodendrocytes to migrate towards the corpus callosum, contributing to myelination [266-268]. Alternatively, they form B2-type cells which give also rise to oligodendrocytes and C-type cells through symmetrical division [269, 270]. Type-B2 cells are located in the brain parenchyma close to Type-A cells [271], and have contact with blood vessels but not the ventricle. These cells proliferate but their function is unclear [272, 273]. Type-C cells divide rapidly to generate A-Type cells when needed [274, 275]. In *in vitro* cultures, the average duration of a single cell cycle is 8h but it can vary [276, 277]. A-type cells, also regarded as neuroblasts, express doublecortin (DCX) and polysialylated neural cell adhesion molecule (PSA-NCAM). In mice, A-type cells migrate through the rostral migratory stream (RMS) to the olfactory bulb, where they integrate into neural circuits, contributing to odour perception [277] [278, 279]. Their journey takes approximately two weeks during which they undergo one to two cell divisions [277]. A-type cells have the potential to give rise to neurons, oligodendrocytes, and astrocytes in the CNS. Differences in the expression and lineage of NSCs have been observed between *in vitro* and *in vivo* environments [3, 280-284]. Liu et al. reported that following stroke, only a fraction of upregulated genes overlap *in vitro* with *in vivo* samples [280]. Other reports indicate that there is a difference between the potential and the actual fate of NSCs *in vivo* vs *in vitro* [285], claiming that NSC *in vivo* can only differentiate into neurons [286] while *in vitro* neurons and oligodendrocytes could be observed [287] (Fig. 9). The specific cytoarchitecture of the niche plays a significant role in niche functions. During embryonic development, radial glia provide the scaffold for CNS formation. They will become a VCAM-expressing subset maintaining stemness [288, 289], and a GFAP expressing pre-B1-type subset [274, 275]. Architectural changes in the niche have been associated with abnormal cell ratios [290, 291] and neurological conditions such as autism [292]. NSCs within the niche are regulated by extrinsic and intrinsic factors, including CSF and

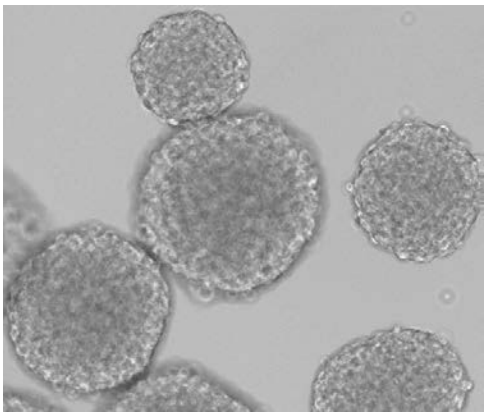


Figure 8 Neurospheres derived from mice SVZ NSC on day 7 after plating.

epithelial cells [293, 294]. Some factors, such as epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and Sex Determining Region Y Box transcription factor (SOX1, 2, 3), maintain stemness, while others guide differentiation and fate decisions [295]. Differences in differentiation potential of NSCs have also been reported between various niches in the CNS. For instance, NSCs derived from the spinal cord exhibit more heterogeneity in function and regulation compared to those from the SVZ [296, 297]. The neurosphere assay, a commonly used method to identify NSCs in vitro, involves the generation of sphere-like structures from activated NSCs using EGF and bFGF (Fig. 8) [298]. These neurospheres can differentiate into neurons and glial cells when transferred to adherent plates without mitogens [13, 299, 300]. Additionally, the presence of NSC markers such as Sox2 can specify NSC populations within the CNS. Accordingly, we used this approach in **Papers I, III** and the **Manuscript**.

1.2.1.1.1 Factors maintaining "Stemness"

EGF is produced by neurons and glial cells [301], bFGF by glial cells [302]. These mitogens are used to keep NSCs in the culture in an undifferentiated, proliferating stem cell-like state [235, 300]. bFGF is involved in early neural development, especially in neural plate formation and patterning [303]. During initiation of astrogliogenesis, a fraction of cells appears to respond to EGF [12, 13], which is a gliogenic transcription factor. These two factors were used in **Papers I, III** and **IV** to maintain the neurosphere cultures.

1.2.1.1.2 Factors inducing cell cycle exit and differentiation

NOTCH is a single-pass transmembrane protein involved in the transition from stem cell maintenance and expansion to differentiation [304, 305]. NOTCH signalling keeps stem cells in the undifferentiated state by repressing neurogenesis via controlling the activity of transcriptional regulators and bHLH family members Hes1 and Hes5 [306, 307]. It has been shown that the C-promoter binding factor 1 (CBF1/RBP-J), another NOTCH effector, is also required to keep NSCs from progressing to neural progenitor states [307]. NOTCH signalling itself is mediated by four different NOTCH receptors which can bind to five different ligands. Of these, only NOTCH-1 and Jagged-1 are expressed in the SVZ [308]. With the initiation of neurogenesis stem cells begin to express higher levels of another NOTCH ligand: Delta-like-1. Adjacent stem cells in the niche sense these signals too and remain undifferentiated or differentiate into astrocytes [309].

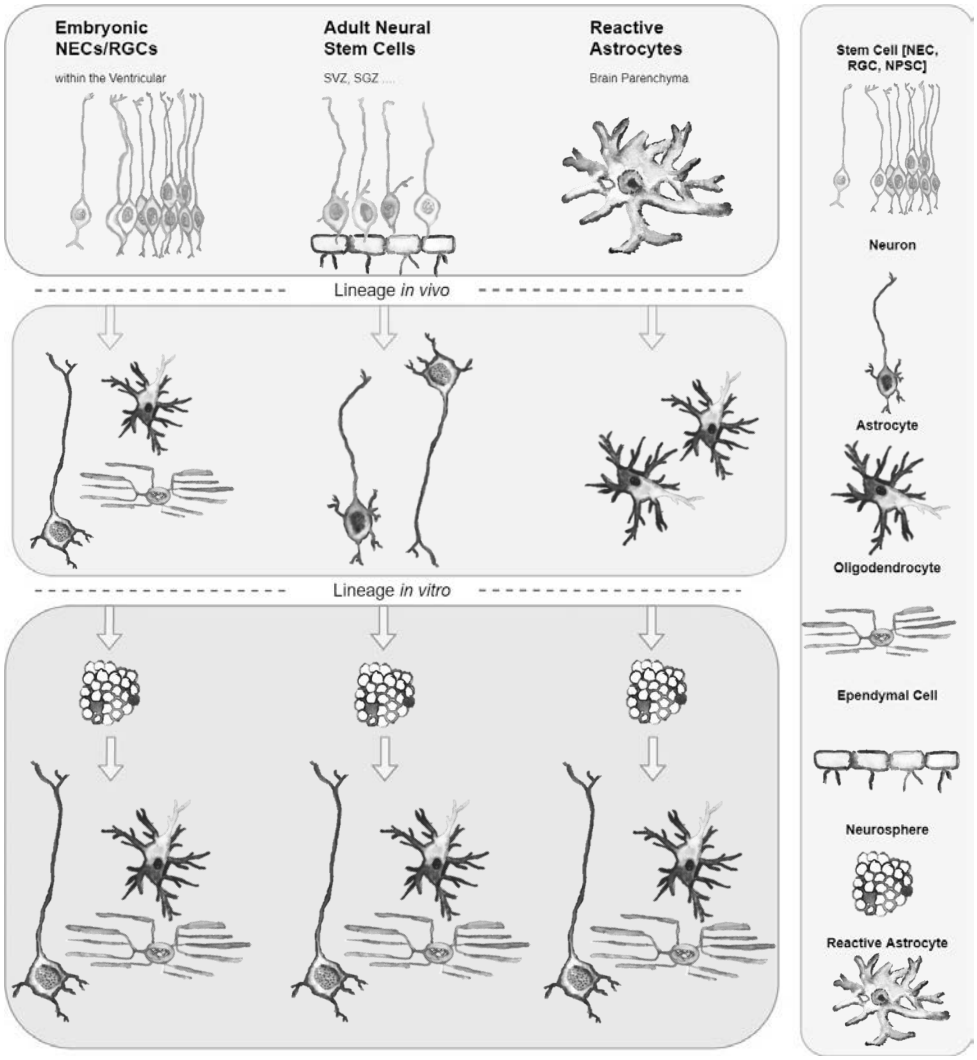


Figure 9 Stem cell lineage - The potential of stem cells may vary from *in vivo* to *in vitro* situations. Stem cells (upper panel) tend to give rise to one specific cell type *in vivo* (middle panel) while they can become different cell types *in vitro* (bottom panel). The first row presents different types of stem cells sources: Embryonic neuroepithelia cells (NEC), Radial glia cells (RGC), adult NSCs from the subventricular zone (SVZ) or subgranular zone (SGZ) as well as reactive astrocytes in the brain parenchyma. The second row represents what these cells predominantly differentiate into *in vivo*, and the third row shows which differentiation pattern is commonly observed *in vitro*. [Adapted from Götz et. al 2015 under the Attribution Creative Commons 4.0 International (CC BY 4.0) [3]]

1.2.1.2 Neural stem cell niches: Spinal Cord

The spinal cord (SC) exhibits considerable activity-dependent plasticity, contributing to learning and motor skill maintenance [310, 311]. Adult stem cells in the mouse SC were identified using the neurosphere assay. Weiss et al. reported in the 1990s that 0.1% of isolated thoracic and 0.6% lumbar SC cells in mice formed neurospheres [235], proving the existence of adult stem cells. These cells were ependymal cells primarily located in the central canal. Other proliferating cells with a more restricted lineage profile can be found in the parenchyma [312-314]. Only 1-10 % of the cells inside the SC-derived neurospheres can give rise to new neurospheres [313-315]. Cells dissociated from these neurospheres are more inclined to differentiate into oligodendrocytes than neurons after SCI, both *in vivo* and *in vitro.* Even after clonal expansion *in vitro* these cells remain a heterogenous population expressing variable levels of markers indicating stem cell (CD133), astrocytic (GFAP, Adhl1l1), radial glia (CD15, Blbp, Glast, RC2) and oligodendrocytic-lineages (NG2, A2B5, PDGFR). Different areas[5] of the SC give rise to varying ratios of neurons, astrocytes, oligodendrocytes, and radial glia. These cells retain their specific Hox-gene expression profile even after several passages in vitro [314, 316], which is a factor we consider in **Paper III**. In contrast to the subventricular zone (SVZ), the central canal lacks a distinct subependymal layer. Ependymal cells, neuronal-like cells, and tanycytes (radial ependymal cells) are dispersed around the central canal in ependymal and subependymal positions, with different functions related to CSF contact and blood vessel connections. Studies in rodents have indicated that GFAP+ cells, which have astrocytic features, may give rise to neurospheres, potentially originating from both the central canal and the parenchyma. Sabourin et al. explored the cell population giving rise to neurospheres in 2009 [314]. They used a hGFAP-GFP transgenic line to examine the hypothesis that GFAP+ cells give rise to neurospheres. Indeed, they could show that >80% of the neurospheres contained at least one or several GFAP+/GFP+ cells. This concords with observations from the SVZ demonstrating stem cell properties in cells with astrocytic features [317]. These cells, however, could also originate from the parenchyma since GFP was not selective for cells in the central canal [314]. In 2008 Meletis et al. examined cells located only in the central canal using FoxJ1[6]

[5] cervical vs lumbar vs brain NSC, lumbar gives rise to 30.8% less radial glia, and 6.9% less neurons compared with cervical NSC

[6] Selected by FoxJ1 expression. FoxJ1 is specifically expressed in epdendymal cells in the adult CNS

expression. They found that 0.2% of the extracted cells could form neurospheres, but they could not detect GFAP+ expression in these cells, concluding that stem cells in the SC are GFAP- ependymal cells [313]. Later transcriptional analyses were however able to show the expression of GFAP in FoxJ1+ cells in the SVZ [318]. The contradiction in these studies could be an indication for several different stem cells populations in the SC with an unclear importance for GFAP as indicator for the presence of stem cells. Most of our knowledge of niche architecture in the SC originates from rodent studies. However, human SC studies from organ-donor patients revealed occluded, disorganized, and hypocellular central canal regions containing GFAP+ filaments and nerve fibres. Neurosphere formation from isolated human central canal cells was much less frequent, indicating differentiation into GFAP+ cells and neurons, but with limited proliferative potential [319]. In rodent studies, a variety of cell populations in the central canal region could be shown. GFAP+ cells are frequently in direct contact with CSF, and a fraction of the cells in contact with CSF expressed Nestin. Neurosphere formation from isolated central canal cells occurred to a much smaller extent (0.001-0.003%), and upon differentiation generated GFAP+ cells and neurons. While proliferation was evident using Ki67 expression, it was not possible to passage these spheres, indicating a lower proliferative potential. In our own hands, neurospheres could be generated from the human filum terminale and have been reported to form neurons [320]. However, the passage of neurospheres generated from human samples was difficult.

1.2.2 Neurons, Oligodendrocytes and Astrocytes

Neurons comprise soma, dendrites, synapses, and typically an axon. Neurons have the ability to transmit electrical impulses through ion channels (Na+ and K+) and the Na+/K+-ATPase pump, while voltage-gated Ca^{2+} channels enable neurotransmitter release for chemical communication [321]. Transcription factors influence neuronal differentiation from NSCs, regulating quiescence and activation. We studied one example, the neuronal repressor hairy and enhancer of split-1 (Hes1) in the **Manuscript.** Hes1 gene deletion promotes formation of neurons, while oscillatory Hes1 expression modulates NSC quiescence by inducing differentiation [309, 322]. Oligodendrocytes play a crucial role in signal transmission by insulating neuronal axons with myelin for fast transmission of electrical signals and for providing trophic support [323]. Oligodendrocytes mature chronologically after astrocytic and neuronal maturation and establish the neural network together with astrocytes and neurons [324]. Oligodendrocyte development is also guided by transcription factors [325, 326],

epigenetic modulation [327-329], translational and post translational modifications [330-332]. Transcription factors (TF) such as *Olig2*, *Sox10* and *Nkx2.2* are key players in oligodendrocyte progenitor cell (OPC) differentiation. *Olig2* and *Sox10* are expressed by oligodendrocytes. OLIG2 induces *Sox10* and a positive feedback loop maintains *Olig2* expression. *Sox10* is also modulated by the nuclear factor of activated T cells (NFAT) protein NFATC2. NFATC2 lifts the inhibitory effect of OLIG2 on SOX10 induced *Nkx2.2* expression and *vice versa* [333]. *Sox10* is additionally controlled by phosphorylation of the TF FOXO1 [334]. Importantly, *Foxo1* can additionally be activated by ROS as will be discussed in later [335, 336]. *Tcf7l2*, restricted to the oligodendrocyte lineage, mediates crosstalk between HDAC1/2 and canonical Wnt signalling to regulate oligodendrocyte differentiation [337, 338]. In addition to its role in neuronal differentiation, Hes1 also plays a role in oligodendrocyte differentiation as an *Ascl1* repressor. Low *Ascl1* expression oscillates in response to *Hes1* in OPCs and leads to OPC proliferation. Conversely, sustained overexpression of Ascl1 decreases oligodendrocyte formation [322]. In addition to the effect of transcription factors, regulatory RNAs such as the long non-coding RNAs lncOL1 and lnc158 promote oligodendrocyte differentiation [339, 340], often by interference with transcriptional inhibitors. In models of CNS demyelination, the differentiation and re-myelination of oligodendrocytes was induced by the micro-RNA miRNA-146 [341-343]. Complete maturation is reached with the formation of myelin and the establishment of interplay between neurons and oligodendrocytes. The process of oligodendrocyte maturation is mainly completed by the end of adolescence. It has been suggested that plasticity is mostly due to myelin exchange, the oligodendrocyte population being stable with an annual exchange rate of only 1/300 [344]. Non-developmental myelination is observed after demyelination [332] and seems to be crucial to motor skill learning [345, 346]. Thus myelin is a dynamic structure throughout life [347], and it responds to neuronal stimulation in particular [348].

Astrocytes provide trophic support to cells in the CNS. They are the first cells arising from NSC during development as radial glia, also providing cues such as EDF and BMP to maintain quiescence in the stem cell niche [349]. Parenchymal astrocytes exhibit neurogenic properties in response to microenvironmental cues like NOTCH and BMP signalling [262]. Astrocytes are the most abundant cell type in the CNS. Their podocytes establish the BBB and fulfil a series of

essential functions maintaining tissue homeostasis[7]. In response to CNS insults, those astrocytes in close vicinity to the insult undergo reactive astrogliogenesis. They respond particularly to cytokines such as interleukins and TNF, which we worked with in **Paper III**, activate the immune response and interact with microglia. Furthermore, the reactive astrocytes near the site of insult undergo significant changes – such as hypertrophy, increased expression of intermediate filament proteins, enhanced proliferation, and secretion of cytokines, chemokines, growth factors, and neurotrophic factors –and contribute to glial scar formation [350]. This response can vary depending on the extent of the insult as well as the brain region affected.

1.2.3 Neural Development versus Adult CNS plasticity

Neural embryonic development is extremely tightly orchestrated and limited to a specific timeframe in which several processes proceed in an organized, partially parallel manner governed by internal and external cues [351]. Neural development is highly conserved between mammals [352]. The process of embryogenesis in the cerebral neocortex is visualized in Figure 10. Neurulation is the process of the neural plate[8], composed of neural epithelia cells (NEC), bending and subsequently fusing to form the neural tube. This occurs in rodents around gestation day (gd) 10 (birth at gd 20-21), and in humans between gd 24-28 [353-355]. After forming the neural tube, NEC convert to radial glia cells (RGC), stretching out from the ventricular zone towards the pial surface, giving the framework for cortical construction. Regions with large neuronal output establish a SVZ-like structure during early embryogenesis [356]. This developmental process is not only governed by chronologically released cues but also by tissue patterning. While NEC can generate all CNS cell types *in vitro* [357, 358], NEC *in vivo* will generate different progeny depending on their position. Additionally, there are several highly conserved signalling pathways governing CNS development. Similarly to adult neurogenesis, the Wnt/β-catenin pathway plays a critical role in neural tube development [359]. Accordingly, disturbance in Wnt signalling has been implicated in several neurodevelopmental disorders such as schizophrenia and autism [360-362]. In the **Manuscript** we see NOTCH signalling being affected by H_2O_2 exposure. NOTCH controls proliferation,

[7] Production of trophic factors such as BDNF, EGF and NGF 321. Pöyhönen, S., et al., *Effects of Neurotrophic Factors in Glial Cells in the Central Nervous System: Expression and Properties in Neurodegeneration and Injury.* Frontiers in physiology, 2019. **10**: p. 486-486.

8 Derivate of the ectoderm, located directly above the notocord

differentiation, and apoptosis in the developing brain [308, 363-365]. The NOTCH receptor interacts with membrane-bound ligands Delta and Jagged on the neighbouring cells, leading to cleavage of intracellular domains which then translocate to the nucleus and act as transcriptional activator for example for Hes1 [365]. Sonic Hedgehog signalling is involved in neural tube patterning, ventral forebrain neuronal differentiation, cerebellar neuronal precursor proliferation and midbrain dopaminergic differentiation [366-369]. Neurotrophic factors such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are involved in neurogenesis too [370]. They bind to tyrosine protein kinase receptors (TrkA, TrkB, TrkC) and to the $p75^{NTR}$ receptor, and mediate various downstream signal transduction cascades [371, 372]. Moreover, increased BDNF levels leads to stimulation of NSC proliferation and formation of neurons in the olfactory bulb (OB) [373, 374]. Neurogenesis in most mammalian brain regions decreases shortly after birth while gliogenesis persists during adulthood [375, 376].

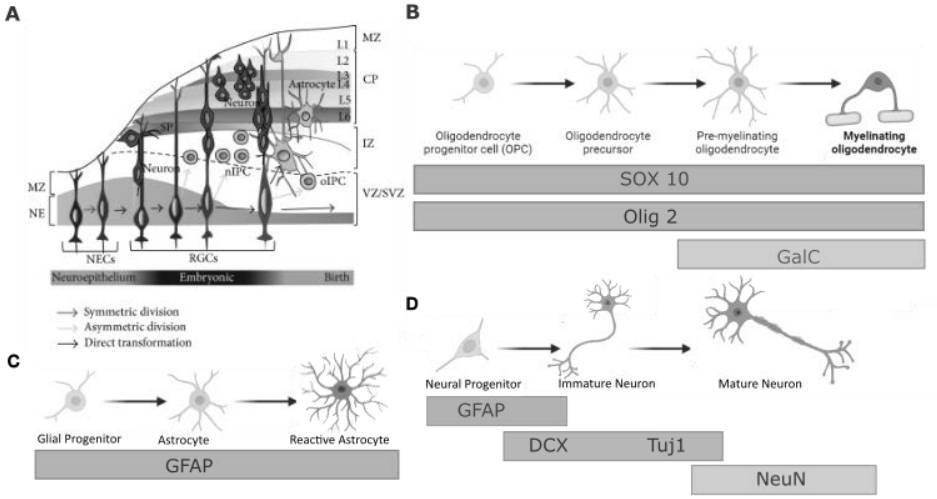


Figure 10 The CNS during embryogenesis - development in a time-dependent manner. **A** Representation of CNS cell genesis during development in humans. From neuroepithelial cells (NEC) located in the neuroepithelium (NE), a series of cell types are produced including radial glial cells which create a scaffold for cells to migrate along generating the different layers of the cortical plate (CP). Neurogenic intermediate progenitor cells (nipc) are formed earlier than oligogenic intermediate progenitor cells (oipc). Nipc subsequently give rise to neurons respective Oligodendrocytes and astrocytes. **B-D** During different stages of development specific proteins are expressed and can be used to indicate celltype and developmental stage. Here the most common marker for oligodendrocyte differentiation is displayed. In this Thesis **B** SOX10, OLIG2 and GALC have been used to identify different maturation stages of Oligodendrocytes **C** GFAP to represent astrocytes and **D** DCX and Tuj1 to identify neurons. Cp - corticalplate; iz - intermediatezone; l1–6 - layers1–6; mz - marginal zone; Rgc - radial glial cells; SVZ - subventricular zone; VZ - ventricular zone. From (zhang and jiao 2015 under the Attribution Creative Commons 3.0 Unported (CC BY 3.0)[5])

Hypothetically, adult neurogenesis is a process that can occur at any given time point and is triggered by external factors. Despite the implicated lack of plasticity in the CNS reported by Cajal [377, 378], Altman later observed the emergence of newly developed neurons in the adult brain of rodents and felines by administering 3H-thymidine and subsequently detecting its incorporation into neurons [9]. This seminal work introduced the concept of adult neurogenesis. Subsequent investigations validated the occurrence of adult neurogenesis in avian species, zebrafish, rodents, and non-human primates. It was only in 1998 that Eriksson et al. provided compelling evidence of adult neurogenesis in the human dentate gyrus through the use of BrdU labelling [379]. Notably, adult neurogenesis exhibits species-specific differences, with neurogenic regions in adult rodents being distinct from those in humans. While adult neurogenesis is robust in certain brain regions of rodents, such as the SVZ and SGZ, the process appears to be primarily an adaptive response in adult humans, activated in specific brain regions under exigent conditions or in response to injury. Human adult neurogenesis has been shown in forebrain samples from epileptic patients [380, 381]. Work from the Frisén Lab at Karolinska Institute using C14 carbon dating to reveal the age of individual cells supports this assumption [382, 383]. Age [384], stress [385], M1 microglial activation [386] and inflammation can inhibit adult neurogenesis or progenitor maturation, while exercise [387], activation of M2 microglia[9] [388] and antidepressant intake [389] seem to promote adult neurogenesis. Neurogenesis in the adult brain is regulated by an abundance of factors and pathways. It is more frequently studied in the SGZ, but the SVZ has also been assessed. It has been shown that in the SVZ the Wnt/β-catenin pathway stimulates NSC proliferation and self-renewal *in vivo* and *in vitro* [390] and so does NOTCH [391]. Sonic Hedgehog has been shown to be required for proper NSC maintenance and neuroblast migration [392]. Additional ways steering NSC activity in the SVZ include growth factors (IGF-1 and BDNF increase neurogenesis [393, 394]), Transcription factors (e.g. CREB is required *in vivo* for neuron al survival and dendritic arborization [395]) and epigenetic regulators (miR-124 promotes neuronal differentiation *in vivo* and *in vitro* [396]).

In the Manuscript we especially focussed on adult oligodendrogenesis in the SVZ. Evidence for post-natal oligodendrogenesis (postnatal day two to three) in the SVZ was provided using retroviral lineage tracing [397, 398], and adult oligodendrogenesis was indirectly shown using

9 M1 & M2 Microglia

BrdU injections and subsequent assessment of BrdU positive oligodendrocytes found in the corpus callosum [399, 400] after demyelination. As previously mentioned, demyelination is a driving factor for adult oligodendrogenesis as it increases the expression of pro-oligodendrogenic transcription factors such as *Olig2*, *Ascl1*, and *Nkx2.2* [401]. It also increases the NSC proliferation followed by a surge in oligodendrogenesis [402]. A similar pattern was evident in post-mortem brains of Multiple Sclerosis (MS) patients [403] together with a decrease in neurogenesis [404]. The NSC multipotency observed *in vitro* has been challenged by *in vivo* data. Live imaging of the SVZ revealed that a single NSC gives rise to either neurons or oligodendrocytes, but not to both, and that expression of *Pax6* and *Olig2* determines a neural vs an oligodendrocytic fate respectively [287, 405]. So far, it has not been possible to demonstrate cortical neurogenesis in humans [406, 407], neither by determining the isotope ^{14}C levels in individual cortical neurons [383] nor by using magnetic resonance spectroscopy [408, 409]. These studies demonstrated the complexity of NSC proliferation and differentiation during development and in adulthood. Our research presented here addressed how inflammatory stimuli can impact NSC biology, whether similar pathways are activated, and if their modulation during pathologic conditions could support regeneration.

1.2.4 Stem cells in in regeneration

While avid regeneration of tissue can be observed in invertebrates and other vertebrates, this process is more restricted in mammals [410]. Yet allogenic and autologous stem cell transplantations are a well-documented procedure in modern medicine and can contribute to alleviation of neurodegenerative diseases [411]. NSCs in adults respond to a wide range of physiological and pathological conditions including ageing, epilepsy, tumour development, drug addiction and infections, with e.g., cell replacement or senescence [412-414]. Cells of the immune system and factors released during inflammation have an impact on stem cell fate [20, 415]. Improved functional recovery after transplantation of NSC has been reported in rodents [416, 417] as we also demonstrated in **Paper I**. To support, stimulate, or to improve the natural regeneration capacity in mammals, various approaches including epidural stimulation, physical exercise and transplantation of stem cells have been tested in rodent models [418, 419]. Transplantations have been explored using a variety of stem cell sources in animals [420], including co-transplantation with ectopic cell types to benefit from their functions [421]. NSCs transplanted into the spinal cord survive to varying degrees depending on the SCI phase during which the transplantation occurs [422-424]. Indeed, transplantations during the intermediate

phase of SCI presented with superior survival compared with the acute or chronic phase [425, 426], but ultimately the best graft survival chances are in the uninjured or normal-appearing parenchyma [427, 428]. Reports of the differentiation potential of transplanted NSCs are diverse. Some studies suggest that NSCs can differentiate into all cell types derived from stem cells in the CNS [429, 430], others report only the formation of astrocytes [431, 432]. It has been unclear in which way the transplanted NSCs affect the inflammatory environment, and which cells are formed that could contribute to functional recovery. It is known that the microenvironment, such as the distance to the injury epicentre, can impact the fate of transplanted NSCs [433]. We demonstrated that transplanted NSCs ameliorated the inflammatory profile *in vivo* and that NSCs gave rise to oligodendrocytes, both processes that could improve hind limb functionality [96]. A few studies reported the formation of functional synapses between host and donor cells [433-435], while others have not observed such interactions [436, 437]. Studies also differ regarding the reported degree of improvement in hind limb function after transplantation, with some showing partial recovery [422, 436, 438] and others reporting no recovery [427, 431, 439]. To assess functional recovery post transplantation in a comprehensive way we applied, additionally to the Basso, Beattie, and Bresnahan-locomotor rating scale, a kinematic evaluation approach measuring several outcomes of hind limb functionality and sensory functions, enabling a more accurate and sensitive assessment of function. Transplanted NSCs can suppress the classical activation of macrophages [440, 441], influencing inflammatory processes and suppressing apoptosis [435, 437]. Most studies show a modest clinical recovery in animal models, mainly resulting from secretion of trophic factors, enhanced remyelination, differentiation of NSCs into astrocytes, oligodendrocytes and neurons, axonal regrowth, and amelioration of inflammation [441-447]. Transplantation of cells in neurodegenerative diseases, such as intrastriatal co-grafts of autologous adrenal medulla and peripheral nerve cells in Parkinson's disease, has also yielded functional improvement in patients [448]. Studies with human fetal spinal cord-derived NSCs and human-induced pluripotent stem cell-derived NSCs have demonstrated the ability to grow across the injury border in rats [443, 449], and a study on human SCI supports the safety of using NSCs in transplantation [450], encouraging further potential in stem cell transplantation. In conclusion, understanding of target specific transplantation strategies and the impact of inflammatory processes on NSC is crucial for improving survival and integration in the pathological environment.

2 AIMS

The overall aim of this thesis is to characterize the effects of inflammatory signals on NSC proliferation, differentiation, and self-renewal, and to study this in the context of regeneration. We addressed these specific scientific questions in each study:

Paper I: Does the transplantation of immune-compatible stem cells contribute to functional recovery?

Paper II: To what extent does irradiation of the juvenile CNS affect the NSC response to a later injury?

Paper III: Can we model the impact of neuroinflammatory astrocytes on motor neuron survival *in vitro* to understand an aspect of neuroinflammation in the brain stem region?

Manuscript: How does H_2O_2, an inflammatory mediator seen in many CNS pathologies, affect NSC differentiation *in vitro* and *in vivo*?

3 MATERIALS AND METHODS

3.1 ETHICAL CONSIDERATIONS

We used various animal models in this thesis to study NSCs in the context of inflammation. Using animal models is one of the biggest ethical issues in my research, but it is currently unavoidable. One big concern is the validity of results obtained from animal studies. An animal model is never identical to the human system the question of the insights gained from it and its applicability to the human situation remains open. Yet there is no good alternative available.

Beside the data generation, part of the research strategy is addressing the suitability of the experimental approach and considering whether the use of particular models justifies the process. Hence, I include the section Ethical Considerations in this thesis:

Methods affecting the well-being of the experimental animals in use are:

- Sacrificing mice, dissecting the subventricular zone in the brain
- Inducing SCI in rats; subsequently transplanting cells, assessing the outcome with immunohistochemically and physical performance-related methods
- Injecting H_2O_2 into the cisterna magna of mice

We do recognize the need to minimize suffering in experimental animals as they are beings worth protecting and considering ethically. I think it is essential to always assess how to make use of animals in the best way. The three Rs – Replacement, Reduction and Responsibility – can guide us. *In vitro* experiments should be preferred whenever possible. We work with primary cell cultures and embryonic stem cells, and thus the need to sacrifice mice arises. In this scenario, no animal experiments and additional suffering are involved, and the number of mice needed is rather low, which I consider a big advantage. We also have to inflict harm when inducing SCI as well as IC. In doing this we always tried to optimize experiments and minimize suffering to the best of our abilities. We additionally developed the kinematic assay as a new approach to score functional recovery with higher sensitivity, and hence minimizing the number of animals needed.

In terms of "publication ethics": the recommendations of the International Committee of Medical Journal Editors on handling authorship apply.

3.1.1 Ethical Approvals

All experiments were conducted in accordance with the ethical permits granted by the Swedish Board of Agriculture´s regional Stockholm County ethics committee (Sweden) and with Swedish legislation and my best knowledge and capabilities.

Table 5 Ethical Permissions presented by Paper.

Ethical approval	Paper	Relevance	Additional Information
N38/16	I	Cell harvesting	
N196/15	I	SCI, cell transplantation	
N12317/17	I	SCI, cell transplantation	
GBG 317-2012	II	Irradiation, Photothrombotic Stroke	
N275/15	III	Cell harvesting	Amendment under #9182-2018
N104/14	III	Embryonic Stem Cells	
N275/15	Manuscript	Cell harvesting	Amendment under #9182-2018
17607-2021	Manuscript	Intra cisternal injections	

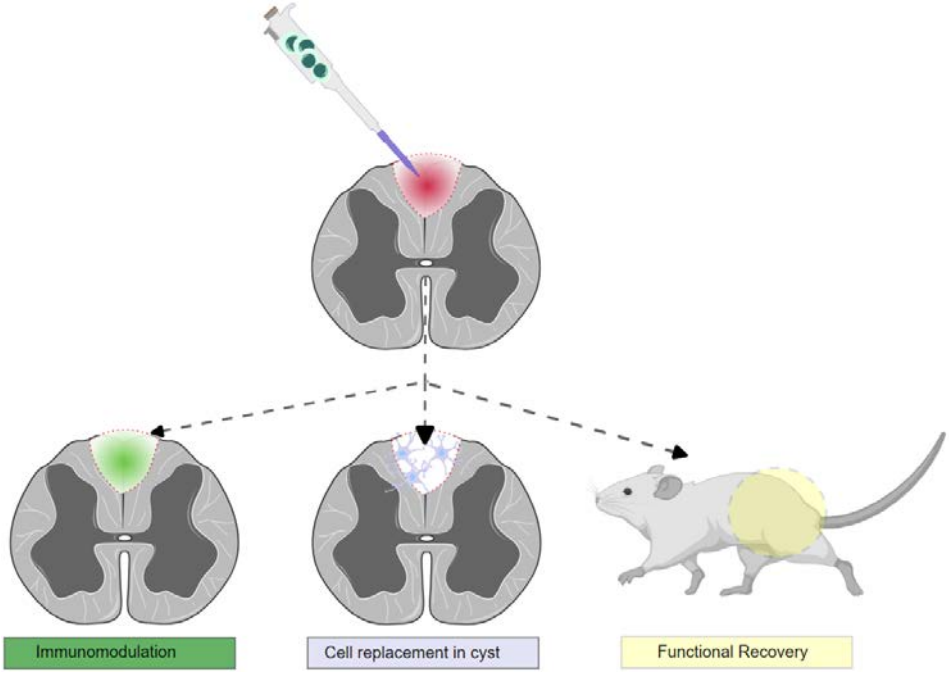
3.2 METHODS AND MATERIALS

For all methodological approaches used to compile this thesis, I here refer to the material and methods sections in Papers I-III and the Manuscript.

4 RESULTS AND DISCUSSION

**All of the following overview images were created using BioRender.com and Inkscape.*

4.1 PAPER I



In this study, we transplanted NSC into the epicentre of an SCI in its acute phase. This enabled us to analyse the impact of NSCs transplanted into SCI on motoric functionality and the inflammatory environment as well as the trajectory of the transplanted cells. These are the main findings:

1.) Amelioration of the inflammatory environment by downregulation of genes related to immune system response and decreased production of cytokines

2.) NSCs differentiate predominantly into oligodendrocytes and contribute to myelination

3.) We observed enhanced recovery of hindlimb function post-transplantation.

Amelioration of the inflammatory environment by downregulation of genes related to immune system response and decreased production of cytokines:

Understanding the microenvironment, especially the immune profile, that transplanted NSC are surrounded by is important to interpret observed changes and processes. To do so we analysed global transcriptomic changes in NSCs sorted three- and four-weeks post-transplantation into the SCI. This revealed downregulation of genes related to cytokine production, immune system response, and cell migration at three weeks, while synaptic signalling-associated genes were upregulated at both three and four weeks. These timepoints were chosen to capture the acute phase after SCI and thus affecting secondary injury mechanisms [451, 452]. In this study we used depletion of NSCs as control group since it was difficult to find a suitable control cell population to inject. The NSC depletion was initiated directly after transplantation to avoid loss of diphtheria-toxin susceptibility. Previous studies have reported that NSC transplantation can improve synaptic connectivity post-SCI, which aligns with our transcriptome findings showing an increase in synaptic signalling post transplantation [422, 434, 435, 438, 453, 454]. The downregulation we observe in the transcriptome of genes involved in cytokine production, immune system response, and cell migration at three weeks (Fig. 11 A). This returns to baseline at four weeks, can be closely followed when looking at the protein expression of cytokines in the CSF which we analysed three-, six- and twelve-weeks post-SCI. We observed significantly lower levels of pro-inflammatory cytokines (interleukin-1a (IL-1a), IL-1b, IL-2, tumour-necrosis-factor-a) in the CSF at three weeks in animals receiving NSCs. This effect was not evident at six- and 12-weeks post-SCI (Fig. 11 B). To our knowledge, a comprehensive assessment of cytokines/chemokines' protein concentration in the CSF after SCI is currently lacking. There are studies on the cytokine profile in the blood post-injury, and its diagnostic potential as an outcome predictor [455]. Some studies have measured cytokine levels in CSF in dogs post-SCI [456], but translating these results into the rat model is not straightforward. Despite the absence of a suitable reference point, accurately predicting outcomes during the acute phase of SCI is vital for effective care planning and rehabilitation strategies, and thus CSF measurements could provide increased diagnostic accuracy.

NSCs predominantly differentiate into oligodendrocytes and contribute to myelination. We were also interested in the differentiation trajectory of transplanted NSCs. We observed long-term survival (12 weeks) of transplanted NSCs, which coincided with downregulation of apoptosis-related genes and the absence of caspase-3-positive NPCs. We also captured upregulation of genes associated with myelination and oligodendrocytes at three- and four-weeks post-SCI, indicating oligodendrocyte differentiation which was confirmed by histological stainings at 12 weeks post-SCI, indicating enhanced myelination (visualized using antibodies against CNPase, MBP) and oligodendrocyte differentiation (visualized using antibodies against CC1, OLIG2) compared to the control group (Fig. 11 C). Long-term survival of transplanted cells was previously reported, thus validating the functionality of our experimental approach [429, 457-460]. The successful long-term survival of transplanted cells and enhanced functional recovery may be attributed to using littermate NSC donors. This immune-compatible model promotes high NSC integration and filling of the cyst cavity. Transplanted NSCs also differentiated into low numbers of astrocytes and neurons, consistent with previous reports of their potential to differentiate into various cell types of neuroectodermal lineage [429, 430, 435]. Glial cell differentiation was predominant in our study, a finding which is supported by others [460, 461]. It also agrees with the phenotypical changes we observed in the Manuscript after exposure to inflammatory stimulants. Differentiation preferences among NSCs vary based on the CNS site of origin [316]. During homeostasis, NSCs from the spinal cord tend to favour gliogenic differentiation, while those from the SVZ exhibit a higher inclination towards neurogenic differentiation. However, under inflammation-induced conditions, differentiation outcomes shift for NSCs from different sites. SC-derived NPCs showing an increased propensity for neurogenic differentiation [297] and SVZ-derived NPCs demonstrating a preference for gliogenic differentiation [404]. Glial scar formation, typically driven by astrocytes, was not assessed in our study. Future research could investigate whether inhibiting astrocyte-mediated glial scar formation enhances functional recovery.

Enhanced recovery of hindlimb function:

Understanding the impact of increased oligodendrocyte formation and myelin production as well as the NSC-modulated inflammatory responses to SCI is ultimately interesting when it improves functionality. Motor function improvement post-transplantation was not a surprise as it has been already reported in several studies [438, 440, 453, 461-463]. We additionally measured factors such as coordination, step cycle process and stepping pattern, and iliac crest

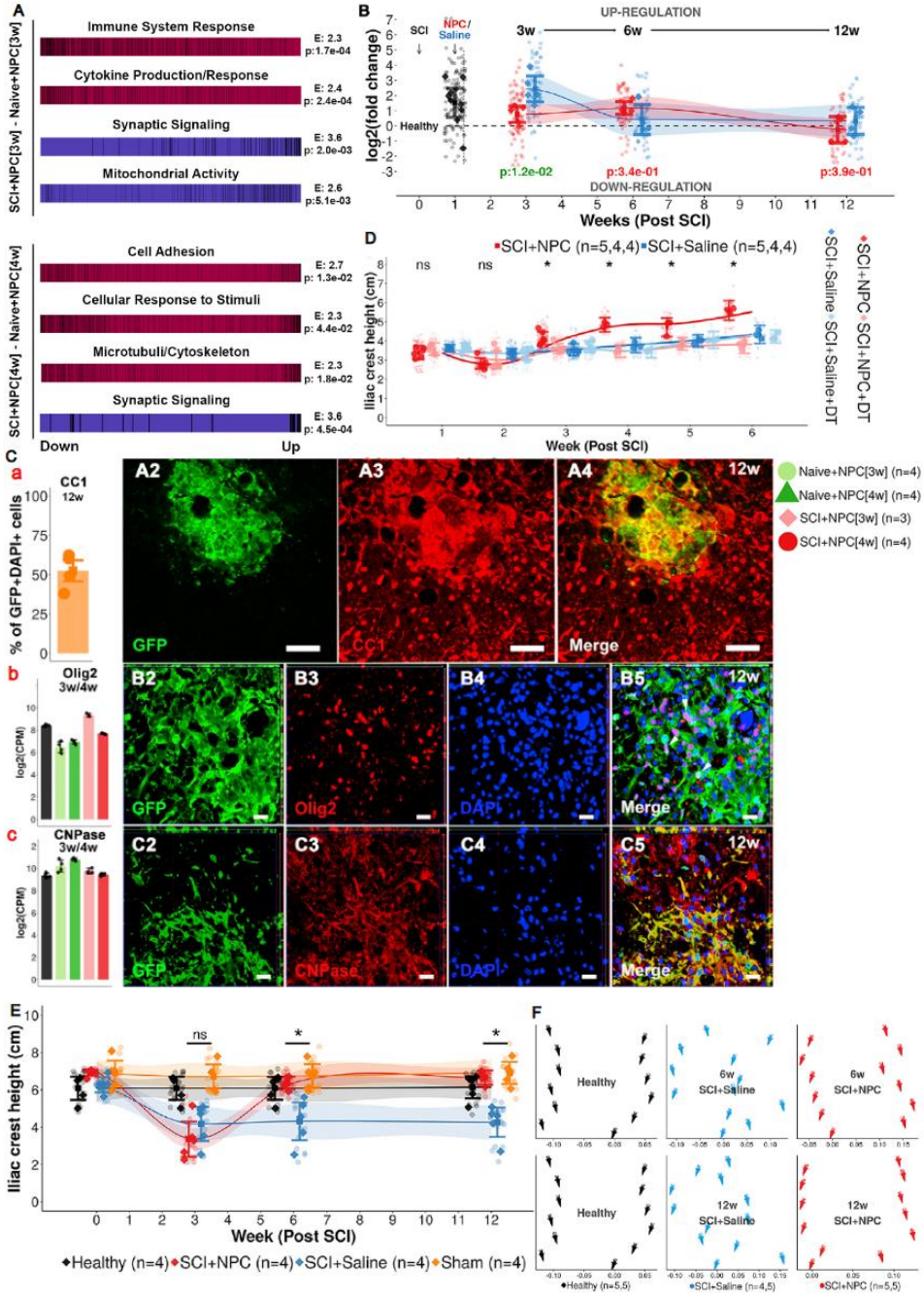
height to obtain a more comprehensive view of hindlimb function. All these factors showed degrees of improvement post-transplantation (Fig. 11 E-F). Additionally, we observe BBB-scale scores returning to saline/sham baseline if we ablated the transplanted NSC using 100 mg/kg diphtheria toxin (DT) via intraperitoneal (i.p.) injections targeting only transplanted cells. These results prove a causal relationship between transplantation of NPCs and enhanced recovery in hindlimb function (Fig. 11 D). Ablation of the transplanted NSCs has been studied previously [464, 465], but not during the acute/subacute phase of SCI using NSC from littermates.

We did not assess the impact of NSC transplantation on autonomic functions in this project, but many others did look especially at bladder function post-SCI and stem cell transplantation with varying outcomes [466]. We noticed for example a lack of bladder control in our animals post-SCI, which ought to be assessed as it is an issue frequently reported by SCI patients [467].

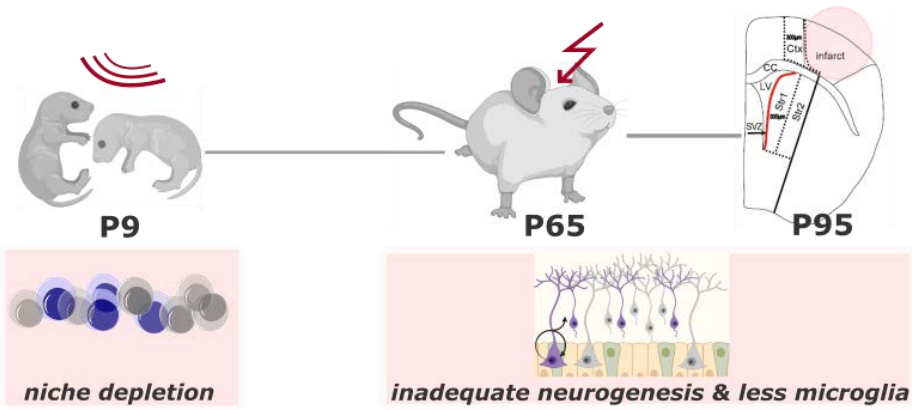
In conclusion, the findings of this study provide evidence supporting the beneficial effects of NSC transplantation in improving motor function and enhancing hindlimb recovery in the acute/subacute phase of SCI, indicating a role for the inflammation modulating capacities of NSC as well as their pro-myelination properties.

Overall, this study contributes to the understanding of the therapeutic potential of NSC transplantation in SCI and provides a platform for further studies to explore the long-term effects and to investigate the broader impact on sensory and autonomic functions.

Figure 11 **A** Barcode plots for top functional categories presented with enrichment score (E) and FDR (p) following competitive gene set testing. **B** Level of pro-inflammation as log2(fold change) in relation to expression in healthy animals and time after SCI. Mean is surrounded by a 95%CI. Each diamond represents one animal. Each dot represents one cytokine/chemokine. p values for two-group comparison are presented at each time point. **C** (a) Quantification of co-localization of GFP and DAPI positive staining (A2–A4) Transversal section of a dorsal horn rostral to an SCI epicenter containing NPCs. (b-c) Gene expression reported using log2(counts per million) in sorted GFP+ NPCs. (B2–B5, C2–C5, D2–D6) Orthogonal projections of transversal sections rostral to SCI epicenter**. D** Iliac crest height over time. Presented as log2(fold change) to expression in healthy animals. Each dot represents one animal. p values for two-group comparison between SCI+NPC and SCI+NPC and DT are not presented 95% CIs are provided instead (*p <0.05; ns, not significant), Technical replicates are reported as diamonds, **E** Iliac crest height over time. Presented as mean surrounded by a 95% CI. Each dot represents one animal. Significance of post hoc test between SCI+NPC and SCI+saline is reported (*p < 0.05; ns, not significant). **F** Placement of the hindlimb paws (average x/y coordinate per treatment group). *As the author of an Elsevier article, one retains the right to include it in a thesis or dissertation, provided it is not published commercially. Permission is not required.*


A
SCI+NPC[3w] - Naive+NPC[3w]
Immune System Response
E: 2.3
p:1.7e-04
Cytokine Production/Response
E: 2.4
p: 2.4e-04
Synaptic Signaling
E: 3.6
p: 2.0e-03
Mitochondrial Activity
E: 2.6
p:5.1e-03
SCI+NPC[4w] - Naive+NPC[4w]
Cell Adhesion
E: 2.7
p: 1.3e-02
Cellular Response to Stimuli
E: 2.3
p: 4.4e-02
Microtubuli/Cytoskeleton
E: 2.3
p: 1.8e-02
Synaptic Signaling
E: 3.6
p: 4.5e-04
Down
Up
B
SCI
NPC/ Saline
UP-REGULATION
3w
6w
12w
log2(fold change)
Healthy
p:1.2e-02
p:3.4e-01
p:3.9e-01
DOWN-REGULATION
Weeks (Post SCI)
D
SCI+NPC (n=5,4,4)
SCI+Saline (n=5,4,4)
ns
ns
Iliac crest height (cm)
Week (Post SCI)
SCI+Saline
SCI+Saline+DT
SCI+NPC
SCI+NPC+DT
C
a
CC1
12w
% of GFP+DAPI+ cells
A2
A3
A4
12w
GFP
CC1
Merge
Naive+NPC[3w] (n=4)
Naive+NPC[4w] (n=4)
SCI+NPC[3w] (n=3)
SCI+NPC[4w] (n=4)
b
Olig2
3w/4w
log2(CPM)
B2
B3
B4
B5
12w
GFP
Olig2
DAPI
Merge
c
CNPase
3w/4w
log2(CPM)
C2
C3
C4
C5
12w
GFP
CNPase
DAPI
Merge
E
Iliac crest height (cm)
ns
*
*
Week (Post SCI)
Healthy (n=4)
SCI+NPC (n=4)
SCI+Saline (n=4)
Sham (n=4)
F
Healthy
6w SCI+Saline
6w SCI+NPC
Healthy
12w SCI+Saline
12w SCI+NPC
Healthy (n=5,5)
SCI+Saline (n=4,5)
SCI+NPC (n=5,5)

4.2 PAPER II



In this study we irradiated animals at postnatal day 9 and introduced a photothrombotic stroke at postnatal day 65. We here were interested in the impact of irradiation of the juvenile CNS on NSC function in response to injury. These are the main findings:

1.) Photothrombotic stroke elicits production of DCX+ neurons in adult mice. This effect was diminished if preceded by irradiation.

2.) Irradiation does not impact the ability of microglia to become activated, but it leads to a reduced number of microglia in the adult CNS.

3.) Irradiation of the juvenile CNS does not impact the infarct area but does affect the relative infarct size after stroke in adult mice.

Photothrombotic stroke elicits production of DCX+ neuronal progenitor cells in adult mice. this effect was diminished if preceded by irradiation, presumably by depletion of the NSC pool.

Understanding the long-term consequences of irradiation is beneficial for optimized subsequent therapy. Here we addressed how NSCs respond to injury after irradiation. We studied this in the adult murine brain using a photothrombotic stroke model to trigger proliferation and migration of neuronal progenitors [111, 468, 469]. We observed that cortical ischemia led to an increase in neuronal progenitors (DCX^+ cells) in the peri-infarct cortex and two striatum regions: one near the SVZ and the other distal but adjacent to the first region. Non-ischemic animals displayed few DCX^+ cells in the cortex. The number of DCX^+ cells significantly increased in the peri-infarct cortex for both irradiated (IR) and non-irradiated (non-IR) after cortical ischemia. When comparing the DCX^+ cell count between IR and non-IR ischemic animals, IR animals exhibited a significantly lower number of DCX^+ cells (Fig. 12 A, B, D-I), suggesting a compromised neuronal response to stroke after IR. There are reports showing that most cells cease to proliferate after irradiation because they start to differentiate instead [470]. This potentially depletes the pool of NSC early on. We hence assumed that irradiation disturbs the SVZ [148] and thereby affects NSC functionality. Irradiation, while effective against cancer cells, is not a targeted method and elicits a significant inflammatory response in the CNS [471] [472]. The impact of radiation on the NSC niche and the subsequent changes in neurogenesis may contribute, if not directly cause, radiation-induced cognitive impairments [473]. It is established in rodents that NSCs respond to damage, such as stroke, and contribute to the repair process that is critical for post-stroke functional recovery [474]. This is a phenomenon we also observed in our **Manuscript** where inflammatory signals led to an increase in proliferation. It has been shown previously that the response to postnatal irradiation is niche-dependent, i.e. with a lack of recovery in the hippocampus, while the SVZ showed not full, but substantial recovery of stem cell activity [475-477]. We could demonstrate here that even with potential recovery of stem cell activity in the SVZ after IR at an early developmental stage, the NSC response to cortical ischemia in adulthood remains compromised. DCX^+ cells represent neuroblasts, late progenitors as well as young neurons. We did not determine if these observations are exclusively based on the depleted pool of stem cells or if IR also exerts an impact on maturation and/or migration of DCX^+ cells. In this study, we have also not addressed if neural progenitors continue to develop into mature neurons, since we have previously shown that maturation of neurons in the stroke-lesioned cortex is extremely limited [478]. We did not examine the effect on oligodendrocytes, and we assessed

astrocyte response in only a rudimentary way by measuring GFAP intensity. Hence we could not evaluate the impact of IR on these cell types. However, it was important to observe that despite a compromised SVZ stem cell pool, a substantial response of neural progenitor cells to stroke was detectable even after brain irradiation at 10 Gy.

Irradiation does not impact the ability of microglia to become activated, but it leads to a reduced number of microglia in the adult CNS.

Since we hypothesized that the local microenvironment of the niche is partly responsible for the observed changes in neural progenitor formation, we were also interested in the function of the brain-resident immune cell. Following brain injury, activated microglia and macrophages (MG/MQ) accumulate at the injury site. We therefore examined the MG/MQ response in the neocortex by evaluating the expression of Iba1, a pan-microglial marker [479, 480]. Here a combination of several microglial markers would be needed to fully characterize the microglial phenotype [481, 482]. Ischemic animals, whether irradiated or non-irradiated, exhibited a significant increase in the number of Iba1+ cells compared to non-ischemic animals. Irradiation led to a significant decrease of Iba1+ cells in the injured cortex compared to the non-irradiated animals (Fig. 12 K). Ischemic animals also displayed a substantial increase in the percentage of Iba1+ cells expressing CD68 compared to non-ischemic animals. CD68 is a glycoprotein that is present on the cell membrane and is expressed by human monocytes and tissue macrophages, serving as an indicator of their phagocytic activity [481]. However, we did not detect a difference in the percentage of Iba1+/CD68+ cells between the irradiated and the non-irradiated animals following ischemia (Fig. 12 L-M). The response of NSCs to injury is influenced by inflammatory signals released by microglia/macrophages [483-486]. Following a stroke, microglia/macrophages undergo proliferation and accumulate at the site of injury [487-489]. These cells release inflammatory molecules e.g. chemokines and cytokines, which promote NSC proliferation and guide their migration towards the injury site [490, 491]. Irradiation can decrease the numbers of microglia/macrophages, particularly during early postnatal stages [492], resulting in a modest inflammatory response to cortical ischemia. We could also show a decrease in microglia number post-inflammation in adult animals. One of our hypotheses was that the reduction in microglial stimulation may contribute to the observed decrease in neural progenitors [468]. A recent study challenged this, as co-cultures of injury/ischemia-induced NSPCs (iNSPC) and microglia/macrophages (MGs/MΦs) significantly decreased proliferation of iNSPCs and reduced differentiation of these cells into functional neurons *in vitro [493].* The interaction of microglia and NSCs in the *in vivo* scenario might differ to some degree from these

in vitro observations. Nevertheless, decreased numbers of DCX^+ and $Iba1^+$ cells could be attributed to other factors, including a direct reduction of the NSC pool in the SVZ due to IR [355, 494, 495], altered vascularization [496, 497] or a combination of the named factors. The age-dependent effect of irradiation can be attributed to the high proliferative capacity of microglia/macrophages during early postnatal stages (postnatal day 5 and postnatal day 14) [62, 468, 498, 499], making them vulnerable to radiation-induced damage.

Irradiation of the juvenile CNS affects the relative infarct size after stroke in adult mice.

The size of injury often correlates with the functional outcome [500]. We also determined the impact of irradiation (IR) on the stroke lesion size. This revealed no statistically significant difference in the infarct volume among the stroke groups (Fig. 12 C). However, animals subjected to irradiation showed a notable decrease in body weight compared to non-irradiated animals, a difference that was not evident prior to irradiation on P9 but persisted after stroke. In this context we re-evaluated the infarct size and observed a significant difference in the relative infarct size compared to the contralateral hemisphere between the animals that experienced ischemic stroke (IS) and those that underwent irradiation followed by stroke (IR + IS) (Fig. 12 J). Moreover, Zhu *et al.* demonstrated increased injury size following irradiation in a hypoxia-ischemia brain injury model [128]. Our own data showed that induction of cortical ischemia, irrespective of irradiation or sham-irradiation, did not affect body weight. Previous reports have documented the negative impact of irradiation on weight gain in rodents [501], as well as in head and neck cancer patients, who experience significant weight loss over time [502]. Female mice were particularly susceptible to irradiation-induced reduction in brain size [503, 504]. The observed differences in body weight suggest a growth delay in both brain and body mass induced by irradiation during the juvenile phase. However, the growth trend after P50 was consistent across all groups, with only a lower starting point in the IR and IR + IS groups. A deeper understanding of the mechanisms underlying IR-induced impairment in the NSC response to the brain injury is crucial for developing interventions that can preserve endogenous regeneration potential or enhance regenerative capacity in brain injuries among cancer survivors [505]. In summary, we demonstrated compromised responses of NSCs to ischemic injury following IR. These results have important clinical implications, particularly for paediatric cancer survivors who are at risk of developing neurovascular diseases such as ischemic stroke [506-508] or who are experiencing cognitive impairments [145, 509, 510],

especially given the impaired endogenous repair capacity resulting from IR may further worsen the outcomes following secondary brain injuries [511].

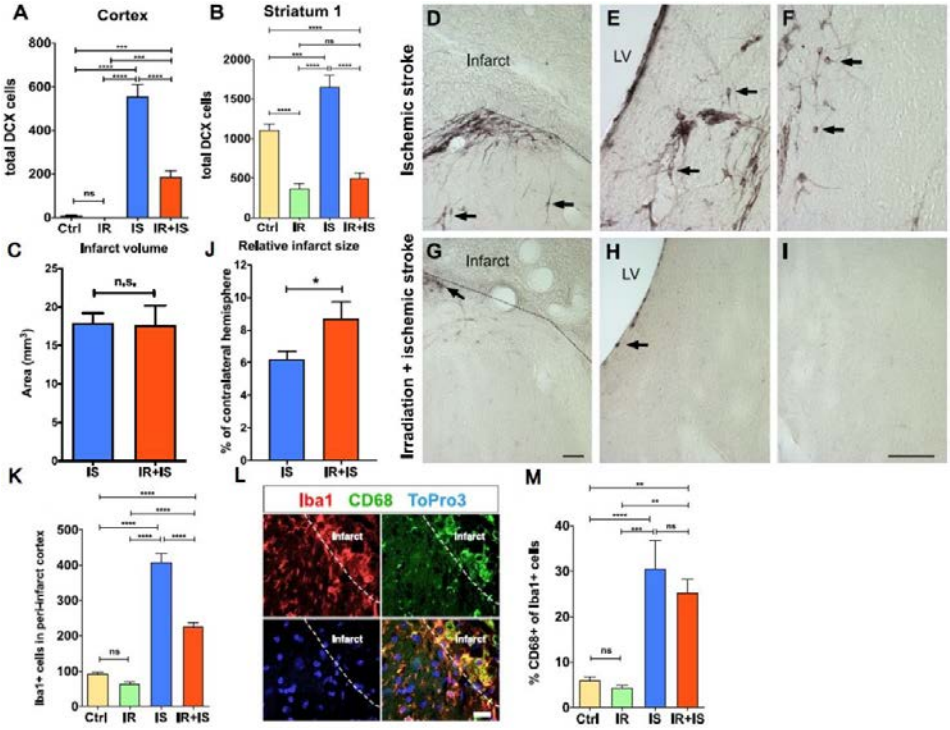
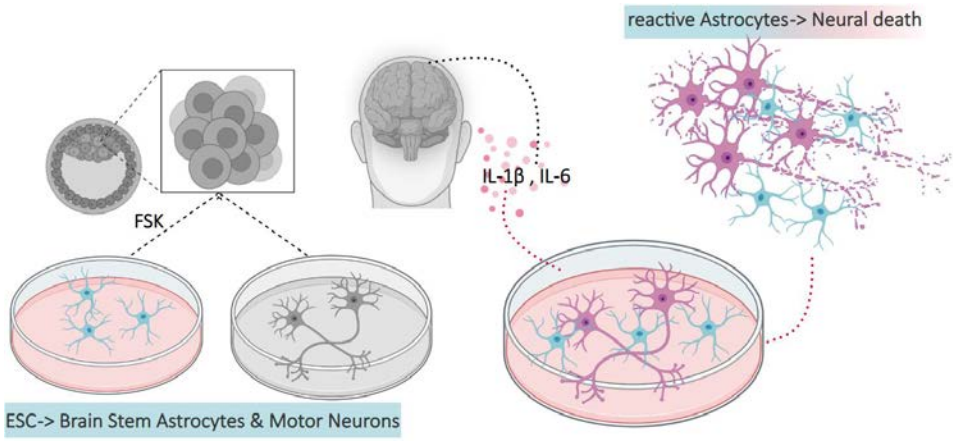


Figure 12 **A** Quantification of DCX+ neuronal progenitor cells in the cortex, **B** Striatum 1 adjacent to the SVZ, Ctrl = Control (n = 15); IR = Irradiation (n = 10); IS = Stroke (n = 11) and IR + IS = Irradiation + Stroke (n = 13), **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001), **C** Measurement of the cortical infarct volume in mm^3 (n = 11 for IS and IR + IS), **D–I** Representative images of DCX+ cells in IS (D–F) and IR + IS (G–I) brain regions. Cortex (D, G), Striatum 1 (E, H), Striatum 2 (F, I), scale bars = 100 µm, **J** Measurement of the relative infarct size in percent of the contralateral hemisphere. (n = 11 for IS and IR + IS), **K – M** Irradiation reduced accumulation of microglia/macrophages in the ischemic cortex. **K** Bar graph shows quantification of Iba$^+$ cells in the peri-infarct cortex (One-way ANOVA, multiple comparison, Ctrl, control; IR, Irradiation; IS, Stroke IR + IS = Irradiation + Stroke, all groups n = 8, **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001). **L, M** analysis of MG/MQ activation as evaluated by co-expression of Iba1 and CD68 (Iba1$^+$/CD68$^+$). **M** Confocal image displays expression Iba1$^+$ (red) and CD68$^+$ (green). ToPro3 nuclear counterstain (blue), merged Scale bar 20 µm. (C) Bar graph shows the percentage of Iba1$^+$/CD68$^+$ of Iba1$^+$ cells in the periinfarct cortex. (One-way ANOVA, multiple comparison, Ctrl = Control n = 15; IR = Irradiation n = 10; IS = Stroke n = 11 and IR + IS= Irradiation + Stroke n= 13, **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001).

4.3 PAPER III



In this study, we used embryonic stem (ES) cells and cytokines relevant to TBI to create an *in vitro* model system representing astrocytes and neurons from a specific environment. This provided us with a comprehensive and applicable model for future investigations into enhancing the survival of neurons in highly susceptible regions of the CNS after events such as traumatic axonal injury (TAI). These are the main findings:

1.) Cytokine stimulation leads to increased c-Jun N-terminal kinase pathway activity, measured by its down-stream product phosphorylated c-Jun, and ES-astrocyte reactivity.

2.) We generated ES culture derived astrocytes with brainstem/rostroventral region identity.

3.) ES-astrocytes activated by disease relevant cytokines demonstrated neurotoxic effects upon co-culture with motor neurons.

Cytokine stimulation leads to increased c-Jun N-terminal kinase pathway activity, measured by its down-stream product phosphorylated c-Jun, and ES-astrocyte reactivity

The neurotoxic astrogliosis subtype can be initiated through the cytokines IL-1α and TNF [512]. IL-1α and TNF activate the c-Jun N-terminal kinase (JNK) pathway [513-515], c-Jun will be phosphorylated, dimerizes and influences transcription [515-517]. The JNK-AP-1 pathway, known for its multifunctionality, has been extensively associated with glial cell response to inflammation [518], particularly in the context of astrogliosis. Two hours after cytokine stimulation we see a significant increase of P-c-Jun which co-localizes with the expression of the mature astrocytic marker GLT-1 (Fig. 13 A-C). To validate shared similarities of ES-astrocytes with *bona fide* astrocytes we compared this result with astrocytes derived from SVZ-derived astrocytes. SVZ-derived astrocytes provide a more representative model of the cells found *in vivo* and which were shown to exhibit glial features [519] and respond to inflammatory stimulus [297]. SVZ-astrocytes exhibited a response similar to ES-derived astrocytes (further referred to as ES-astrocytes). We observed the upregulation of phosphorylated–c-Jun (P-c-Jun) through the JNK pathway activation and the co-localization of P–c-Jun with GLT-1 in response to neuroinflammatory stimuli. Overall, these results indicate that the mouse ES-astrocytes exhibited similarities to astrocytes *in vivo* in terms of functionality. The activation of a neurotoxic phenotype in astrocytes possibly represents a secondary mechanism contribution to CNS trauma-induced neuronal loss. The JNK pathway was suggested earlier as a downstream mediator for astrogliosis in e.g. epilepsy [520], but it is possible that additional signalling pathways are involved in mediating the neurotoxic effects by astrocytes. We conducted hypothesis-generating RNA sequencing of ES-astrocytes and ES-motor neurons following co-culture and stimulation with IL-1β and IL-6[10]. We detected changes in genes associated to MYC-regulation, cell cycle regulatory mechanisms, and endoplasmic reticulum stress (Fig. 13 D-F), as well as genes related to TBA [521] and TAI [522] via their protein products. These results show a comprehensive effect, involving a variety of pathways and processes, on astrocyte reactivity in our model system. It is however a correlation and does not give a mechanistic answer as to how neurotoxicity is generated. It is further important to realize the limitation of the ES approach as there are notable differences between the astrocytic genomes of mice and humans, particularly in terms of inflammatory responses. Employing hiPSCs in future studies

[10] These cytokines are disease relevant in the context of TBI as our group has shown earlier and where hence selected for stimulation. I will refer to them further in the next section.

would allow us to generate human-specific cell models. Moreover, we would obtain more accurate and relevant data regarding the mechanisms underlying astrogliosis and its involvement in inflammatory processes in humans.

ES derived astrocytes display brainstem/rostroventral region identity.

The patterning of the developing CNS is achieved through a complex interplay of spatial and temporal cues [523] starting with neurogenesis, which is subsequently followed by gliogenesis [524]. Creating astrocytes with a specific regional identity enables us to study regional effects in vitro. It has been shown that retinoic acid and Sonic hedgehog [525] generate brainstem and rostroventral spinal cord motor neurons. We applied this knowledge and generated region-specific Hb9+ motor neurons from embryonic stem cells (Fig. 13 L). Hb9 is a TF that plays a vital role in the consolidation of spinal motor neuron (MN) fate throughout the developmental processes [526, 527]. Hence, we used Hb9 to verify motor neuron identity. Our neuronal cultures were heterogenous and contained different types of neurons as well as about 5% glial progenitors. This was an outcome previously reported [528]. Recapitulating the during physiological brain development occurring gliogenic switch [524] to generate glial cells, we continued the culture past motor neuron formation over multiple passages in an FBS-containing medium that induces differentiation. We could differentiate these glia cells into mature astrocyte-like cells when exposed to Forskolin (FSK), these peptides having been demonstrated to be involved in astrocyte maturation [529-534] (Fig. 13 G). To address the brain stem identity of the generated ES- astrocytes, we analysed the transcriptome of ES-astrocytes and compared it to the generated ES-motor neurons and SVZ-derived astrocytes. The comparison of rostro-caudal positional identity genes (Hox) between the ES-astrocytes and motor neurons did not show any significant differences, suggesting a shared origin. Within these genes, all four paralogs of Hox 4 (Hoxa4-Hoxd4) were identified, thereby establishing the cells' identity in the brainstem and rostral spinal cord along the rostro-caudal axis [535-537]. The positional identity of ES-astrocytes in the ventral region of the brainstem and rostral spinal cord was additionally confirmed by the upregulation of Nkx6.1, a gene known to be specific to brainstem astrocytes [537, 538] (Fig. 13 H). Consistent with this, control astrocytes obtained from stem cells originating from the SVZ did not show any expression of Nkx6.1. We also conducted a comparison between the differentially expressed genes and the essential microarray data of astrocyte-enriched genes from Cahoy et al. [539]. While these cells demonstrated many characteristic features of authentic astrocytes, it is important to consider that there may be differences between these cells which we did not analyse. We chose to

generate ventral brainstem/rostroventral spinal cord specific ES-astrocytes and ES-motor neurons as this area is particularly susceptible to trauma [158]. Within this specific region, the impairment of astrocytic function has been associated with the loss of motor neurons in amyotrophic lateral sclerosis [540]. This does not exclude the importance of our results for other CNS diseases. The interaction between reactive astrocytes and neurons is important in a variety of pathologies. IL-1α, TNF, complement, the cytokines we used, have been recently studied in the context of forebrain [512] astrocyte-mediated neurotoxicity and spinal hiPSC [541] astrocyte-mediated neurotoxicity. Reactive astrocytes exhibit profound regional differences [542, 543] and different types of injury elicit distinct genetic signatures of astrogliosis [544]. A recent single-cell study on cortical astrocytes post-stimulation revealed nine gene clusters that exhibit both regional and reactive characteristics [545], and it has previously been shown by analysing the secretome that regionality plays a substantial role in functionality [546]. We are to our knowledge the first to specifically address brainstem/spinal (motor) neurons and brainstem/spinal astrocytes. Here we here generated ES-astrocytes with positional identity in the ventral region of the caudal brainstem, strengthening the biological relevance of our findings. Their regional identity also limits their generalizability to other regions of the CNS and further applicability must be carefully tested.

ES-astrocytes activated by other disease relevant cytokines conferred a neurotoxic effect upon co-culture with motor neurons.

Reactive astrocytes have been implicated in various neurological disorders. Previous studies have demonstrated a neurotoxic "A1" effect of reactive astrocytes on cortical neurons when stimulated with cytokines [512], and similar observations were made in hiPSC-derived astrocytes with a spinal identity [541]. Additionally, deletion of A1-inducing cytokine genes *in vivo* has a positive impact on neuronal survival indicating causality [512]. However, it remains unclear whether this neurotoxic effect extends to motor neurons in the brainstem and spinal cord and if other disease relevant mediators can elicit neurotoxicity. To examine the neurotoxic effect of IL-1α and TNF in cells with brainstem and spinal cord regionality we exposed ES-astrocytes and co-cultured them with ES-motor neurons we generated. ES-motor neurons exhibited cell death, whereas ES-interneurons remained unaffected by co-cultivation with reactive astrocytes. Moreover, we revealed crucial dissimilarities in the neurite characteristics of ES-motor neurons. Neurite outgrowth (Fig. 13 J) is one of the most commonly measured indications of neural cell health and function [547]. ES-motor neurons displayed aberrant features compared to unexposed ES-neurons in all measured features (Fig. 13 I-J). These

findings provide compelling evidence for the induction of neurotoxic phenotype in ES-astrocytes by IL-1α and TNF, particularly affecting ES-motor neurons in our co-culture model. This suggests that brainstem and spinal cord specific ES-astrocytes have the capacity to undergo a neurotoxic transformation, leading to a decrease in the survival of neurons with corresponding regional identity. TBI prompts a plethora of cellular injury mechanisms [548] and is accompanied by neurotoxicity [549]. We hypothesized that factors found in TBI might play a role in inducing the neurotoxic effect of ES-astrocytes on ES-motor neurons. We based our choice of molecules to induce reactivity astrocytes on a recent study [521] investigating proteins associated with severe TBI in humans. We observed the highest upregulation of the JNK pathway, as indicated by P-c-Jun levels, in cells treated with IL-1β and IL-6 [Fig. 13 B, p= 0.035]. IL-1β and IL-6 are considered key neuroinflammatory mediators in both experimental and clinical TBI contexts [550]. Given their potential interdependence [551], we chose to evaluate them together in our experimental setup. IL-1β and IL-6 activated ES-astrocytes also exhibited neurotoxic properties as assessed by decreased number of ES-motor neurons (Fig. 13 K). This demonstrated that our ES-derived co-culture system effectively models astrocyte-mediated neurotoxic effects using clinically relevant neuroinflammatory stimuli.

Understanding the complex communication between astrocytes and motor neurons in the context of regional identity may offer potential targets for therapeutic interventions aimed at mitigating neuronal loss and preserving motor function in relevant neurological conditions. There have been approaches to modulate astrocyte responses to reduce their neurotoxic actions. One potential candidate for such modulation is transforming growth factor-beta (TGF-β) [552], which is upregulated during severe TBI in humans [521]. In our hands, the induction of astrocyte reactivity using TGF-β did not induce astrocyte-mediated neurotoxicity (Fig. 13 K). Additionally, a recent study demonstrated that a combination of three cytokines, including TGF-β1, improved outcomes following TBI [157]. Paradoxically, the same study also showed that IL-6 was beneficial for TBI outcome [157]. This contrasts with our findings, where a combination of IL-1β and IL-6 resulted in astrocyte-induced neurotoxic effects. Also, previous research implicated IL-6 in neurological dysfunction following mild TBI [553]. Further investigation is required to determine whether these observations are attributed to a differential impact of IL-6 on peripheral and CNS immune cells.

Modulating astrocytic neuroinflammation may represent a potential therapeutic approach prior to the replacement of damaged CNS neurons. We think that our results provide a valuable platform for studying the underlying mechanisms and potential therapeutic interventions for

region specific astrocyte-mediated neurotoxicity in the context of neuroinflammation. In conclusion, our findings suggest that region-specific ES-astrocytes have the capacity to undergo a neurotoxic transformation in response to the disease-relevant cytokines, leading to the loss of region-specific motor neurons. This model could be used to elucidate the role of astrocytes in neurotoxicity and their potential as therapeutic targets in neurological disorders.

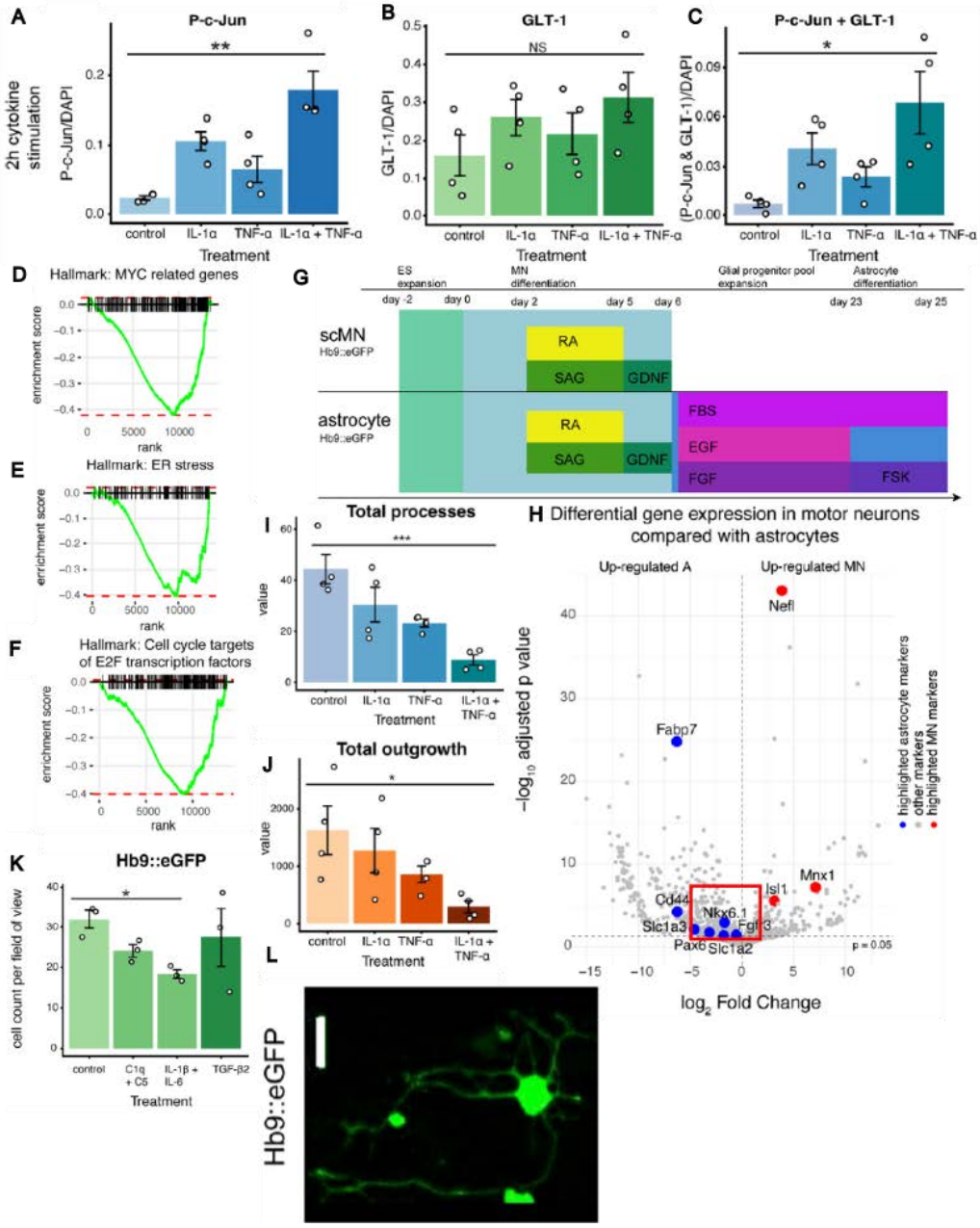
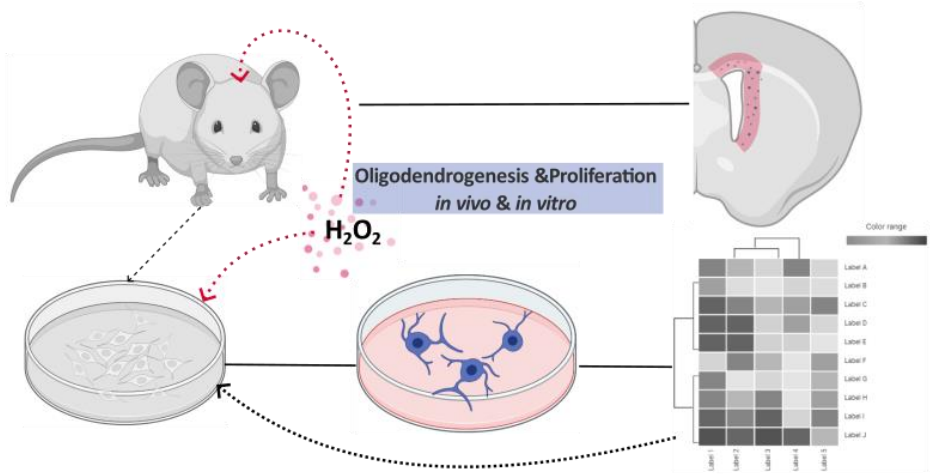


Figure 13 **A** At 2 h, P–c-Jun was increased in treated groups, **B** while GLT-1 expression was similar across treatment groups, **C** indicative that the maturity state of the cells was not altered, and that the astrocyte-like cells were afflicted by this stimulus **E-F** tentative pathways in data set of which some **(F)** are also implicated in the c-jun N terminal kinase pathway **G** Differentiation of embryonic stem cells into brainstem/spinal motor neurons and astrocytes. By mimicking neurogenesis in vivo, brainstem/spinal motor neurons followed by astrocyte-like cells from the same regional niche were generated **H** Hypothesis-generating polyA + bulk-RNA-sequencing of FACS-sorted reactive astrocytes, red rectangle indicating Nkx6.1, a brainstem astrocyte specific gene **I - J** presentation of altered neurite morphology in surviving and dying motor neurons **K** astrocyte-mediated neurotoxic effect on motor neurons following astrogliosis induced by IL-1β and IL-6, **L** Hb9 expressing motor neurons, scale bare 25µm.

4.4 MANUSCRIPT



Here we assessed the impact of H_2O_2 on NSCs, a molecule readily released during inflammation and implicated in various CNS pathologies. These are the main findings:

1.) Exposure to H_2O_2 increased the proliferation of NSCs and the number of oligodendrocytes both *in vitro* and *in vivo.*

2.) Transcriptomal analysis indicated that H_2O_2 induces a shift from neuronal development to differentiation with focus on the plasma membrane and cell projection organisation.

3.) Preliminary data – screening for the candidate genes to engage in H2O2 signaling.

Exposure to H2O2 increased the proliferation of NSCs and the number of oligodendrocytes both in vitro and in vivo.

Why did evolution provide the CNS with poor scavenging processes and high susceptibility to ROS? The brain is not only subjected to the adverse effects of ROS but also benefits from the role of ROS as a signalling molecule. Oxygen levels are crucial factors influencing brain development and neurogenesis during embryogenesis until adulthood, as well as during inflammation accompanying CNS pathologies [554]. Among the by-products generated during oxygen metabolism and during immunoreactions mainly released by microglia, H_2O_2 has emerged as a key factor. H_2O_2 signalling has been linked to NSC survival, proliferation, and differentiation. However, the downstream effects of H_2O_2 are complex since they are dependent on the context and H_2O_2 concentration. Low levels of H_2O_2 within the physiological range promote NSC proliferation and differentiation, suggesting its engagement in neurogenesis and brain development. Excessive or prolonged exposure to high levels of H_2O_2 can induce oxidative stress and damage cellular components, leading to impaired NSC function and subsequent neurodegeneration.

NSC niches within the CNS are characterized by a unique microenvironment that maintains low oxygen (O2) conditions [555]. This niche plays a crucial role in regulating NSC behaviour and fate determination. Interestingly, while being surrounded by low oxygen in the niche, proliferative NSCs exhibit high endogenous levels of ROS [556]. We wanted to explore the effect that pathological concentrations of H_2O_2 exert. We therefore exposed primary NSCs to 100 µM of H_2O_2, a concentration reported in wound healing [557] and CNS pathologies [182]. Our undifferentiated cultures exhibited 17.7% proliferating cells 1h after the exposure (Fig. 14 A). Using flow cytometry, a more sensitive quantification method, we also confirmed a significant increase in EdU+ cells 8h post-H_2O_2 exposure. Additionally, when compared to unexposed controls, we observed 4 % more $SOX10^+$ cells in the H_2O_2-exposed cultures after 1h, and up to an 8 % increase after 8 h exposure (Fig. 14 B). EDU is incorporated into newly synthesized DNA during the S phase of the cell cycle hence not capturing all proliferative cells but giving a good estimate of proliferative activity [558]. Sox10 has been reported to direct NSC to an oligodendrogenic fate [559]. $SOX10^+$ NSCs predominantly differentiated into oligodendrocytes, but there is still a possibility for cells to differentiate into neurons and astrocytes, which is reported to be dependent on developmental stage and brain region [560-562]. We therefore also assessed the phenotype profile of differentiated cells in the respective cultures 7 days after H_2O_2 removal. We detected a significant increase in GALC expressing cells

in the group exposed for 8h to H_2O_2 (Fig. 14 C, G). GALC is a lysosomal protein, part of myelin. GALC is abundant in differentiated, axon-myelinating oligodendrocytes [563]. This increase concords with previous research from our group conducted in rats [221]. Our new observations suggest the existence of a conserved mechanism across different species, thereby emphasizing the robustness of the obtained results. We furthermore assessed the effect of H_2O_2 on NSCs *in vivo* by injecting 10 mM of H_2O_2 into the cisterna magna. This is a way to access the cerebrospinal fluid to deliver ROS to the SVZ, which is steadily flushed with CSF. It is also a standard practice to measure inflammatory markers in the CSF due to its accessibility [521], and the CSF is a promising path for drug delivery to less accessible sites in the CNS [564]. We hence believed this to be a good route for delivery of H_2O_2. However, we were aware that, despite intra-cisternal injections (IC) being minimally invasive, we could not exclude inflammatory signals provoked by the injection itself. Therefore, the control animals also received an intra cisternal injection. We applied a much higher concentration *in vivo* than we used *in vitro* to account for more complex antioxidant defence line in a tissue context, and due to the fact that CSF in mice is turned over 12-13 times a day, thus limiting the exposure from a single injection [565]. Despite these limitations we observed an increase in the number of $OLIG2^+$ oligodendrocytes in the area surrounding the ventricles, as well as increased expression of Ki67 seven days after H_2O_2 injection (Fig. 14 D, E, F). This data shows that the effect of H_2O_2 exposure *in vitro* is translatable into the *in vivo* situation. It will still be necessary to double-stain the $KI67^+$ cells with a NSC marker, e.g. SOX2, or birthdate these cells using EDU pulsing, to confirm the identity of the cells. In prior investigations, it was demonstrated that within MS lesions of human post-mortem brain samples, the SVZ exhibited an elevated level of proliferation. Notably, a subset of these neuroplastic cells expressed SOX10 and OLIG2, which were detectable in demyelinated MS lesions [403]. Similar findings were observed in murine models [399, 400]. Enhancing the production of OPCs and mature oligodendrocytes by amplifying mechanisms already in place holds potential for facilitating regeneration, as newly generated oligodendrocytes are known to produce myelin in greater quantities compared to old ones [344, 566]. There are, however, reports using human post-mortem tissue from MS patients contradicting the importance of newly formed oligodendrocytes in regeneration [567]. Gaining a comprehensive understanding of the connection between inflammation and the potential for regeneration is crucial for the development of therapeutic strategies aimed at promoting repair and recovery in neurological conditions. Hence, we need to assess the integration and functionality of the cells we observe.

Transcriptome analysis revealed that H_2O_2 induces a shift from neuronal development to differentiation with focus on plasma membrane and cell projection organisation.

We extracted RNA from undifferentiated NSCs in culture directly after 1 hour and 8 hours of H_2O_2 exposure, respectively, along with corresponding control cultures to assess the direct effects of H_2O_2 exposure on global transcriptome changes. Using the DESeq2 Package in R-Studio we analysed differential gene expression between these groups, identifying several hundred genes that surpassed the ± logFC expression (FC =2) cut-off. Five clusters were detected. When assessing expression patterns over time, two types of patterns emerged. Clusters 2,3 and 5 display similar expression patterns, as do clusters 1 and 4 (Fig. 14 H-I). We analysed these as functionally corresponding groups. Hence, we refer now to group 1 (clusters 1 and 4) and group 2 (clusters 2,3 and 5). It was apparent that clusters contained in group 2 seem to be in accordance with observed phenotypical changes, such as increased proliferation and formation of oligodendrocytesCluster 5 is overrepresented in the control and remains at control levels at 1h but decreases at 8h. It represents nervous system development and neurogenesis and scores highest in the biological regulation categories – indicative of a more stem cell like state as we expect in control samples. These categories decrease at 8h – a timepoint which we believe represents a shift towards a differentiation state. At the 8h timepoint cluster 2 and 3 show an overrepresentation of genes involved in metabolic processes and cellular components categories related to membranes. This could be indicative of a switch to more differentiated cells such as glia cells. When considering oligodendrocytes and especially membrane production in the form of myelin sheets, it is indicative for their differentiation [568, 569] and requires high metabolic effort [570]. In our hands, the exposure to H_2O_2 for 8h provided a more pronounced effect on oligodendrocyte differentiation (Fig. 14) which could be related to the changes represented by transcriptome analysis.

Preliminary data – screening for the candidate genes to engage in H2O2 signalling.

We furthermore tried to identify potential candidate genes guiding oligodendrocyte differentiation post-H_2O_2 exposure. We used Ingenuity Pathway Analysis (IPA) to aid with the interpretation and analysis of biological pathways, networks and molecular interactions (Fig. 14 F). Mapping all significant genes from our Deseq2 transcriptome analysis to identify genes associated with oligodendrogenesis, oxidative stress, stem cell differentiation and their potential upstream regulators provided us with a list of candidates containing genes involved in oligodendrocyte lineage development such as NOTCH, TCF7L2 as well as ROS sensors Nrf2, HMOX1, FOXO1. Interestingly most of these genes are found in clusters 3 and 5 belonging to

group 1 (Fig. 14 I). As of now, Hmox1 presents the most suitable candidate gene as we could validate its expression using qPCR. Heme oxygenase–1 (Hmox1) is an enzyme involved in heme metabolism, known to be responsive to ROS. Hmox1 expression has been shown to act neuroprotective in stroke [571] and Hmox1$^{-/-}$ presents with increased levels of demyelination in a multiple sclerosis model (experimental autoimmune encephalomyelitis (EAE)) [572]. It is not yet known if there is a connection between Hmox1 expression, ROS and the induction of oligodendrogenesis.

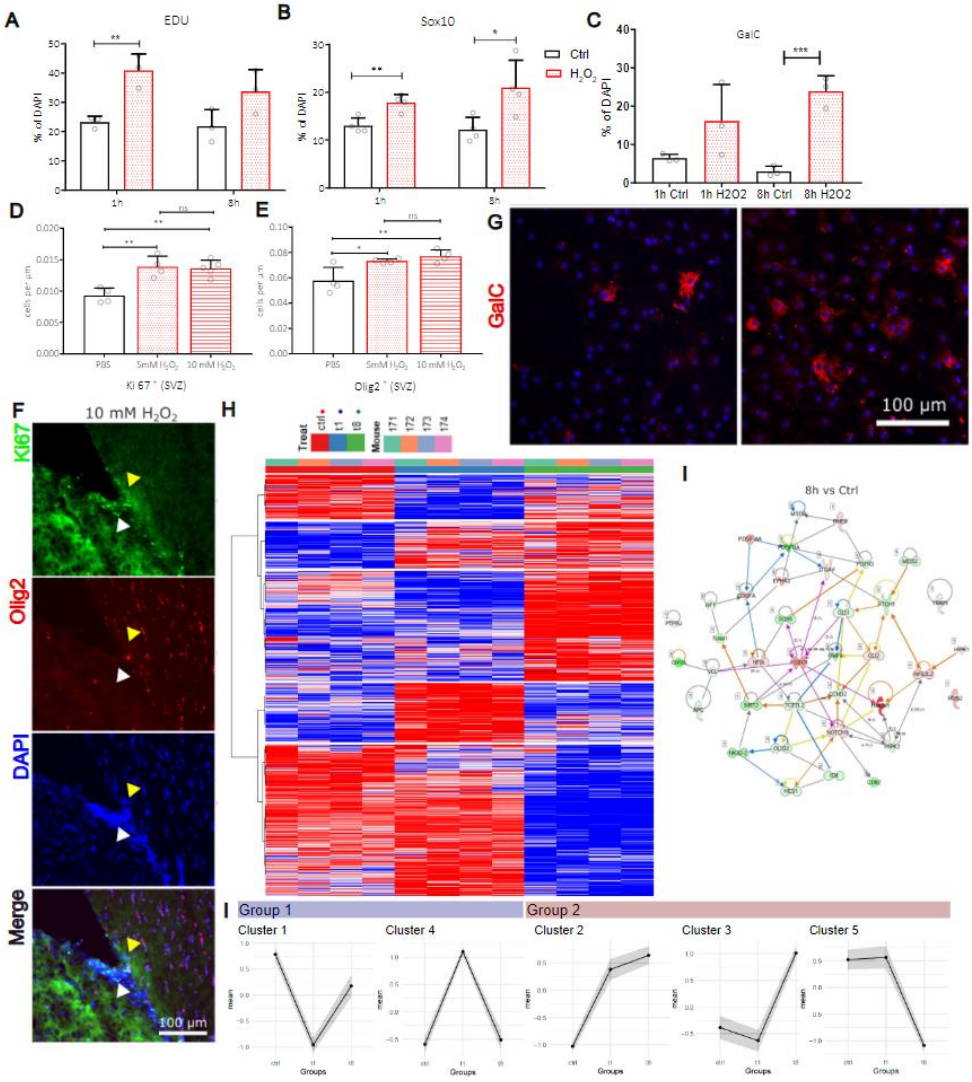


Figure 14 **A** Quantification of the percentage of EdU+ cells (EdU+/DAPI+), **B** Quantification of the percentage of $SOX10^+$ cells ($SOX10^+$/$DAPI^+$) yellow arrowheads point out SOX10 positive staining, **A-B** present undifferentiated cultures treated with 100 µM H_2O_2 for 1h respective 8h **C** Quantification of the percentage of $GALC^+$ cells ($GALC^+$/$DAPI^+$), **C** presents differentiated cultures treated with 100 µM H_2O_2 for 1h respective 8h A-C control (white columns) and H_2O_2-exposed cultures (red columns). Bars represent mean±SEM. n=3, **p>0.01, ***p>0.001, **D** Quantification of the number of Ki67+ cells ($Ki67^+$/$DAPI^+$) per µm at 7d post ICJ (Ctrl n= 4, 5 mM H_2O_2 n= 4, 10 mM H2O2 n= 5), **E** Quantification of the number of $Olig2^+$ cells (Olig2+/DAPI+) per µm at 28d post ICJ (Ctrl n= 4, 5 mM H_2O_2 n= 4, 10 mM H_2O_2 n= 4), **F** coronal section from animal injected with 10 mM H_2O_2 F are example images for tissue harvested 7 days post injection. $KI67^+$ cells in green, $OLIG2^+$ cells in red, nuclear counterstain using DAPI in blue and all channels merged. **G** Phenotypical representation of differentiated cells positive for GALC after exposure to H_2O_2 for 8h (left) compared to control (right image). $GALC^+$ cells (red) and DAPI nuclear counterstaining (blue). **H** Heatmap presentation of RNAseq data, red represents overexpression, blue underexpression **I** Kinetic development of gene expression changes over time, clusters grouped together based on their expression dynamic **J** IPA derived network of genes involved in oligodendrogenesis and/or ROS response at 8h H_2O_2 exposure vs Ctrl.

Overall, our findings shed light on the intricate network of gene interactions involving oligodendrocyte lineage development and ROS sensors, but which of these genes are directly implicated in the changes we observe post-H_2O_2 exposure remains to be elucidated.

5 SIGNIFICANCE AND FUTURE PERSPECTIVE

Paper I: We demonstrated that NSCs can modulate the inflammatory environment they have been introduced into, as well as generate differentiated cells, both of which improve tissue functionality. We analysed the effect of transplantation 8-10 days post-SCI targeting the acute phase of SCI. Access to greater numbers of NSCs generated from autologous as well as allogenous cells shortly after SCI is required but will be difficult in clinical practise. The NSC culture protocol we used spans approximately 2 weeks and receiving suitable NSCs to initiate a culture is improbable. It would be interesting to challenge the knowledge gained from paper I using other more practical cell types, as the extraction of NSCs is usually not an option. Co-transplants with other cell types [448] or transplantations into the sub-acute phase of SCI, accommodating for culturing times, would be a future step to make a transition into therapy more feasible. Additionally, the assessment of glial-scar formation in our experiments would be essential. Transplanted NSCs also differentiated into astroglia, known to form glial scars post injury [350]. Understanding the extent of this process and attempting to modulate the astrocyte formation from NSCs could lead to further improvement of recovery. Eventually, the assessment of autonomic functions, such as bladder control, post-transplantation would be important. Losing autonomic functions is one of the burdens post SCI [573] and improving these deficits would contribute to higher quality of life [574].

Paper II: We have also observed that the stem cell response to insults in the CNS is limited if animals were exposed to irradiation in juvenile stages of life, indicating potential long-term consequences of irradiation in terms of recovery after damage. This paper would benefit from a more in-depth analysis of the astroglia response to stroke post-IR as an unchanged GFAP response in the penumbra does not reflect all aspects of astrocyte functionality. Examining the trophic support that the astrocytes provide post irradiation could contribute to the understanding of the decreased proliferation we observed. Gene ontology analysis at different timepoints post irradiation and stroke would uncover the changes these cells undergo in a more comprehensive fashion. In paper II we did not assess oligodendrocytes. Oligodendrocytes contribute to signal conduction [323] as well as the immune environment [49]. In respect to my manuscript, I think it would be essential to also assess the impact of irradiation on oligodendrocyte formation and function. Furthermore, interventions managing irradiation-induced inflammation [575] directly after IR should be tested to understand cognitive impairments and CNS pathologies post-IR.

Paper III: We created a region-specific model for neuroinflammation, which might become a useful tool to study the effects of inflammatory mediators. Furthermore, it is novel to recreate an anatomical CNS niche *in vitro*, giving us the possibility to characterize processes in a setting closer to the *in vivo* situation. This provides a possibility to study interactions between astrocytes and neurons in a specific region of the CNS. The characterisation of the neurotoxic phenotype adopted by astrocytes is incomplete. Elucidating the exact mechanism that astrocytes exert on neurons and the potential intervention remains to uncovered [512, 576]. It would also be interesting to address if it is only the astrocytes near the inflammation which transition into a neurotoxic phenotype. What will be the consequences of inhibited neuronal death *in vivo* post injury? Is it beneficial to support survival of potentially damaged neurons?

Manuscript: Here we assessed the impact of pathological concentrations of the inflammatory mediator H_2O_2 on NSC differentiation and demonstrated increased proliferation and formation of oligodendrocytes *in vitro* as well as *in vivo*. We identified transcriptional changes in treated cells supporting these observations. In the future, we plan to further elucidate the pathways involved in mediating these effects and to identify the key genes involved in driving the oligodendrocytic fate. This could be addressed by modifying gene expression through *in vivo* using transfections or transduction using viral vectors. We furthermore need to confirm that the formed oligodendrocytes we see *in vivo* arise from stem cells and not from e.g. OPCs using for example EdU labelling. It would be here also beneficial to know if long-term exposure of cells *in vivo*, e.g. with the help of a pump, elicits the same or different cellular responses. It would be interesting to see if it is possible to steer NSC development to e.g., replace cells needed or affected during a disease.

Inflammation is a complex physiological response that plays a crucial role in tissue homeostasis, immune defence, and repair. More importantly, in neurodegenerative diseases, stroke, and traumatic brain injury, inflammation is a hallmark feature that has a significant impact on NSC function. Excessive and/or chronic inflammation can have detrimental effects on NSCs, which are responsible for neurogenesis and maintaining neural tissue integrity. Understanding the influence of inflammation on NSCs helps unravel the intricate mechanisms underlying neurodevelopment and adult neurogenesis as well as helping us to better understand the pathophysiological mechanisms involved in the CNS injury, repair, and regenerative processes. In this thesis, I have explored the interaction between NSCs and inflammatory processes and their mediators. I believe that our data have provided valuable and novel insights into the regulation of NSC proliferation, differentiation, and migration, thereby shedding light on brain

plasticity and cognitive function. This knowledge is critical for developing new strategies to modulate inflammation, to protect NSCs, and to enhance endogenous repair mechanisms in diseased or injured CNS.

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