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FIBROSIS IN THE CENTRAL NERVOUS SYSTEM: THE ROLE OF PERIVASCULAR CELLS

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Cover illustration: Chaos or Concert – The fibrotic injury core under construction during the subacute phase, five days after complete spinal cord crush injury in mouse; Perivascular cells leave the vasculature, proliferate and become reactive/myo-fibroblasts. Labelled were all fibroblasts and mural cells with Pdgfrb+ expression (EGFP), GLAST+ derived fibrotic cells (tdtomato), Myofibroblasts and smooth muscle cells (Transgelin - SM22), Endothelial cells (Podocalyxin), Nuclei (DAPI) – here presented with inverted colours – by Fraulein

Fibrosis in the central nervous system:
The Role of Perivascular Cells
Thesis for Doctoral Degree (Ph.D.)

By

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To Helena, Astrid and Greta

“Not everything that counts can be counted,
and not everything that can be counted counts.”

WB Cameron

ABSTRACT

Regeneration in the adult mammalian central nervous system (CNS) is very limited. One limiting factor is the formation of chronic scar tissue, which inhibits axonal regeneration and functional recovery. While scar formation has been recognized for more than a century, research on the origin and function of the fibrotic scar component has been mostly neglected. The objective of this thesis is to thoroughly characterize the origin of fibrotic scar forming stromal cells and their transformation upon spinal cord injury. Furthermore, we aimed to decipher if fibrotic scar formation and the contribution of perivascular cells to fibrosis is a general mechanism, in brain and spinal cord and in response to different kinds of lesions. And ultimately, the goal was to determine, if modulation of fibrotic scarring represents a therapeutic potential to achieve functional recovery after CNS injury.

The Göritz laboratory previously established GLAST$^+$ perivascular cells, named type A pericytes, as the origin of fibrotic scar tissue after spinal cord injury. In **paper III**, we employed genetic *in vivo* lineage tracing to heritable label the GLAST (Glutamate aspartate transporter) expressing subpopulation of Pdgfrβ (Platelet-derived growth factor receptor beta) positive perivascular cells in combination with single-cell RNA sequencing to characterize the cells at the molecular level. Our results show that GLAST$^+$ perivascular cells encompass pericytes and perivascular fibroblasts, based on their transcriptome. To distinguish between pericytes and perivascular fibroblasts, we genetically labeled perivascular fibroblasts, using an inducible *Col1a1-CreER^{T2}* transgenic mouse line. Our results show that GLAST$^+$*Col1a1*$^+$ fibroblasts are more readily observed along larger diameter penetrating blood vessels in the spinal cord white matter, while GLAST$^+$*Col1a1*$^-$ pericytes partially cover the abluminal surface of smaller vessels in the arteriole-capillary transitional zone. Importantly, both populations contributed to stromal fibroblasts in fibrotic scar tissue. Remarkably, cells derived from perivascular fibroblasts contributed mostly to the white matter portion of the scar, while pericyte progeny mainly contributed to grey matter areas, together establishing heterogeneity of fibrotic scar composition.

The aim of **paper II** was to determine fibrotic tissue formation in response to different kinds of lesions in brain and spinal cord, in mice and humans. Furthermore, we asked to which extend GLAST$^+$ perivascular cells contribute to fibrotic tissue in different parts of the CNS and in response to distinct lesions. For this, we compared fibrotic tissue formation as well as the contribution of GLAST$^+$ perivascular cells after complete crush-, contusion- (paper III), dorsal hemisection- (paper I), and dorsal funiculus incision spinal cord injury, large cortical, cortico-striatal brain stab wound lesions, striatal-, cortical- and striatal-cortical ischemic stroke lesions, experimental autoimmune encephalomyelitis-induced lesions and the Gl261 glioblastoma model. In all lesion models and pathologies investigated, we found stromal tissue formation. However, the cellular arrangement and ECM distribution was lesion dependent. In all lesion models in which stromal fibroblasts accumulated outside the vessel wall, the vast majority was derived from GLAST$^+$ perivascular cells, except for the Gl261 glioblastoma model. We also showed that stromal tissue is formed upon spinal cord injury, multiple sclerosis, stroke and glioblastoma in humans and that a subset of pdgfrb$^+$ perivascular cells in the human brain and spinal cord expresses GLAST (*SLC1A3*). Our results show that fibrotic scarring by GLAST$^+$ perivascular cells is conserved throughout the CNS.

In **paper I,** we investigated the therapeutic potential of mitigating fibrotic scarring after spinal cord injury by genetic reduction of GLAST$^+$ perivascular cell proliferation. We demonstrate that decreased fibroblast accumulation is attended by reduced deposition of

extracellular matrix in the injury core, modulated glial scar architecture and diminished inflammation, leading to increased regeneration of corticospinal- and raphespinal tract axons. Furthermore, regenerated corticospinal tract axons functionally integrate caudal to the lesion as shown by electrophysiologic recordings upon optogenetic activation. Mice with reduced perivascular cell-derived scarring and the highest number of regenerated axons showed best recovery of sensorimotor functions.

In summary: Various CNS pathologies trigger fibrosis by perivascular fibroblasts and a subset of pericytes in a region-dependent manner. Interfering with the scarring process, to moderately reduce fibrotic scarring by $GLAST^{+}$ perivascular cells, may represent a strategy to improve functional recovery after several detrimental CNS maladies.

POPULAR SCIENCE SUMMARY OF THE THESIS

The central nervous system is made up of brain and spinal cord. It coordinates all activities and processes information throughout the body. Information travels by long nerve fibres called axons which transmit messages to neurons or to the periphery. This is why injuries to the spinal cord, the major connection between brain and peripheral organs, lead to partial or complete paralysis or loss of sensation. Unfortunately, not much is known about how to repair damage to the central nervous system. Mammals have a very limited capacity to regenerate injured tissue. While small incisions might heal completely, larger injuries often lead to the replacement of functional structures by connective tissue – a process called fibrosis.

A severe injury of the spinal cord destroys axon connections and in an attempt to repair the damage, a complex cellular response is induced that results in the formation of a scar. Although nerve fibres can regrow to a certain extent, the scar tissue and the environment around it prohibit functional nerve fibre regeneration. Only recently we learned that the core of the fibrotic injury that impairs regeneration is produced by a specific type of cells that normally resides around blood vessels in the central nervous system.
Our understanding of brain and spinal cord pathologies has been greatly enhanced by studying animal models. Since it is more or less impossible to analyse cellular and molecular details of central nervous system maladies directly in human. In this thesis, we worked with different mouse injury and disease models to understand the effects of fibrosis after damage to the central nervous system.

First, we wanted to know more about the types of cells that sit around the vasculature in the central nervous system, to be able to specifically target the ones which produce fibrotic tissue. We isolated cells that wrap blood vessels in the spinal cord and used a method that is called RNA single cells sequencing. This technique allows us to obtain information about all genes that are active at a certain timepoint. By these means we could study single cells and identify groups depending on how similarly they activate their genes. Interestingly, we discovered that the cells that can form fibrotic tissue can be divided into two cell types: fibroblasts residing around large blood vessels and a subpopulation of pericytes on smaller vessels. We showed that both are active in fibrosis but each of the cell types contributes specifically to one part of the scar.

Further, we were interested in understanding if fibrotic scarring also happens in other injuries or diseases of either brain or spinal cord and if fibroblasts and pericytes were involved in this. We studied different types of traumatic spinal cord and brain injuries as well as stroke, a model for multiples sclerosis and brain tumors. After most of the injuries or diseases, cells rapidly detached from the blood vessels, and increased extensively in number. Only within two weeks the number of cells could increase 100 times. But we also found that each and every type of lesion has its specific characteristics. For instance, stroke in one region of the brain led to cell proliferation but no fibrotic scar formation, whilst the type of brain cancer we studied only generated a small response of the fibroblasts we followed. Intriguingly, we found a similar cell type in human spinal cord and brain showing that fibrotic scarring is also a mechanism that takes place in human injuries and diseases.

Last but not least, we tried to find out if reducing the number of fibrotic cells could reduce scar tissue and help regenerating destroyed nerve fibres. By using a specific genetic technique to reduce the number of cells to be responsible for the formation of the scar. We found that the fibrotic tissue after spinal cord injury was reduced which translated into a different composition of the injured area as a whole. Strikingly, this generated an environment that

allowed some axons to grow through the injured tissue and we were able to proof that these nerve fibres were functional and improved the paralysis symptoms of the research animals. Altogether this work shows that fibrotic scaring through cells surrounding blood vessels happens in different central nervous system diseases, and that two different cell types are responsible for this process: fibroblasts and pericytes. By knowing which cells to target, we open the door to new research avenues leading to improving regeneration in common diseases of the central nervous system which are otherwise almost impossible to treat nowadays.

POPULÄRWISSENSCHAFTLICHE ZUSAMMENFASSUNG DER DOKTORARBEIT

Fibrose im zentralen Nervensystem: Die Rolle Perivaskulärer Zellen

Gehirn und Rückenmark gehören zum zentralen Nervensystem. Dieses koordiniert die Aktivitäten und Prozesse des gesamten Körpers. Informationen zwischen den Nerven, oder zwischen Nerven und der Umgebung werden durch lange Nervenfasen weitergeleitet, die man Axone nennt. Daher führen Schäden des Rückenmarks, der Hauptverbindung des Gehirns mit den anderen Organen, teilweise oder ganz zu Lähmungen oder Gefühlstaubheit (Querschnittslähmung). Leider wissen wir noch nicht sehr viel darüber, wie wir Schäden des zentralen Nervensystems heilen können. Säugetiere können verletztes Gewebe nur in sehr begrenztem Umfang reparieren. Während kleine Wunden komplett verheilen können, führen größere Verletzungen zum Austausch von funktionierendem Gewebe mit Bindegewebe (auch Narbengewebe), in einem Prozess, den man Fibrose nennt.

Eine schwere Verletzung des Rückenmarks zerstört Axonverbindungen. Bei dem Versuch, den Schaden zu reparieren, wird eine komplexe Zellantwort in Gang gesetzt, die häufig zur Bildung einer Narbe führt. Obwohl die Nervenfasen nach einem Schaden etwas wachsen können, verhindert die Narbe und das sie umgebende Milieu funktionierende Regeneration. Erst kürzlich haben wir herausgefunden, dass der innerste Teil der Narbe, welcher Regeneration behindert, durch spezielle Zellen gebildet wird, die normalerweise eng entlang der Blutgefäße des zentralen Nervensystems sitzen.

Unser Verständnis über Krankheiten das Gehirns und des Rückenmarks verdanken wir zu einem großen Teil Tierexperimenten, da es weitestgehend sehr schwierig ist zelluläre und molekulare Zusammenhänge und Details des zentralen Nervensystems direkt am Menschen zu untersuchen. In dieser Studie haben wir verschiedene Mausmodelle für Krankheiten und Verletzungen benutzt, um den Einfluss der Narbe nach Schädigung des Nervengewebes zu untersuchen.

Zuerst wollten wir mehr über die Zellen, welche die Blutgefäße des zentralen Nervensystems umschließen, herausfinden, um dann später genau die Zellen behandeln zu können, die Fibrose bilden. Dazu haben wir die Zellen vom umgebenden Gewebe und den Blutgefäßen gelöst und eine Methode benutzt, die sich RNA Einzelzellsequenzierung nennt. Diese erlaubt es die Aktivität der Gene in sehr vielen einzelnen Zellen gleichzeitig zu messen. Damit können wir bestimmte Zellgruppen anhand der Genaktivität identifizieren und einzelne Zellen genauer untersuchen.

Dabei haben wir herausgefunden, dass zwei verschieden Zelltypen zur Bildung der Narbe nach Schädigung des Rückenmarks beitragen. Zum einen Fibroblasten, die wir an größeren Blutgefäßen im zentralen Nervensystem finden und zum anderen Pericyten, die auf kleineren Gefäßen mehr im inneren Teil (der grauen Substanz) des Rückenmarks sitzen. Wir konnten aufzeigen, dass beide aktiv zur Fibrose beitragen.

Außerdem wollten wir wissen, ob fibröse Narbenbildung auch in anderen Verletzungen oder Krankheiten des Hirns oder Rückenmarks durch Fibroblasten oder Perizyten gebildet wird. Dazu haben wir verschiedene Arten von Hirn- und Rückenmarkschäden, sowie Schlaganfall, ein Modell für Multiple Sklerose und Hirntumore analysiert. In den meisten dieser Verletzungen oder Krankheiten lösten sich Pericyten und Fibroblasten von den Blutgefäßen und hundertfachten sich innerhalb von nur zwei Wochen. Gleichzeitig haben wir aber auch gesehen, dass jede Gewebeschädigung ihre Eigenheiten hat. Ein Schlaganfall in einer Hirnregion etwa führte zu Zellwachstum, aber ohne, dass diese die Blutgefäße verließen. Im Hirntumor trugen die Zellen der Blutgefäße nur wenig zur Fibrose bei.

Wir haben auch Gewebe vom Rückenmark und Hirn des Menschen untersucht und konnten Fibroblasten/Pericyten ähnlich zu denen der Mäuse finden. In Gewebeproben von Personen mit Querschnittslähmung, Hirnschlag oder Multipler Sklerose fanden wir vergleichbares Narbengewebe zu dem der Maus.

Im letzten Teil dieser Studie wollten wir wissen, ob sich durch die Reduzierung von Fibroblasten und Pericyten das Narbengewebe so verändern lässt, dass Nervengewebe neu gebildet werden kann. Dazu haben wir ein spezielles genetisches Modell verwendet, das es ermöglicht, die Anzahl der sich teilenden Fibroblasten uns Pericyten zu verringern. Dabei fanden wir heraus, dass sich weniger Narbengewebe bildet und sich dadurch die Zusammensetzung der gesamten Wunde verändert. Bemerkenswerterweise konnten einige Axone durch die verringerte Narbe hindurch wachsen. Wir konnten zeigen, dass diese auch funktionierende Verbindungen mit anderen Nerven eingehen konnten, was tatsächlich zu einer Verbesserung der gestörten Feinmotorik der Versuchstiere führte.

Zusammenfassend zeigt diese Arbeit, dass Fibrose in verschiedenen Krankheiten des zentralen Nervensystems durch Zellen entlang der Blutgefäße gebildet wird und zwei verschiedene Zelltypen dafür verantwortlich sind: Fibroblasten und Pericyten. Damit ebnen wir den Weg zur Identifizierung von spezifischen Behandlungsmöglichkeiten mit diesen Zellen als Ziel. Zusammen mit anderen Therapien kann uns das ein Stück weiter auf dem Weg zu verbesserter Regeneration des Nervensystems bringen.

List of scientific papers

I. David O. Dias, Hoseok Kim, **Daniel Holl**, Beata W. Solnestam, Joakim Lundeberg, Marie Carlén, Christian Göritz† and Jonas Frisén†
Reducing Pericyte-derived Scarring Promotes Recovery after Spinal Cord Injury
Cell; 2018; 173: 153-165

II. David O. Dias *, Jannis Kalkitsas*, Yildiz Kelahmetoglu, Cynthia Perez Estrada, Jemal Tatarishvili, **Daniel Holl**, Linda Jansson, Shervin Banitalebi, Mahmood Amiry-Moghaddam, Aurélie Ernst, Hagen B. Huttner, Zaal Kokaia, Olle Lindvall, Lou Brundin, Jonas Frisén and Christian Göritz
A Pericyte Origin of Fibrotic Scar Tissue Across Diverse Central Nervous System Lesions
Nature Communications; 2021; 12, 1-24

III. **Daniel Holl**, Wing Hau, Shervin Banitalebi, Jannis Kalkitsas, Soniya Savant, Enric Llorens-Bobadilla, Yann Herault, Guillaume Pavlovic, Mahmood Amiry-Moghaddam, David Oliveira Dias and Christian Göritz
Pericytes and perivascular fibroblasts contribute to central nervous system fibrosis in a region dependent manner
(*Manuscript*)

† denotes Co-corresponding authors
* denote equal contribution

Scientific papers not included in the thesis

Matthijs C Dorst, María Díaz-Moreno, David O Dias, Eduardo L Guimarães, **Daniel Holl**, Jannis Kalkitsas, Gilad Silberberg†, Christian Göritz†; Astrocyte-derived neurons provide excitatory input to the adult striatal circuitry; *Proc Natl Acad Sci;* 2021; 118(33)

Contents

List of abbreviations

ACTA2	Alpha-actin, also alpha smooth muscle actin (αSMA)
APOE	Apolipoprotein E
BBB	Blood brain barrier
BM	Basement membrane
BSCB	Blood spinal cord barrier
cAMP	cyclic Adenosine monophosphate
CNS	Central nervous system
CSF	Cerebrospinal fluid
CSPG	Chondroitin sulphate proteoglycan
CST	Corticospinal tract
CSV	Cerebrospinal fluid
DCN	Decorin
dMCAO	Distal middle cerebral artery occlusion
DNA	Deoxyribonucleic acid
EC	Endothelial cell
ECM	Extracellular matrix
FACS	Fluorescence-activated cell sorting
FN	Fibronectin
GFAP	Glial fibrillary acidic protein
GJA1	Gap junction alpha-1 protein, also connexin 43 (Cx43)
GJB6	Gap junction beta-6 protein, also connexin 30 (Cx30)
GLAST	Glutamate aspartate transporter 1, gene name Slc1a3
LUM	Lumican
MCAO	Middle cerebral artery occlusion
MS	Multiple Sclerosis
NG2	Neuron-glial antigen 2 (gene name Cspg4)
NG2-glia	also Oligodendrocyte precursor cell, OPC
OPC	Oligodendrocyte precursor cell, also NG2-glia
Osm	Oncostatin M
Osmr	Oncostatin M receptor
PDGFRA	Platelet-derived growth factor receptor alpha (gene name Pdgfra)
PDGFRB	Platelet-derived growth factor receptor beta (gene name Pdgfrb)
PVF	Perivascular fibroblasts
PVM	Perivascular macrophages
PVS	Perivascular space
RNA	Ribonucleic acid
RST	Raphespinal tract
SCI	Spinal cord injury
SPP1	Secreted phosphoprotein 1, also Osteopontin
TAGLN	Transgelin, also SM22
TBI	Traumatic brain injury
TGFβ1	Transforming growth factor beta
TGFβR1	Transforming growth factor beta receptor I
TNF-α	Tumor necrosis factor (alpha)
UMAP	Uniform Manifold Approximation and Projection for Dimension Reduction
vSMC	Vascular smooth muscle cells

1 INTRODUCTION

Wound healing is a highly orchestrated process, involving the cooperation of several different cell types in a very specified order.

After the initial damage bleeding has to be stopped by vessel constriction and blood coagulation (hemostasis). Quick thereafter, follows the infiltration of immune cells to prohibit infection and clear up the injury area (inflammation). This sets the stage for cells proliferation, and the deposition of extracellular matrix setting the scaffold for regeneration (proliferation). If everything has worked out so far, the wound contracts and closes, the matrix is remodelled and the tissue architecture is restored (remodelling) (1).

Unfortunately, the regenerative capacity in human is very limited. While small incisions e.g. in the skin might heal completely, larger damages lead to the replacement of functional tissue and the accumulation of extracellular matrix. The excessive production of connective tissue is called fibrosis, and scarring when it appears in response to an injury. In many organs formation of scar tissue can be well tolerated. However, in the central nervous system formation of fibrotic scar tissue has wide-ranging consequences.

The central nervous system only works as a network of interconnected neurons which gather, send and process signals within the brain and throughout the body. A destroyed connection between brain and the periphery e.g. the spinal cord can lead to long-lasting functional impairment. Damage to spinal cord or brain results in death of neurons and destroys the connection between them. Equally affected is the surrounding tissue including glial cells and the vasculature. Initially more destruction can be avoided by the rapid formation of scar tissue and sealing of the injured area, but in the long run, it is exactly this seal of non-neural tissue and the environment around it that blocks regeneration of functional neuronal connections.

While fibrotic processes in other parts of the body have been studied extensively (2), the knowledge about scar formation in brain and spinal cord is very limited. Only recently it was discovered that cells residing in the perivascular niche of the central nervous system are responsible for fibrotic scarring (3). This observation boosted the interest to study fibrosis in the context of many different maladies of the central nervous system. A view over the horizon and learning from what is known about scar formation and tissue healing in other contexts might accelerate the identification of novel targets and treatment options for many devastating pathologies.

This thesis attempts to discuss heterogeneity of perivascular cells in brain and spinal cord and hopefully contribute to the information needed to identify targets for the treatment of severe central nervous system maladies.

2 LITERATURE REVIEW

2.1 Perivascular cells and perivascular space in the CNS

The CNS vascular tree (Figure 1) is composed, like in other organs, of an endothelial tube, the surrounding extracellular matrix (ECM) and a heterogenous population of perivascular cells. The endothelial tube is organised in arteries, arterioles, interconnected capillaries, venules, and veins. One specialty of the CNS vasculature is that vessels are additionally ensheathed by a layer of astrocyte endfeet along the parenchymal basement membrane, the glia limitans perivascularis an integral part of the blood brain barrier (BBB) and the meningeal epithelium on larger vessels (4).

The cerebrospinal fluid (CSF) fills the perivascular space (PVS) between glia limitans, pia mater and the vascular basement membrane. The PVS surrounding penetrating vessels is called Virchow-Robin space (5), containing perivascular macrophages (PVM) (6–8) which could be discerned from microglia in parabiosis and lineage tracing experiments (9). Further along the vascular tree, the meningeal layer discontinues and the glia limitans becomes the sole barrier between the PVS and the parenchyma (10,11).

Pericytes cover the abluminal surface of CNS microvessels and are embedded in the endothelial basement membrane. Characteristically they share the same developmental origin with vascular smooth muscle cells which envelop the endothelium of larger vessels and together they are called mural cells (12,13). Perivascular fibroblasts (PVF) are located around large vessels passing through the pia mater, in connection to smooth muscle cells (14–17), but cover as well penetrating arterioles and venules (16,18).

Demarcation, functional differences, marker profiles and potential heterogeneity of both mural cells and PVF are subject of ongoing discussion which I will address in this thesis.
In the following sections I will restrict the term “perivascular cells” to mural cells and perivascular fibroblasts (16,19,20).

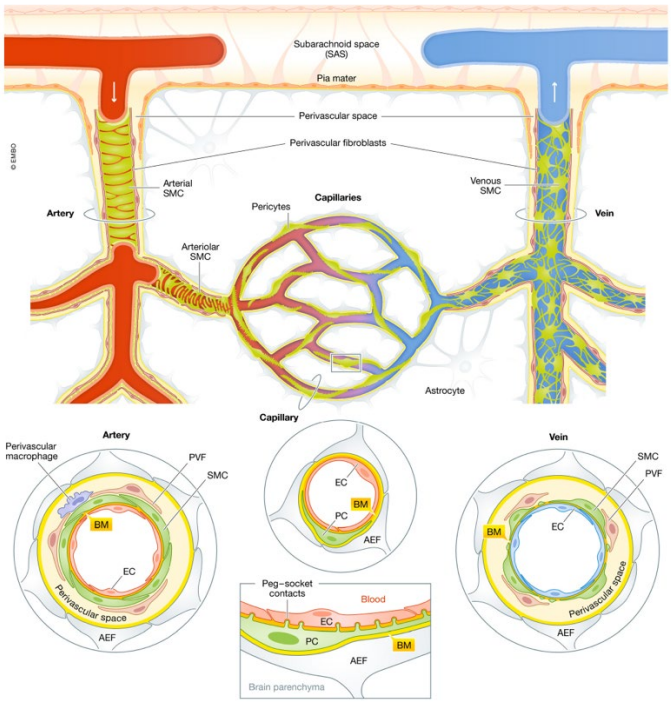

Figure 1 - The vasculature of the brain
Schematic sagittal view through the cortical vasculature. Penetrating pial arterioles and veins branch from feeding pial arteries/veins of the subarachnoid space (SAS) and penetrate perpendicular to the surface into the cortex. They are covered by smooth muscle cells (SMC) and initially surrounded by the pia mater, encased by the parenchymal basement membrane and astrocyte endfeet (AEF) (glia limitans). There is a continuous transition from smooth muscle cells to pericytes as the vessels split into smaller arterioles. Pericytes (PC) are encased in the endothelial basement membrane and cover the capillary vasculature. At locations where the basement membrane is interrupted, pericytes and endothelial cells form peg-socket contacts. Perivascular fibroblasts (PVF) are positioned abluminal to mural cells (if present) of larger penetrating vessels and the vasculature of the transition zone to the capillary bed. Perivascular macrophages reside in the perivascular space (PVS).
Reproduced from Lendahl et al. 2019 (275).

2.2 Origin of perivascular cells in the CNS

The origin of perivascular cells does not necessarily have to influence their functionality or persistent differences in the adult organism. However, given the functional differences of distinct cell populations in specific circumstances, for example upon injury, identification of the cellular origin can help understand inherent cell heterogeneity.

2.2.1 Mural cells

While endothelial cells of brain and spinal cord derive completely from the mesoderm (21,22), the origin of mural cells is not finally resolved. CNS mural cells proliferate and expand following vascular sprouts from embryonic day 11.5 (E11.5) in mouse. During embryonic development, mural cells mostly cover arteries (23,24)and pericytes and vascular smooth muscle cells cannot be told apart (25). Mural progenitor cells might even appear before endothelial cell differentiation (26,27). Studies in mouse, chicken and zebrafish conclude that the majority of CNS mural cells stems from the neural crest, especially in the fore- and midbrain (28–31), while other studies find heterogeneity in the developmental origin and suggest a mesodermal origin, especially of hindbrain mural cells (23,28,31). In particular, pericytes are thought to be derived either from neural crest (ectoderm) or mesothelium (mesoderm) depending on the context and organ where they develop (12). Interestingly, in recent years, different studies have discussed the possibility of pericytes having a hematopoietic origin (32,33).

2.2.2 Fibroblasts

Fibroblasts are a heterogeneous cell population and they differ depending on the organ and location within the tissue (34–37). Tissue resident fibroblasts maintain ECM and tissue structure and remain quiescent (non-proliferative) until stimulated (38). Their developmental origin is from primary fibroblasts, derived from the primary mesenchyme before separation into endoderm and mesoderm (38).

Besides the heterogeneity of tissue fibroblast populations, the picture becomes even more complex when including perivascular cells that contribute to fibrotic tissue formation upon injury but are not *per se* fibroblasts. These could be pericytes, pericyte-like cells, mesenchymal stem cells or other “mesenchymal” cells with varying differentiation status (3,38–46). These fibroblast-like cells can have manifold origin and might, for example, be generated through epithelial- (47) or endothelial- to mesenchymal transition (48).

Myofibroblasts (49,50) describe a reactive state of stromal cells with a transient expression of a contractile apparatus (49,51–53). Importantly, also in a reactive state there is heterogeneity of different fibrogenic populations (54) where myofibroblasts constitute only a subset or transient state.

With regards to the CNS, literature about fibroblast origin is more limited and covers mostly meningeal fibroblasts. This is in part due to the fact that the existence of perivascular fibroblasts in the brain only recently received broader attention. Particularly, single cell RNA sequencing studies revealed CNS fibroblast populations in both mouse (20,55–57) and human (58,59).

Most brain developmental studies focus on the origin of bone and meninges, which similarly to mural cell development, indicate an anterior neural crest origin of meninges and a mesodermal origin for the posterior regions in quail-chick and mouse embryos (21,60,61). Fibroblasts of the three meningeal layers and different brain regions are heterogeneous and show distinct gene expression patterns (62). Meningeal fibroblast markers appear later in

development than mural cells, from E12 to E14, in a ventral to dorsal manner (62,63). Remarkably, *Col1a1*-expressing perivascular fibroblasts on penetrating cerebral vessels first appear postnatally (64). While at birth *Col1a1*-expressing cells are almost absent in the mouse brain, their numbers increase steadily during the first 21 days. The first fibroblasts can be detected along vessels, close to the pial surface and later further within the brain (64). This suggests that perivascular fibroblasts could be meningeal-derived and either migrate along the vasculature or follow the vasculature in the growing brain in the early postnatal development (64,65). However, it cannot be ruled out that existing perivascular cells, such as mural cells, start to express *Col1a1* during postnatal development. Besides *Col1a1*, PVFs share gene expression of other genes, such as *Pdgfra*, with pial fibroblasts (62,64). At the same time, PVFs also have certain expression patterns in common with mural cells (20). Studies of mural cell colonisation during CNS vessel development are mainly based on *Pdgfrb* expression (23,66) and might as well cover PDGFRß$^{+}$ perivascular cells that later during development acquire a fibroblast identity, that is, *Col1a1* or *Pdgfra* expression.

It is important to point out that developmental origin is not the only factor contributing to cellular identity or function. The microenvironment or the specific niche may have a crucial role in different perivascular cell types once they are differentiated. Nonetheless, it would be relevant to understand when fibroblasts and mural cells diverge. Current literature still allows for speculation, and it could be that both fibroblasts and mural cells are derived either from neural crest or mesenchymal origin and differentiate into different cell types solely depending on their location. Another alternative could be that mural cells throughout the CNS derive from the neural crest (30) while fibroblasts are of mesodermal origin. Moreover, fibroblasts and mural cells could be derived from multiple sources and/or recruited at different timepoints.

Another important consideration when exploring the origin of perivascular cells in the CNS is the actual position along the neural tube in the embryo. The neural tube, along which the different parts of the CNS develop, spans the whole anterior-posterior axis of the early embryo which might subsequently translate into regional differences in the developing vasculature (Kurz, 2009). It is therefore important to acknowledge these facts when comparing data from spinal cord and brain or even between different spinal cord and brain regions.

2.3 Functional aspects of vasculature and perivascular cells in the CNS

In contrast to other organs, the entrance of blood plasma, nutrients or immune cells to the central nervous system is highly limited and regulated. The function of the blood brain and blood spinal cord barrier (BBB and BSCB, respectively) is based on the interaction of endothelial, perivascular and glial cells and their non-cellular component, the basement membrane (BM) (67). Next, I will summarise some aspects of the BBB/BSCB.

2.3.1 Blood brain barrier

Paul Ehrlich described in 1885 the lack of staining penetrating the brain in his studies about the oxygen requirements of the organism (68). Later, injections via the cerebrospinal fluid stained structures of the central nervous tissue but not other organs (69). These observations opened a new area of studies focusing on brain perfusion. Max Lewandowsky suggested a barrier function between brain capillaries and cerebrospinal fluid, which required much higher drug concentrations in the periphery to reach the same effect or clinical symptoms in the brain as if injected in the subarachnoid space (70). Detailed electron microscopy studies,

in conjunction with the injection of horseradish peroxidase, led to the identification of tight-junctions between endothelial cells as a physical barrier between the vasculature and the adjacent brain tissue (71). Besides the tight junction dependent limit of paracellular flow of solutes, brain endothelial cells (ECs) allow only restricted transcellular vesicle-mediated transport (72).

The first cellular barrier is of great importance for the integrity of the BBB. However, the same degree of importance should be awarded to the non-cellular components of the BBB. Endothelial, perivascular cells and glial cells produce basement membranes which are specific forms of ECM interacting and communicating with the underlying cells to maintain the barrier function (73,74).

The terms basement membrane (BM) and basal lamina are used side by side in many publications about the BBB. Both terms seem clearly defined but do not describe exactly the same in different tissues or contexts. The BM includes all layers of ECM on the basal side of epithelial or endothelial cells while basal lamina does not include the fibronectin rich "lamina reticularis" (75). Differentiation of the different BM layers can only be achieved with electron microscopy and might partly be tissue preparation artefacts (76,77). BMs are not exclusive for the vasculature, they also define epithelia and encapsulate tissues (78)(79). Moreover, BMs of adjacent tissues can fuse and form stable connections, for example during the development of glomeruli in kidney, the alveoli in lung (80,81) or the vasculature of the central nervous system (67).

The basement membrane of the CNS vasculature has a specific ECM composition based on type IV and XV/XVIII collagens, laminins, nidogen, perlecan, fibronectins (FNs) and tenascins (82). Together, these ECM proteins give structural support, enable cell anchoring and mediate signal transduction. Nonetheless, blood vessels of the CNS vascular tree differ in size, wall thickness and have different BM composition. Especially the composition of different laminin isoforms has been studied in detail (83,84). For instance, the endothelial BM contains mainly laminins α4 and α5 (α2 in larger vessels). Additionally, blood vessels in the CNS are ensheathed by the laminin α2 rich parenchymal BM (19,83–85) (figure 2). This specific BM contributes to the restricted permeability of the CNS vasculature (14) and is produced by both pericytes and astrocytes (86,87). The parenchymal BM is associated to astrocyte endfeet and partly microglial processes (88,89), it is part of the glia limitans, a thin barrier surrounding the brain and the spinal cord on the external side, towards the subarachnoid space (glia limitans superficialis) and internally around the vasculature (glia limitans perivascularis) (90). Astrocytic endfeet constitute a continuous layer covering microvessels in the brain (91), forming a perivascular space, which is essential for water and metabolite exchange, as well as signalling along the vasculature (92).

In bigger vessels the different basement membranes are separated. Pial arteries are composed of a monolayer of endothelial cells, vascular smooth muscle cells (tunica media), connective tissue (tunica adventitia) and are lined with pial cells. In penetrating arterioles, the pial cells are adjoined by the astrocytic endfeet and form together the parenchymal basement membrane (14,84,85). Following the vascular tree, there are no more pial cells in smaller arterioles and vascular smooth muscle cells are replaced by pericytes and the parenchymal and vascular BM appear as one (4). In capillaries, vascular and parenchymal basement membranes are fused and, together with endothelial tight junctions, the high number of pericytes limits transcellular diffusion of solutes (93). In postcapillary venules, parenchymal and vascular BM might be separated, at least in disease where a perivascular space can appear (94) (Figure 2). Extravasation of immune cells happens mainly at the post capillary venules

through receptor mediated adhesion and diapedesis first into the perivascular space and then with the help of matrix metalloprotease by breaking the perivascular glia limitans (95).

In homeostasis mural cells regulate vessel permeability and BBB integrity. Mice with deficiency of Pdgfβ (platelet-derived growth factor beta) or Pdgfrβ (platelet-derived growth factor beta receptor) lack pericytes completely in the CNS, which leads to vessel dilation and haemorrhage and is embryonic lethal in genetic mouse models (24,96). In adult mice pericytes are especially required to regulate transcytosis (19). Animal models with varying numbers of pericytes show that the absolute coverage determines vascular permeability (97). Pericytes are defined by being embedded in the vascular BM ("basal lamina") (98). It is not known to which extend PVF contribute to the BM and BBB function.

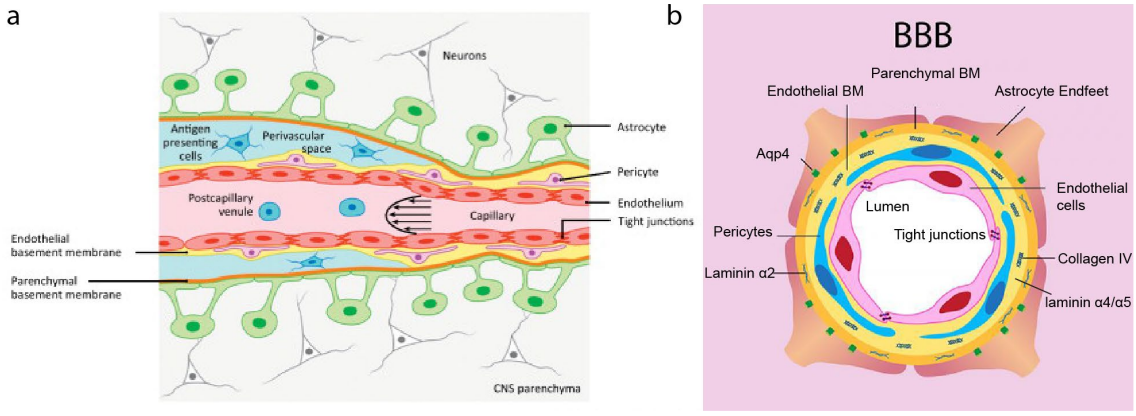

Figure 2 - Illustration of Blood brain barrier
(a) Transition from capillary to postcapillary venule, parenchymal and endothelial basement membrane separate and open up for the perivascular space. Pericytes are embedded in the endothelial basement membrane.
(b) Coronal view through capillary. Endothelial cells with tight junctions are the primary barrier, covered by the endothelial basement membrane which encases pericytes and is rich in laminin α4/α5 and collagen IV. The parenchymal basement membrane contain laminin α2 and separates astrocyte endfeet from the vasculature/perivascular space. a) Reprinted from (420), with permission from Elsevier; b) modified (421)

2.3.2 Blood spinal cord barrier

Many of the results of studies on the BBB can be transferred to the BSCB, which has very similar in function and morphology. As a matter of fact, many previous studies have referred to both anatomical structures as the same or rather as the BSCB being an extension to the BBB. Some findings point towards a slightly higher permeability of the BSCB in comparison to the BBB, due to reduced expression of tight and adherence junction proteins (reviewed by (99)), which might have implications especially in pathophysiology (100). Upon spinal cord injury it takes only a few minutes until the BSCB breaks down (101) with restauration only in the chronic phase after injury (102).

2.4 Extracellular Matrix

Besides the compact BM (see above), the CNS extracellular space is bound together by a complex network of highly organised interstitial ECM (103,104). In contrast to the BM, the interstitial ECM contains less fibrous collagen and instead more adhesive fibronectin and more proteoglycans, hyaluronan and tenascins (105–107).

2.4.1 Wound healing and fibrosis

In the first phase after an injury the existing ECM matrix is destroyed, and growth factors and cytokines infiltrate from the broken vasculature. Plasma proteins form a fibrin enriched primary ECM which is stabilised by fibroblasts. Resident and invading cells use this net to migrate and proliferate (108). Cytokines, like PDGF (Platelet-derived growth factor) promote cell proliferation and the production of versican and hyaluronan rich ECM (109,110). This creates an environment for inflammatory cells to accumulate (111,112). With the initial invasion of immune cells, fibroblasts become activated, transform into myofibroblasts (expression of α-SMA) and migrate along the primary fibrin matrix to the lesion site (113). Myofibroblasts are the main cell type to contribute to collagen rich ECM deposition and wound closure (114). Activation and proliferation of myofibroblasts is promoted by TGF-β (Transforming growth factor beta), PDGF (Platelet-derived growth factor beta) and IGF-1 (Insulin-like growth factor-1) signalling and mechanical tension (108,115). Fibronectin is readily produced and secreted by fibroblasts (116). Binding to integrin cell surface receptors mediates self-association into an insoluble form and matrix assembly (117). Continuous polymerisation and an intact fibronectin matrix are required for the assembly of other ECM proteins, especially collagen-I (118). In wound healing this matrix of fibrin, fibronectin and collagens is permanently assembled and disassembled and forms a scaffold for myofibroblast mediated wound contraction (119,120). In fibrosis, fibrillar collagen rich ECM is overproduced and functional tissue is permanently replaced by a fibrotic scar (113).

2.4.2 Collagens

Collagens makes up around 30% of the total protein mass in mammals and they are the major structural extracellular matrix protein (121). Expression of collagen (especially *Col1a1* and *Col1a2*) is commonly used as markers for fibroblasts (18,122). Until today, 28 different collagen types make up the collagen superfamily (named with roman numbers I-XXVIII) and their common feature is the presence of homo- or heterotrimeric triple helices formed by alpha polypeptides (123,124). Different collagen types are produced by various cell types and have diverse functions. However, the variety of different collagen types goes beyond different alpha polypeptides, isoforms and structures, since there is a long list of other molecules containing collagen motifs as well (121).

Collagens can be classified by the 3D structure they form. For instance, types I, III and V (mainly produced by fibroblasts) together with II, XI, XIV and XVII have one major triple helical domain to form fibrils, which can be detected as bands in electron microscopy (121,125).

Type I collagen heterotrimers are usually assembled by two α1 and one α2 chains. Absence of α2 ends up in formation of α1 homotrimers, impairing fibril formation and decreasing degradability (126,127). The sequence of the α2 chain permits specifically heterotrimerisation (128) and absence or inactivation of pro- α2(I) collagen, for example due to mutations in the *COL1A2* gene, leads to severe disease (*e.g.* reduced body size, hyperelastic tissue or osteogenesis imperfecta) due to the formation of α1 homotrimers in human and experimental mouse models (129–131).

Type I collagen is a rather stable protein with a low turnover rate under physiological conditions. A study in healthy volunteers concluded a fractional synthesis rate for dermal collagen of around 2% per day (132), which can be compared to more than 10% for albumin (133). However, upon injury the production of collagen can be upregulated dramatically within a few days (134,135). The fast regulation of collagen production cannot solely be

explained by upregulated transcription, which would not allow for such a rapid response on its own (136,137). Experiments in hepatic stellate cells show that post-translational mRNA stabilisation plays an important role in the quick increase in collagen production upon cell activation (138). This stabilisation is linked to binding of collagen mRNA to non-muscular myosin (139), suggesting a link between acquisition of motor function/cell migration and collagen production in myofibroblasts upon injury.

Other cell types different from fibroblasts also produce collagens. For example, collagen IV is mostly produced by epithelial and endothelial cells, and is together with laminin, nidogen and perlecan the main component of the basement membrane (140). Type IV belongs together with VI, VIII, and X to the group of network-forming collagens (124).

2.5 Functional aspects of perivascular cells in CNS injuries

2.5.1 Central nervous system scar formation

Lesions to the CNS often cause neuronal death and functional impairment. Brain and spinal cord can be damaged by trauma (spinal cord injury: SCI, traumatic brain injury: TBI), vascular disorders (stroke, hemorrhage), tumors (like glioblastoma), autoimmune disorders (demyelinating diseases like multiple sclerosis: MS), infection (encephalitis) or degeneration (like Alzheimer's disease). Common amongst most CNS insults is a limited regeneration capacity (141), while partial or full regeneration is possible in other parts of the adult human body (142), including the peripheral nerve system (PNS) (143). Aspects responsible for this phenomenon are both limited intrinsic regenerative capacity of adult neurons and the formation of chronic glial and fibrotic scars with a complex immune response (144–146).
Following, I will highlight some aspects of spinal cord injury response and conclude with reflections on other CNS injuries.

2.5.1.1 Spinal cord injury

Responses to both acute CNS injuries and chronic diseases trigger multicellular effects with complex interactions of local neural-lineage and non-neural cells, as well as blood or bone marrow-derived cells (146,147). Traumatic spinal cord injury as a result of an incision, crush, contusion or compression serves in many studies as a model for the analysis of the mechanisms and multicellular interactions in the repair process (147).

The different events following injury are complex and have to be considered in their spatio-temporal context.

From a temporal point of view, the events that follow an acute spinal cord injury can be distinguished in three interlinked phases of response (summarized in figure 3). Most of the research about spinal cord injury has been performed using mice and rats, therefore the sequences described below are generally based on small rodent research. Even between mice and rat there are differences for example regarding immune cell infiltration (148):

- **Acute phase**: The primary damage of the spinal cord is characterized by tissue disruption and cell death of neuronal, glial and non-neuronal cells and rupture of the vasculature (149–151). Following this initial event, a secondary injury with tissue swelling, local ischemia, oxidative damage, hypoxia and inflammation takes place (152,153). The acute phase lasts between 24 and 48 h from the moment of injury.

- During the **subacute phase**, from about two to fourteen days after injury, glial and non-neural cells interact with infiltrating immune cells and become reactive. Local astrocytes, ependymal cells, oligodendrocyte precursor cells (OPCs), perivascular fibroblasts and pericyte-derived cells proliferate, contain inflammation and initiate wound healing (3,154–157). At this stage, the glial and fibrotic scars are formed and deposited ECM starts to replace functional tissue. By the end of the subacute phase a glial barrier encases the fibrotic core containing fibroblasts and infiltrated immune cells (3,158,159). Re-establishment of the vasculature is initiated in the first week after injury, but blood vessel and BSCB integrity are not completely recovered yet (151,160–163).

- From about two weeks following the initial insult, the injured region transitions to an intermediate/**chronic phase** marked by tissue remodelling and formation of a persistent CNS scar (145,164,165). The scar tissue condenses and the composition of glial and fibrotic ECM changes (166,167). The acute phase immune reaction abates, but a pro-inflammatory environment persists (168–170)

From a functional or spatial point of view the mature lesion can be subdivided into different, communicating compartments. Distal to the injury core persists spared neural tissue, intermingled with reactive astrocytes, followed by the glial scar, forming a border which encloses the fibrotic core:

2.5.1.1.1 Neural tissue

Injury-induced cell death happens mainly within the first day. It starts within minutes and peaks around 8 hours after injury (151,171). Neuronal death happens either directly through the mechanical impact of the damage, as a secondary response due to exposure to toxic debris and excitotoxic glutamate release (172) or due to prolonged ischemia (173). Dying astrocytes and neurons release high levels of glutamate which cannot be cleared by the remaining astrocytes (174,175). This glutamate accumulation can lead to neuronal receptor overactivation, massive calcium influx and finally excitotoxic death (176). Immune cells that are recruited to the injury core for clearance of cellular debris extend the reaction and induce further damage (177) by releasing cytokines like TNF-α (Tumor necrosis factor) directly after injury, which potentiates the damaging glutamate effect (178).

Upon injury, axons that are not severed directly go into a metastable state with axonal swelling and start to exhibit dystrophic growth cones (179). The environment that forms around the injury site by the astrocyte-derived glial scar induces a dystrophic growth state. Proximal axons stabilise in a steady position outside the injury core, after phases of dieback and extension (180,181) where they can persist for years (182). The distal side of the axon can remain functional over several weeks but starts to degrade in a process termed Wallerian degeneration (160,183).

Axonal regeneration capacity is limited (184). Spontaneous sprouting is possible (185,186), although not enough for functional recovery on its own. Sensory axons have a greater regenerative ability than motor axons (185–188). Axons either have to cross the lesion core or in incomplete injuries the spared neuronal tissue. Alternatively, severed fibres of the corticospinal tract must first contact intraspinal neurons that bridge the lesion, which on their part must form contacts with transected tracts (189).

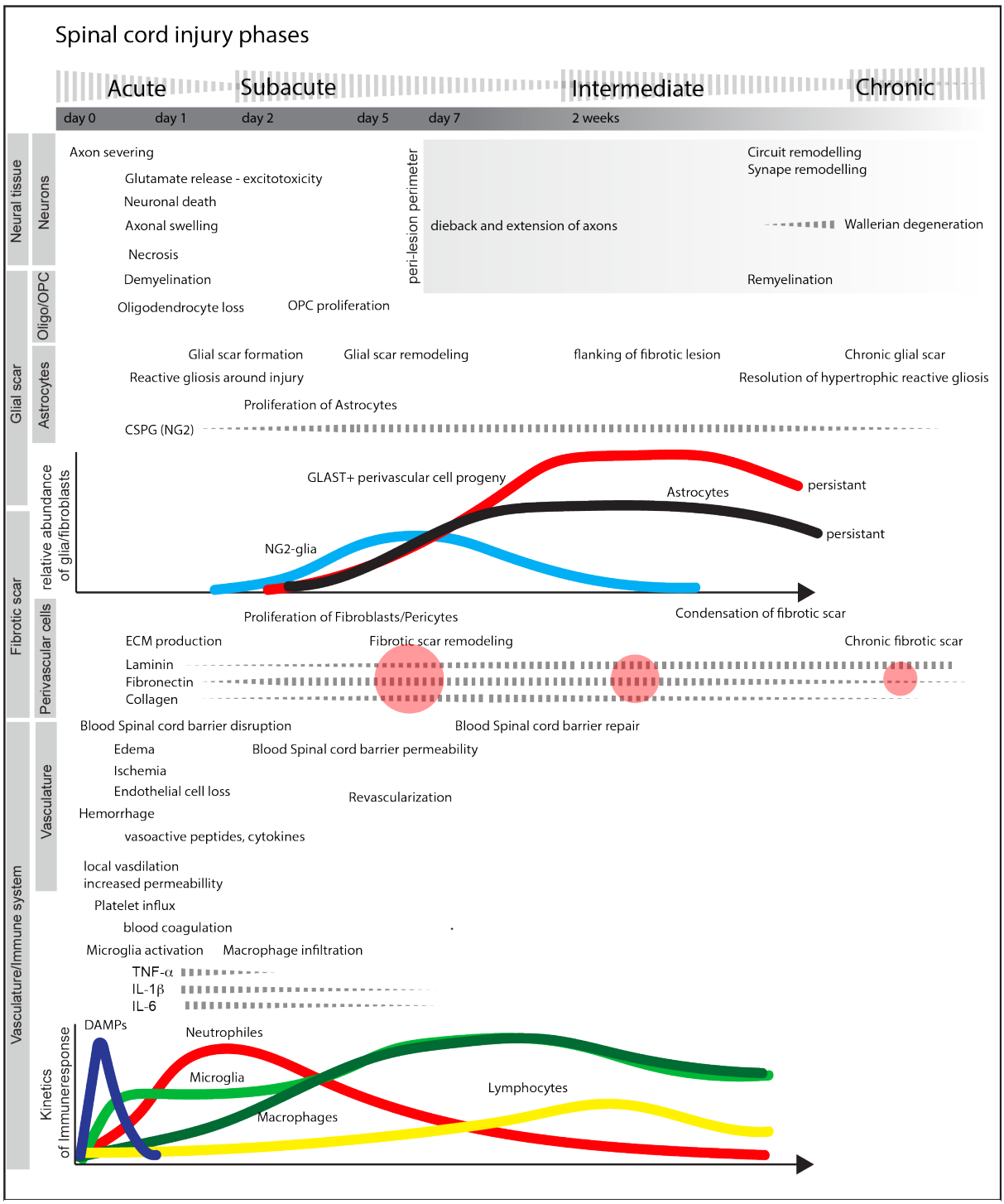

Figure 3 - Phases and time course of spinal cord injury pathophysiology.
This scheme illustrates the time course of the events after spinal cord injury, separated according to the different chronic injury compartments. Of note, in the acute and subacute phase the different parts of the injury scar have not formed yet. Neural and non-neural cells interact with each other and the microenvironment and events overlap spatial and temporal. Insult to the spinal cord triggers a complex sequence of events. In the **acute phase** cell death and inflammation are predominant, followed by the **subacute phase** with cell proliferation and tissue replacement. About two weeks after the initial insult the **intermediate phase** starts, characterized by tissue remodeling and transitions into the **chronic phase** with a persistent glial and fibrotic scar.
Adapted in parts from (147) with addition of dynamics of: Laminin (163), Fibronectin (116), CSPG (247,422), Collagen (163), GLAST+ perivascular cells (3), Astrocytes(157), NG2-glia (155), Immune cells (227), DAMPs (218);
Red circles: Relative size of lesion core area – complete spinal cord crush (Paper II).

2.5.1.1.2 Glial scar

Astrocytes are the main glial cell type and are crucial for both neuronal function and the BBB/BSCB, and upon injury, astrocytes become reactive and form an outer layer enveloping the fibrotic injury core, known as the glial scar (190).

Astrocytes react within hours to proinflammatory cytokines (156) by upregulating activation markers (191), intermediate filament proteins, such as glial fibrillary acidic protein (GFAP) and Nestin (192) and by becoming hypertrophic (154). Among other factors, astrocyte reactivity is triggered by the release of TNF-α from activated microglia (193). After injury astrocytes start proliferating and double in number within two weeks in the region closest to the injury core (154). In the early subacute phase, reactive astrocytes (194) promote tissue repair by containing inflammation (195) and formation of a barrier towards the fibrotic compartment. In the maturing glial scar reactive astrocytes produce ECM proteins, mainly chondroitin and keratan sulphates, which have an inhibitory effect on axon growth (158,159,196). However, chondroitin sulphate proteoglycans (CSPGs) are not produced solely by astrocytes (197).

Astrocytes compact towards the lesion centre within the first two weeks (195). A dense glial-fibrotic scar border is formed where astrocytes meet the collagen-rich fibrotic core of the scar, and their processes extend perpendicular to the injury site (154,196). Similarly, ephrin type-B receptor 2 (Ephb2) expression on meningeal fibroblasts has been shown to interact with astrocytic ephrin B2 (Efnb2) promoting glial-fibrotic scar border formation (198,199). Newly formed, reactive astrocytes are not only present in direct opposition to the fibrotic core, but they have also been found as far as 2 mm from the injury centre in the mouse spinal cord crush injury model (154).

Depending on the type of damage to the spinal cord (200), scar-forming astrocytes derive from local astrocytes, as well as ependymal cells with stem cell potential (157,201,202).
NG2-glia are another component of the glial scar (203–205). They proliferate strongly in the first 10 days after injury (155,206) and accumulate in the lesion penumbra (207). NG2-glia express CSPGs (like NG2) and have therefore, on one hand been considered to hamper axon regeneration (208,209). However, other studies show that they rather might stabilize axons (181).

Even though the glial scar appears as a detrimental barrier for axonal regeneration (190,210), astrocyte reaction to injury is crucial for tissue repair and to limit leucocyte infiltration into spared tissue, demyelination and further neuronal death (195,211,212). Inhibition of astrocytic barrier formation or chronic scarring neither improves injury restoration, nor promotes it spontaneous regrowth of axons through the lesion core (154,165,197,211,213–215).

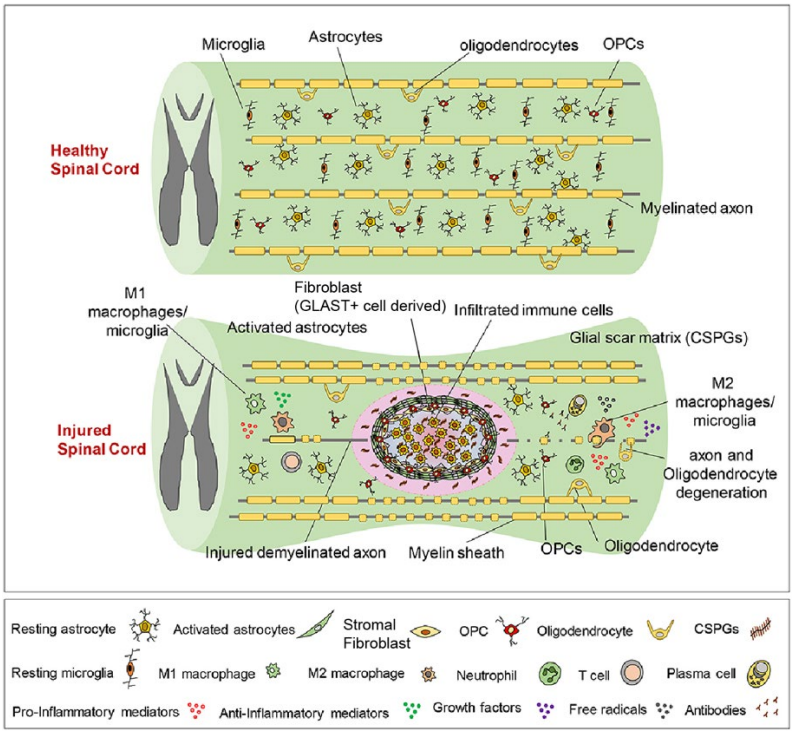

Figure 4 - Spinal cord injury scar formation
Schematic depiction of uninjured and injured spinal cord after traumatic injury. Upon injury astrocytes and microglia get activated and non-resident immune cells infiltrate the lesion. GLAST$^+$ perivascular cells leave the vasculature, proliferate and become stromal fibroblasts, forming the fibrotic lesion core. Reactive astrocytes and NG2-glia/OPCs form an outer glial scar border and enclose the fibrotic tissue and infiltrated immune cells. This inhibits primarily the spread of inflammation but hinders together with the fibrotic core, regeneration in the chronic phase. Oligodendrocyte cell death leads to axon demyelination and degradation.
Modulated version of figure by (148)

2.5.1.1.3 Immune reaction

Damage to blood vessels and disruption of the BSCB allows the infiltration of cells and even large molecules within the first hours post-injury (216).

Immediately after injury, cell damage and death results in the exposure of negatively charged surfaces and the activation of the contact system. This further activates pro-inflammatory and pro-coagulation pathways (217), as well as the release of danger-associated molecular patterns (DAMPs) (218) such as ATP (219), dsDNA, RNA or endogenous proteins like IL-1α (220) or Hmgb1 (221). Within hours after injury, proinflammatory triggers initiate leucocyte adhesion to endothelial cells and transmigration into the tissue (222). DAMPs promote rapid microglia reaction and migration towards the damaged area (223), which in the very early stages is beneficial to prevent injury expansion (224). Newly formed microglia can be detected 2 days after injury and cell proliferation peaks one week after injury (225). In the early sub-acute phase microglia are distributed over the injury core and locate at the border between the fibrotic and glial scar, within two weeks (225).

During the first hours after injury, TNFα, IL-1α -expressing microglia and astrocytes, appear at the injury site (226), release proinflammatory cytokines and promote extensive infiltration of further immune cells, such as neutrophils, within 12 hours (227,228). After a rapid upregulation of proinflammatory cytokines, TNFα levels decrease within two days and IL-1 α and IL-6 within the first week (226,229). This process is followed by an upregulation of chemokines and consequent recruitment of monocytes, T-cells, and dendritic cells within the first 48h (227). Blood-derived macrophages arrive 2-3 days after injury, peaking at one week and persist during the chronic phase (230,231). Microglia and macrophages clear neurotoxic myelin, tissue debris and dying cells (232,233). Only a minority of macrophages show an anti-inflammatory M2 pattern. The injury environment favours pro-inflammatory M1 polarisation of macrophages, which might favour secondary damage (232,234,235), by the release of reactive oxygen species or other cytotoxic byproducts (236). After the initial beneficial debris clearance in the injured tissue, macrophages turn into foam cells and maintain a proinflammatory phenotype, mostly due to the large amounts of phagocytosed

myelin debris (170). The chronic persistence of macrophages (232,237,238) at the injury site contributes to the creation and maintenance of an anti-regenerative environment (168,169). Lymphocyte accumulation at the injury site seems to differ substantially between different species. While T-cell numbers peak in the rat within the first week, they appear much later in mice (239). There is also another peak of pro-inflammatory cytokines two weeks after injury in mice (226).

The beneficial or detrimental nature of the different waves of immune reaction upon spinal cord injury are still debated (218), as well as substantial differences in immune cell composition and cytokine composition between species and even different mouse strains (227,238)

2.5.1.1.4 Fibrotic scar

The injury core is mostly composed of stromal fibroblasts, infiltrating immune cells and ECM deposits (145,240). Until recently, the CNS appeared to be exempt of resident fibroblast-derived fibrosis upon injury and disease. Most research focused on the glial scar and astrocyte-derived gliosis as the main barrier for axonal regeneration upon injury (190,211). Although a connective tissue scar surrounded by gliosis and reactive astrocytes has been described early on in the brain (241,242) its origin was mainly thought to be derived from the surrounding meninges in penetrating injuries (190,243) and astrocytes in injuries with intact meninges (240). ECM deposition in the perivascular space in lesions within the CNS has been described as well (244), but until recently the origin of this fibrotic tissue remained ambiguous.

Göritz *et al*. identified a $GLAST^{+}$ subpopulation of pericytes as the origin for stromal fibroblasts upon spinal cord injury. While it is now widely accepted that perivascular cells are the major contributors to fibrotic scar tissue in several CNS diseases (18,122), there is an ongoing debate about the expansion and origin of these cells (see Paper III and discussion). Under homeostatic conditions $GLAST^{+}$ perivascular cells (genetically labelled using GLAST-$CreER^{T2}$ transgenic mice) represent around 10% of the total $PDGFR\beta^{+}$ perivascular cell pool. From three days after spinal cord injury, they detach from the vascular wall, rapidly proliferate and reach the highest cell density around 2 weeks after injury (3)(Paper II). After one week post-injury the fibrotic core, mainly formed by these newly generated stromal cells and immune cells, starts to be surrounded by the glial scar, which limits further expansion of the lesion (212,213), followed by scar maturation and condensation.

After spinal cord injury the major structural component, hyaluronan, is degraded (245) and proteoglycans, potent inhibitors of axon regrowth are released (158). During the subacute and chronic phases reactive fibroblasts produce a dense ECM network and functional tissue is replaced by a fibrotic scar. The composition resembles that of the BM containing fibrous collagens, fibronectin and laminin (116,166,240,246). Virtually all stromal cells express *Col1a1* and PDGFRβ after injury (3,122).

Fibroblasts deposit large amounts of soluble fibronectin in the acute/early subacute phase (day 3 or earlier). However, polymerisation to fibrillar matrix fibronectin upon binding to integrin receptor, happens later and fibronectin amounts in the lesion peak at 7 days, but remain high for at least two weeks (116).

Several growth inhibitory molecules can bind to the deposited ECM, like semphorin 3a, heparan/keratan/chondroitin sulphate proteoglycans, tenacin-C or EphB2 (198,247–249) and reach high concentrations in the lesion core (116,240). Not all ECM components necessarily

inhibit regeneration, and several studies aim to modulate matrix components to benefit axon growth and tissue regeneration (240,250).

Targeting or removal of ECM components of the lesion core appears promising to improve axon regeneration (251–253). Transient inhibition of fibroblast proliferation with cAMP, inhibition of Collagen IV synthesis pharmacologically (iron chelator 2,2′-dipyridyl - DPY) or with antibodies against Col IV are examples for treatments that promoted CST axon regeneration and improved motor function (252,254–256). Another iron-chelator (deferoxamine) has shown promising results by reducing fibrosis in rat spinal cord injury (257) with additional anti-inflammatory and neuroprotective effect (258,259). Like in other organs (260) TGFβ signalling supposedly plays an important role in the regulation of CNS fibrosis (261). Inhibition of either TGFβ1 or TGFβR1/2 diminishes fibrotic scarring in brain injuries (262,263).

In human spinal cord injuries, cystic, fluid filled, cavities can be formed (post-traumatic syringomyelia), surrounded by glial and fibrotic scar tissue, establishing yet another layer of barrier for axonal regeneration (264,265). Intraspinal expanding cysts, might be diagnosed even years after the initial insult and worsen autonomic, motor or sensory functions. Cyst formation can be modelled in rat spinal cord injury but does not appear in mice (266–268).

2.5.2 Perivascular cells in other CNS injuries/diseases

2.5.2.1 Stroke

Cerebral stroke is induced by interrupted blood supply to the brain, either due to a lack of blood flow (ischemic stroke) or bleeding (hemorrhagic stroke) and leads to cell death in the affected tissue. Several models for cerebral ischemic stroke exist (269). Transient occlusion of the mouse middle cerebral artery (MCAO) mimics a common ischemic stroke type in human (270) and does not require craniectomy. PDGFRß-expressing cells accumulate in the infarcted area in mouse models of focal cerebral ischemia and human stroke (271–274) Similarly to the situation in spinal cord injury, the fibrotic ischemic core is suggested to be derived from perivascular cells, contains macrophages and is surrounded by a glial scar (64,274,275).

2.5.2.2 Traumatic brain injury

Traumatic brain injury (TBI) can be induced by mechanical external force to the brain, leading to tissue deformation, alternatively by incision of the brain. Animal models for TBI include weight drop, fluid percussion, blast or stab wound injuries (276). Neuronal death, like in spinal cord injury, is induced by direct mechanical damage, excitotoxicity (277) or due to secondary injury by BBB disruption, reduced blood flow or ischemia (278,279), edema and inflammation (280) even long after the initial insult (281,282). Perivascular cells react within hours after the impact (283), leave the blood vessels and accumulate in the trauma zone (284,285). Fibrotic scar formation appears both in non-penetrating (284) and penetrating injuries (Paper 2) and depending on the injury model meningeal or perivascular cells have been suggested as source for fibrosis (263,283,285,286).

2.5.2.3 Brain tumors

Brain tumor types are diverse and depend on the cell type of origin in the CNS (287). Glioblastoma is one of the most common, aggressive and lethal CNS tumors (288,289). A well characterized and broadly used model in glioblastoma research is transplantation of GL261 cells into the mouse brain. The GL261 line was derived from cells of chemically

induced tumor in mouse and resembles ependymoblastomas histologically (290). Pericytes play an important role in the tumor microenvironment and have been shown to modulate immune response and support tumor growth (291,292). Brain tumor stroma has increased ECM density in comparison to intact tissue (293) and high numbers of potentially perivascular-derived stromal cells and myofibroblasts (294).

2.5.2.4 Multiple sclerosis

Multiple sclerosis (MS) is an autoimmune disease where the immune system attacks the myelin sheath of axons leading to permanent damage or deterioration of nerves. Experimental autoimmune encephalomyelitis (EAE) is a commonly used model to mimic MS in mice (295). ECM deposition in the lesion has been described in mice and human demyelinating diseases (296,297) and scar formation in the white matter of spinal cord and brain are persistent and characteristic for MS (298). Recent studies identified perivascular cells as the origin of scar formation in the EAE mouse model (18,299) (paper II).

2.6 Heterogeneity of perivascular cells in the CNS

2.6.1 Functional considerations

In 1923 Zimmermann described pericytes as representatives of vascular smooth muscle cells on pre-capillary arteries, capillaries and post-capillary venules in different organs and species. He also raised the question whether pericytes are capable of contraction and blood flow regulation. He further showed large morphological variation of different pericyte types which, until today, gives rise to speculation about different functions and whether cells in the transition zone between different vessel types can be defined as pericytes or rather vascular smooth muscle cells (300).

Despite being present in numerous tissues, pericyte-vessel coverage differs considerably in different organs. In the CNS, vessels have the highest coverage of pericytes which is crucial for the control of vascular permeability, blood brain barrier formation and function (97). The role of pericytes in cerebral blood flow regulation has been debated intensively during recent years (301–305). Much of the discussion about pericyte contribution to vasoconstriction or dilation has focused on the detection or absence of a contractile apparatus in pericytes. While αSMA is clearly expressed in vascular smooth muscle cells and in adjacent pre- and postcapillary pericytes of the CNS, expression on pericytes on smaller calibre vessels has been disputed (12,306–308). A reason for the lack of αSMA antibody labelling on capillary pericytes might be due to the fast degradation of actin filaments with common staining methods (309). The minimal prerequisite for contraction in vSMC and non-muscle cells is the coordinated activity of myosin II along the filamentous-actin framework (310). In our dataset (Paper III), most of the genes related to actomyosin (GO term 0042641) are exclusively expressed by vascular smooth muscle cells, but pericytes show for example expression of *Myh9*, *Myl9*, *Myl2a/b* or *Mylk* suggesting the presence of one part of the mechanical apparatus.

However, most of the controversy about pericyte capability to regulate blood flow concerns the transition zone between arterioles and capillaries in the CNS and is rather a question of nomenclature and demarcation between pericytes or vascular smooth muscle cells (311) (summarised in figure 5). In some studies, all vessels branching from penetrating arterioles are considered capillaries and therefore the mural cells on them as pericytes (301,303,312,313). Others differentiate between arterioles, pre-capillary arterioles and capillaries (302,304,305). One suggestion is to describe all cells on pre-capillary arterioles,

capillaries and post-capillary venules as pericytes. However, there is a need to define different sub-classes, to make it easier to describe morphological and accompanying functional characteristics (311). Nonetheless, this process is still ongoing and most controversy exists in the transition zone between arterioles and capillaries.

Pericytes on the first branches coming off the penetrating arterioles, have thin processes ensheathing the vasculature, an ovoid soma and express medium levels of αSMA (309,314). They could be considered as pericytes/SMC hybrid (315,316) and have been named ensheathing (314,317) or transition (318,319) pericytes. On the following pre-capillary arterioles/capillaries, follow α-SMA negative pericytes with different morphology, they have been described as “thin strand” and “mesh” pericytes which have a lower endothelial tube coverage than ensheathing pericytes (314,315). Post-capillary venule pericytes are similar to mesh pericytes with shorter branches (316,319), followed by venular star shaped SMC (302).

Irrespective of the exact definition of the mural cells in pre-capillary arterioles, a recent study shows that even pericytes on the smallest blood vessels have the capability to regulate blood flow, but that their contraction and dilation occurs in a much slower time scale than in perivascular cells around bigger vessels, with higher αSMA content. The authors use optogenetic activation of pericytes and two photon microscopy in their analysis and carefully differentiate pericyte subtypes, by vessel size and morphology. By inhibiting the actomyosin contractile apparatus pharmacologically, the optogenetic capillary constriction could be inhibited further, underlining that capillary pericytes have the machinery necessary to modulate the vascular tone (305).

The use of two photon microscopy contributed significantly to a better understanding of the role of cerebral pericytes *in vivo* and allows for a more detailed definition of the different perivascular cell types with regards to morphology, vessel size and vessel order/branching points. At the same time, most of the studies focus, due to accessibility, on the mouse cortical vasculature. If capillary pericytes in other parts of the CNS or in humans fulfil the same function or if there is functional heterogeneity remains unknown.

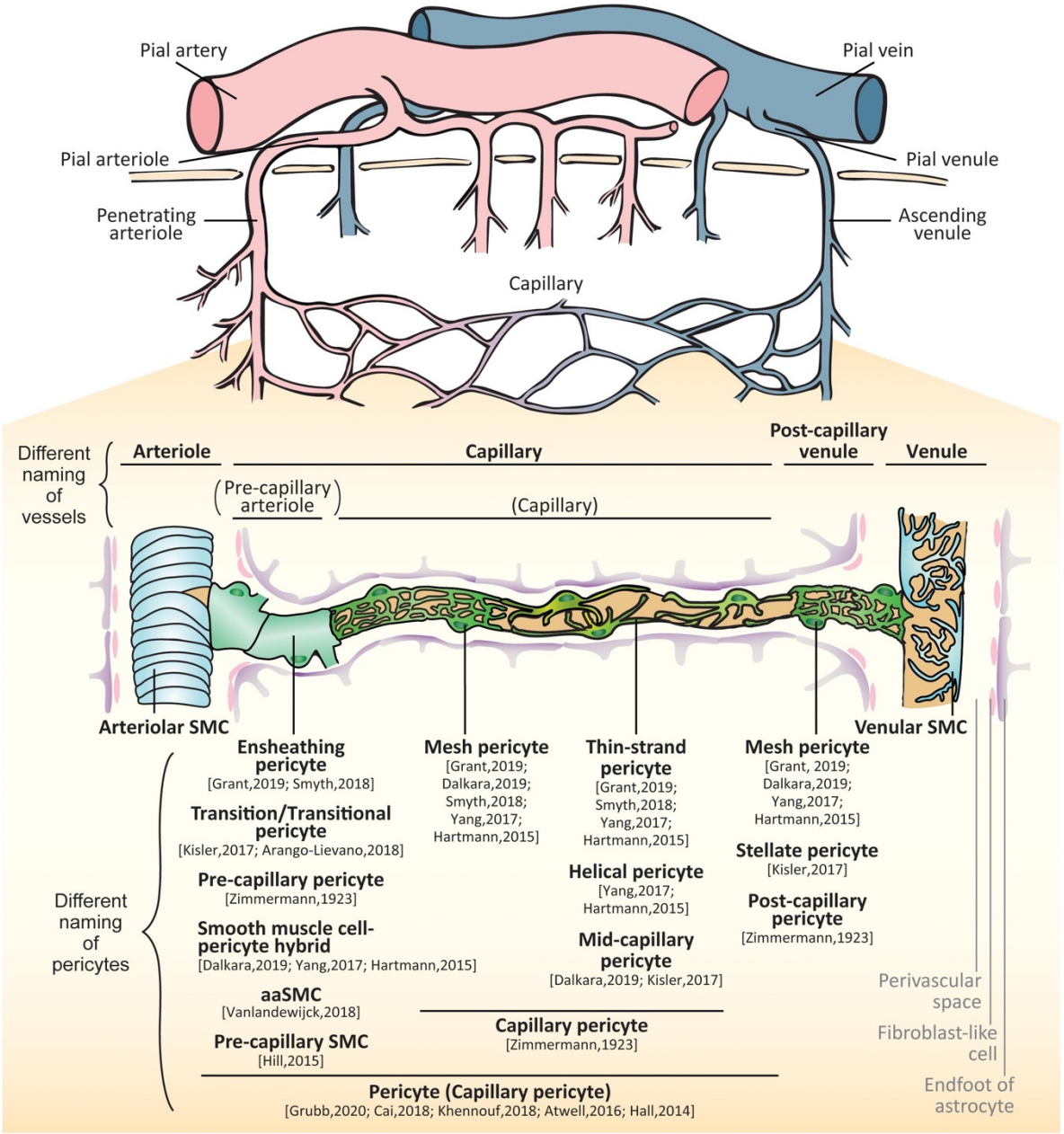

Figure 5 - Mural cells along the Vasculature of the brain
Pial arterioles branch from pial arteries and penetrate perpendicular to the surface, along the pia mater into the parenchyma. Vessel diameter decreases as smaller arterioles split and branch further, forming the capillary bed. Capillaries connect into venules, which lead the blood back to pial veins. Arterioles and veins are covered by ring forming arteriolar and star shaped venular smooth muscle cells respectively. Both, definition of the vessels in-between and in conjunction with that naming of the mural cells on them is object for debate. One suggestion shown here is to name vessels branching from the penetrating arteriole as pre-capillary arteriole and only the smallest vessels capillaries, followed by post capillary venules. The perivascular cells on the pre-capillary arterioles have pericyte and smooth-muscle cell characteristics. Differentiation and names for the different cell types are discussed in the literature review and summarised in this scheme. Reprinted from (13) © 2020 Uemura, Maki, Ihara, Lee and Trojanowski.

2.6.2 Transcriptomic considerations

Within only a few years the view on cellular heterogeneity has changed dramatically due to the application of single cell sequencing technology on almost every organ of mouse, human and other species.

Single cell RNA sequencing of the CNS allows categorising cells in a completely new way and finding new relationships between different cell types. Early whole brain datasets (57,320) contain perivascular cells but rather as a marginal note. Partly due to lower abundance and limited capture-efficiency in droplet based methods, but also because detachment of cells embedded in the vascular BM requires stronger tissue dissociation methods (20,37,59). To specifically focus on vasculature and its associated cells in the CNS, different studies have used reporter mice targeting the vasculature and FACsorted (Fluorescence-activated cell sorting) specifically for cell types of interest, on their own or in combination with vessel enrichment methods during cell dissociation (18,20,321).

Four recent publications focused on cerebral vascular heterogeneity in humans and employed a large number of samples from both healthy brain and brains affected by different vessel related diseases (58,59,322,323) (Table 1).

Author	Method	Samples	Populations	Focus disease	Total cells	Vasc. Cell
Wälchli *et al.*	Single cells; FACS-enrichment for endothelial cells other cell types from unsorted pool	8 fetuses (5 fetal brains, 7 peripheral), 41 adult brain specimens (derived from 39 individual adult patients)	Fibroblast, Pericyte, SMC - one population each in embryonal, adult brain and throughout different disease models - focus of the text and analysis mostly on endothelial compartment	Fetal vs adult brain and 8 vascular diseases/ adjacent tissues	599215	
Yang *et al.*	Single nuclei; VINE-seq (vessel isolation and nuclei extraction for sequencing)	25 samples: the hippocampus of 9 individuals with AD and 8 age- and sex-matched individuals with no cognitive impairment, 4+4 superior frontal cortex	2 fibroblast, 2 pericyte, 2 arterial SMC, arteriolar SMC	Alzheimer's disease	143793	37921
Garcia *et al.*	Single nuclei; blood vessel enrichment (BVE) protocol	17 temporal lobe surgical resections of patients with intractable epilepsy, selecting only healthy tissue distal to epileptic foci + in silico data re-analysis from post mortem brain	3 fibroblast, 2-3 pericyte, separation in aSMV and vSMC	Huntington's disease	84350	4992
Winkler *et al*.	Single cells; Large arteries and veins micro-dissected, and smaller vessels (arterioles, capillaries, and venules) enriched	Cerebral cortex tissue from patients undergoing tailored lobectomies for epilepsy	2 fibroblast, 1 pericyte, 2 fibromyocyte, 7 SMC	Human brain arteriovenous malformations	181388	55981 (norm); 57842 (avm)

Table 1 – Summary of the experimental set up and detected populations in 4 human brain single cell sequencing publication, focus disease of the paper, total and vascular cell number; all used 10x Genomics Chromium for sequencing.

The study of Wälchli *et al*. compares healthy fetal and adult human brain cells with cells from other tissues and brain samples from eight different vascular diseases. The study has so far been deposited on Biorxiv and has not been peer reviewed yet. There is no particular focus on the perivascular cells in this version of the article and the relevant cell numbers are rather small in relation to 600 thousand sequenced cells in total, as no enrichment method for mural cells or fibroblasts was applied. Furthermore, potentially interesting clusters showing expression of vSMC or PVF related genes like *ACTA2*, *TAGLN* and *APOE* are interpreted by the authors as cells under endothelial to mesenchymal transition, although evidence for this is rather weak in the related experiments and one could rather speculate about a possible endothelial contamination in the mural cell population (323). Therefore, the focus of the following comparison will be on the other three studies (For summary see figure 6). Yet, it will be interesting to use the raw data for deeper comparison once it is published.

Both Yang *et al*. and Garcia *et al*. performed single nuclei sequencing, after vessel enrichment. The authors of the first study analysed hippocampi and frontal cortices of patients with Alzheimer's disease and healthy control subjects without cognitive impairment (58). The second study used dissected healthy brain tissue from frontal lobe surgery of patients with epilepsy and compared it with cells derived from Huntington's disease brains, as well as re-analysed an earlier published dataset (59).

Yang *et al*. classified two different pericyte populations along functional categories: Matrix (M-) pericytes, which show differential ECM gene expression (*COL4A1, LAMA4*) and transport (T-) pericytes with high expression of small-molecule transmembrane transporter genes (*SLC20A2, SLC6A1, SLC1A3*). The authors did not find indication for zonation of pericyte populations and assumed that pericytes of both categories distribute over small and large diameter vessels. The researchers uncovered gene expression differences between meningeal (*KCNMA1, SLC4A4*) and perivascular fibroblasts (*LAMA2, FBLN*), and concluded that the ECM gene profile indicates that perivascular fibroblasts are responsible for CNS scar formation (58).

Garcia and colleagues identified two different vSMCs and two (3, together with other analysed datasets) pericyte subpopulations, which distribute according to the underlying vasculature zonation. Arteriolar vSMCs (*ACTA2, MYH11*) are followed along the vascular tree by pericyte 2 (*GRM8, PDGFRB*) and pericyte 1 (*SLC38A5, SPOCK1*) subpopulations on capillaries and venular vSMCs (*MYOD, CD74*). Furthermore, they also distinguished three different fibroblast populations, which all express VEGF-VEGFR2 signalling genes supporting their perivascular location. Fibroblasts type I (*ABCA10, FBLN1*) are considered to be the major ECM producing injury-responsive cells. A pseudotime trajectory between fibroblast subpopulations (text and figure are not congruent regarding which population) and pericyte cluster 2 indicate a close relationship between these cell types (59).

The last study, from Winkler *et al*., distinguishes 7 vSMC subpopulations, with an overarching gene expression profile similar to the other studies (*CCN1, TAGLN, MYH11*), and one pericyte population (*HIGD1B, ATP1A2, KCNJ8*). They introduce also fibromyocytes (2 subpopulations) with a low expression of *TAGLN* and *ACTA2* and high expression of *DCN* and *LUM*, which cluster separately from fibroblasts (2 subpopulations) and vSMCs.

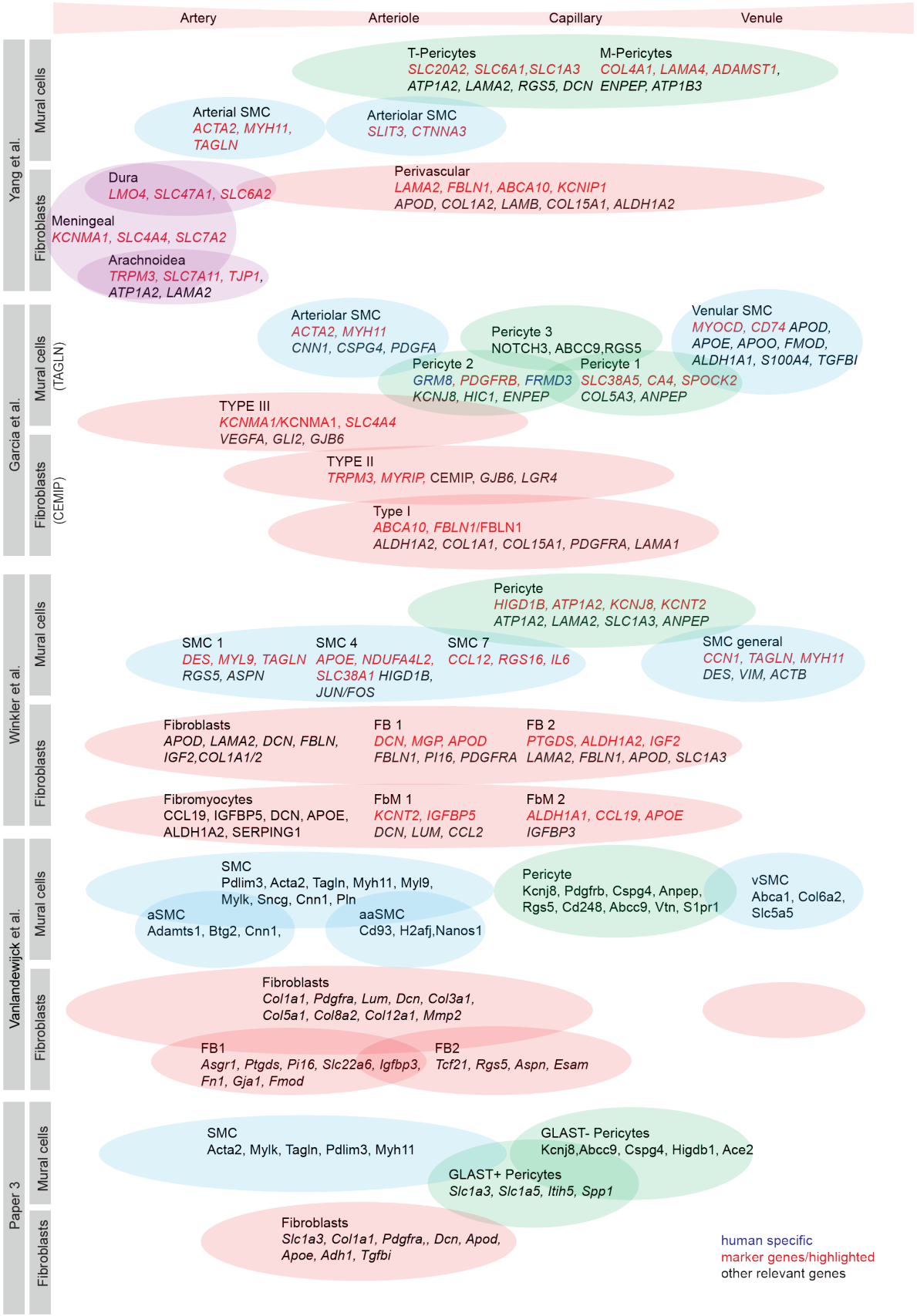

Figure 6 - Graphic summary of recent human brain RNA-single cell sequencing studies in comparison with mouse
Three independent human brain single cell studies with focus on the vasculature identified different subpopulations of perivascular cells (Yang et al.; Garcia et al.; Winkler et al.). The different identified cell populations are represented with respective marker/highly expressed genes and ordered according to their potential location along the vascular tree and relationship of the population to each other. Populations are compared with perivascular cells from the dataset by Vanlandewijck et al. in relation to mouse spinal cord dataset presented in paper III.
Vascular smooth muscle cells (green); Pericytes (green); Fibroblasts/Perivascular fibroblasts/Fibromyocytes (red); Meningeal fibroblasts (purple).

All studies refer to the mouse brain perivascular cell dataset of Vanlandewijk *et al*. (20) and compare to the cell definition and zonation described there. While there is quite some overlap of genes and general vSMC (*ACTA2, TAGLN, MYH11*), pericyte (*RGS5, KCNJ8*) or fibroblast (*COL1A1, DES, DCN*) markers, all studies find also substantial differences between mice and humans and confirm markers that are exclusively expressed in humans (59). Depending on the subdivision of different clusters some markers end up in different populations, accentuating the picture of a vast perivascular heterogeneity in the CNS.

Winkler *et al*. speculate that fibromyocytes are derived from vSMCs, but their pseudotime analysis cannot resolve this. The gene expression pattern rather resembles a close relationship to fibroblasts and potentially an activated state (myofibrolast), however in dimension reduction fibroblasts and fibromyocytes clusters are clearly separated (322).

Particularly interesting is the distribution of Apolipoproteins D and E. In mice, both *Apod* and *Apoe* appear to be suitable markers for fibroblasts (18,20,324), while in human single cell experiments interpretations deviate. Differential expression is either mainly found in vSMCs (*APOD, APOE* (59)), fibromyocytes (*APOE* (322)) or ultimately in perivascular fibroblasts (*APOD* (58,322). *APOE* in humans occurs in three major genetic variants and expression of *APOE4*ε4* is a well-studied genetic risk factor for Alzheimer's disease, linked to Aβ aggregation and tau pathology (325). A main research focus has been astrocytic APOE production and lipidation, but expression in mural cells is also described and studied (326). Apoe expression is upregulated in several CNS lesions like SCI, TBI or stroke (327–329). Therefore, identification of the cell type(s) which express apolipoproteins poses relevance for the understanding of different types of CNS maladies.

Furthermore, Garcia, Yang and Winkler find high SLC1A3 expression in fibroblasts, as well as pericytes (see discussion).

Regarding perivascular fibroblasts Garcia *et al*. defines three different populations (fibroblast type I, II and III) while Yang *et al*. finds one (plus meningeal fibroblasts). There is a high similarity between fibroblast type I (59) and perivascular fibroblasts (58). Makers of these ECM-producing fibroblasts can be found over all 4 fibromyocyte and fibroblast populations (322). Furthermore, the main markers for the fibroblast population III (*KCNMA1* and *SLC4A4*) (59) are also defined as makers of meningeal fibroblasts (58).

Perivascular fibroblasts, pericytes and smooth muscle cells share the microenvironment in close connection to the vasculature, express common markers and share common gene expression signatures underlining their close connection. A growing body of studies defines differences between the cell types but leaves also a lot of room for heterogeneity and distinct subpopulations in homeostasis and disease. The studies included in this thesis define distinct perivascular cell populations and their contribution to different CNS pathologies.

3 RESEARCH AIMS

The overarching aim of this thesis was to well characterise a specific subset of perivascular cells, investigate their contribution to fibrosis in diseases and injuries of the central nervous system and to analyse if they are a suitable target for therapy.

The specific aims of the constituent papers were:

Paper I: To study if the reduction of fibrotic scarring through a subpopulation perivascular cells benefits regeneration as well as functional recovery upon spinal cord injury and thus poses a target for therapy.

Paper II: To investigate if fibrotic scar formation is a general mechanism across different central nervous system maladies and the contribution of perivascular cells.

Paper III: To characterise populations of perivascular fibrotic scar forming cells in homeostasis and after spinal cord injury.

4 METHODOLOGICAL CONSIDERATIONS

All methods used in this thesis are described in detail in the individual papers I-III. Following are some considerations about the mouse lines that were used in the papers included in this thesis:

To genetically target and manipulate GLAST$^+$ perivascular cells, we used the following tools (Figure 7): For fate mapping of GLAST$^+$ cells, we used BAC transgenic mice that express CreER^{T2} recombinase under the promotor for GLAST (330) and crossed them with a Rosa26-EYFP (enhanced yellow fluorescent protein) reporter line (331): *GLAST- CreER^{T2};R26R-EYFP* (**paper I and II**) or Rosa26-tdTomato reporter line (332): *GLAST- CreER^{T2};R26R-tdTom* (**paper II and III**).

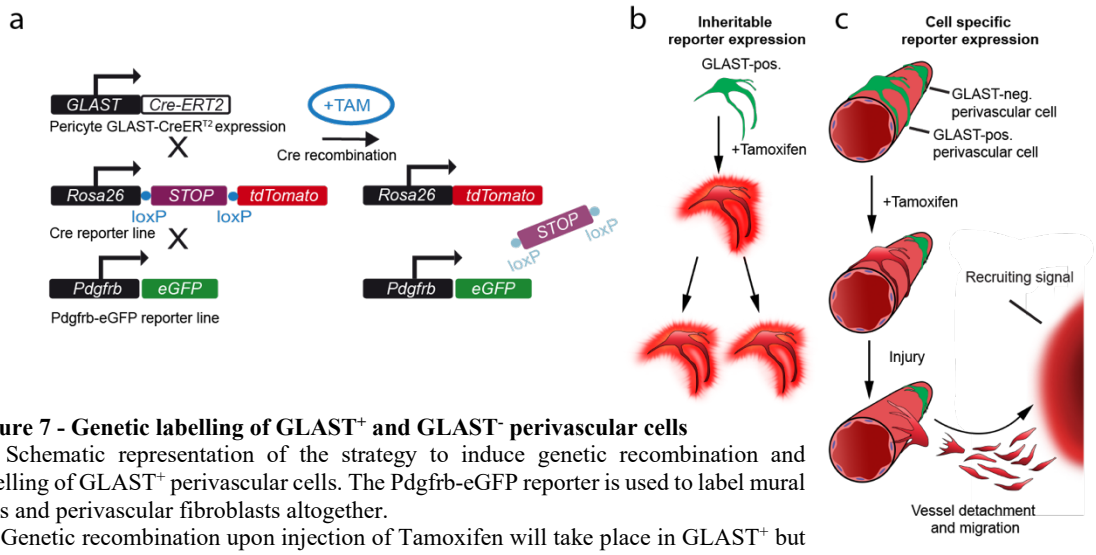

Figure 7 - Genetic labelling of GLAST$^+$ and GLAST$^-$ perivascular cells
(**a**) Schematic representation of the strategy to induce genetic recombination and labelling of GLAST$^+$ perivascular cells. The Pdgfrb-eGFP reporter is used to label mural cells and perivascular fibroblasts altogether.
(**b**) Genetic recombination upon injection of Tamoxifen will take place in GLAST$^+$ but not GLAST$^-$ cells and turn on fluorescent tdTomato reporter expression. All perivascular cells express Pdgfrb and therefore recombined cells are double positive eGFP$^+$;tdTomato$^+$, while GLAST$^-$ remain only eGFP$^+$. The progeny of recombined cells inherits the recombination and stable expression of tdTomato.
(**c**) GLAST$^+$ perivascular cells can be traced using double reporter expression and differentiated from other single eGFP$^+$ perivascular cells.
(The same strategy was used with Col1a1-CreER transgenic mice).
Modified with friendly permission from original illustration by Jannis Kalkitsas.

To inhibit proliferation of GLAST$^+$ cells, we crossed *GLAST-CreER^{T2};R26R-EYFP* mice with "rasless-mice" (Figure 8), carrying a complete knockout for *Hras* and *Nras* and a conditional Cre-dependent knockout for *Kras* (333), which are critical for cell proliferation: *GLAST-CreER^{T2};rasless;R26R-EYFP*.

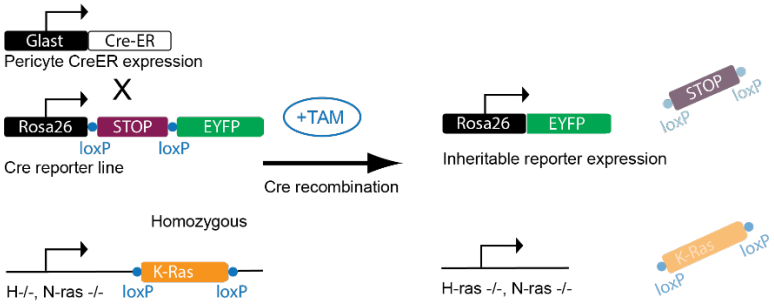

Figure 8 - Genetic strategy to label and genetically inhibit the proliferation of GLAST$^+$ perivascular cells

GLAST as marker for perivascular cells

The sodium-dependent glutamate/aspartate transporter (GLAST) also known as excitatory amino acid transporter 1 (Eaat1) is encoded by the *Slc1a3* gene. Transporters appear as trimers (334) and function as co/counter-transporter for glutamate, sodium, potassium, and protons (335) and are essential for extracellular glutamate homeostasis in the CNS (336). In brain and spinal cord. GLAST has been mainly studied and used as maker for astrocytes (337), but is also expressed in cells of several other organs (338) with important functions in stem cell niches (339).
We use *GLAST- CreER^{T2}* transgenic mice crossed with Rosa26-enhanced yellow fluorescent protein (331) or Rosa26-tdTomato reporter lines (340) to label and lineage trace GLAST^{+} cells. About 10% of all Pdgfrβ^{+} perivascular cells in mouse spinal cord, cortex and striatum are recombined (3)(Paper 2). However, in line with other studies using GLAST as maker, recombined cells can also be found in a subpopulation of astrocytes, ependymal and neural progenitor cells and associated to the meningeal vasculature (3,330,341)(paper II).

Ependymal cells and astrocytes do not participate in stromal scar formation (157). In papers I-III we use Pdgfrß co-staining or *Pdgfrb-eGFP* reporter mice (340) in combination with lineage tracing to identify GLAST^{+} perivascular cells. Recombined ependymal cells and astrocytes do not express Pdgfrß (3,201). In spinal cord we do not detect significant Pdgfrβ expression in astrocytes of *Pdgfrb-eGFP* reporter mice and we did not fetch an astrocyte subpopulation when sorting and sequencing *Pdgfrb-egfp* cells (Paper III). However, a population of astrocytes with Pdgfrß expression exists mainly in brain (20,342–344) which has to be carefully considered not at least in injury experiments.

Lineage tracing with CreER^{T2} lines

The Cre/loxP system is a widely used powerful system for gene editing, it allows to express reporter- or transgenes in specific tissue or cells. Cre recombinase expressed under a promotor of interest recognises and excises DNA flanked by loxP sites. When used in reporter mice the loxP sites flank a stop cassette, activating the expression of a downstream reporter gene (345,346). In addition to cell specific labelling the CreER system adds a temporal component. Cre is linked to a mutated estrogen receptor (ER) which inhibits entry to the nucleus. Application of the synthetic ER antagonist tamoxifen allows translocation to the nucleus and Cre mediated gene editing (347).

We use this system to inheritably label cells that express GLAST or *Col1a1* at the timepoint of tamoxifen injection (adult mice – Paper I-III). To be certain that tamoxifen left the system at the timepoint of surgery or induction of injury we apply a one week “clearing” period, to circumvent recombination in cells which sporadically might express the gene of interest due to the insult (202) or modulation of injury response.

CreER lines have been reported to be “leaky” with reporter gene expression independent of promotor specific or tamoxifen dependent Cre recombination (348,349). The Ai14 tdtomato reporter constructs is more prone to be recombined at basal CreER^{T2} levels due to leakage than the R26R-EYFP reporter (348). This is especially critical if cells are recombined early during development and give rise to significant reporter positive progeny. We have occasionally detected recombination in cells of mice that were not treated with tamoxifen, especially in mice with tdtomato reporter. In our experiments we use both tdtomato and EYFP mice with uniform results. Throughout the different studies included here we used both CreERT2+ control mice with injection of the solvent oil/ethanol instead of tamoxifen and CreER^{T2}- controls.

5 ETHICAL CONSIDERATIONS

Most of the work presented in this thesis would have not been possible without the use of animal models. Mice are specifically valuable and have many advantages for the research in life-science in general and in the study of SCI and brain in particular. Mice and humans share the same complex physiology and they are closer related than other common non-mammalian model organisms (e.g. worm, fly). Almost everything we know about the CNS comes from initial observations in model organisms. The mouse genome is completely sequenced and more and more detailed atlases for protein and gene expression of all organs and cell types are published. Furthermore, a huge toolbox to remove, target, modify or activate cells, genes or proteins exists. This allows to address questions in great detail.

One aim of this study was to analyse the contribution of a specific cell-type to fibrosis in the CNS. Genetic labelling and manipulation of this specific cell type and its progeny is one the of the methods that is only possible in research model organisms. The complexity of the disease/injury progression in the CNS after e.g. spinal cord injury cannot be modelled, even in the best tissue culture, organoid or microfluidic devices available today (350). Several different local and infiltrating cell types contribute to a very specific microenvironment that drives scar formation. As discussed in this thesis, small changes of the cellular composition have huge effects on the whole pathologic progression. Further, we are studying regeneration of neurons with the cell body far away from the initial injury site in a completely different environment, which cannot be modelled in a culture system. The animals we use in our injury models receive the best possible pre- and post-operative care in close cooperation with veterinarians and caretakers. We always aim to optimise procedures and reduce the number of animals needed. The findings of our research set the focus on fibrotic scarring in human CNS pathologies and initiated studies to translate the results into a human setting to find new treatment strategies. CNS injuries and diseases, lead to long lasting harm, pain or costs for the healthcare system. Any improvement towards regeneration or enhanced quality of live is a huge step for everyone suffering from these pathologies.
Therefore, I consider it justifiable to use mice in our research.

Another ethical consideration I would like to point out is the use of resources.
The earth´s resources are limited and humanity is “sleepwalking into climate catastrophe” (351).

Life science research has very specific and high requirements regarding e.g. hygiene, clean and controlled experimental conditions, prevention of contaminations or infections. This requires appropriate equipment, research facilities and large amounts of energy. The benefit of research in most cases justifies the extra use of materials, energy and transport of valuable reagents or samples. However, there is still a lot of room for improvement when it comes to waste handling, energy consumption and not least logistics. We have a shared responsibility to carefully evaluate the use of our common goods and to reduce our carbon footprint.

6 RESULTS

Regeneration in the adult mammalian CNS is very limited. One limiting factor is the formation of chronic scar tissue, inhibiting axonal regeneration for functional recovery. Scar tissue, formed after adult spinal cord lesion, compartmentalises into a fibrotic scar core, immediately surrounded by a glial border (154). While the glial component of the CNS scar was intensively studied, little was known about the fibrotic part. In 2011, Göritz *et al*., established a subpopulation of GLAST$^+$ perivascular cells, named type A pericytes, as the origin of fibrotic scar tissue after spinal cord injury (3).

Building on this finding, we consequently investigated if the reduction of fibrotic scarring by GLAST$^+$ perivascular cells has a therapeutic potential to improve functional recovery (**paper I**), if GLAST$^+$ perivascular cells are a general source of fibrotic scar tissue throughout the CNS (**paper II**) and what are the anatomical and molecular characteristics of GLAST$^+$ perivascular cells and how they change in response to injury (**paper III**).

In **paper I,** our aim was to investigate if modulation of fibrotic scarring by GLAST$^+$ perivascular cells could improve axonal regeneration and functional recovery after spinal cord injury.

Inhibition of GLAST$^+$ cell proliferation leads of fibrotic scar tissue reduction

To address this question, we inhibited the proliferation of GLAST$^+$ perivascular cells in response to injury by simultaneous deletion of H-, N- and K-RAS genes. Using this method, we could reduce the number of stromal cells and the amount of fibrotic extracellular matrix deposited at the lesion site dependent on recombination efficiency. We focused on mice with a moderate recombination efficacy, in which the lesions are closed, but the fibrotic scar density was significantly reduced. Mice with the highest recombination efficiency (*i.e.*, mice exhibiting the strongest inhibition of proliferation) showed a tissue defect, characterised by the lack of the stromal scar component and the formation of a cavity and were therefore excluded from further analysis (paper I, figure S1).

Gene expression analysis indicated that GLAST$^+$ perivascular cells deposit fibrotic ECM at the lesion site and that a reduction of GLAST$^+$ perivascular cell proliferation consequently prohibited the injury-induced upregulation of fibrosis associated genes (paper I, figure 1). The deposition of fibrotic ECM molecules was reduced in line with the reduction of stromal fibroblast cell numbers (paper I figure S2).

Reduced fibrotic scar density enables axon regeneration across the lesion site

To test the influence of reduced fibrotic scar density on spontaneous axonal regeneration, we applied a dorsal hemisection injury model, transecting corticospinal- (CST) and raphespinal- (RST) tracts. In comparison to control mice with unaltered fibrotic scarring, mice with reduced fibrotic scar density had fewer dystrophic retraction bulbs, more axons reaching the lesion centre and importantly, a significantly higher number of axons crossing the injury site (paper I figure 2). This axonal regeneration was persistently observed in chronic stages after spinal cord injury. Eighteen weeks after injury we detected traced CST axons caudal to the lesion in different grey matter regions, underlining that they are not representing spared axons. Likewise, the number of RST axons increased either due to sprouting of spared axons or regeneration. In control mice and in mice with tissue defects, fewer or no CST and RST axons were detected caudal to the lesion.

Regenerated axons are functional and integrate into the local circuitry caudal to the lesion

To test whether the regenerated axons can form functional synapses with neurons caudal to the lesion site, we paired optogenetic activation of CST axons with electrophysiologic recordings. Photoactivation of regenerated axons caudal to the lesion induced neuronal activity in the local grey matter circuitry. These results indicate that regenerated axons formed synapses and functionally integrated into the local circuitry caudal to the lesion (paper I figure 6).

Reduction of fibrotic scarring promotes sensory-motor function recovery after spinal cord injury

The read out for CST-mediated function is fine motor coordination. We assessed the motor-sensory function using the gridwalk task and measured coordination assessing the regularity index from catwalk analysis. Both sensory-motor tests showed a significant functional improvement over the control group from about 7 weeks post injury, a time point when axons had crossed the injury site (summarised in figure 9 below). To investigate if the observed axonal regeneration leads to functional improvement, we photoactivated neurons in the sensorimotor cortex in awake injured animals to trigger involuntary motor output mediated by CST fibres. After the experiment we quantified the number of regenerated CST axon fibres. Induced motor output was strongest in mice with a higher number of regenerated CST axons, suggesting that axonal regeneration may be the reason for the improved functional recovery (paper I figure 7).

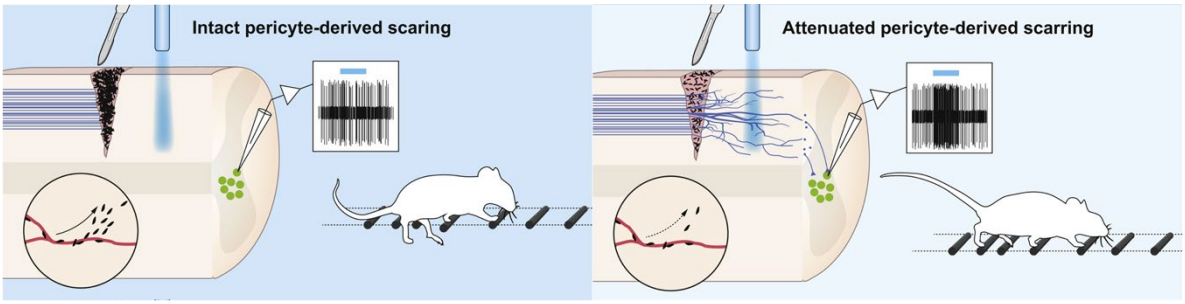

Figure 9 - Schematic summary of paper I
Left: Mice have impaired motor-sensory function after dorsal hemisection spinal cord injury. Fibrotic scar formation inhibits axonal regeneration. Right: Reduction of GLAST^{+} pericyte/fibroblast derived cell proliferation reduced fibrotic scar formation. This promoted axon regeneration and formation of synapses with local spinal neurons (functional integration), leading to improved fine motor coordination.

In **paper II,** we investigated the extent and distribution of fibrotic tissue formation in response to different kind of lesions in brain and spinal cord in mice (summarised in figure 10 below) and humans. Furthermore, using a genetic lineage-tracing strategy, we asked to which extent GLAST$^+$ perivascular cells contribute to fibrotic tissue throughout the CNS and in response to different lesions.

GLAST$^+$ perivascular cells are the main source of fibrotic scar-forming cells in penetrating and non-penetrating injuries in the adult spinal cord and brain

We were able to confirm that the same response as in the aforementioned penetrating spinal cord injury models occurred after full spinal crush (**paper II**) and contusion (**paper III**), two injury models that do not involve meningeal incision. As in the spinal cord, GLAST$^+$ perivascular cells represented about 10% of the total number of PDGFRß$^+$ cells (paper II figure 2h) in the uninjured brain (cerebral cortex and striatum) and shared a similar marker profile (paper II figure S3). Upon large cortical and cortico-striatal stab wound injuries GLAST$^+$ perivascular cell progeny left the blood vessel wall, contributed to the majority of stromal fibroblasts and continuously persisted in the fibrotic injury core (paper II figures 2 and 3). Smaller stab wounds restricted to the cerebral cortex generated only a mild fibrotic response surrounded by reactive astrocytes (paper II figure S9).

GLAST$^+$ perivascular cells contribute to distinct CNS lesions in an injury-dependent manner

In experimental autoimmune encephalomyelitis (EAE), a model for demyelinating diseases, such as multiple sclerosis (MS) (299), progeny of GLAST$^+$ perivascular cells left the vasculature and were the major contributor to stromal fibroblasts in chronic EAE scars. In contrast to the injury models described earlier, stromal fibroblasts intermingled with reactive astrocytes and immune cells and were not separated from surrounding tissue by a defined glial scar border (paper II figure 4) (352). In tumour stroma, GLAST$^+$ perivascular cells proliferated and expressed the myofibroblast marker αSMA but were not the major component (paper II figure 7). The response of lineage traced GLAST$^+$ perivascular cells to ischemic lesions in the brain was particularly intriguing. In response to cortical stroke GLAST$^+$ perivascular cells proliferated, detached from the blood vessel wall, and gave rise to the majority of stromal fibroblasts that occupied the fibrotic ischemic core (paper II figure 6), comparable to our observations following spinal cord injury or large stab wounds. In contrast, response to striatal ischemia was distinctive. The number of GLAST$^+$ perivascular cell progeny increased but reached its maximum at 5 days after injury, with the vast majority remaining associated with the vasculature and lacking αSMA expression (paper II figure 5). In line, ECM deposition was much lower in striatal than in cortical lesions and no sharp fibrotic-glial scar border was formed after striatal stroke (paper II figure 8).

Fibrotic scarring is observed in human CNS pathology and the human brain and spinal cord comprise a *PDGFRb*$^+$ *GLAST*$^+$ perivascular cell population

Human spinal cord injury lesions contain large areas of fibrotic scar tissue surrounded by reactive glia. In MS patients we detected increased perivascular aggregates of PDGFRß$^+$ cells, with a substantial number of cells detached from the vasculature. Tissue fibrosis was associated with demyelinated regions in the spinal cord. In subcortical ischemic stroke and brain tumor tissue PDGFRß$^+$ stromal cells were mostly associated with the vasculature (paper II figure 10). Interestingly, we identified a population of *PDGFRB* mRNA$^+$ perivascular cells co-expressing *SLC1A3* (also known as GLAST) mRNA residing in the healthy human spinal cord and brain vasculature, similar to the mouse (paper II figure 9).

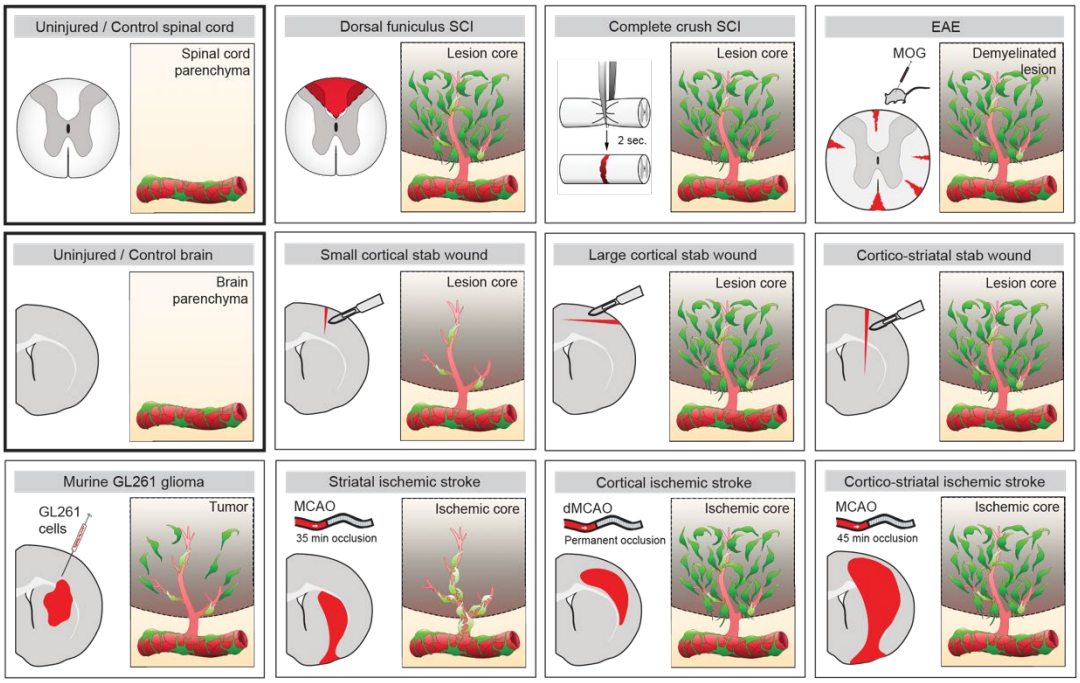

Figure 10 – GLAST$^+$ pericyte/fibroblast derived cell response in different injury and disease models. GLAST$^+$ pericytes and fibroblast leave the vasculature, proliferate and contribute to the fibrotic injury core in penetrating and non-penetrating spinal cord injury models, a model for demyelinating disease (EAE), large cortical and cortico-striatal stab wounds as well as cortical and cortico-striatal ischemic stroke. In striatal ischemic strokes perivascular cells proliferate but do not leave the vasculature. In a model for Glioblastoma only few GLAST$^+$ derived cells contribute to the stroma. Small stab wounds do not trigger a reaction. Reproduced from (423).

The objective of **paper III** was to further define GLAST$^+$ perivascular cells regarding their anatomical and molecular characteristics and compare them with other CNS perivascular cells both under homeostatic conditions and after injury.

GLAST$^+$ perivascular cells comprise pericytes and perivascular fibroblasts

To define GLAST$^+$ perivascular cells by gene expression profiling, we employed *GLAST-CreER^{T2};R26R-tdTom* mice crossed to a *Pdgfrb-eGFP* reporter line. We used PDGFRβ as a pan- perivascular cell marker. Using this mouse line, we isolated GLAST$^+$, PDGFRβ$^+$ (tdTom$^+$EGFP$^+$) cells as well as GLAST$^-$, PDGFRβ$^+$ (tdTom$^-$EGFP$^+$) cells and subjected them to single cell RNA sequencing. Subsequent bioinformatics analysis resulted in the identification of two different GLAST$^+$ perivascular cell populations in the adult uninjured mouse spinal cord, which are best defined as pericytes and fibroblasts. Comparison to GLAST$^-$ PDGFRβ$^+$ cells shows overlap between GLAST$^+$ and GLAST$^-$ pericytes, while GLAST$^+$ fibroblasts clustered separate (paper III figure 1).

Perivascular fibroblasts can be targeted with an inducible Col1a1-CreER^{T2} transgenic line and differ in several aspects from GLAST$^+$ pericytes

We found *Col1a1* to be specifically expressed in the fibroblast fraction of GLAST$^+$ perivascular cells. To better characterise the anatomical distribution of fibroblasts in the mouse CNS and their response to injury, we established a tamoxifen-inducible *Col1a1-CreER^{T2}* transgenic mouse line crossed to the *Rosa26-tdTomato* reporter (*Col1a1-CreER^{T2};R26R-tdTom)* to label and lineage trace GLAST$^+$*Col1a1*$^+$ perivascular cells (paper III figure 2). Our characterisation showed that *Col1a1*$^+$ perivascular fibroblasts (PVF) are more readily observed along large diameter penetrating blood vessels in the spinal cord white matter. In contrast, GLAST$^+$ *Col1a1*$^-$ pericytes lined smaller arteriolar vessels down to the

capillary bed and were accordingly more abundant in grey matter regions (paper III figure 3). At the ultrastructural level GLAST$^+$ pericytes were found juxtaposed to endothelial cells, embedded in the vascular basal lamina. Remarkably, they exhibited less finger shaped processes, distinguishing them morphologically from GLAST$^-$ pericytes (paper III figure 4). In contrast, PVF's were found along larger penetrating vessels.

GLAST$^+$ pericytes and fibroblasts contribute to fibrotic scar tissue in a region dependent manner

As described above (**papers I and II**) fate mapping studies employing GLAST-CreER^{T2};*R26R-tdTomato* mice showed that GLAST$^+$ perivascular cells contributed to the majority of scar-forming fibroblasts across different CNS lesions. Interestingly, comparison of the response of GLAST$^+$ and Col1a1$^+$ perivascular cells to injury in two different spinal cord lesion models by lineage tracing, showed that GLAST$^+$ pericytes and perivascular fibroblasts contributed to different portions of the fibrotic scar. In line with their anatomical position along the vascular tree in the uninjured spinal cord, our results suggest that perivascular fibroblasts (GLAST$^+$*Col1a1*$^+$ cells) contribute more substantially to fibrotic scarring in white matter regions of the spinal cord while GLAST$^+$*Col1a1*$^-$ pericyte derived cells preferentially locate in the grey matter portion of the fibrotic scar (paper III figures 5 and 6).

Different fibroblast subsets can be distinguished at day 5 post spinal cord injury

GLAST$^+$ perivascular cell-derived progeny displayed a clear fibrotic transcriptional profile at 5 days after spinal cord injury. However, while all lineage traced cells showed high expression of ECM genes, one subpopulation exhibited a myofibroblast signature. Furthermore, we identified a population of activated GLAST$^-$ pericytes, which upregulated a fibrosis gene profile after injury, but did not cluster with the fibroblast populations.

7 DISCUSSION

The occurrence of fibrotic scarring in response to various CNS pathologies is increasingly acknowledged. We and others have found strong evidence that perivascular cells are the major contributor to stromal fibroblasts and fibrotic ECM in several CNS lesion models (18,122,299,353–355).

Pericytes, Fibroblasts or both?

Following the initial discovery, which identified GLAST$^+$ perivascular cells in the adult spinal cord as the origin of stromal fibroblasts upon injury (3), several studies identified perivascular cells in the adult CNS with a fibroblast signature. Not least, single cell RNA sequencing enabled the unbiased identification of perivascular cell types and subpopulations in different organs, including the CNS (18,20,356,357). As a consequence, perivascular fibroblasts have been assumed as a source of fibrotic cells upon injury (18). Both CNS fibroblasts and pericytes express *Slc1a3* (20), the gene encoding for GLAST, suggesting that GLAST$^+$ perivascular cells represent a pool of pericytes and fibroblasts. Therefore, this allows for two hypotheses: i) only perivascular fibroblasts are the source of fibrotic tissue or ii) both fibroblast and pericyte populations contribute to scarring.

In paper III we identified, with single cell RNA sequencing, that GLAST$^+$ perivascular cells indeed encompass two populations: pericytes and fibroblasts. Fate-mapping of fibroblasts using a newly generated *Col1a1-CreER^{T2}* mouse line, revealed that both, GLAST$^+$ pericytes and fibroblasts contribute to fibrotic scar formation after SCI.

The definition of perivascular fibroblasts and scar-forming pericytes depends largely on their anatomical location in the CNS vasculature. The distribution of PVF along the vascular tree is a current subject for debate.

Confocal imaging of *Pdgfra*-H2BGFP reporter mice (histone-2B fused GFP reporter) identifies perivascular fibroblasts on all vessel types except capillaries in the adult mouse cerebral cortex within the Virchow-Robin space. In opposition to mural cells, they are "loosely" adhered to the vasculature (20). In the adult mouse spinal cord, *Col1a1*-GFP$^+$ cells are associated with large penetrating vessels (18). Two-photon imaging microscopy shows that *Col1a1-GFP* and *Col1a2*-expressing perivascular cells (genetically labelled with a *Col1a2-CreER^{T2}* mouse line) can be detected on arterioles, larger ascending venules, as well as in the arteriole-capillary transition zone (16). This is in line with our results, detecting GLAST$^+$ perivascular cells in the transition zone between arterioles and capillaries, but not on the smallest capillaries. In addition, our results showed that *Col1a1*$^+$ PVFs (identified using a *Col1a1-CreER^T* transgenic line) were only found on lower order penetrating arterioles and venules (**paper III, figure 3**). Following the position of CNS *Col1a2*-expressing PVFs, during four weeks Bonney *et al*. found that PVFs somata shifted more dynamically on the vasculature than those of mural cells (16) which are encased in the vascular basement membrane (358). However, PVFs do not migrate along the vasculature (16,358).

The *Col1a2-CreER^{T2}* mouse line recombines 90% of *Col1a1*-GFP$^+$ cells and 5% were NG2$^+$. After EAE, 80% of the scar-forming *Col1a1*-GFP$^+$ cells are derived from lineage traced *Col1a2*$^+$ *PVFs* (18). Single cell RNA sequencing of *Col1a1*-GFP$^+$ cells from the spinal cord 30 days post EAE induction confirms that most GFP$^+$ cells present a fibroblast signature, except a small subpopulation showing more pronounced mural cell signature.

The contribution of *Col1a2*$^{+}$ cells to fibrotic scarring after EAE is in line with our results (**paper II, figure 4**). EAE leads mainly to scar formation in the ventral and ventrolateral white matter. We showed that contribution to the fibrotic scar is dependent on the insult location (**paper III, figure 5 and 6**) and would assume that the PVF population and not pericytes is the major contributor to EAE lesions.

Although both Col1α1 and Col1α2 are needed to form type I collagen heterotrimers, their transcription profile in perivascular cells differs. In the dataset of Vanlandewijk *et al*. *Col1a1* is expressed solely in fibroblasts, while *Col1a2* expression can be found in all perivascular cell types. However, collagen production is not mainly regulated at the transcriptional level (136,138). It will be interesting to investigate if the *Col1a2-CreER*^{T2} mouse line labels mural cells, and specifically GLAST^{+} pericytes.

The overlap between *Col1a1-CreER*^{T2} and *Col1a2-CreER*^{T2} derived cells could be assessed by crossing the *Col1a1-CreER*^{T2} line to the same Col1a1-GFP (359) reporter mouse that has been used by Dorrier *et al*. Functionally, the overlap of the two populations could be evaluated by using grey and white matter specific injuries in the *Col1a2-CreER*^{T2} mouse, as we did in figures 5 and 6 of paper III.

Most of the current controversy is due to the varying definition of pericytes. The lack of a single unique pericyte marker for tissue staining has contributed to the shifting interpretations of what defines a pericyte. However, a long-standing benchmark has been that pericytes are enveloped in the endothelial BM ("a basal lamina which is continuous with the basal lamina of the capillary") (98). Göritz *et al*. showed that perivascular cells in smaller blood vessels labelled by the GLAST-CreER^{T2} transgenic line are surrounded by basal lamina in homeostasis and break through the basal lamina upon injury. We showed that if GLAST^{+} pericytes did not intersect with GLAST^{-} pericytes, they were directly abluminal to the endothelial tube (**paper II figure S1 and paper III figure 4**). Moreover, we also found morphological differences between GLAST^{+} and GLAST^{-} pericytes. GLAST^{+} pericytes presented a homogenous surface with short processes, while GLAST^{-} pericytes exhibited finger shaped processes (**paper III figure 4)**. In zebrafish a similar morphology is described as "awkward hug" and attributed to PVFs (360). Interestingly, the same study shows that a subpopulation of these cells gradually downregulates col1a2-GFP expression, induces pdgfrb-expression and acts as pericyte precursor. Early ablation of col1a2 cells leads to loss of PVF and substantial reduction of pericytes (360), indicating a lineage relationship between PVF and pericytes in zebrafish.

The perivascular niche, providing a basement membrane, has been proposed to create a beneficial environment for stem and progenitor cells (361–364). Furthermore, it contains stromal cells of varying differentiation status (365–368) with morphology and marker profile similar to pericytes (40). Based on these elements, we could hypothesise that the CNS contains a population of pericytes or pericyte-like-cells, which can functionally serve as fibroblast progenitor cells, different from PVFs around larger vessels, and contribute to scar-forming fibroblasts upon injury.

Heterogeneity of pericyte populations

Besides molecular marker combinations and cell definition at the ultrastructural level, RNA single cell sequencing has accelerated the identification of cell types and characterisation of cellular heterogeneity. Nonetheless GLAST^{+} and GLAST^{-} pericytes could not be differentiated by single cell RNA sequencing (**Paper III, figure 1**) (20).
However, we speculated that there might be a gene signature gradient within the pericyte population with higher expression of vSMCs related genes on one side, and fibroblast related

genes on the other side, which can be visualised in dimension reduction plots (**Paper III, figure S2**). However, single cell RNA sequencing cluster analyses and data visualisation with dimension reduction techniques, like UMAP (Uniform Manifold Approximation and Projection for Dimension Reduction), depend on many parameters. One should be cautious that cluster sizes and distances between clusters infer no (or only limited) meaning in case of UMAP, but it usually gives a good impression on the global structure (369). Further the complexity of the dataset and the sequencing depth are important parameters that influence clustering. Other recent single cell studies reinforce the link between pericytes and fibroblasts in the CNS. A study of cellular heterogeneity upon spinal cord injury shows a gene expression gradient with fibroblasts on one side, pericytes in the middle and vSMCs on the other side (356), in line with our results (paper III, figure S2). A recent single cell RNA sequencing study of human brain identified 2 pericyte and 3 fibroblast populations. Pseudotime analyses revealed a transcriptional transition between one of the pericyte and fibroblast populations, interpreting this fibroblast population as an "intermediate state" (59).

All three most recent publications on human brain single cell RNA sequencing with focus on the vasculature identify high *SLC1A3* expression in both fibroblast and/or pericyte populations (58,59,322). This is in line with our identification of a population of *SLC1A3*$^{+}$*PDGFRB*$^{+}$ perivascular cells in the human cortex and spinal cord using *in situ* hybridisation (**paper II, figure 9**).

It is striking that GLAST^{+} and GLAST^{-} pericytes inhabit a similar niche and that their transcriptome cannot be told apart. The close relationship between these two populations could suggest that they share a common origin or that one cell type is the progenitor of the other (360).

Another line of thought is that GLAST^{+} and GLAST^{-} pericytes could have different developmental origins (neural crest or mesenchymal) and/or they are recruited to the vasculature at different timepoints, which defines their identity, morphology and transcriptome in convergence with the niche they share. Nonetheless, upon injury, their origin dependent underlying programs are activated, and they react according to their lineage of origin. In bone and adipose tissue, it has been described that phenotypically similar cells, originating from different progenitors can re-activate lineage specific programs following their underlying epigenetic properties. (370–373).

Relation of perivascular fibroblasts to astrocytes

Besides *Slc1a3*, GLAST^{+} PVFs express *Gja1* (gene encoding for connexin 43, Cx43) and *Gjb6* (gene encoding for connexin 30, Cx30) in mice (**paper III, figure S1**) and in humans, markers commonly expressed by astrocytes (58). Connexins assemble to form gap junctions and mediate direct intercellular communication, with Cx30 and Cx43 being required for BBB integrity (374). Endothelial cells communicate with pericytes (besides N-cadherin adhesion and growth factor signalling) via gap junctions (374). Connection of pericytes with endothelial precursor cells via Cx43 is crucial for vasculogenesis (26). Cx30 has been shown to be expressed in a *Pdgfrb*$^{+}$ perivascular cell subpopulation (375). Cx30 and Cx43 are characteristic for astrocytic end-feet, enwrapping the CNS vasculature and crucial for their BBB function (376). Ultrastructural analysis of perivascular cells labelled in GLAST-CreER^{T2} and Col1a1-CreER^{T2} mice exposed protrusions of PVFs into the astrocytic end-feet on arterioles and venules (**Paper III; figure 4**), extending the mutual surface area. Expression of connexins and formation of gap junctions between PVFs and astrocyte end-feet, pericytes and/or endothelial cells indicates an integral function within the BBB.

Mechanisms of perivascular cell recruitment

The mature fibrotic scar core is mainly constituted by macrophages, stromal fibroblasts and fibrotic ECM (353). We showed that reduction of perivascular cell-derived scarring led to a decreased accumulation of $CD68^+$ macrophages/microglia upon SCI (**paper I, figure S3**). During scar formation, the recruitment of scar-forming perivascular cells is temporally synchronised with the infiltration of monocyte-derived macrophages into the lesion site. As the scar matures and becomes compartmentalised, scar-forming fibroblasts and macrophages cluster in the lesion core (3,232,377) (**paper II**). Depletion of macrophages in spinal cord injury leads to reduction of scar-forming fibroblasts and ECM deposition (377,378), further underlining the interdependence of fibrotic and immune cells during the formation of fibrotic scar tissue (355).

A recent single cell RNA sequencing study, analysing interactions of all cellular components in spinal cord lesions, identified that the fibroblast-macrophage axis has the highest number of ligand-receptor interaction pairs. Interestingly, *Spp1* and *Apoe* were the genes with the highest interaction scores (356). Both genes were highly differentially expressed between pericytes and PVFs and we have highlighted *Spp1* as one of the genes which potentially differs between $GLAST^+$ and $GLAST^-$ pericyte populations (**paper III**). Milich *et al*. further identified oncostatin M (Osm) signalling as a potential activator of fibroblasts and astrocytes by myeloid cells. Upon spinal cord injury *Osm* and Oncostatin M receptor (*Osmr*) expression is upregulated in the whole lesion site (379). Osm belongs to the IL-6 family of cytokines, which play important roles in cell communication, and is highly expressed in myeloid cells upon injury (356). Osm signals through a receptor complex, including Osmr and glycoprotein 130 (gp130) (380), and both genes are mainly expressed by fibroblasts, pericytes and astrocytes (356). Genes related to the oncostatin M signalling pathway were upregulated in all fibroblast populations, 5 days after spinal cord injury (**paper III, figure 7**). OSM application upon spinal cord injury and in an EAE model reduced immune cell infiltration, lesion size and improved functional outcome (379,381), supporting an important role of this pathway and the immune cell – fibroblast axis in CNS injuries.

Immune cell infiltration dynamics vary in different CNS injury models (382). A rapid activation of resident microglia is characteristic for ischemic stroke (383) and in early stages more pronounced than infiltration of macrophages (384,385). Neutrophil infiltration is delayed in comparison to traumatic injuries and peaks only 2 days after the insult, with neutrophils mainly accumulating in the perivascular space (386–389). Differences exist as well between different stroke models. While in a transient MCAO model, microglia exceeds macrophage response and neutrophil extravasation is apparent, only few neutrophils extravasate in the ischemic tissue of permanent MCAO (385,388).

In the cortical and striatal stroke models analysed by us, we found variations in fibrotic tissue formation in the ischemic lesion core, which could be related to the differences in immune cell recruitment. Using the GLAST-$CreER^{T2}$ line, we showed that $GLAST^+$ perivascular cells and progeny accumulated around the striatal vasculature in a transient MCAO ischemic stroke model, in which the lesion is restricted to the striatum. In contrast, permanent occlusion of the distal middle cerebral artery (dMCAO), which resulted in a lesion restricted to the cerebral cortex, induced a fibrotic response similar to traumatic CNS injuries, with $GLAST^+$ perivascular cell-derived progeny leaving the blood vessel wall, proliferating and accumulating in the ischemic lesion core (**paper II figure 5 and 6**). Although $GLAST^+$ perivascular cells and progeny did not leave the blood vessel wall after striatal ischemic stroke, we observed a high proliferation rate. This could be a response to a pro-angiogenic and anti-inflammatory effect of activated microglia (390). Accumulation of $PDGFR\beta^+$

pericytes around the vasculature has been shown to support BBB function after ischemic stroke (391).
Interestingly, in cortico-striatal stroke lesions, GLAST$^+$ perivascular cell-derived progeny migrated out from the vascular wall in the cortex and striatum and contributed to robust fibrotic scar tissue formation (**paper II figure S11**). This observation suggests that an external stimulus, such as immune cell infiltration or an altered microenvironment, may activate perivascular cell migration and stimulate ECM production.

ECM deposition was higher in lesions with perivascular cells leaving the vasculature, compared to striatal stroke, in which lineage traced GLAST$^+$ perivascular cells proliferated but did not leave the blood vessel wall. Inhibiting proliferation of GLAST$^+$ perivascular cells reduced deposition of fibrotic ECM (**paper I, figure 1, S2; paper II, figure 8**). Moreover, cells that left the vasculature expressed αSMA and vimentin indicating a transition to a myofibroblast phenotype (**paper II figure 6, paper III figure S4**). Altogether, these results strongly indicate that the amount of deposited ECM is dependent on the detachment of GLAST$^+$ perivascular cells and progeny from the vascular wall. It is intriguing that GLAST-CreER^{T2} derived cells in striatal strokes are instructed to proliferate, but not to leave the vasculature and deposit ECM. This suggests that GLAST$^+$ perivascular cells inhere different injury response programs dependent on external stimuli.

CNS fibrotic scarring a conserved mechanism

PDGFRβ can be considered a pan-perivascular cell marker, as vSMC, pericytes and PVF readily express both *Pdgfrb* mRNA and the receptor. Of note, there is a gradient of *Pdgfrb* gene expression, with highest levels in pericytes, lower expression in PVF and decreasing levels in vSMC on bigger vessels at the same time as *Acta2* expression increases (paper 3) (20). PDGFRβ also labels stromal fibroblasts after injury throughout all studies. With the same PDGFRβ labelling strategy we were also able to verify fibrotic scaring in human spinal cord injury, multiple sclerosis, stroke and tumor (**paper III, figure 10**). The identification of fibrosis e.g. in multiple sclerosis is supported by others (244,392). In opposition to mouse, rats form large cysts after spinal cord injury and have long been considered not to produce fibrotic scar tissue, but it has been shown otherwise recently (116,393). This indicates that CNS fibrotic scarring is a mechanism conserved between rodents and human.

The fibrotic scar - interaction of injury compartments and therapeutic target

Using the GLAST-CreER^{T2} mouse line, we showed that small stab lesions to the cerebral cortex only induced a limited response from GLAST$^+$ perivascular cells (**paper II, figure S9**), in analogy to a study with lineage tracing of *Tbx18*$^+$ perivascular cells (394). This, yet again, raises the question about what is needed to induce a robust fibrotic reaction in the CNS. There is doubtless a high number of GLAST$^+$ perivascular cells in the adult mouse cortex (**paper II, figure 2)**, and large stab wounds and cortical strokes trigger a strong fibrotic response. It could be that the manifestation depends on the number of closely affected fibrogenic perivascular cells. The small stab lesions run parallel to the cortical penetrating arterioles (16) and might therefore transect or affect only few vessels with a substantial pool of GLAST$^+$ pericytes or fibroblasts.

Besides limited fibrosis in response to small cortical stab lesions, there is also no sharp glial scar border formed. When inhibiting the proliferation of GLAST$^+$ perivascular cells, and consequently reducing the number of stromal fibroblasts and fibrotic scarring, upon SCI we observed a reduced and less complex glial compartment (**paper I, figure S3**). This indicates that alterations to the fibrotic compartment of the scar also influence the glial scar and the formation of a sharp border between these two lesion components. In a study analysing

fibrotic core-astrocyte interactions, Hara *et al*. identified type I collagen-mediated transformation of astrocytes from reactive to a scar-forming phenotype, via N-cadherin signalling (196). There is no doubt about *Col1a1/2* expression in scar-forming fibroblasts and abundance of type I collagen in the fibrotic core in different CNS injuries (**paper I, II, III**). However, for example in EAE lesions, with pronounced scar-forming fibroblast proliferation and type I collagen deposition, astrocytes react differently, intermingle with immune cells and fibroblasts, but do not form a sharp border around fibrotic regions (18) (**paper II, figure 4**).

On the one hand, several studies report that reduction of glial and fibrotic scarring, as well as a reduced immune response, are beneficial for functional recovery after CNS injuries (377,395–401). On the other hand, selective ablation or modulation of astrocytes, ependymal cells or microglia can have a negative effect on regeneration (197,211,213,402–404). Lesion size alone is a poor indicator of functional outcome (405) and injury components affect each other and might have beneficial and detrimental effects on tissue regeneration. Therefore, complete ablation of one cell type will probably not be a solution for improved functional outcome. All interventions must most likely be titrated in a spatio-temporal manner.

Complete inhibition of proliferation by GLAST$^+$ perivascular cells resulted in virtually no fibrotic scarring and led to the formation of a tissue defect, which was detrimental for regeneration (**paper I, figure S1**). An independent study with a different animal model, leading to almost complete ablation of fibrotic scarring confirms increased functional impairment (155). These observations indicate that fibrotic scarring is needed to re-establish tissue integrity during the early phases of lesion formation. Antifibrotic treatment might therefore be most beneficial in the late subacute/chronic phase. In our bulk sequencing experiment of the SCI lesion site two weeks after dorsal hemisection many fibrosis and ECM genes are still highly expressed (**Paper I, figure 1**). This indicates continuous expression of ECM which can be potential targeted for intervention. Conversely reduction of collagen IV in rat only showed a positive effect in the acute/early subacute phase but not after five days or four weeks respectively (255,406). This could be because collagen IV is not produced later, or the treatment has an effect on cyst cavity formation. Fibrosis has been extensively studied in several other organs and many other approaches exist to target scarring that could be applied to the CNS. Inhibition of TGFβ1 or TGFβR1 has reduced fibrosis in brain lesions (262,395) and e.g. Pifernidone a drug that potentially acts through TGFβ1 (407) inhibition has shown promising neuroprotective results in a multiple sclerosis trial (408,409).

Nonetheless, persistent fibrotic scar tissue represents a barrier for regeneration in the chronic phase after injury. Attenuation of fibrotic scar tissue generation enabled regeneration of CST axons and regeneration/sprouting of RST axons caudal to the lesion (**paper I**). We chose to use a dorsal hemisection model to ensure that all CST axons are transected by the injury, allowing us to assess faithful CST axon regeneration (410–412). Serotonergic RST axons are unmyelinated and more prone to regenerate or sprout after SCI (413). Because of their sprouting ability, we cannot be certain that the increased number of RST fibres caudal to the lesion stems from regenerated axons. However, the density of RST axons was higher in mice with reduced scarring. Animals with the largest number of regenerated fibres caudal to the lesion showed the best sensorimotor performance (**paper I, figure 7**).

Together, our results identify perivascular-derived fibrotic scar formation as a relevant therapeutic target to improve recovery after CNS injuries.

8 CONCLUDING REMARKS AND POINTS OF PERSPECTIVE

In my thesis I have addressed the role of two perivascular cell types to detrimental fibrotic tissue deposition in different CNS injuries and diseases. The work presented here supports the central role of perivascular fibroblasts and pericytes for scarring and highlights them as potential targets to improve brain and spinal cord regeneration.

Paper I demonstrates that reduction of GLAST$^+$ perivascular cells in spinal cord injury reduces fibrotic scarring, modulates the lesion structure and aids axonal regeneration.
We can show that newly formed nerve fibres are functionally integrated and promote functional recovery after spinal cord injury.

This shows that perivascular fibroblasts and pericytes can be modulated to achieve beneficial scar alteration and that already a moderate reduction of cells improves functional outcome. We also found that complete abolishment of the fibrotic scar core is not beneficial for regeneration. In parallel to studies of other lesion components like astrocytes, microglia or macrophages (197,211,213,402–404,414–417) this underlines that modulation has to happen in a titrated, timed and coordinated fashion, because each lesion component contributes with both detrimental and beneficial components. The fibrotic compartment is necessary to initially seal the lesion, but after tissue reorganisation the persistent chronic scar inhibits regeneration. All in all, this highlights the GLAST$^+$ perivascular cell population as one potential target for future treatment of spinal cord injury.

Paper II broadens the picture and sheds light on several unanswered questions. We conclude that fibrotic scarring is a general mechanism throughout several spinal cord and brain injuries or diseases and that GLAST$^+$ perivascular cells are responsible for fibrosis. Moreover, it establishes the connection to human CNS maladies and identifies fibroblasts in similar non-neural fibrotic scar tissue. The findings further shift the focus towards perivascular cell derived fibrosis in the CNS as a critical component across several CNS disorders (409). Fibrosis has been intensively studied in other organs and different tools to modulate scar forming fibroblasts exist and represent potential treatment options.

Paper III determines that CNS fibrotic scar forming cells have a dual origin. Both a GLAST$^+$ subpopulation of pericytes and GLAST$^+$ perivascular fibroblasts contribute to the fibrotic core after spinal cord injury in a region dependent manner. Further, stromal fibroblasts comprise heterogenous subpopulations in the subacute phase after injury.

PVF and pericytes with comparable profile to mouse GLAST$^+$ perivascular cells can be found in human CNS (58,59,322). We are only starting to understand the heterogeneity of CNS perivascular cell populations, but translation of our results might help to identify mechanisms and target cells to modulate fibrosis in several human CNS maladies.

Taken together our studies highlight the detrimental character of fibrosis in the CNS, elucidate the cells of origin and shows opportunities for beneficial modulation of scarring.
We identified perivascular cell derived fibrosis in several different injury models, but also cleared differences between the lesion types and CNS regions. Understanding why some injuries and diseases trigger bona fide perivascular cell derived fibrosis, others only cell proliferation on the vasculature and some none at all, will clearly bring us closer to the mechanism behind CNS fibrosis.

The initial identification of GLAST$^+$ perivascular cells as major contributors to the fibrotic injury core, instigated many new studies about fibrosis in the CNS and a scientific discussion: are pericytes or perivascular fibroblasts the origin of CNS fibrotic tissue (353,409,418,419)? The answer we presented here is: both.

However, careful comparison of the mouse lines used in the different studies is needed. It will be crucial to compare sequencing results in depth and to sequence perivascular fibroblasts and GLAST$^+$ pericytes on their own and compare them to the whole GLAST$^+$ population. It will be crucial to study and compare the exact location along the vasculature in a comparable manner and to benchmark results from different parts of the CNS. For the time being GLAST$^+$ and GLAST$^-$ pericytes cannot be differentiated on the transcriptomic level. Potentially deeper sequencing of a larger population could resolve the tendentious differences we describe. As discussed, different origins during development might explain the different activation upon injury of superficially similar cells. These differences might be hidden in the epigenome of the cells, which deserves a closer investigation.

This lifts another, more general question. Does it matter, what the origin of stromal cells in the injury core is and if these cells are called fibroblast, perivascular fibroblast, injury induced mesenchymal stem cell, pericyte or something different? In principle, the name itself is of little importance, what matters is the function of different cell types and how we can use the detailed description of each and every population to develop treatment strategies for injuries and diseases, which lead to long lasting harm, pain or costs for the healthcare system. Nonetheless, a common nomenclature is critical to communicate with each other. Only if other researchers in the community understand which exact cell type an experiment or a publication is about, strategies to modulate or target these cells can be developed together. This is even more essential when information about different cell populations becomes more detailed and subdivisions more refined, down to the single cell level, with the help of present transcriptomics and proteomics tools.

New names for perivascular cells along the CNS vasculature have been suggested (311) and more will be needed. Until an accepted nomenclature is in place, the detailed description of the analysed populations is crucial, if possible, with regards to specific features, morphology, transcriptome, proteome or location along the vasculature.

If GLAST$^+$ perivascular cells are PVF, fibroblast-like pericytes or pericyte like-fibroblasts might appear trivial, but this difference could be the key to identify cell specific mechanisms, and ultimately the identification of new therapeutic approaches.

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Thank you so much **Urban** for accepting the task to be my co-supervisor. I think there is still a lot to discuss about CNS fibroblasts, maybe after the defense...

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Und nicht zuletzt natürlich dir, mein Kind
Wenn′s brenzlig wird, wenn's schief geht, wenn die Welt zusammenfällt
Eltern, die aus diesem Holze sind
Eltern, die aus diesem Holz geschnitten sind” (Reinhard Mey)

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