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***tPA mediated activation of PDGF-C/PDGFR α
signaling in the CNS***

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tPA mediated activation of PDGF-C/PDGFR α signaling in the CNS

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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“The important thing is to not stop questioning. Curiosity has its own reason for existing.”
- Albert Einstein

ABSTRACT

The platelet derived growth factors (PDGFs) and their tyrosine kinase receptors play an essential role during development, in adult tissue homeostasis and in several pathological events. PDGF-CC, a ligand for PDGFR α , is secreted as an inactive dimer. Proteolytic activation of PDGF-CC is mediated by the serine protease tissue plasminogen activator (tPA) and subsequently this allows binding of the active PDGF-CC dimer to and signaling through PDGFR α . In the blood tPA is involved in fibrinolysis and recombinant tPA is the only FDA approved treatment of acute ischemic stroke. However, thrombolytic therapy with tPA is highly limited due to an increased risk of intracerebral hemorrhage and it has been hypothesized that this is caused by unique activities of tPA in the neurovascular unit (NVU).

To better understand the biological function of tPA and PDGF-CC signaling in the central nervous system (CNS) we first characterized the effect of tPA ablation on brain development in mice (**Paper I**). We found that tPA deficient mice presented with rearrangements in the cerebrovascular tree, including a shift towards small diameter vessels as compared to wild-type mice. Additionally, we found ventricular malformations, including asymmetry of the lateral ventricles and a distorted ependymal lining. Since PDGF-C deficient mice have previously been described with asymmetric lateral ventricles, this potentially provides a first *in vivo* link between tPA and PDGF signaling in CNS development.

In **Paper II** we aimed to identify the mechanism underlying abnormal ventricular development in PDGF-C deficient mice. Our findings suggest that PDGF-CC/PDGFR α signaling controls radial glia migration and differentiation, subsequently affecting ependymal development and maturation. This in turn might explain the ventricular expansion and asymmetry associated with PDGF-C ablation.

tPA mediated activation of PDGF-CC and subsequent PDGFR α signaling has been associated with increased cerebrovascular permeability in CNS disorders. However, activation of PDGF-CC by tPA *in vitro* has been described as inefficient, thus we investigated potential co-factors needed for tPA mediated activation of PDGF-CC in the NVU in **Paper III**. We found that Mac-1 on microglia is required to facilitate efficient activation of PDGF-CC by tPA. Subsequently this enhanced PDGFR α phosphorylation in the NVU, resulting in loss of BBB integrity and intracerebral hemorrhage. In support of Mac-1 being a co-factor for PDGF-CC/PDGFR α activation, we found that Mac-1 deficient mice showed decreased BBB permeability and smaller infarct size in an experimental model of ischemic stroke.

Previous studies have shown that the tyrosine kinase inhibitor imatinib improves neurological and functional outcome after ischemic stroke via inhibition of PDGFR α signaling in both mice and patients. In **Paper IV** we described the mechanism how imatinib ameliorates stroke pathology. In the acute phase we found that imatinib preserved BBB integrity and reduced reactive gliosis, whereas in the chronic phase blocking PDGFR α signaling in the NVU in ischemic stroke moderated scar formation.

Collectively these findings will help us to better understand the role of tPA in the NVU and provide new insights regarding the role of PDGF-CC/PDGFR α signaling in CNS development and in ischemic stroke. Ultimately this might lead to novel treatment strategies to improve outcome following ischemic stroke.

WISSENSCHAFTLICHE ZUSAMMENFASSUNG

Die PDGFs (Platelet Derived Growth Factor) gehören zu einer Familie von Wachstumsfaktoren, bestehend aus PDGF-AA, PDGF-BB, PDGF-CC und PDGF-DD und den dazugehörigen Tyrosinkinase-Rezeptoren PDGFR α und PDGFR β . PDGF-CC wird als inaktives Dimer sezerniert und muss vor der Signaltransduktion durch PDGFR α , von dem gewebespezifischen Plasminogen Aktivator (tPA) aktiviert werden. tPA ist bekannt durch seine Rolle in der Fibrinolyse. In dieser klassischen Funktion wird tPA zur Auflösung von Blutgerinnseln eingesetzt. Tatsächlich ist tPA die einzige FDA anerkannte medikamentöse Behandlung von akutem ischämischem Schlaganfall. Die Anwendung ist jedoch aufgrund des Risikos von Hirnblutungen sehr limitiert. Dieses Risiko wird auf besondere, teils unbekannt Funktionen von tPA im Gehirn und auf die Blut-Hirn-Schranke zurückgeführt.

Um diese besondere Funktion von tPA im Gehirn besser zu verstehen, charakterisierten wir in **Paper I** tPA defiziente Mäuse. In diesen Mäusen führt das Fehlen von tPA zu einer Neuorganisation des zerebralen Blutgefäßsystems, einschließlich einer Verlagerung zu kleineren Gefäßdurchmessern. Zusätzlich fanden wir eine Fehlbildung des zerebralen Ventrikelsystems in Form von vergrößerten, asymmetrischen lateralen Ventrikeln. Ähnliche Fehlbildungen wurden zuvor auch in PDGF-C defizienten Mäusen beschrieben. Dementsprechend zeigen diese Erkenntnisse eine erste Verbindung von tPA und PDGF-C in der Entwicklung der zerebralen Ventrikel auf. Die molekularen Hintergründe, durch die das Fehlen von PDGF-C zu ventrikulären Fehlbildungen führt, sind nicht bekannt und wurden in **Paper II** erforscht. Wir zeigen, dass vermutlich fehlerhafte Zellmigration und Zelldifferenzierung zu mangelhafter Entwicklung und Reife von ependymalen Zellen entlang der Ventrikelwand führen und folglich zu ventrikulären Fehlbildungen.

Aktivierung von PDGF-CC durch tPA und anschließende Signaltransduktion durch PDGFR α wird mit erhöhter Permeabilität der Blut-Hirn-Schranke in diversen Erkrankungen des zentralen Nervensystems (ZNS) verbunden. Die Aktivierung von PDGF-CC durch tPA *in vitro*, außerhalb eines lebenden Organismus, wird jedoch als mangelhaft beschrieben. In **Paper III** untersuchten wir, welche möglichen Co-Faktoren um die Blut-Hirn-Schranke in einem lebenden Organismus die Aktivierung von PDGF-CC unterstützen könnten. Wir fanden heraus, dass LRP1 und Mac-1 auf Mikroglia zur leistungsfähigen Aktivierung von PDGF-CC durch tPA notwendig sind. Zusätzlich zeigten wir, dass das Fehlen von Mac-1, Mäuse vor erhöhter Permeabilität der Blut-Hirn-Schranke schützt und auch zu kleineren Schlaganfällen führt. Dementsprechend stellt die Voraussetzung dieser Co-Faktoren eine Limitation für die Aktivierung von PDGF-CC durch tPA dar und bietet eine neuartige Möglichkeit, um die momentan vorhandene Behandlung von ischämischem Schlaganfall zu verbessern. Zusätzlich konnten wir kürzlich in einer klinischen Studie zeigen, dass der Tyrosinkinase Inhibitor Imatinib durch Hemmung der PDGFR α Signaltransduktion den Krankheitsverlauf eines ischämischen Schlaganfalls abschwächt und zu verbessertem neurologischen Ausgang führt. Der darunter liegende Mechanismus ist jedoch weitgehend unbekannt. In **Paper IV** beschreiben wir, dass die Hemmung von PDGFR α durch Imatinib die Integrität der Blut-Hirn-Schranke schützt und Narbenbildung so wie auch reaktive Gliose in Mäusen mit experimentellem ischämischem Schlaganfall mildert.

Diese Erkenntnisse helfen die Rolle von tPA um die Blut-Hirn-Schranke besser zu verstehen und bieten neue Einblicke in die Funktion von PDGF-CC/PDGFR α Signaltransduktion in der Entwicklung des ZNS und bei ischämischem Schlaganfall. Die Schlüsselrolle der Blut-Hirn-Schranke bietet eine Möglichkeit zu verbesserten Behandlungsstrategien und dadurch verbesserten Krankheitsausgang für Schlaganfall Patienten.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, which will be referred to by their roman numerals

- I. **C. Stefanitsch**, A. E. Lawrence, A. Olverling, I. Nilsson, L. Fredriksson
tPA Deficiency in Mice Leads to Rearrangement in the Cerebrovascular Tree and Cerebroventricular Malformations
Frontiers in Cellular Neuroscience, 2015, Volume 9, Article 456
- II. **C. Stefanitsch**, L. Fredriksson
Platelet-derived growth factor-CC regulates ependymal cell maturation in mice
Manuscript
- III. E.J. Su, C. Cao, L. Fredriksson, I. Nilsson, **C. Stefanitsch**, T.K. Stevenson, J. Zhao, M. Ragsdale, Y. Sun, M. Yepes, C. Kuan, U. Eriksson, D. K. Strickland, D.A. Lawrence, L. Zhan
Microglial-mediated PDGF-CC activation increases cerebrovascular permeability during ischemic stroke
Acta Neuropathologica, 2017, 134(4), 585-604
- IV. M. Zeitelhofer, **C. Stefanitsch**, M.Z. Adzemovic, E.J. Su, S. Lewandowski, L. Muhl, I. Nilsson, D. A. Lawrence, L. Fredriksson
PDGFR α antagonism reduces reactive gliosis and the scar formation after ischemic stroke in mice
Manuscript

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LIST OF ABBREVIATIONS

ALS	amyotrophic lateral sclerosis
ASMA	alpha smooth muscle actin
BBB	blood-brain barrier
BCR-ABL	breakpoint cluster region protein - abelson murine leukemia viral oncogene homolog
CCL	C-C motif ligand
CCR	C-C-motif-chemokine-receptor
c-kit	KIT proto-oncogene receptor tyrosine kinase
CML	chronic myeloid leukemia
CNS	central nervous system
CPEB protein	cytoplasmic polyadenylation element binding protein
CSF	cerebrospinal fluid
CSPG	chondroitin sulfate proteoglycans
CUB	complement C1r/C1s, Uegf, Bmp1
CX3CR1	C-X3-C motif chemokine receptor 1
ERG	ETS-related gene
ETS	erythroblast transformation specific
FDA	food and drug administration
GAP	glyceraldehyde 3-phosphate
GIST	gastrointestinal stromal tumors
GFAP	glial fibrillary acidic protein
GFD	growth factor domain
GFP	green fluorescent protein
Glut	glucose transporter
Grb2	growth factor receptor-bound protein 2
ICH	intracerebral hemorrhage
IFN- γ	interferon-gamma
IL	interleukin
iNOS	inducible nitric oxide synthase
Jam3	junctional adhesion molecule C
L-LTP	late phase long-term potentiation
LRP	low-density lipoprotein receptor-related protein

Mac-1	macrophage-1 antigen, integrin α M β 2, CD11b/CD18
MCAO	middle cerebral artery occlusion
MMP	matrix metalloproteinase
Mpdz	multiple PDZ domain protein
NFIX	nuclear factor 1 X-type
NG2	neuron-glia antigen 2
NMDA	N-methyl-D-aspartate
NRP1	neuropilin 1
NVU	neurovascular unit
OPC	oligodendrocyte precursor cell
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate-buffered saline
PDGF	platelet derived growth factor
PDGFR	platelet derived growth factor receptor
PI3K	phosphatidylinositol 3-kinase
PLC- γ	phospholipase C- γ
RFP	red fluorescent protein
RGMa	repulsive guidance molecule a
ROS	reactive oxygen species
SDF-1	stromal cell-derived factor-1
SH2-domain	Src homology 2 domain
SHP-2	cytoplasmic SH2 domain containing protein tyrosine phosphatase
Stat	signal transducer and activator of transcription
SVZ	subventricular zone
TGF- β	transforming growth factor- β
TH cells	T helper cells
TNF	tumor necrosis factor
tPA	tissue plasminogen activator
TSG-6	tumor necrosis factor-stimulated gene-6
uPA	urokinase plasminogen activator
VEGF	vascular endothelial growth factor
ZO1	zona occludens protein-1

1 INTRODUCTION

1.1 The Platelet-Derived Growth Factor (PDGF) Family

The Platelet-Derived Growth Factor (PDGF) family consists of five known disulphide-bonded dimers formed from four different polypeptide chains, PDGF-A, PDGF-B, PDGF-C, and PDGF-D, and their tyrosine kinase receptors, PDGFR α and PDGFR β [1].

The PDGF ligands belong to the superfamily of Vascular Endothelial Growth Factors (VEGFs). These factors all contain a growth factor domain (GFD) with a pattern of eight highly conserved cysteine residues necessary for dimerization, receptor binding and activation [2]. PDGF-A and PDGF-B polypeptides contain N-terminal pro-domains, which need to be intracellularly removed by either furin or related proprotein convertases in order for these factors to become active [3]. Further, PDGF-A and PDGF-B polypeptides exhibit a C-terminal basic retention motif that binds and interacts with extracellular matrix components via electrostatic interactions between the retention motif and negatively charged groups in heparin sulfate [1, 2]. It was long believed that PDGF-AA and PDGF-BB dimers were the only ligands for PDGFR α and PDGFR β , respectively, but the fact that PDGF-A deficient mice displayed a less severe phenotype [4, 5] than PDGFR α deficient mice [6] implied the existence of an additional ligand for PDGFR α . Search for such a ligand led to the discovery of PDGF-CC [7], and later also to the rather unexpected discovery of a novel ligand for PDGFR β , PDGF-DD [8].

PDGF-CC and PDGF-DD are secreted as full-length inactive dimers with N-terminal CUB-domains linked to the respective GFD via a hinge region of 80-90 amino acids [7, 9]. The CUB-domains need to be proteolytically removed in order to allow receptor binding of the active dimer and subsequent signaling. The GFD, the hinge region as well as the CUB-domain are required for specific interactions with the proteolytic enzymes and cleavage occurs extracellularly after secretion of the inactive dimers. Full length PDGF-CC is proteolytically processed by tissue plasminogen activator (tPA) [9, 10] (Fig.1), whereas PDGF-DD is cleaved by the other known plasminogen activator, urokinase plasminogen activator (uPA) [11, 12] or matriptase [13].

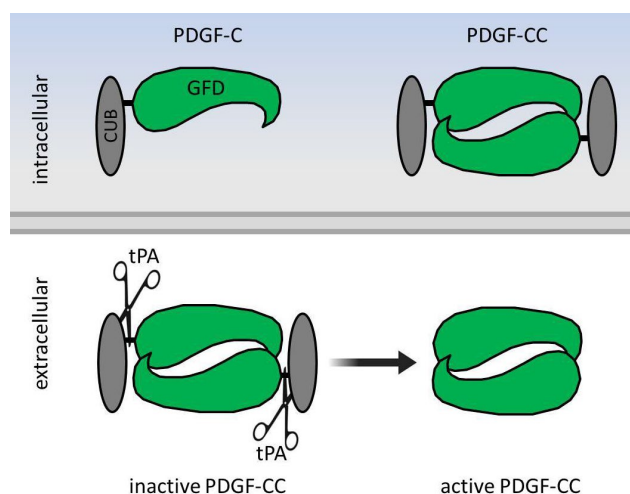


Fig.1: Extracellular processing of PDGF-CC.

PDGF-CC is produced and secreted as an inactive dimer requiring extracellular proteolysis for activation. Full length PDGF-CC is proteolytically cleaved by tPA, resulting in an active growth factor domain (GFD) dimer able to bind and stimulate PDGFR α signaling.

The PDGF receptors are tyrosine kinase receptors of ~170 and 180 kDa [2]. Each PDGF receptor contains an intracellular tyrosine kinase domain, a trans-membrane domain and five extracellular immunoglobulin-like domains (illustrated in Fig.2). The extracellular domains are involved in ligand binding and receptor dimerization, which subsequently leads to conformational changes and auto-phosphorylation of tyrosine residues in the intracellular domains. This intracellular phosphorylation enables interaction with different families of SH2-domain containing molecules, including members of the PI3-kinase family, Phospholipase C- γ (PLC- γ) and the Src family, as well as Grb2, SHP-2, GAP and Stat molecules [2]. Following activation of the receptor, clathrins are recruited, thereby leading to internalization of the receptor from the cell surface by endocytosis for either degradation or recycling [14].

In vivo experiments have shown that, after intracellular processing and secretion, the PDGF-AA dimer signals via PDGFR α and PDGF-BB mainly via PDGFR β [2], whereas, after extracellular proteolysis, PDGF-CC signals via PDGFR α [7]. PDGF-DD signals via PDGFR β [8], however *in vivo* this has so far only been shown in pathology such as fibrotic processes, various cancers and cardiovascular disease [1, 15-17] and, unlike the other PDGFR β ligand, PDGF-BB, PDGF-DD seems to be dispensable for murine embryonic development [18]. The differential effect of PDGF-DD and PDGF-BB signaling through PDGFR β might be explained by the recent findings that Neuropilin 1 (NRP1) is a co-receptor for PDGF-DD/PDGFR β signaling but not for PDGF-BB/PDGFR β signaling [19]. *In vitro* PDGF-AB and PDGF-BB (in addition to PDGF-AA and PDGF-CC) have been demonstrated to bind PDGFR α . Furthermore, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD have been shown to bind to a heterodimerized PDGFR $\alpha\beta$ receptor complex [20] (see Fig.2 for schematic illustration of the ligand binding profile to the PDGFRs). The physiologic relevance of these *in vitro* findings has however yet to be determined.

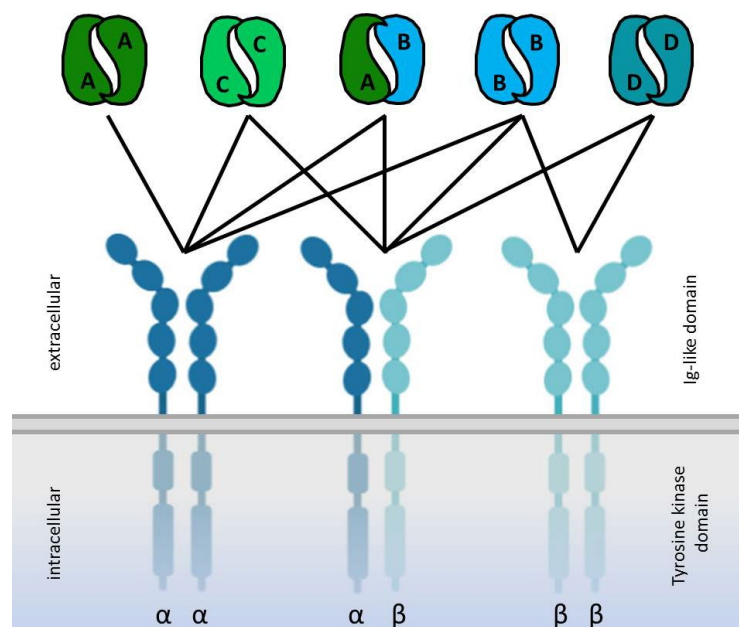


Fig.2: Receptor interactions of the PDGFs

PDGF-AA, PDGF-BB and PDGF-CC homodimers as well as the PDGF-AB heterodimer signals via PDGFR α whereas PDGF-BB and PDGF-DD signals via PDGFR β . PDGF-CC, PDGF-BB and PDGF-DD homodimers as well as the PDGF-AB heterodimer have been shown to interact with PDGFR $\alpha\beta$.

PDGFR α , but not PDGFR β , signaling has been linked to primary cilia [21, 22]. Primary cilia are signaling hubs on eukaryotic cells that coordinate signaling pathways in cell-cycle control, migration, differentiation during development and tissue homeostasis [23, 24]. Along these lines, activation of PDGFR α on primary cilia has been found, via activation of Akt and the Mek1/2-Erk1/2 pathways, to control cell migration and differentiation processes [21, 24, 25]. Defects in assembly or function of primary cilia lead to numerous developmental disorders and diseases potentially due to aberrant signal transduction [24].

1.1.1 Roles of PDGF-CC and PDGFR α Signaling in Embryonic Development

PDGFs act primarily as paracrine growth factors and their expression is spatio-temporally regulated during development and disease. The expression patterns of the PDGF ligands and their receptors during embryonic development have been shown to be distinct but partially overlapping [1]. Deletion of the genes encoding PDGFR α and its ligands PDGF-AA and PDGF-CC in mice have provided valuable information about their biological function during embryonic development. Knockout of PDGF-A [4, 5, 26] or PDGFR α in mice [6] is lethal, although deletion of PDGFR α results in a phenotype similar, but more severe than the phenotype caused by ablation of the PDGF-A gene [6]. PDGFR α signaling was found to be critical for embryonic development in mice, as all PDGFR α deficient embryos die at mid-gestation. It was established that PDGFR α signaling is required for the migration of various cell populations during developmental processes, including migration of cranial neural crest cells and spreading of oligodendrocyte precursors in the spinal cord [6]. The documented phenotypes associated with PDGFR α ablation included, in addition to the defects found in PDGF-A deficient mice [5], skeletal defects such as spina bifida (incomplete closure of the neural tube), cleft palate and abnormalities of the rib cage, but also, hemorrhaging and subepidermal blistering [6]. The discrepancy between the phenotypes of PDGF-A and PDGFR α deficiency could not be explained by PDGF-BB signaling through PDGFR α , as double PDGF-A/B null embryos did not display the additional PDGFR α defects [7]. This therefore led to the search for, and later the identification, of PDGF-CC as a novel PDGFR α ligand [7]. Ablation of PDGF-C in 129S1/Sv mice has since been shown to cause perinatal lethality due to feeding and respiratory difficulties associated with a complete cleft of the secondary palate, with 95% of the pups dying at postnatal day (P) 1 and none surviving until weaning [27]. The PDGF-C deficient mice displayed subepidermal blisters and spina bifida occulta, thus resembling some of the phenotypes reported for the PDGFR α knockouts that had not been associated with PDGF-A ablation. Combined deletion of PDGF-A and PDGF-C was found to recapitulate the PDGFR α knockout phenotype, indicating that PDGF-AA and PDGF-CC are the major PDGFR α ligands in murine embryonic development [27]. It has later been established that targeted deletion of PDGF-C in mice results in different phenotypes depending on the genetic background of the mice. In a C57BL/6 background, deletion of PDGF-C was reported to be compatible with postnatal life, although loss of PDGF-CC signaling was found to result in various central nervous system (CNS) abnormalities [28] (discussed in section 1.1.3).

1.1.2 Physiological Roles of PDGF-CC and PDGFR α Signaling in the Adult

Due to the importance of PDGF-CC and PDGFR α signaling during embryonic development and pathology, much less focus has been on understanding the role of this signaling system in adult physiology. In most adult tissues the expression of the PDGFR α is usually low, as it is mainly needed to maintain tissue homeostasis. However, during various tissue remodeling processes, such as wound healing and angiogenesis, as well as inflammation and disease, PDGFR α expression increases [1]. Due to the generally low activity of PDGFR α signaling in the adult, pharmacological usage of imatinib, a tyrosine kinase antagonist of ABL, c-kit and the PDGFRs, was considered as safe. Consequently, imatinib is today successfully used to treat patients suffering from myelodysplastic/myeloproliferative diseases associated with PDGFR α gene rearrangements as well as for treatment of BCR-ABL driven chronic myeloid leukemia (CML) and c-kit positive gastrointestinal stromal tumors (GIST) [29, 30]. Nevertheless, several short-term side effects occur with imatinib treatment, including edema, muscle cramps, nausea, diarrhea, fatigue, and headache, as well as long-term effects such as cerebral edema, multiple sclerosis, hepatic- and pulmonary toxicities, dermatitis and pancreatitis [31]. Whether this is due to imatinib's effect on PDGFR α signaling or its activity against c-kit or ABL is not established.

In conditional knockout mice, where PDGFR α was completely inactivated in adult mice but intact during development, it was shown that PDGFR α plays a crucial role in connective tissue remodeling and wound healing [32]. This, by controlling deposition of collagen and fibronectin in extracellular matrix production. Further these mice revealed decreased angiogenesis and improper fibroblast recruitment [32]. Taken together this suggests various different roles of PDGFR α signaling in adult physiology. Corresponding conditional knockout studies to investigate the role of PDGF-CC in the adult have yet to be performed and as a result less is known about this factor's role in physiologic processes.

1.1.3 Expression and Function of PDGF-CC and PDGFR α in the CNS

1.1.3.1 Expression

High expression of PDGF-C transcripts have been found in many organs including the brain [33]. During CNS development PDGF-C is found in neuronal progenitors of the developing brain, in the choroid plexus and vascular elements of the pia mater, as well as in the neural tube and in the ventral horn of the spinal cord [33]. Ding et al. also reported PDGF-C expression in the cerebellum and neurogenic derivatives of neural crest cells [34]. In the adult murine brain PDGF-C is highly expressed on both transcriptional and protein level in the cortex, the hippocampus, the amygdala and the septum separating the lateral ventricles [28]. In single cell analyses of the neurovascular unit (NVU), PDGF-C transcript was found to be highly expressed in arteriolar and arterial smooth muscle cells, as well as in astrocytes and vascular fibroblast like cells [35, 36]. In addition, PDGF-C expression was found in non NVU cells, with the highest expression recorded in oligodendrocyte precursor cells (OPCs) and less in mature oligodendrocytes [35, 36].

In the CNS, high expression of PDGFR α is found in OPCs and glia cells but also in cells undergoing neuronal development and neurons of the olfactory bulb, the cerebral cortex, the hippocampus as well as in neuroepithelial cells during early development [37, 38]. PDGFR α expression is also found in perivascular cells in the NVU [28, 39-41]. The identity of the PDGFR α expressing cells in the NVU is however a matter of some debate [28, 35, 39, 42, 43]. Immunofluorescent studies suggest that PDGFR α in the NVU is expressed on perivascular astrocytes along arterioles and pre-capillary arterioles throughout the CNS [28, 39, 44], and single cell sequencing suggests these cells to be vascular fibroblast-like cells [35]. Nevertheless, ablation of PDGFR α expression from the perivascular pool can be achieved in mice utilizing site-specific recombination with glial-fibrillary acidic protein (GFAP)-driven Cre expression [40].

1.1.3.2 Function

During CNS development PDGF-CC has been reported to play a role in cerebral vascular development. This since PDGF-C deficiency in C57BL/6 mice was found to cause an overall increase in vascular density and diameter as well as incorrect investment of vascular smooth muscles cells around vessels [28]. In addition, cerebral ventricular malformations, such as asymmetry of the cerebral lateral ventricles, displaced septum and an abnormal ependymal lining were noticed [28]. The mechanism how aberrant PDGF-CC signaling leads to abnormal ventricular development remains elusive and is further investigated in Paper II.

Deletion of PDGF-C in mice is associated with a milder form of skeletal defect (spina bifida occulta) [27] than the skeletal defect associated with ablation of PDGFR α (spina bifida) [6]. However, deletion of one PDGFR α allele in the PDGF-C deficient mice resulted in manifestation of the spina bifida phenotype [45]. This incomplete closure of the neural tube is not normally seen in PDGFR α heterozygous mice, indicating a gene dosage effect of PDGFR α signaling in neural tube closure. In addition, these double mutant *Pdgfc*^{-/-};*Pdgfra*^{GFP/+} mice also displayed abnormal meninges with neuronal over-migration in the cerebral cortex [45].

In the adult CNS, proliferation, differentiation and migration of OPCs has been shown to be stimulated by PDGFR α [46], and later it was found in *in vitro* experiments that microglia produce factors stimulating oligodendrocyte development via PDGFR α signaling [47]. Interestingly, PDGF-CC was mentioned as a crucial factor for OPC recruitment and activation in mice injected with glioma cells [48], although the effect of PDGFR α signaling on OPC differentiation and migration is normally ascribed to the effect of PDGF-AA signaling [5].

In addition, PDGFR α signaling is well known to regulate blood-brain barrier (BBB) integrity [39, 49] and in this context PDGFR α signaling is controlled by tPA mediated proteolytic activation of PDGF-CC [39] (discussed in further detail in section 1.3).

Collectively, this illustrates that PDGF-CC and PDGFR α are important for normal CNS development and that this signaling pathway is involved in physiological CNS processes in adult mice, including OPC proliferation, differentiation and migration [46-48], as well as BBB regulation [39].

1.2 The Multiple Roles of tPA

tPA is a multidomain trypsin-like serine protease best known for its role in fibrinolysis in blood plasma, where it converts plasminogen into the broad specificity protease plasmin [50-52]. Plasmin in turn controls fibrin degradation and the conversion of latent matrix metalloproteinases (MMP) into active MMPs leading to degradation of extracellular matrix [50-52]. tPA consists of 530 amino acids and is composed of a finger domain, a growth factor domain, two kringle domains and the protease domain [52]. The protease domain is the active site of tPA and consists of the amino acids serine, aspartic acid and histidine and is located in the C-terminal region [52]. Activation of the plasminogen system by infusion of plasminogen activators like tPA is one approach for thrombolytic therapy to achieve lysis of blood clots. Recombinant tPA is currently the only FDA approved treatment of acute ischemic stroke, but evidence suggests a role of tPA in hemorrhagic transformation of an ischemic stroke due to unique activities in the NVU [39, 53]. The NVU is a functional unit of endothelial cells, forming the blood vessel lining, neurons and non-neuronal cells, including vascular mural cells, astrocytes and microglia, on the abluminal side (Fig.3). The close proximity of these different cell types is critical for paracrine regulations and normal CNS function including neurovascular coupling and vascular integrity [54].

1.2.1 Expression and Function of tPA in the CNS

tPA is mainly produced and secreted from endothelial cells, but this pool of tPA is nearly exclusively released into the bloodstream [52]. In the CNS, tPA is also synthesized in neurons, where it is stored in vesicles and released in response to neuronal activity to regulate various biological responses, including cerebrovascular responses [55]. In the adult murine brain, tPA is highly expressed in the cortex, the amygdala, the mossy fiber pathway of the hippocampus, as well as the cerebellum and the hypothalamus [56]. High expression of tPA is also reported in the human CNS, where tPA is found in neurons of many different brain structures, including in the hippocampus and amygdala of the limbic system. High expressions were found in the human hippocampus, especially in pyramidal neurons of the cornu ammonis and granule neurons of the dentate gyrus. Additional to neuronal expression, tPA is found in microglia and astroglia and has been associated with gliosis in disease [57]. tPA activity and expression in the CNS is regulated by its inhibitor neuroserpin and by binding of the cytoplasmic polyadenylation element binding (CPEB) protein leading to extension of tPA mRNA polyadenylation and an increase in tPA protein synthesis [55, 58, 59].

1.2.2 Roles of tPA in CNS Development

During development tPA is involved in neuronal development and survival [60], migration [61, 62], and cerebrovascular as well as cerebroventricular development (Paper I [41]).

In *in vitro* studies it has been shown that granule neurons possess binding sites for tPA [63] and that tPA is released at the neuronal growth cone [64], suggesting a role of tPA in neurite growth and migration. In neural progenitor cells this was later shown to be via Wnt/ β -catenin signaling [65].

Further, PC12 cells, a pheochromocytoma cell line from the adrenal gland, showed faster migration and regeneration of neurites when transfected to overexpress tPA [62] and tPA deficient mice displayed delayed migration of cerebellar granule neurons [61], suggesting a direct role of tPA in neuronal migration. It should be noted that tPA overexpression in mutant mice has been described to result in dendritic underdevelopment, thus challenging the above *in vitro* results [60].

1.2.3 Physiological Roles of tPA in the Adult

In the adult CNS, tPA has been reported to be involved in many different processes, including neurovascular coupling [40, 66], excitotoxicity [67-69], neurometabolic effects [70, 71], regulation of neuronal activity via the N-methyl-D-aspartate (NMDA) receptor [69, 72], microglial activation [73-75] and regulation of vascular permeability [39, 76]. High expression of tPA in the mossy fibers of the hippocampus suggests a role of tPA in memory and learning [58]. Further, tPA expression is increased during activity-dependent forms of synaptic plasticity [77] and in Purkinje neurons of rats during motor learning [78]. Accordingly, tPA deficient mice show learning deficits in both rate and extent [79, 80] and stimulation with tPA enhances late phase long-term potentiation (L-LTP), synaptic formation and strength, the underlying mechanisms of learning and memory [81].

It has been suggested that the pleiotropic effects reported for tPA in physiologic and pathologic processes in the CNS, may be a consequence of its role in the NVU regulating cerebrovascular responses and subsequently parenchymal homeostasis [55, 59]. The effect of tPA in the NVU has been shown to be mediated by a plasminogen-independent activation of PDGF-CC on the abluminal side of the vessel wall. This, since injection of tPA into the blood of unchallenged mice does not increase BBB permeability, whereas injection into the cerebrospinal fluid does [39]. Further, this activity of tPA in the NVU was shown to be independent of uPA, plasminogen and MMP-9, thus suggesting another substrate in tPA-mediated regulation of cerebrovascular permeability [76]. *In vitro* it was shown that tPA is capable of cleaving latent PDGF-CC in a plasminogen-independent manner, generating an active PDGF-CC ligand that can bind and activate PDGFR α [9] (Fig.1 and 2). This tPA mediated activation of PDGF-CC was later confirmed *in vivo*, when it was shown that tPA-induced cerebrovascular changes are facilitated by activation of PDGF-CC and subsequent PDGFR α signaling on perivascular astrocytes [39].

Further, it has been shown that tPA is released into the perivascular space in response to neuronal activity, where it is thought to control the process of neurovascular coupling, possibly through a PDGF-CC/PDGFR α dependent mechanism [55, 66]. Neurovascular coupling describes an increase in local cerebral blood flow in response to increased neuronal activity, which is critical for energy supply to activated neurons and clearance of metabolic waste products [82]. Underlining a role of tPA in neurovascular coupling is the fact that penetrating arterioles have been shown to control this process and tPA has been shown to be expressed in close proximity to arterioles in the CNS [40, 66]. Additionally, tPA has been reported to reduce vessel reactivity to increased luminal pressure and vasoactive mediators, and tPA can directly reduce cerebral vascular resistance and systemic blood pressure [55]. Under pathologic conditions the role of tPA mediated activation of PDGF-CC/PDGFR α in the NVU has been extensively studied and is discussed in further detail below.

1.3 tPA and PDGF-CC/PDGFR α Signaling in CNS Pathology

During CNS development both tPA and PDGF-CC have been associated with cerebroventricular formation in mice [28, 41], providing a first link between tPA and PDGF-CC signaling *in vivo*. In addition, aberrant PDGFR α signaling has been associated with hydrocephalus formation [83], the most well characterized form of cerebroventricular malformation in patients (also referred to as ventriculomegaly). These findings are intriguing because the molecular mechanisms underlying brain ventricle formation remain poorly understood.

In adult mice, tPA mediated activation of PDGF-CC/PDGFR α has been shown to be involved in several neurological disorders, including ischemic stroke [39] (discussed in further detail in section 1.3.2), spinal cord injury [84], traumatic brain injury [85], multiple sclerosis [86], ALS [87], and seizures [40]. For all these disorders inhibition of PDGF signaling has been shown to reduce BBB permeability and restore vascular barrier properties, leading to improved outcome of the disease.

1.3.1 Hydrocephalus / Ventriculomegaly

Hydrocephalus is a CNS disorder characterized by expansion of the cerebral ventricles and accumulation of cerebrospinal fluid (CSF). Accumulation of CSF leads to increased intracranial pressure and subsequent compression of the surrounding tissue [88]. It is a common neurological disorder associated with cognitive and physical impairment, creating a yearly economic burden in the United States of \$2 billion for pediatric hydrocephalus alone [89].

The etiology of hydrocephalus is widely discussed and related to multiple causes. So far hydrocephalus has been linked to hemorrhage and infections [90, 91], overproduction [92-94] or disturbed absorption of CSF [94-96] as well as disturbances in CSF flow due to an obstruction [96-99] or defect motile cilia structure and function on ependymal cells [100].

CSF is produced by highly specialized epithelial cells in the choroid plexus in the lateral ventricles. From the lateral ventricles CSF circulates through the interventricular foramina to the third ventricle and via the cerebral aqueduct to the fourth ventricle before reaching the subarachnoid spaces, where it is reabsorbed at the arachnoid granulations into the internal jugular vein by a pressure-dependent gradient [101, 102]. CSF circulation is thought to be achieved by propulsion of motile cilia on ependymal cells lining the ventricular system, pulmonary respiration and cardiac pulsations [102]. Thus, venous insufficiency [103], arteriovenous malformations [104, 105] and cardiac failure [106] have been described to contribute to hydrocephalus development. Further, altered CSF absorption in patients and in mice deficient in the water channel aquaporin-4, has been linked to hydrocephalus [94, 95, 107]. Another reason for disturbed CSF flow resulting in hydrocephalus formation is an obstruction of the narrow passage through the cerebral aqueduct. This is for example seen in *Wrp* deficient mice, where abnormal cell migration and astrogliosis has been shown to lead to blockage of the cerebral aqueduct and subsequently to hydrocephalus [98]. Obstruction in the aqueduct due to increased secretory activity of the subcommissural organ has also been associated with hydrocephalus [97].

CSF is mainly composed of water, transporting proteins, ions, neurotransmitters and glucose [102] and a change in the osmotic gradient of CSF due to excess molecules can result in hydrocephalus formation [108]. Decreasing CSF protein levels has been successful to reverse or prevent post hemorrhagic hydrocephalus in several cases [109, 110]. Interestingly, patients with hydrocephalus present elevated levels of VEGF in the CSF [111] and VEGF infusion and increased VEGF signaling leads to ependymal changes and hydrocephalus in rats [111, 112].

In addition to the above discussed causes of hydrocephalus, signaling on non-motile primary cilia has gained increasing interest. This, since patients with the ciliopathy Laurence-Moon-Biedl syndrome or Bardet-Biedl syndrome present with occasional hydrocephalus [113, 114]. Primary cilia serve as a signaling sensor extending from nearly all cell types [115] and several studies have linked impaired signaling on primary cilia to hydrocephalus development [83, 116-119]. PDGFR α signaling has been shown to depend on primary cilia [21] and it has been demonstrated that aberrant PDGFR α signaling in primary cilia on neuronal progenitor cells leads to hydrocephalus formation [83]. This, due to a decrease of PDGFR α /NG2 positive progenitor cells in the subventricular zone (SVZ) [83]. Disruption of the SVZ, including loss of neural progenitor cells, has repeatedly been associated with hydrocephalus [120]. As discussed above, PDGFs and their tyrosine kinase receptors play an important role during development [1] and ablation of PDGF-CC has been shown to result in ventricular malformations such as asymmetry of the lateral ventricles and hypoplastic development of the septum separating the lateral ventricles [28]. In addition, our findings presented in Paper I show that mice deficient in tPA, the protease responsible for PDGF-CC cleavage and activation, display enlarged asymmetric lateral ventricles as well [41]. Collectively, this suggests a role of tPA mediated activation of PDGF-CC/PDGFR α signaling in normal ventricle development and that defective signaling might be involved in hydrocephalus formation, although the precise mechanism has yet to be determined.

1.3.1.1 The Role of Ependymal Cells in Hydrocephalus Development

Ependymal cells act as a barrier between the CSF and the brain parenchyma, are involved in CSF homeostasis and provide structural and metabolic support to cells in the SVZ. Mature ependymal cells carry an apical cluster of motile cilia contributing to CSF propulsion [121-123]. Many groups have linked hydrocephalus development to altered ependymal motile cilia function and mutations in genes that disrupt cilia structure and function lead to impaired CSF flow and consequently to expansion of the ventricles [118, 119, 124-128].

Besides impaired ependymal motile cilia, ependymal cell maturation and integrity have been associated with hydrocephalus development. This, since loss of junctional proteins such as Jam3 and Mpdz in mice has been described with hydrocephalus [129, 130]. Interestingly, both Jam3 and Mpdz deficient mouse models show signs of astrogliosis as a repair mechanism to ependymal defects and ependymal denudation, which in Mpdz deficient mice resulted in a narrowed cerebral aqueduct [130].

Ependymal cells arise from a subpopulation of radial glia cells [131] and Six3 has been shown to be required for ependymal cell differentiation and maturation. Thus, Six3 deficiency results in aberrant

ependymal cell maturation and consequently in abnormal neuroblast migration and differentiation and hydrocephalus development [132]. Further, mice deficient for the transcription factor NFIX, which has been described to promote neural stem cell differentiation, displayed ependymal deficits including partial loss of the ependymal layer lining the cerebral ventricles [133]. This is especially intriguing, since ependymal denudation has also been reported in humans with hydrocephalus [134-136]. Additionally, overexpression of Nestin (a marker for radial glia) has been associated with immature glial cells and ependymal cell loss has been reported in postmortem hydrocephalic brains [137].

Taken together, this underlines the importance of ependymal cell maturation and integrity in the context of hydrocephalus development.

1.3.2 Ischemic Stroke

Stroke is a leading cause of morbidity and mortality. 40% of stroke patients remain with moderate functional impairments and 15-30% with severe disability, resulting in a tremendous economic burden [138].

The majority of strokes are ischemic, with hemorrhagic bleeding stroke accounting for approximately 10-13% of all strokes [138]. An ischemic stroke is caused by a thrombus blocking the cerebral blood flow to an area of the brain causing oxygen deprivation resulting in cell death. Hemorrhagic strokes generally have a worse prognosis than ischemic strokes [139], and a hemorrhagic conversion of an ischemic stroke increases stroke severity and worsens outcome [140]. Recombinant tPA is currently the only FDA approved thrombolytic treatment of acute ischemic stroke, but its use is greatly limited due to the requirement of administration within 4.5 hours after the onset of symptoms. This, because of concerns of increased BBB permeability and transformation to a hemorrhagic stroke, which is currently affecting about 7% of treated patients [140, 141]. Hemorrhagic transformation of an ischemic stroke is defined by bleeding with petechial hemorrhage in the infarction area and parenchymal hematoma accompanied by neurological deterioration [142]. The risk to develop hemorrhagic complications has been linked to the amount of time passed before treatment, symptom severity, age, blood glucose levels, thrombocytopenia, hypertension, congestive heart failure and leukoaraiosis [140, 142]. Additionally, besides the risk of hemorrhagic complications, tPA has been reported to promote neuronal degeneration after cerebral ischemia [143]. Taken this into account, less than 5% of stroke patients are eligible for tPA therapy [141]. However, intravenously administered tPA for the treatment of ischemic stroke is associated with improved functional outcome [144] and if administered within 90 minutes from stroke onset tPA treatment allows nearly complete recovery [141]. The American Heart Association/ American Stroke Association predicate that the beneficial effect outweighs the risk and strongly recommend tPA treatment of eligible patients despite the risk of hemorrhagic complications, but also emphasize the urgent need for further research on the treatment of ischemic stroke [145].

1.3.2.1 Blood-brain Barrier Dysfunction in Ischemic Stroke

The mechanism how thrombolytic tPA might lead to hemorrhagic complications is not fully understood, but it appears to be due to the unique activities of tPA in the CNS, including cleavage and activation of PDGF-CC [55].

tPA deficient mice display significantly smaller strokes compared to wild-type controls but after intravenous administration of tPA, stroke volume and neuronal damage increase to a comparable volume as in wild-type controls [143]. This, as well as tPA activity found in the ischemic area of tPA deficient mice after intravenous administration of tPA, suggests that exogenous tPA administered for thrombolysis can cross the BBB and exacerbate stroke volume and neuronal damage [143]. Additional to a smaller stroke volume, tPA deficient mice exhibit decreased Evan's blue extravasation after ischemic stroke as compared to wild-type controls indicating that these mice are protected from stroke induced BBB leakage [76]. Also, plasminogen deficient mice are not protected against BBB permeability induced by intraventricular injection of tPA and an NMDAR antagonist cannot preserve BBB integrity either [76]. Thus, these studies reveal that tPA is both necessary and sufficient to induce opening of the BBB in a plasminogen- and NMDAR-independent manner [76]. About the same time as these studies were published, PDGF-CC was identified as a new substrate for tPA [9]. However, these *in vitro* studies revealed that activation of PDGF-CC by tPA was rather inefficient and LRP1 (low-density lipoprotein receptor-related protein-1) was shown to enhance tPA mediated PDGF-CC activation [39]. Later it was also demonstrated that the integrin Mac-1 on microglia acts together with LRP1 to facilitate tPA mediated activation of PDGF-CC in the NVU [146]. Further, activation of PDGF-CC by tPA has been shown to impair BBB integrity following ischemic stroke in mice, via activation of PDGFR α on perivascular astrocytes (Fig.3), and treatment with the PDGFR α antagonist imatinib has been shown to reduce this increase in BBB permeability [39]. This has later been confirmed by different groups [147-149] and a phase II clinical study recently revealed that imatinib is both safe and tolerable and reduces neurological disability in patients receiving intravenous tPA treatment after stroke [150].

In pathological conditions like ischemic stroke, increased BBB permeability has been shown to be mediated by tPA in response to the increased energy and metabolic demand in neurons following ischemia [55]. Excessive BBB disruption subsequently leads to extravasation of substances like fibrin(ogen) from the blood stream and infiltration of leukocytes from the vascular space into the brain parenchyma, triggering an inflammatory response and an increase in MMP9 activity [151, 152]. Interestingly, increased MMP9 activity has been described in the context with tPA induced hemorrhage and inhibition of MMP9 directly, or inhibition of the MMP9 pathway, results in decreased BBB disruption after ischemic stroke in rabbits, mice and rats [153-157]. BBB disruption is thought to occur in phases, with MMP9 being involved in a second phase in both animal models [158] and patients [159] and infiltrating neutrophils have been described as a source of MMP9 involved in the later phase of BBB opening [160].

Based on these findings, changes in the cerebrovascular permeability have been hypothesized to underlie the neurotoxic effects associated with thrombolytic tPA in the CNS after ischemic stroke. Following intravenous treatment with thrombolytic tPA after ischemic stroke, this exogenous tPA presumably enters into the brain parenchyma through a compromised BBB and exacerbates BBB

breakdown via intensified PDGF-CC cleavage and PDGFR α signaling, thereby leading to severe tissue injury. In line with these findings, higher levels of PDGF-CC in the plasma of stroke patients have been associated with an increased risk of hemorrhagic transformation [55]. Controversially, a neuroprotective role of tPA during stroke has been proposed. This role of tPA was ascribed to tPA-induced increase in GLUT3 expression and glucose uptake, thereby meeting the increased metabolic demand of neurons during ischemia [70], although this needs to be further investigated in *in vivo* models.

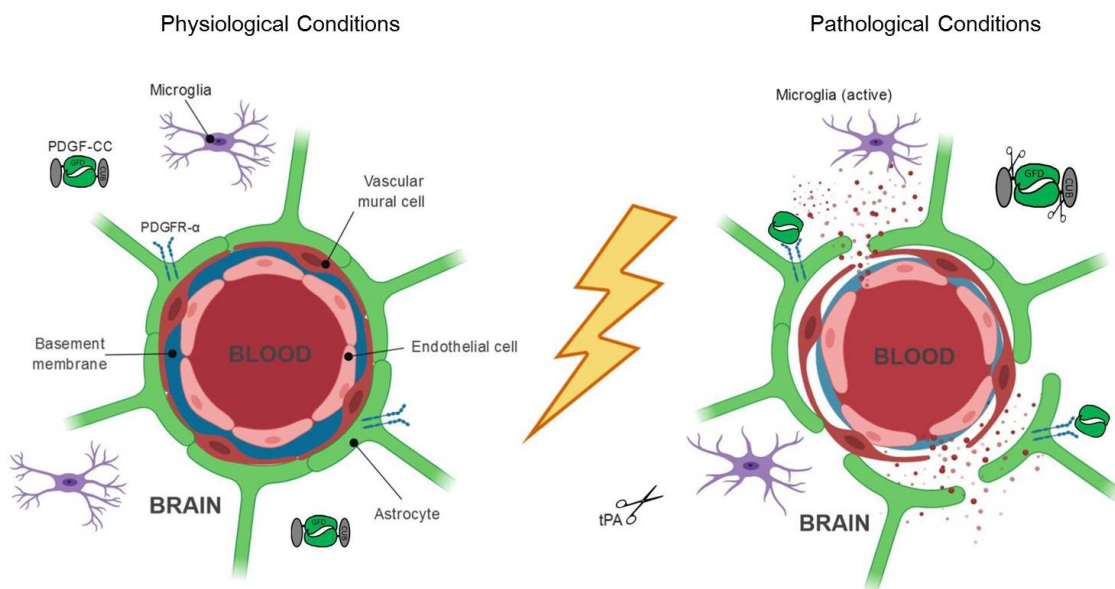


Fig3: The neurovascular unit and blood-brain barrier disruption following ischemic insult.

The neurovascular unit consists of endothelial cells, vascular mural cells, astrocytes and microglia in a functional interaction with neuronal cells. Upon an ischemic insult, increased activity of tPA leads to removal of the PDGF-CC CUB-domains and subsequent PDGFR α signaling. This results in vascular mural cell and astrocyte detachment and consequential BBB breakdown and release of blood born substances into the brain parenchyma.

1.3.2.2 Immune Response in Ischemic Stroke

Inflammation plays an important role in the pathology of ischemic stroke, as the brain responds to the ischemic insult with an acute and a subacute / chronic immune reaction, critical to tissue damage and healing. Thus, immune deficient animals have been described with reduced stroke size after transient middle cerebral artery occlusion (tMCAO) [161-164] and accordingly neuroimaging of stroke patients associated increased numbers of peripheral leukocytes and neutrophils with larger infarct volumes [165].

The inflammatory response to ischemic stroke is characterized by pro-inflammatory molecules released from the injured tissue triggering an immune response and by a rapid activation of resident microglia. This initial phase is followed by a subacute phase defined by infiltration of circulating immune cells such as neutrophils, T-cells, B-cells and monocytes / macrophages in both animal models of ischemic stroke [146, 166-168] and in humans [169-171]. Interestingly, a study recently described an effect of thrombolytic tPA on the systemic immune response after tMCAO in mice but

whether this affects the number of infiltrating immune cells into the ischemic brain parenchyma remains to be seen [172].

Resident microglia have a controversial dual role in the acute response after ischemic stroke, as microglia polarization produces either the pro-inflammatory M1 or the neuroprotective M2 phenotype [173]. Microglial polarization towards the pro-inflammatory M1 phenotype has been described to be mediated by ischemic neurons releasing Fas ligand [174]. Thereafter M1 microglia contributes to neuronal damage and apoptosis as M1 microglia produces pro-inflammatory mediators such as TNF- α (tumor necrosis factor- α), ROS, interleukin-1 β , CD14, CCL2 and iNOS [175, 176]. Further it has been shown that microglia promote increased BBB permeability after ischemic stroke by enhancing tPA mediated activation of PDGF-CC [146] and by tPA-mediated upregulation of MMP9 [177]. On the contrary, M2 microglia has been associated with neuroprotective effects after ischemic stroke, since M2 activated microglia release anti-inflammatory cytokines such as IL-4, IL-10 and transforming growth factor- β [178, 179]. This is supported by the fact that IL-4 deficiency in mice leads to worsened outcome accompanied by increased inflammation after tMCAO [180].

As early as 30 minutes after an ischemic insult, neutrophils infiltrate the ischemic brain, peaking 3 days after cerebral ischemia in mice and rats [168, 181]. Neutrophils produce ROS and release pro-inflammatory molecules such as IL-1 β , IL-6, IL-8, CCL2 and TNF- α [182]. Thus, pharmacologic depletion of neutrophils has been associated with decreased infarct volumes [183-187] and decreased edema after reperfusion injury [183]. Infiltrating neutrophils are a direct source of MMP9 in the ischemic brain [160], thus potentially contributing to increased BBB permeability in both animal models [158] and stroke patients [159].

T-cells contribute in the subacute phase 72 to 96 hours post occlusion [188] and T-cell deficient animals display reduced stroke size after tMCAO [161-164]. However, the role of T-cells in ischemic stroke progression is rather perplexing. This, since CD4+ and CD8+ T-cells have been associated with a pro-inflammatory response [163, 189], whereas regulatory T-cells have been described as neuroprotective, as they countervail the pro-inflammatory cytokines TNF- α and IFN- γ via IL-10 [190]. In fact, depletion of regulatory T-cells in mice elevates pro-inflammatory cytokine expression in the ischemic brain and results in increased infarct size [190]. Additionally, CD4+ TH2 cells have been reported to secrete anti-inflammatory cytokines (IL-4, IL-5, IL-10, IL-13) whereas CD4+ TH1 cells may contribute to stroke pathogenesis by secreting pro-inflammatory cytokines such as IL-2, IL-12, IFN- γ and TNF- α [189]. Also, docking of regulatory T-cells at the injured vessel wall, mediated by CCR5, is protective against early BBB disruption following ischemic stroke, attributed to inhibition of neutrophil release of MMP9 [191].

B-cells were found to be protective after ischemic stroke, as intravenous injection of IL-10 secreting B-cells reduced both infarct size and infiltration of inflammatory T-cells [192]. Cognitive impairment and dementia has however been proposed to be mediated by B-cell infiltration after stroke [193].

Overall, the inflammatory response after ischemic stroke is defined by complex cellular interactions and shows both beneficial and adverse effects on stroke pathology.

1.3.2.3 The Glial Scar

Upon CNS injury, reactive astrocytes, NG2 glia / OPCs, meningeal and vascular derived fibroblasts and microglia form the glial scar around the area of tissue damage.

CNS injury is accompanied by cell death, BBB damage and inflammation. Microglia are among the first cells responding to CNS injury by proliferation and migration to the site of injury, where they are involved in phagocytosis of cellular debris, regulation of neuronal excitability and trophic neuronal support [194]. Extracellular matrix, consisting of collagens, laminins, and glycoproteins such as chondroitin sulfate proteoglycans (CSPGs), both support and inhibit CNS regeneration and begins to form shortly after injury [195].

A second proliferative phase is characterized by cell proliferation and migration. Astrocytes become highly proliferative and undergo morphological changes resulting in cellular hypertrophy [196]. Hypertrophic reactive astrocytes form a reticular structure and synthesize and deposit CSPGs in the extracellular matrix [196]. Reactive astrocytes are crucial in scar formation since mice lacking GFAP and vimentin display less organized glial scarring [197]. NG2 glia is, like astrocytes, characterized by hypertrophy, increased proliferation and expression of proteoglycans following CNS insult [198] and participates in scar formation by stabilizing dystrophic axons within the hostile lesion environment and differentiate into oligodendrocytes, thereby contributing to remyelination [194, 196]. NG2 positive OPCs migrate towards the lesion, orchestrating formation of a structurally layered scar [196]. Fibroblast-related cells such as perivascular fibroblasts, meningeal fibroblasts and pericytes further contribute to scar formation [199]. Additionally, neural stem cells give rise to neural progenitors migrating to the injury sites [195].

In a third phase of tissue remodeling a compact scar has formed, building a structural and functional barrier sealing the lesion and protecting the surrounding parenchyma [195].

Often the glial scar, and the created inhibitory environment, has been viewed as limiting to CNS regeneration. As mentioned above, reactive astrocytes synthesize and deposit high amounts of CSPGs, including growth-inhibitory factors, thereby inhibiting neurite regeneration and functional recovery after CNS injury [194, 196]. CSPGs have also been reported to prevent oligodendrocyte maturation and thus remyelination in a mouse model of demyelination and spontaneous remyelination [200]. Further, endothelin-1, which is secreted by reactive astrocytes, acts as a negative regulator of NG2 glial differentiation and functional remyelination [201]. Moreover, NG2 positive cells have been described to form synapse-like contacts, trapping axons and creating an additional barrier to regeneration [202].

While the glial scar shows negative effects on regenerative processes, it is crucial to restrict the area of damage as well as the inflammatory response and maintain extracellular ion and fluid balance, thus protecting the surrounding healthy CNS tissue [203]. Reactive astrocytes were shown to have a role in BBB repair, leukocyte infiltration, demyelination as well as in neuronal and oligodendrocyte death, resulting in enhanced recovery after spinal cord injury [204]. Further, scar forming reactive astrocytes have been shown to restrict inflammation and decrease lesion volume in a STAT3-dependent mechanism after spinal cord injury [205, 206]. Additionally, reactive astrocytes have been

described to secrete TNF-stimulated gene-6 (TSG-6) within the glial scar, coordinating the formation of a hyaluronan-rich matrix and suppressing neuroinflammation [207].

In ischemic stroke, astrocytes move towards the lesion resulting in accumulation of reactive astrocytes, microgliosis and glial scar formation in animal models of MCAO [208] as well as in the human brain after ischemic stroke [209]. This forms a compact border around the lesion, restricting the damaged area. Reactive astrocytes display altered expression of molecules involved in cell structure, gene transcription, intracellular signaling and energy metabolism, protecting neurons from oxidative stress and providing important metabolic support during cerebral ischemia [210]. Both reactive astrocytes and microglia secrete stromal cell-derived factor-1 (SDF-1), attracting neuroblasts towards the ischemic area [210]. However, as discussed, glial scar formation also interferes with neurite regeneration and functional recovery. In response to ischemic stroke, astrogliosis has been described to be mediated by repulsive guidance molecule a (RGMa) through TGF β 1 and knockdown of RGMa resulted in abolished hypertrophy, cell migration and CSPG secretion [208]. Furthermore influencing astrocyte activation by knockdown of apoptosis signal-regulating kinase 1 in mice with tMCAO, resulted in reduced glial scar formation, correlating with extended neurites and advanced functional recovery [211].

In conclusion, the glial scar has been associated with both beneficial and disadvantageous effects, but whether these diverse functions offer potential therapeutic targets needs to be further investigated. However, current knowledge is suggesting that intervention of specific molecular pathways rather than blocking the scarring event itself will have greater therapeutic potential.

2 AIMS OF THIS THESIS

In the neurovascular unit tPA mediates activation of latent PDGF-CC for subsequent PDGFR α signaling. This has previously been implicated with increased cerebrovascular permeability and hemorrhagic complications in ischemic stroke.

In this thesis we aim to describe the physiological role of tPA and PDGF-CC/PDGFR α signaling in the murine brain in development and disease.

Specific aims include:

Paper I

To further investigate the biological function of tPA in CNS development and thoroughly characterize the *tPA*^{-/-} mouse with focus on the cerebrovasculature and the ventricular system.

Paper II

To explore the role of PDGF-CC/PDGFR α signaling in cerebroventricular development and ependymal cell maturation.

Paper III

To elucidate the interplay of microglial Mac-1, LRP1 and tPA for tPA mediated PDGF-CC activation and subsequent PDGFR α signaling in ischemic stroke.

Paper IV

To identify the molecular mechanisms by which imatinib treatment improves stroke outcome by influencing BBB integrity and scar formation in ischemic stroke.

3 RESULTS AND DISCUSSION

3.1 Paper I

tPA Deficiency in Mice Leads to Rearrangement in the Cerebrovascular Tree and Cerebroventricular Malformations

In this paper the role of tPA during CNS development was investigated. Our previous work has shown that activation of PDGF-CC and subsequent signaling via its receptor PDGFR α is catalyzed by tPA mediated cleavage of latent PDGF-CC [9]. Further studies have implicated this signaling pathway to regulate BBB integrity [39]. To better understand the role of tPA and PDGF-CC/PDGFR α in cerebrovascular regulation we conducted a detailed analysis of the cerebrovasculature in brains from adult tPA deficient (*tPA*^{-/-}) mice.

Using immunofluorescent staining analyses, we could demonstrate that life-long deficiency of tPA is associated with rearrangements in the cerebrovascular bed towards smaller diameter vessels. This rearrangement in the cerebrovascular tree was further characterized by an increased number of ERG positive endothelial cells. This was intriguing, since ERG has been associated with vascular development [212, 213] and has been shown to control vascular permeability and junctional integrity [214]. Accordingly, we found that the increased number of ERG positive cells in tPA deficient mice was accompanied by enhanced junctional expression of ZO1.

To further evaluate the effect of tPA deficiency on the cerebrovasculature we investigated vascular mural cell coverage in *tPA*^{-/-} mice. Mural cells such as pericytes and vascular smooth muscle cells are known to play an important role in BBB maintenance and in regulation of blood flow [215, 216] and tPA has been described to play a key role in neurovascular coupling [66]. We found a decrease in the number of vascular smooth-muscle cell covered, large diameter vessels, resulting in an apparent shift in the size towards more ASMA (alpha smooth muscle actin) positive, small diameter, vessels. However, the cerebral capillary bed appeared to have normal coverage of pericytes.

Since previous work has shown that tPA mediated activation of PDGF-CC and subsequent PDGFR α signaling on perivascular astrocytes regulates cerebrovascular permeability, we investigated PDGFR α expression in *tPA*^{-/-} mice. Interestingly, we found a significant reduction of perivascular PDGFR α expression in *tPA*^{-/-} mice as compared to wild-type controls. Together, the cerebrovascular rearrangement described in tPA deficient mice, could partially explain the protection of BBB integrity observed in *tPA*^{-/-} mice [76].

In addition to cerebrovascular changes, we noted that tPA deficient mice displayed asymmetric lateral ventricles, similar to what had previously been reported for PDGF-CC deficient mice on C57BL/6 background [28]. This provided a first *in vivo* link between tPA and PDGF signaling during CNS development. However, PDGF-CC deficient mice had been reported with denudation in their ependymal lining, which was not seen in the ependyma of *tPA*^{-/-} mice, even though the ependyma in these mice was abnormal and distorted and did not form a uniform single layer. Further, we also

found an increased expression of the tight junction protein ZO1 and GLUT1 in the irregular ependymal lining of tPA deficient mice as compared to wild-type controls.

We conclude from these studies that tPA plays an important role in the development of normal cerebral vascularization and normal cerebral ventricular formation. This study enhances our understanding of the role of tPA in the CNS and might help to explain how tPA is involved in regulation of cerebrovascular integrity and vascular barrier defects in CNS pathology.

3.2 Paper II

Platelet-derived growth factor-CC Regulates Ependymal Cell Maturation in Mice

We have previously reported malformations of cerebral lateral ventricles and loss of neuroependymal integrity in adult PDGF-C deficient mice. These findings were especially intriguing, since this had previously not been linked to PDGFR α signaling, and since the molecular mechanisms underlying brain ventricle formation and ependymal differentiation still remain poorly understood. Loss of neuroependymal integrity has been linked to hydrocephalus and spina bifida, a neural tube defect associated with dysfunctional PDGFR α signaling. This strongly implicates PDGF-CC/PDGFR α signaling in brain ventricle and ependymal development.

Here we intended to reveal the molecular mechanism underlying abnormal ventricular development in PDGF-C deficient mice. We first examined whether the expansion of the lateral ventricles in adult PDGF-C deficient mice might be due to an obstruction of flow in the ventricular system. By visualizing the CSF flow in the ventricular cavities using Evans blue dye, we found that the dye circulated through the lateral and third ventricle as well as the cerebral aqueduct to the fourth ventricle, indicating no obstruction of flow in the ventricular system.

To determine whether the ventricular abnormalities were present already from birth, or acquired during postnatal development, we investigated the prevalence of the cerebral ventricular defects in PDGF-C deficient mice during perinatal development. We found a higher proportion of asymmetric lateral ventricles in PDGF-C deficient mice compared to wild-type controls as early as postnatal day 7. This asymmetry persisted in the deficient mice at later postnatal stages, whereas in wild-type mice we did not detect any asymmetric lateral ventricles.

The cerebral ventricles are lined with a layer of cuboidal, ciliated ependymal cells propelling CSF. Previously PDGF-C deficient mice were described with ependymal denudation in the lateral ventricles [28]. Thus, we investigated whether these ependymal defects are acquired during ependymal cell maturation postnatally. We found delayed maturation of ependymal cells in the lateral ventricles of PDGF-C deficient mice, coinciding with an undulation and denudations of the ependymal lining. Further, the ependymal defects were accompanied by mild astrogliosis, a repair mechanism for ependymal defects and ependymal cell dysfunction [217].

During ependymal cell maturation tufts of motile cilia form on the apical surface of ependymal cells [131]. Loss or dysfunction of ependymal cilia has been associated with hydrocephalus formation due to impaired CSF propulsion, resulting in CSF accumulation and subsequent ventricular expansion [119, 124, 127, 128]. However, in *Pdgfr^c*^{-/-} mice we found significantly shorter motile cilia, appearing disorganized instead of well-organized in tufts as seen in wild-type mice. Since ventricular asymmetry in the PDGF-C deficient mice was found to occur prior to ependymal cell maturation and motile cilia formation, we speculated that impaired ependymal lining and motile cilia structure might only exacerbate ventricular expansion. Given that ependymal cells arise from a subpopulation of radial glia cells between E14 and E16 [131], we investigated whether PDGF-CC might influence radial glia differentiation resulting in impaired ependymal cell development. By immunofluorescent staining, we found Nestin positive cells (a marker for radial glia) mislocalized within the lateral ventricular lumen.

Taken together this suggests that PDGF-CC/PDGFR α signaling in the developing murine brain might influence radial glia differentiation and migration, leading to altered ependymal cell maturation and motile cilia formation, resulting in asymmetric and enlarged ventricular development.

3.3 Paper III

Microglial – mediated PDGF-CC Activation Increases Cerebrovascular Permeability during Ischemic Stroke

Previously, we reported that tPA acting on the parenchymal side of the NVU mediates activation of latent PDGF-CC and subsequent PDGFR α signaling, resulting in increased BBB permeability and intracerebral hemorrhage (ICH) [39]. However, tPA mediated activation of PDGF-CC *in vitro* has been shown to be inefficient, suggesting co-factors to enhance the activation of PDGF-CC in the NVU.

Here we investigated possible co-factors needed for efficient activation of latent PDGF-CC by tPA. Earlier studies had shown that one of these co-factors was the endocytic receptor LRP1 [39, 76, 218]. Using a PAE- α cell line with stable expression of recombinant PDGFR α , our studies demonstrated that together with LRP1, the integrin Mac-1 on microglia is necessary to facilitate efficient tPA mediated activation of PDGF-CC. In various immunofluorescence analyses we showed that in the NVU, Mac-1, LRP1 and PDGFR α are present in close vicinity of each other, promoting the hypothesis that Mac-1 and LRP1 act as co-factors for tPA mediated PDGF-CC activation.

Following ischemic stroke, it has been shown that microglia upregulate expression of Mac-1 and that this upregulation coincides with a tPA dependent increase in cerebrovascular permeability. Here we showed that intraventricular injections of tPA in Mac-1 deficient mice did not increase BBB permeability as compared to wild-type controls, whereas injections of active PDGF-CC did. Thus, this suggested that Mac-1 is required for tPA mediated activation of PDGF-CC and that activated PDGF-CC acts downstream of Mac-1 and tPA in the control of BBB permeability *in vivo*. In

an experimental model of ischemic stroke (MCAO), Mac-1 deficiency was found to be associated with less Evans blue extravasation and preserved BBB integrity compared to wild-type mice. We investigated whether this was due to Mac-1 acting as a co-factor for tPA mediated activation of PDGF-CC in the NVU, and subsequently injected tPA or active PDGF-CC in the cerebral ventricles one hour after MCAO. Active PDGF-CC, but not tPA, was able to increase BBB permeability in Mac-1 deficient mice, whereas intraventricular injections of tPA and active PDGF-CC both showed additional increase in BBB permeability in wild-type mice. Further, following MCAO, Mac-1 deficient mice showed significantly less PDGFR α phosphorylation around vessels in the ischemic penumbra as compared to wild-type mice. This supports that tPA mediated activation of PDGF-CC is dependent on Mac-1 and thereby is involved in controlling cerebrovascular permeability via PDGFR α .

In order to see whether Mac-1 on resident microglia or on infiltrating leukocytes is responsible for mediating PDGF-CC activation by tPA and subsequent BBB disruption in stroke, CX3CR1-GFP/CCR2-RFP (R/G) mice were used. These mice express GFP in microglia and macrophages under the control of the CXCR1 promotor and RFP in monocytes and macrophages under the control of the CCR2 promotor, thus offering a good tool to distinguish microglia, monocytes and monocyte-derived macrophages and follow the recruitment into the ischemic penumbra. This revealed microglia as the prominent cell type present prior to and six hours after MCAO. 24 hours after MCAO, monocytes associated with vessels and infiltrating into the brain parenchyma were found, although at low numbers. The timing of the various events occurring post MCAO suggests that resident microglia is the cellular source of Mac-1 facilitating tPA mediated activation of PDGF-CC in the NVU and subsequently leading to loss of BBB integrity during ischemic stroke. To confirm this, bone marrow from Mac-1 deficient mice or wild-type mice was transplanted into bone-marrow irradiated wild-type or Mac-1 deficient mice respectively. We found that Mac-1 deficient mice receiving wild-type bone marrow did not show increased BBB leakage and that Mac-1 deficient bone marrow transplanted to wild-type mice did not protect from BBB disruption. This is in line with the observation that early activation of PDGF-CC is facilitated by Mac-1 on resident microglia rather than on circulating leukocytes.

Late thrombolysis after ischemic stroke significantly increases the risk of ICH. Accordingly, wild-type mice have an increased incidence of spontaneous ICH following thrombolysis with tPA five hours after MCAO, whereas Mac-1 deficient mice are resistant to the development of ICH, even with late tPA treatment for thrombolysis.

Taken together, we show that Mac-1 together with LRP1 facilitates PDGF-CC activation by tPA in the NVU, thus providing a limiting factor for PDGF-CC/ PDGFR α signaling and a way to regulate BBB permeability and stroke volume.

3.4 Paper IV

PDGFR α Antagonism Reduces Reactive Gliosis and the Scar Formation after Ischemic Stroke in Mice

Previous studies have demonstrated that the tyrosine kinase inhibitor, imatinib, attenuates BBB breakdown, reduces stroke volume and improves the outcome after ischemic stroke via inhibition of PDGFR α signaling, both in experimental mouse models [39] and in human patients [150]. However, the underlying mechanism of how imatinib ameliorates stroke pathology has not been described.

To investigate molecular changes in the NVU that are induced by ischemic stroke, we performed differential gene expression analyses on vascular fragments isolated at different time points after induction of ischemia and compared this to vessels isolated from sham-operated murine brains. We identified 691 differentially expressed transcripts in the NVU three hours post MCAO and 1417 differentially expressed transcripts 24 hours post MCAO as compared to sham-operated mice. The majority of the upregulated pathways were associated with inflammatory responses, including granulocyte adhesion, pro-inflammatory mediators, the acute phase response, integrin signaling as well as the complement system. Transcripts associated with immune cell recruitment and cell activation were primarily upregulated 24 hours post MCAO, suggesting immune cell recruitment as a central response at later time points. This is in line with previous reports, describing immune cell recruitment to be more prominent at 48 to 96 hours post MCAO [188]. However, three hours post MCAO most of the regulated genes were found to be associated with vascular damage and BBB function, including tight- and adherens junction signaling, peptidases and various transporters, suggesting a crucial role of pathways related to vascular damage in the early response to MCAO.

Differential gene expression analyses of vascular fragments isolated from imatinib and PBS treated mice after MCAO revealed 122 and 85 differentially expressed transcripts three and 24 hours after MCAO, respectively. These were mostly representing mechanisms associated with immune cell activation and recruitment, brain metabolism and the injury response and were normalized in imatinib treated mice as compared to PBS controls.

Considering that our gene expression data identified that the immune cell response was affected by imatinib treatment, we further examined immune cell infiltration using immunofluorescence stainings. We found that imatinib had limited effect on immune cell infiltration but instead revealed that imatinib treatment influenced reactive gliosis (acute phase), microglia activation (subacute phase) and the scarring response (late subacute to chronic phase) after MCAO. In the ischemic border PDGFR α positive NG2-glia appeared condensed and hypertrophic as early as three hours post MCAO. However, in imatinib treated mice PDGFR α positive cell condensation appeared to be inhibited. Further, imatinib attenuated astrocyte activation and diminished the increased expression of GFAP found in PBS treated control animals. This is intriguing, since activation of NG2-glia and astrocyte activation are hallmarks of the reactive gliosis response to injury. Additionally, we found that imatinib influenced scar formation. In PBS treated mice the PDGFR α positive scar was unstructured and enlarged as compared to imatinib treated mice, where a significant reduction of the scar thickness was evident. This is interesting, since the role of reactive gliosis and scar formation in functional recovery and disease outcome is controversial. Often the glial scar has

been described as limiting to functional recovery after CNS injury due to inhibition of neurite regeneration [194, 196]. However, the glial scar is crucial to restrict the area of damage as well as the inflammatory response in order to protect healthy CNS tissue [203]. Thus, a structured scar with reduced thickness as seen after imatinib treatment, might be beneficial for recovery and add to the improved neurological and functional outcome seen with imatinib treatment after ischemic stroke [39, 150].

Further we described the kinetics of the loss of BBB integrity and showed that imatinib attenuates cerebrovascular permeability early after MCAO. Three hours post MCAO, when we detected the highest level of BBB permeability, we found a significant decrease in BBB dysfunction in imatinib treated mice as compared to PBS controls. At 24 hours post MCAO BBB leakage was less dominant, but still approximately 45% decreased in imatinib treated mice as compared to PBS treated mice. Further, we found imatinib treatment to preserve PDGFR α and GFAP expression in perivascular astrocytes, whereas we found it downregulated in the ischemic center of PBS treated control mice three hours after MCAO.

Collectively we provide valuable insight in the molecular response to ischemic stroke and in the mechanism by which imatinib improves stroke outcome.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

In the work included in this thesis, we describe the physiological role of tPA (Paper I) and PDGF-CC (Paper II) in CNS ventricular and vascular development as well as the mechanism of action of tPA mediated PDGF-CC activation in the NVU in an experimental model of ischemic stroke (Papers III and IV). The results obtained in these studies offer new insights to tPA mediated PDGF-CC/PDGFR α signaling in development and disease.

In Paper I, we describe an important role of tPA in the development of normal cerebral vascularization and ventricular formation. This paper provides a first *in vivo* link between tPA and PDGF-CC in CNS development and enhances our understanding of the role of tPA in the CNS, especially in regulation of cerebrovascular integrity. Since our studies revealed that the ventricular malformations in tPA deficient mice did not entirely phenocopy the ventricular malformations seen in PDGF-C deficient mice, further studies are needed to fully understand these processes.

In Paper II, we describe a new mechanism involved in ependymal cell development and subsequently in malformation and expansion of the cerebral ventricles. This study broadens our knowledge of PDGF-CC/PDGFR α signaling in CNS development and potentially offers new opportunities to understand radial glia differentiation and migration as well as ependymal cell development. In future experiments it will be very interesting to determine the cellular identity of the highly proliferative cell type underlying the undulating ependymal cell lining, and whether they might stem from the subpopulation of Nestin⁺ radial glia mislocalized within the ventricular lumen of PDGF-C deficient embryos at E14.5 and E16.5. These mislocalized Nestin⁺ cells do not express PDGFR α , suggesting that compromised PDGF-CC/PDGFR α signaling impairs radial glia differentiation, and potentially also migration, at an earlier developmental stage. Utilizing PDGF-C deficient/PDGFR α ^{GFP/+} double mutant mice will provide further insight into whether and how this signaling system regulates these processes.

In Paper III and Paper IV, we provide novel knowledge on the activation of PDGF-CC in the NVU and suggest a mechanism by which blocking PDGF-C/PDGFR α signaling with imatinib improves stroke outcome. Our data in Paper III identify Mac-1 as a key player in the activation of PDGF-CC. Characterizing these essential interactions in the NVU is critical to understand the regulation of BBB integrity in ischemic stroke and might open new doors to potential future therapies. This however needs further investigation. Towards this end we report in Paper IV that imatinib treatment attenuates BBB permeability and reactive gliosis in the acute phase after ischemic insult and controls scar formation at later time points. These data provide valuable insight to further elucidate when treatment to restore BBB integrity might be most effective in ischemic stroke patients. Future in depth analyses of the differential glial scar formation, including cellular composition and origin, as well as effect on tissue regenerative processes such as neurite regrowth and angiogenesis, will help to further advance our understanding on the role of this pathway in ischemic stroke. In addition, it will be interesting to determine the timing and/or contribution of the various biochemical and cellular responses, and subsequently the effect of blocking the respective responses, on disease outcome. This, by the use of delayed imatinib treatment following ischemic insult, inflammation-modulating treatments as well as genetic approaches to target the various compartments of PDGFR α

expressing cells. Last, future studies using a monoclonal anti-PDGF-CC antibody to prohibit PDGF-CC/PDGFR α signaling [219, 220] will reveal if all processes affected by imatinib are indeed driven by PDGFR α signaling and might offer a new, more specific way to treat ischemic stroke, but also other CNS disorders in which the BBB integrity is compromised and where scarring plays a crucial role.

“If our brains were simple enough for us to understand them, we’d be so simple that we couldn’t”
- Ian Stewart

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