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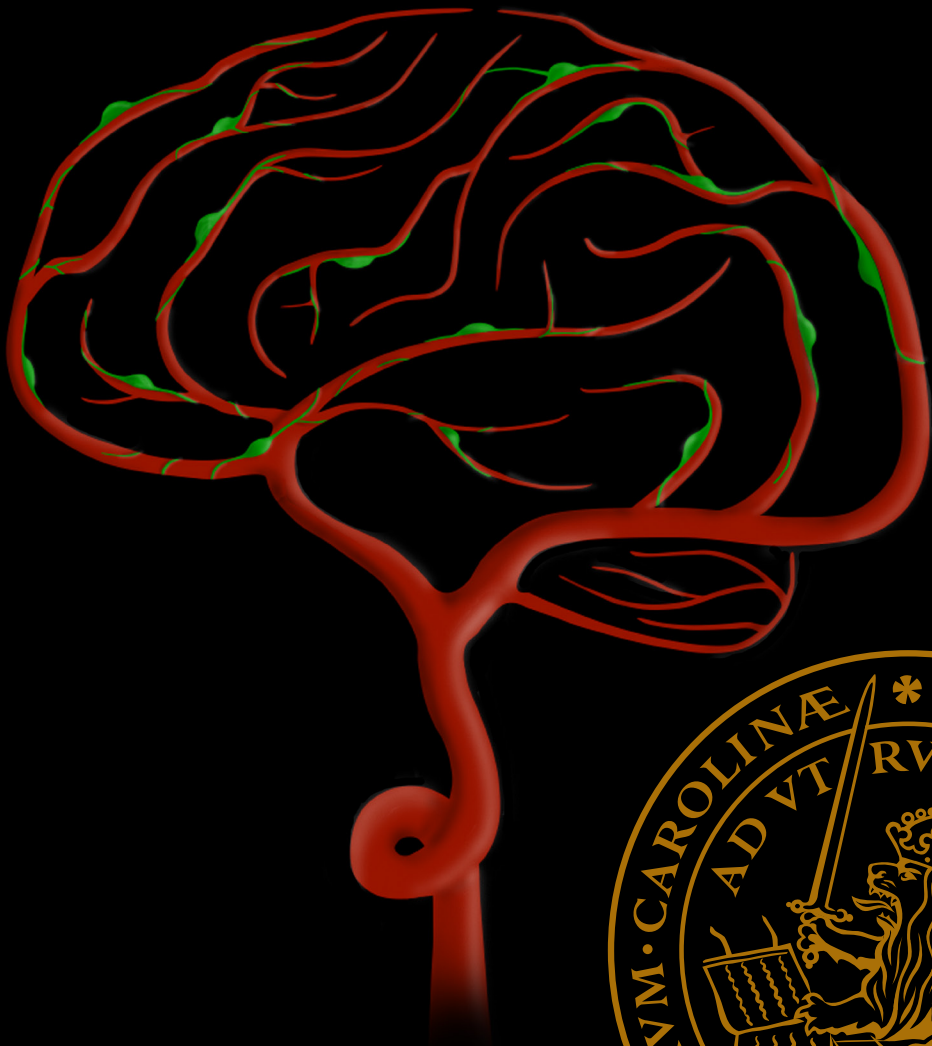
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# Pericytes in Neurodegeneration

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# Pericytes in neurodegeneration

Thomas Padel



**LUND**  
UNIVERSITY

DOCTORAL DISSERTATION

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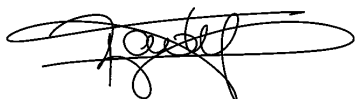
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Abstract  <p>The brain microvasculature is formed of different cell types, where endothelial cells, forming the vessel tube, are encircled by pericytes. Both cell types are ensheathed in a basal lamina, and covered by astrocyte end-feet and neurons, forming altogether the neurovascular unit. Brain pericytes play a pivotal role in angiogenesis, blood-brain barrier (BBB) maintenance and clearance of brain byproducts. Pericytes express numerous markers, such as (i) Regulator for G-protein signaling 5 (RGS5) and Chondroitin sulphate proteoglycan NG2 (NG2), upregulated during vascular remodelling, and (ii) Platelet-derived growth factor receptor beta (PDGFR<math>\beta</math>), a specific receptor for PDGF-BB, secreted by endothelial cells for pericyte recruitment on the blood vessels.</p> <p>The neurovasculature has recently gained attention in neurodegenerative disorders such as Parkinson's (PD) and Huntington's (HD) disease, where blood vessel alterations, BBB disruption and cerebral blood flow abnormalities were reported and suggested to contribute to disease onset and progression. In PD and HD, there is a need for neurorestorative therapies, as only symptomatic treatments are currently available in both rodent models of PD and in PD patients. PDGF-BB infusion into the brain lateral ventricle has shown promising results, although the mechanism behind the PDGF-BB-mediated neurorestorative effect remains unclear.</p> <p>Therefore, this thesis aimed at (i) investigating the pericyte alterations during PD and HD pathology progression, and (ii) examining the pericyte changes upon PDGF-BB treatment in PD. For this, we used a specific transgenic knock-in mouse expressing the green fluorescent protein gene under the <i>Rgs5</i> promoter, which was subjected to a partial 6-hydroxydopamine (6-OHDA) lesion of the nigrostriatal pathway. Moreover, we also used a transgenic mouse model of PD, overexpressing the human <i>alpha-synuclein</i> (<i><math>\alpha</math>-syn</i>) gene under the mouse <i><math>\alpha</math>-syn</i> promoter. To examine pericyte changes in HD, we crossed the R6/2 mouse model of HD with Rgs5 transgenic mice.</p> <p>In the <i><math>\alpha</math>-syn</i> transgenic PD mouse model, an increase in vascular density occurs in the striatum at the early stage of the disease, while blood vessel regression is observed later, when behavioural deficits were observed. The vascular regression was also coupled with an increased PDGFR<math>\beta</math><sup>+</sup> pericyte number. The latter was also detected in the striatum of the partial 6-OHDA lesion mouse model of PD, together with an increase in the RGS5<sup>+</sup> and NG2<sup>+</sup> pericyte numbers. Interestingly, PDGF-BB treatment not only induced neurorestoration of the nigrostriatal pathway, associated with behavioural recovery, but also normalized the number of RGS5<sup>+</sup> and NG2<sup>+</sup> pericytes. Moreover, <i>in vitro</i>, PDGF-BB-stimulated human brain pericytes released several growth factors as well as a greater quantity of microvesicles containing similar growth factors. In HD, vascular changes occurred early during disease progression, with an increase in PDGFR<math>\beta</math><sup>+</sup> pericyte number at early stage, when no motor deficits are present, which persisted at later stage of the disease, associated with behavioural impairment, in the R6/2 mouse striatum and the HD post mortem brain. In particular, RGS5<sup>+</sup> pericyte number increased solely at earlier disease stage, while NG2<sup>+</sup> pericyte numbers were increased only at late stage, in HD.</p> <p>In summary, this thesis highlights pericyte changes occurring during PD and HD progression, with some pericyte alterations even preceding neuronal cell loss and behavioural impairment. The brain vasculature, and more specifically brain pericytes, may therefore be a potential cell target for future therapies in PD and HD.</p>			
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# Pericytes in Neurodegeneration

Thomas Padel



**LUND**  
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Department of Clinical Sciences  
Faculty of Medicine  
Lund, 2017

Cover by Thomas Padel, representing pericytes on the brain vascular tree,  
emerging from the darkness of neurodegeneration

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*À ma famille*

*De deux choses lune, l'autre c'est le soleil*

Jacques Prévert (1946)



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## Original papers included in this thesis

- I. A partial lesion model of Parkinson's disease in mice – Characterization of a 6-OHDA-induced medial forebrain bundle lesion  
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*Behavioural Brain Research* (2015); 284: 196-206.  
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- II. Platelet-derived growth factor-BB has neurorestorative effects and modulates the pericyte response in a partial 6-hydroxydopamine lesion mouse model of Parkinson's disease  
**Padel T.**, Özen I., Boix J., Barbariga, M., Gaceb A., Roth M., Paul G.  
*Neurobiology of Disease* (2016); 94: 95-105.  
Doi: 10.1016/j.nbd.2016.06.002
- III. Pericytes secrete pro-regenerative molecules in response to Platelet-derived growth factor-BB  
Gaceb A., Özen I., **Padel T.**, Barbariga M., Paul G.  
*Journal of Cerebral Blood Flow & Metabolism* (2017).  
Doi: 10.1177/0271678X17719645.
- IV. Early vascular changes in an alpha-synuclein mouse model of Parkinson's disease  
**Padel T.**, Roth M., Li J-Y., Paul G.  
*Manuscript*
- V. Brain pericyte activation occurs early in Huntington's disease  
**Padel T.**, Roth M., Li J-Y., Björkqvist M., Paul G.  
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## Paper not included in this thesis

Brain pericytes acquire a microglial phenotype after stroke  
Özen I., Deierborg T., Miharada K., **Padel T.**, Englund E., Genové G., Paul G.  
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## Populärvetenskaplig sammanfattning

Vaskulaturen i hjärnan är en mycket specialiserad struktur. Blodkärlen i hjärnan måste kunna leva upp till de krav som hjärncellerna ställer när det gäller tillgången till syre och näringsämnen, samt skydda dem mot toxiner som cirkulerar i blodet. För detta har hjärnan utvecklat en specialiserad barriär, dels för att förhindra att toxiner i blodet tränger in i hjärnan och dels för att släppa ut överflödiga molekyler i blodflödet, den så kallade blod-hjärnbarriären (BHB).

Blodkärl bildas av endotelceller, som i sin tur är omgivna av pericyter, en celltyp som projicerar långsgående och tunna processer längs blodkärlen. Andra nervcellstyper lindar in detta komplex som då tillsammans bildar den neurovaskulära enheten.

Pericyter har många olika roller i hjärnan. De deltar i bildandet och mognaden av nya blodkärl, en process som kallas angiogenes. Normalt sett utsöndrar endotelceller specifika faktorer för att rekrytera pericyter till blodkärlen. Den huvudsakliga signaleringsmolekylen är Platelet-derived growth factor (PDGF-BB), som känns igen av pericyter tack vare deras uttryck av PDGF-receptor beta (PDGFR $\beta$ ). Detta möjliggör för pericyterna att täcka och hjälpa till göra blodkärlen mer mogna. Pericyter i hjärnan spelar också en viktig roll för att bibehålla BHB, samtidigt som de besitter egenskaper för att aktivt frisläppa toxiska molekyler i blodet.

För att upprätthålla de olika vaskulära aspekterna uttrycker pericyter i hjärnan många proteiner. Exempelvis uttrycker de pericyter som är involverade i bildning av blodkärl typiska markörer som Regulator for G-Protein signaling 5 (RGS5) och Chondroitin sulphate proteoglycan NG2 (NG2). Mer mogna pericyter som är associerade med stabilisering av blodkärl uttrycker istället PDGFR $\beta$ .

Pericyter är inblandade i många sjukdomar i hjärnan, men deras förändringar i specifika neurodegenerativa sjukdomar är ännu inte helt utredda. Neurodegenerativa sjukdomar kännetecknas av en progressiv förlust av specifika neuroner, vilket leder till motoriska, kognitiva och psykiatriska symptom. Bland dessa påverkar Parkinsons sjukdom (PS) och Huntingtons sjukdom (HS) tillsammans miljontals människor över hela världen och idag finns det ingen behandling för att stoppa eller återställa cellförlusten i dessa sjukdomar. Men i djurmodeller av PS ledde infusion av PDGF-BB i hjärnan till en återställning av nervceller, vilket ledde till en förbättring i beteende. Den första kliniska studien i PS där patienter fick PDGF-BB gav också lovande resultat, även om mekanismerna kring den fördelaktiga effekten av en behandling med PDGF-BB fortfarande behöver undersökas.

På sista tiden har intresset för vaskulaturen i hjärnan ökat inom neurodegenerativa sjukdomar. I hjärnor från både PS och HS observeras förändringar i blodkärlen,

skador på BHB och förändringar i cerebralt blodflöde. Vidare visade en tidigare studie att vaskulära störningar ledde till nervcellsförlust, vilket tyder på att vaskulaturen i hjärnan kan ha en nyckelroll i sjukdomsstarten och förloppet i neurodegenerativa sjukdomar. Att förstå och rikta insatser mot de vaskulära förändringarna i hjärnan i PS och HS kan bidra till att sakta ner eller till och med stoppa sjukdomsförloppet.

I denna avhandling undersökte vi därför hur hjärnans pericyter förändras i PS och HS och hur PDGF-BB-behandling i PS skulle påverka hur dessa pericyter agerar.

Vi identifierade först att vaskulaturen i hjärnan i PS är dynamisk över tid. I ett tidigt stadie och innan några motorsymptom var uppenbara ökade blodkärlens densitet i hjärnan i PS. Och tvärtom, när symptomen var synliga, minskade blodkärlens densitet. Detta var associerat med en ökning av antalet pericyter som uttrycker RGS5 eller NG2. Behandling med PDGF-BB ledde till en återställning av nervcellsförlusten som förekommer i hjärnan i PS, samt till en minskning av antalet pericyter som uttrycker RGS5 eller NG2. Vidare, när mänskliga pericyter odlas i en petriskål och stimuleras av PDGF-BB, utsöndrar de många faktorer som är kända för att stödja nervcellers överlevnad.

Således kan behandling med PDGF-BB i PS förändra proteinuttrycket hos pericyter och leda till ett mer moget tillstånd, vilket kanske kan bidra till blodkärlens mognad och vidare leda till den observerade återställningen av nervceller.

I HS förekommer liknande kärlförändringar i hjärnan. Blodkärlens densitet ökar innan symptom observerats och kvarstår vid ett sent stadium av sjukdomen. Antalet pericyter ökade redan i ett tidigt skede, redan innan någon nervcellsförlust observerats. Detta var associerat med fler RGS5<sup>+</sup> pericyter, vilket kan indikera stöd för de nybildade blodkärlen i HS. Och tvärtom, när beteendeförändringar var märkbara ökade antalet NG2<sup>+</sup> pericyter. I HS verkar förändringar i pericyter ske redan innan förändringar i beteende vilket tyder på att den här celltypen kan anpassa sig till tidiga och subtila förändringar i hjärnan som sker i HS. Pericyter ändrar också deras uttryck av markörer över tid, vilket ytterligare indikerar deras känslighet för förändringar i hjärnan i HS.

Pericyter i hjärnan är en mycket dynamisk celltyp som anpassar sig till sin omgivande mikromiljö. Deras respons i hjärnan i PS och HS, som föregår förlusten av nervceller och beteendemässiga förändringar i HS, kan indikera en möjlig kompensationsmekanism för de observerade vaskulära störningarna. Att inrikta sig på vaskulaturen i hjärnan, och mer specifikt på pericyter, kan vara av stort intresse i framtiden för att hitta nya behandlingar för neurodegenerativa sjukdomar.

## Résumé scientifique

Le système vasculaire cérébral est une structure extrêmement spécialisée. Les capillaires sanguins doivent à la fois assouvir les besoins en oxygène et nutritifs des cellules neurales, ainsi que protéger ces dernières des toxines circulantes dans le sang. Pour cela, le système nerveux central a développé une « protection » particulière afin d'empêcher le passage de ces dites toxines dans le cerveau, mais aussi de permettre l'évacuation, dans le sang, de toutes les molécules non nécessaires au bon fonctionnement cérébral. Cette structure est appelée la barrière hémato-encéphalique (BHE).

Plus précisément, les cellules endothéliales forment les capillaires sanguins et sont entourées de péricytes, ces derniers projetant de fins prolongements cellulaires dans le sens de la longueur du vaisseau. Plusieurs autres types de cellules recouvrent ce complexe, formant alors l'unité neurovasculaire.

Les péricytes possèdent de nombreux rôles dans le cerveau. Ils participent à la formation et la maturation des vaisseaux sanguins, processus appelé angiogenèse. Pour se faire, les cellules endothéliales sécrètent des facteurs de croissance afin de recruter les péricytes sur les vaisseaux sanguins. La principale molécule de signalisation est le PDGF-BB (pour « Platelet-derived growth factor-BB »), reconnue par les péricytes grâce à leur récepteur PDGFR $\beta$  (pour « Platelet-derived growth factor receptor beta »). Cette interaction moléculaire permet aux péricytes de recouvrir les capillaires sanguins et d'induire leur maturation. Les péricytes cérébraux ont aussi un rôle important dans la maintenance de la BHE, et sont capables d'évacuer dans le sang certains résidus secondaires, toxiques pour le cerveau.

Afin de compléter correctement tous ces rôles, les péricytes cérébraux expriment de nombreuses protéines. Par exemple, les péricytes impliqués dans la formation des vaisseaux sanguins expriment des marqueurs cellulaires spécifiques comme RGS5 (pour « Regulator for G-protein signaling 5 ») et NG2 (pour « Chondroïtine sulfate proteoglycan NG2 »). À l'inverse, les péricytes plus matures sont associés à la stabilisation des vaisseaux sanguins et produisent PDGFR $\beta$ .

Bien que relativement bien élucidée dans de nombreuses maladies cérébrales, la réponse des péricytes lors de certaines maladies neurodégénératives reste encore floue. Les maladies neurodégénératives sont caractérisées par une perte progressive de certains types de neurones, causant alors des symptômes moteurs, cognitifs et psychiatriques. Parmi elles, la maladie de Parkinson (MP) et de Huntington (MH) affectent ensemble des millions de personnes dans le monde. De nos jours, aucun traitement ne peut arrêter la progression de ces maladies, ou restaurer la perte des neurones. En revanche, chez des modèles animaux de la MP, l'infusion de PDGF-BB dans le cerveau induit une restauration des neurones,

associée à une amélioration des symptômes moteurs. Un premier essai clinique impliquant le traitement au PDGF-BB chez des patients atteints de la MP a d'ailleurs donné des résultats prometteurs. Cependant, le mécanisme permettant la restauration des neurones perdus n'a pas encore été entièrement clarifié.

Récemment, un intérêt est né pour le système vasculaire cérébral dans le cerveau de patients atteints de la MP et de la MH. En effet, des aberrations vasculaires, associées à une rupture de la BHE ainsi qu'à des variations du débit sanguin cérébral, ont pu y être observées. De plus, une étude a démontré que l'induction de perturbations vasculaires entraînait la perte de certains types de neurones. Ceci laisse supposer que le système vasculaire cérébral possède un rôle clef dans le déclenchement et la progression des maladies neurodégénératives. Comprendre et cibler les mécanismes impliqués dans ces anomalies vasculaires pourrait éventuellement permettre le ralentissement, voire l'arrêt complet de la progression de ces maladies.

Dans cette thèse, nous avons donc examiné la réponse des péricytes dans la MP et la MH, mais aussi comment ceux-ci sont influencés par le traitement au PDGF-BB dans la MP. Pour cela, plusieurs modèles animaux de la MP et de la MH ont été utilisés.

Tout d'abord, nous avons observé que chez la souris rendue parkinsonienne, le système vasculaire cérébral évolue à mesure où la maladie progresse. En effet, bien avant l'apparition des premiers symptômes moteurs, la densité de vaisseaux sanguins a déjà augmenté. Inversement, lorsque ces symptômes sont visibles, celle-ci a diminué. Cette dernière découverte est par ailleurs accompagnée d'une augmentation du nombre de péricytes exprimant RGS5 ou NG2, indication d'un remaniement vasculaire. Dans le cerveau de la souris parkinsonienne, le traitement au PDGF-BB a restauré la perte de neurone observée et, curieusement, a aussi diminué le nombre de péricytes exprimant RGS5 et NG2. Ceci indiquerait une maturation des péricytes, et a fortiori une stabilisation de la vascularisation cérébrale. En outre, *in vitro*, la stimulation des péricytes par le PDGF-BB a entraîné une augmentation de la sécrétion de plusieurs facteurs de croissance, connus pour leurs capacités à promouvoir la survie des neurones.

Le traitement au PDGF-BB provoquerait donc la maturation des péricytes et des vaisseaux sanguins, ce qui pourrait contribuer indirectement à la restauration des neurones dans la MP.

En ce qui concerne la MH, des remaniements au niveau du système vasculaire cérébral apparaissent aussi chez le modèle murin. La densité de vaisseaux sanguins a augmenté avant même l'arrivée des premiers troubles comportementaux, persistant durant toute la progression de la maladie. Étonnamment, le nombre de péricytes est aussi plus important en stade précoce,

lorsqu'aucune perte de neurone n'est observée. Plus particulièrement, un nombre plus important de péricytes exprime RGS5, indiquant un possible soutien des péricytes pour les vaisseaux néoformés. À l'inverse, lors de l'apparition des symptômes, le nombre de péricytes exprimant NG2 a cette fois-ci augmenté. Par conséquent, chez la souris modèle de la MH, les changements concernant les péricytes émergent avant même l'apparition des premiers symptômes, ce qui laisse supposer que ce type de cellule s'adapte aux altérations subtiles et précoces de la MH. Les péricytes changent l'expression de leurs marqueurs protéiques durant la progression de la maladie. Ceci supposerait une sensibilité face aux variations cérébrales impliquées dans la MH.

Les péricytes cérébraux représentent un type cellulaire très dynamique, s'ajustant rapidement à leur microenvironnement. Leur réaction, précédant la perte de neurones et l'arrivée des premiers symptômes moteurs lors de la MP et la MH, pourrait être un moyen de compenser les perturbations vasculaires observées. Cibler le système vasculaire cérébral et, plus particulièrement, les péricytes, serait donc intéressant dans le but d'explorer de nouveaux traitements pour les maladies neurodégénératives.



# Abbreviations

3-NPA	3-nitropropionic acid
6-OHDA	6-hydroxydopamine
AD	Alzheimer's disease
Alb	Albumin
Alk	Activin receptor-like kinase
AMPH	Amphetamine
Ang	Angiotensin
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AUC	Area under the curve
A/P	Antero-posterior
$\alpha$ SMA	Alpha smooth muscle actin
$\alpha$ -syn	Alpha-synuclein
BAC	Bacterial artificial chromosome
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BM	Basement membrane
CAG	Cytosine-adenosine-guanine
CBF	Cerebral blood flow
Coll-IV	Collagen-IV
Conc	Concentration
Ctrl	Control
DA	Dopamine
DAT	Dopamine transporter
D/V	Dorso-ventral
EB	Evans blue
EC	Endothelial cell
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
ERK	Extracellular signal-regulated kinases
Fb	Fibrin
Fbg	Fibrinogen
FITC	Fluorescein isothiocyanate
Gal-3	Galectin-3
GDNF	Glial cell line-derived neurotrophic factor
GFP	Green fluorescent protein
GLUT1	Glucose transporter 1
Hb	Hemoglobin
HD	Huntington's disease
HTT	Huntingtin
i.c.v.	intracerebroventricular
i.p.	intraperitoneally
IHC	Immunohistochemistry
Lam	Laminin
L-DOPA	L-3,4-dihydroxyphenylalanine
LRRK2	Leucine rich repeat kinase 2
MFB	Medial forebrain bundle
MMP	Matrix metalloproteinase
MPP+	1-methyl-4-phenylpyridinium ion
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MSC	Mesenchymal stem cell
MSN	Medium spiny neuron
MV	Microvesicle
M/L	Medio-lateral
NG2	Chondroitin sulfate proteoglycan NG2
NGF	Nerve growth factor
Pi3K	Phosphatidylinositol-3-OH kinase
PBS	Phosphate buffer saline
PD	Parkinson's disease
PDCLX	Podocalyxin
PDGF-BB	Platelet-derived growth factor-BB
PDGFR $\beta$	Platelet-derived growth factor receptor beta
Ret	Reticulin
RGS5	Regulator of G-protein signaling 5
ROC	Receiver operating characteristic
RT	Room temperature
SMC	Smooth muscle cell
SNpc	Substantia nigra pars compacta
TG	Transgenic mice overexpressing alpha-synuclein
TGF $\beta$	Transforming growth factor beta
TGF $\beta$ R	Transforming growth factor beta receptor
TH	Tyrosine hydroxylase
TJ	Tight junction
VEGF-A	Vascular endothelial growth factor A
VEGFR2	Vascular endothelial growth factor receptor 2
WB	Western blot
WT	Wild-type
ZO	Zona occludens



# Introduction

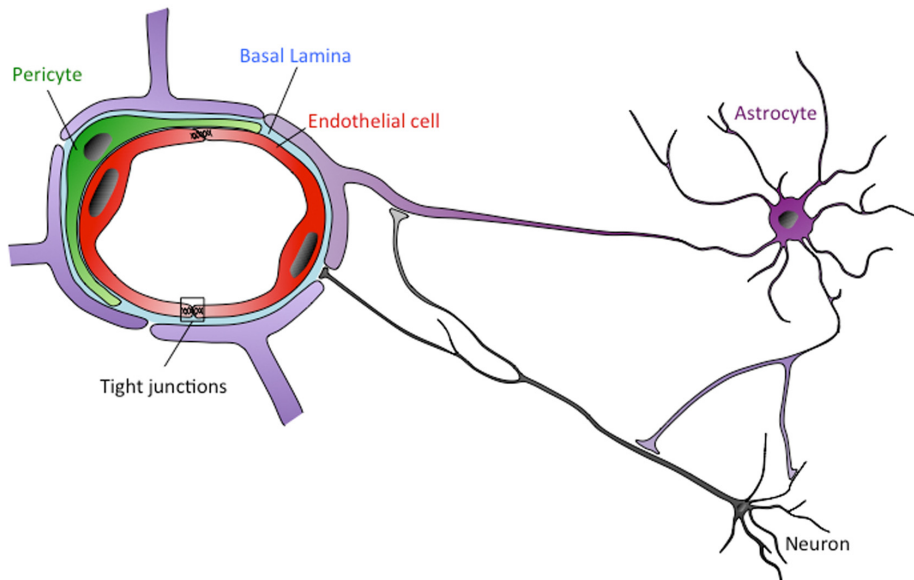
Microvascular changes in neurodegenerative disorders have recently attracted attention. Increasing data suggests that the brain vasculature is altered upon neurodegeneration, and that vascular changes may be actively participating in disease progression.

## The neurovascular system

While representing solely 2% of the total weight in humans, the brain consumes 20% of the total oxygen at rest, and is restricted to a high glucose-based diet [1, 2]. Therefore, the vasculature has adapted to meet the important energy demands of the brain, resulting in a 400 mile-long capillary network in the brain [3].

The brain has a variety of needs, preventing the passage of toxic products from the blood stream, but also demanding large amounts of oxygen and nutrients from the very same source. Moreover, the fine regulation of the cerebral blood flow (CBF) to adapt to the regional needs of brain cells requires a fast and dynamic response of the brain vasculature [4]. Therefore, a tight regulation of the vasculature is necessary for proper brain functions, and to selectively transfer molecules from the blood to the brain. This occurs through the involvement of several different cell types (endothelial cells (EC), pericytes, astrocytes and neurons) that together form the neurovascular unit (Figure 1) [5]. This highly specialized structure allows for an extremely regulated border between the blood circulation and the brain parenchyma: the blood-brain barrier (BBB) [6].

As part of the BBB, brain EC forming the vessel tube possess numerous differences compared to EC from other organs, such as specific tight junctions (TJ) between EC and the lack of fenestration [7, 8]. TJ constitute the main impermeable structure of the BBB, formed by a complex assembly of several proteins, including the Occludin, Claudin and Zona occludens (ZO) proteins [9-12].



**Figure 1. Schematic cross section of a brain capillary with the different neurovascular unit components**  
 In the brain, the neurovascular unit is composed of endothelial cells attached to each other by specific tight junctions. Surrounding the endothelial cells are the pericytes, ensheathed in the basal lamina. Several astrocyte end-feet, as well as neurons, envelop this structure.

On larger blood vessels (such as arteries and veins), EC are encircled by smooth muscle cells (SMC), while at the capillary level, EC are surrounded by pericytes [13, 14]. Both EC and pericytes are ensheathed in a basal lamina that is composed of extracellular matrix proteins, providing cell adherence but also promoting TJ formation [15, 16]. Surrounding the capillaries are the astrocytes, projecting filopodia with specialized end-feet apposed to the blood vessels. They play a dual role, as they maintain the TJ integrity [17] in addition to adjusting the CBF [18].

A number of brain disorders have been associated with BBB disruption, such as stroke [19] and more recently even several neurodegenerative diseases [20-22]. Specifically, brain pericytes are a particularly dynamic and versatile cell type, presenting numerous functions in the brain.

## Pericytes

Pericytes were first described as “Rouget cells” in 1873 [23], and renamed later in 1923 by Zimmermann according to their position at the abluminal surface of the blood vessels [24]. While ubiquitously present in all organs, the pericyte density is

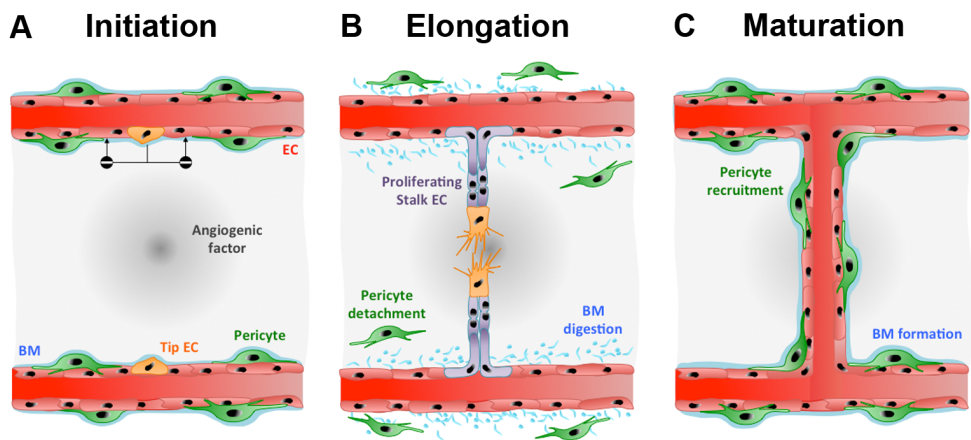
highest in the central nervous system, being between 1:1 and 1:3 pericyte-EC ratio, as compared to a 1:100 ratio in skeletal muscle, for instance [25, 26].

## Multiple functions of pericytes in the brain

### *Angiogenesis*

In the embryo, vascular development occurs early to meet the needs of the growing organs for oxygen and nutrients. This *de novo* blood vessel formation is termed vasculogenesis. For this, endothelial precursor cells (angioblasts) develop from embryonic stem cells, and then spread through the entire embryo [27]. Angioblasts then further differentiate into EC and form a first blood vessel system.

This primitive vascular plexus will, however, become rapidly insufficient as the embryo develops. Hence, there is a need for formation of new blood vessels from this pre-existing vessel network, a process called angiogenesis. Sprouting angiogenesis is the most characterized vessel formation mechanism and can be summarized in three steps (Figure 2).



**Figure 2. Mechanisms involved in sprouting angiogenesis**

(A) Initiation of angiogenesis occurs by induction of a tip endothelial cell (EC) by angiogenic factors, which inhibits nearby EC to also differentiate into tip EC. (B) The basement membrane (BM) is digested and pericytes detach from the blood vessel. EC tip filopodia sense the angiogenic gradient to provide guidance of the vessel sprout, while stalk EC proliferate and elongate the immature vessel. (C) When two tip EC meet, pericytes are recruited and a new BM is formed, maturing the newly formed blood vessel.

First, an induction occurs from angiogenic cues sensed by EC. Typically, a hypoxic environment triggers neighbouring cells to secrete angiogenic factors, such as Vascular endothelial growth factor A (VEGF-A) [28]. This angiogenic molecular gradient is perceived by capillary EC, where the EC located at the highest concentration become the tip EC, i.e. the EC directing the vessel sprout to

the angiogenic factor source [29]. To regulate angiogenic sprouting, a negative feedback loop is activated, where the tip EC stimulates the Notch/Delta signaling pathway in the nearby EC, inhibiting their tip EC conversion potential [29], and becoming stalk EC instead.

Second, pericytes detach from the blood vessels and the tip EC secretes enzymes such as Matrix metalloproteinases (MMP) to digest basal lamina proteins [30], allowing the vessel sprout to elongate through the parenchyma. Moreover, the tip EC possesses numerous filopodia, which sense the VEGF-A gradient so as to specifically guide the vessel to the VEGF-A source [31]. Following the tip EC, stalk EC divide and elongate the vessel sprout [32]. When two tip EC meet, the immature capillary is formed.

Third, capillary maturation occurs through pericyte recruitment onto the blood vessel, where their cross-talk with EC will induce the formation of a new basal lamina [33, 34]. Specifically, tip EC secrete Platelet-derived growth factor-BB (PDGF-BB), which is recognized with high affinity by PDGF receptor  $\beta$  (PDGFR $\beta$ )-expressing pericytes. This leads to pericyte recruitment which stabilizes the blood vessel [35].

Under physiological conditions, angiogenesis occurs mainly during embryonic development, and in very restricted areas of the body during adulthood [36, 37]. In the adult brain, however, angiogenesis is very limited and only stimulated under pathological conditions [38].

### *Blood-brain barrier integrity*

BBB maintenance consists of the tight control of the brain permeability through TJ protein expression and arrangement, as well as the regulation of both EC transcytosis and active transporters expressed at the neurovascular unit, e.g. glucose and iron transporters in EC and water channel in astrocytes [39].

Both during development and adulthood, brain pericytes are crucial for BBB integrity, as pericyte deficiency in mice leads to decreased pericyte coverage, which was associated with increased capillary diameter and BBB leakage [13, 14]. Pericyte-deficient mice did not show any changes in TJ protein expression, but rather promoted disorganization of the TJ disposition in EC [13, 14]. Pericytes have, however, the capacity to strengthen the BBB, expressing the Glial cell line-derived neurotrophic factor (GDNF) to increase Claudin-5 expression in EC [40].

Interestingly, pericyte loss induces an increased EC transcytosis as well as a decrease in transporter expression in EC (Transferrin receptor) and in astrocytes (Aquaporine 4) [13]. Overall, pericytes participate in the BBB maintenance, implicated not only in TJ regulation, but also in the control of vesicle trafficking in EC and active transporter expression at the neurovascular unit [13].

### *Secretory properties*

Brain pericytes have been described to share common characteristics with mesenchymal stem cells (MSC) [41]. This includes (i) similar marker expression, e.g. Aminopeptidase N (CD13) [42] and Nestin [43] (ii) their capacity to differentiate into the osteoblast, chondroblast and adipocyte lineages *in vitro* [44], (iii) their location in the perivascular space of different organs including the brain [45] and (iv) their similar migratory response to PDGF-BB [46].

MSC possess an important pro-regenerative capacity as well as a role in trophic support for neighbouring cells [47]. For this, MSC use paracrine secretion of a broad range of trophic factors, e.g GDNF, Brain-derived neurotrophic factor (BDNF), Nerve growth factor (NGF) and VEGF [48]. MSC also release microvesicles (MV) containing proteins and RNAs for cell-to-cell communication [49]. However, whether pericytes also share this secretive capacity remains largely unknown.

### *Clearance*

Brain pericytes present several surface markers to capture and remove toxic byproducts from the brain. For instance, they express special receptors to actively discharge cholesterol metabolites into the blood stream [50], as well as the receptor for  $\beta$ -amyloid, one of the main proteins implicated in Alzheimer's disease (AD) pathology [51]. The mechanisms of pericyte clearance have only just begun to be clarified, and may have implications especially in neurodegenerative diseases.

### *Cerebral blood flow regulation*

Pericytes have been suggested to participate in the CBF regulation, as they can express contractile proteins in the brain [52]. Hall and colleagues reported that pericytes stimulated by either noradrenaline or glutamate promoted blood vessel constriction and dilation, respectively, which were also observed in ischemic conditions [53]. However, a year later, a second study claimed that SMC, and not pericytes, control CBF [54]. These opposite conclusions may be a direct consequence of the difficulty to define, identify and target pericytes in the brain.

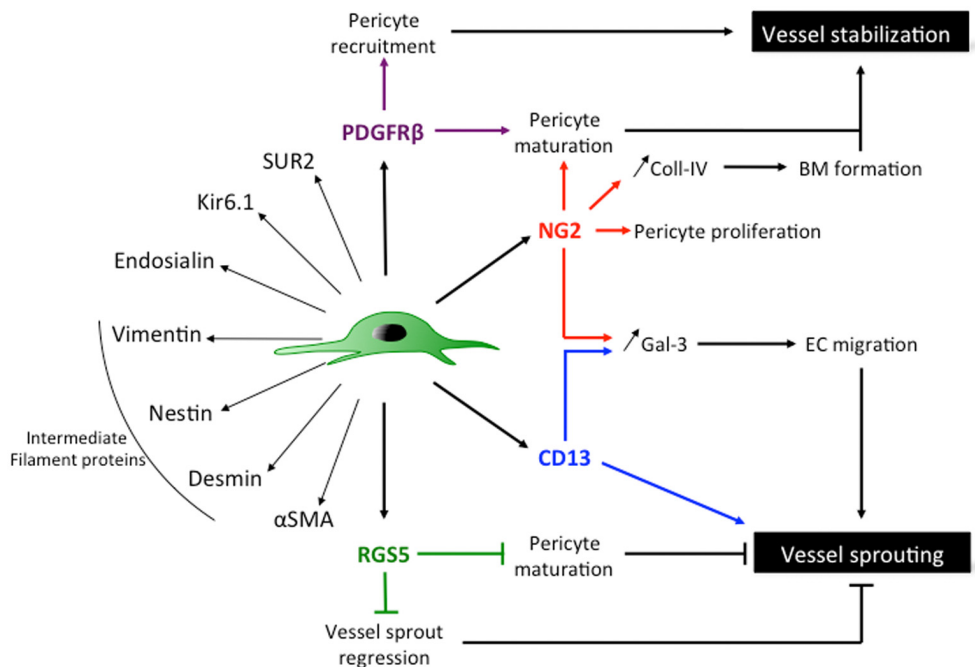
## **Pericyte identification**

Characterization of pericytes is usually based on their location, their morphology and their dynamic protein expression profile. Capillary pericytes are generally defined surrounding the blood vessels, covered by the basal lamina, and with a flat cell soma from where extensive longitudinal and thin processes span over several EC [55]. However, under pathological conditions, pericytes are shown to acquire a



more bulging cell soma with shorter processes, typical of activated and migratory pericytes [55, 56].

The diversity in brain pericyte markers may depend on their different origins during development [57, 58], or their location along the vasculature. Indeed, some reports described that pre-capillary arteriole pericytes may express the contractile protein Alpha smooth muscle actin ( $\alpha$ SMA) and the Chondroitin sulfate proteoglycan NG2 (NG2), acquiring a mesh morphology [54, 59]. However, capillary pericytes are devoid of  $\alpha$ SMA [59, 60], although a report has shown that capillary pericytes can also express this protein when cultured *in vitro* [61]. Pericytes on post-capillary venules express only  $\alpha$ SMA and not NG2 [45, 62].



**Figure 3. Schematic representation of different markers expressed by pericytes**

PDGFR $\beta$  is the main marker used for pericyte identification, participating in pericyte recruitment onto the blood vessels and promoting vascular and pericyte maturation. NG2<sup>+</sup> pericytes are involved in vessel sprouting, through Galectin-3 (Gal-3)-mediated EC migration, as well as pericyte proliferation. However, NG2 is also an indicator of pericyte maturation, as NG2<sup>+</sup> pericytes secrete Collagen-IV (Coll-IV), contributing to the basement membrane (BM) formation and vessel maturation. CD13 is increased in pericyte during angiogenesis, and participates also in EC migration through Gal-3 expression. RGS5 expression in pericytes inhibits vessel sprout regression and pericyte maturation, thereby promoting vascular remodeling. To a lower extent, the pericyte dynamic marker expression profile includes also  $\alpha$ SMA, the intermediate filament proteins Desmin, Nestin and Vimentin, as well as Endosialin, the potassium channel Kir6.1 and SUR2.

Among the markers identified, some pericyte subpopulations express the intermediate filament proteins Desmin and Nestin [55]. Moreover, PDGFR $\beta$ , Regulator of G-protein signaling 5 (RGS5) and the transmembrane protein CD13, were also identified as pericyte markers [63-65] (Figure 3).

PDGFR $\beta$  is the most widely utilized pericyte marker and has been employed in numerous pericyte studies in different brain pathologies such as stroke [56], AD [22], and other brain disorders [66-68]. PDGFR $\beta$  is a transmembrane receptor with tyrosine kinase activity, where each phosphorylated tyrosine residue transduces different signaling pathways (see below). *Pdgfrb* knockout transgenic mice display a drastic reduction of pericyte coverage, leading to microhemorrhages. This highlights the importance of PDGFR $\beta$  for pericyte recruitment onto the blood vessels [69, 70]. Therefore PDGFR $\beta$  expression in pericytes reflects a more mature, vessel-stabilizing pericyte state.

RGS5 is a 200 amino acid protein member of the large RGS family, located in the inner side of the cytoplasmic membrane [71]. RGS5 plays a main role in inhibiting G-proteins, thus impairing G-protein-coupled receptor signaling [72]. Upregulation of RGS5 in pericytes is associated with reduced vessel sprout regression, thereby contributing to capillary elongation during angiogenesis [73]. This is further supported by a developmental study, showing that RGS5 is highly expressed in both pericytes and SMC of the embryo (highest expression level between E14.5 and E17.5 in mice), most likely as a result of the extensive angiogenic response occurring during development [74]. However RGS5 expression is drastically reduced to barely a small proportion of capillary pericytes in the adult brain, upregulated only during vascular remodeling [75] and under pathological conditions such as stroke or tumor [56, 68]. Moreover, tumors lacking RGS5 present a more stabilized vasculature, a decreased vessel leakage and more mature pericytes, suggesting that RGS5 expression in pericytes is also associated with the aberrant angiogenic vascular response occurring in cancer [76]. In line with *in vivo* studies, *in vitro* experiments reported that RGS5 inhibited phosphorylation of some downstream targets of the PDGF-BB/PDGFR $\beta$  pathway [74]. This further indicates an active role of RGS5<sup>+</sup> pericytes during angiogenic sprouting, as opposed to the vessel stabilization induced by the PDGFR $\beta$  signaling pathway.

NG2 is a membrane protein part of the proteoglycan family, where the transmembrane protein unit is coupled with a glycosaminoglycan on the extracellular side of the cytoplasmic membrane [77]. NG2 is expressed by mural cells during vascular remodeling [78], and specific *Ng2* knock-out in pericytes leads to a decreased neovascularization, with reduced proliferation of pericytes and EC [79, 80]. Furthermore, NG2 binds to Galectin-3 on EC, promoting vessel tube formation and EC migration *in vitro* [79]. Brain pericytes have also been

shown to increase NG2 expression under pathological conditions [56, 68]. Thus, pericytes may not only play a role during blood vessel maturation, but also during vessel sprouting, requiring RGS5 and NG2. The expression of those two proteins reflects a more activated state of pericytes in response to vascular changes [75, 78, 81]. NG2 is also used as a marker for mature pericytes, as NG2-expressing pericytes were shown to stimulate Collagen-IV expression for basement membrane (BM) formation [82]. Thus, NG2 expression in pericytes may play a dual role in vascular remodeling and vessel stabilization.

CD13 is a zinc-dependent ectoenzyme, i.e. a membrane protein with an enzymatic site facing the external microenvironment. CD13 is involved in the degradation of extracellular matrix molecules, such as Collagen-IV, as well as neuropeptides and several cytokines and chemokines [83]. CD13 was identified as a marker for pericytes and vascular SMC in the brain, where Kunz and colleagues (1994) showed that CD13 expression was found at the capillary level and associated with BBB integrity [64]. Furthermore, the expression of CD13 was described to coincide with angiogenesis, as (i) CD13 expression increases upon VEGF and other angiogenic factor stimulation [84], (ii) *Cd13* mRNA silencing impairs EC tube formation *in vitro* [84] and (iii) *Cd13* knock-out mice display decreased vessel density [85]. Moreover, CD13 is also upregulated during inflammation and tumor neovascularization [86, 87], with an *in vitro* study indicating CD13 to mediate angiogenesis through Galectin-3 [88]. Thus, it may be that CD13 and NG2 expression in pericytes trigger similar signaling pathway targets in EC during angiogenesis.

Finally, additional proteins were found to be expressed by some, but not all, pericytes, e.g. Endosialin [89], SUR2 [90], the potassium channel Kir6.1 [90] and Vimentin [52]. However, thus far, no pan-marker is available to identify the entire pericyte population [55].

## **Molecular regulators of pericytes in the neurovasculature**

### *PDGF-BB/PDGFR $\beta$ pathway*

PDGF-BB was first identified in platelet-containing serum, inducing SMC proliferation [91]. PDGF-BB is a member of the PDGF family, composed of four different protein sub-units (A, B, C and D), which can homo or heterodimerize to form five growth factor complexes (PDGF-AA, -BB, -AB, -CC and -DD) [92]. Each PDGF isoform binds specifically to PDGF transmembrane receptors composed of PDGFR $\alpha$  and/or  $\beta$  sub-units, leading to the receptor dimerization ( $\alpha\alpha$ ,  $\alpha\beta$  or  $\beta\beta$ ). While both PDGF-A and -B isoforms bind to the  $\alpha$ -receptor sub-unit, PDGF-B binds with high affinity to the  $\beta$  receptor sub-unit [93].

In the brain, PDGF-BB secretion by EC is crucial for pericyte recruitment. Knockout of *Pdgf-b* is lethal and such transgenic mouse embryos display hemorrhages [94]. More specifically, the PDGF-BB C-terminal is composed of positively charged amino acids, which bind to the negatively charged proteins of the extracellular matrix [92]. This retention motif is essential for proper pericyte recruitment, as it allows PDGF-BB to be kept within the microenvironment of the producing tip EC [95]. Knockout mice for this specific *Pdgf-b* gene region are viable, but develop a substantial decrease in pericyte coverage of the vasculature [95].

When PDGF-BB binds to PDGFR $\beta$ , homodimerization of the receptor occurs, thereby leading to the receptor autophosphorylation on several tyrosine residues. Each tyrosine residue phosphorylation will activate a specific downstream signaling pathway. For instance, SHP2 phosphatase forms a complex with PDGFR $\beta$  specifically through the phosphorylated tyrosine residue Y1009, activating the Extracellular signal-regulated kinase (ERK) pathway [96]. This induces cytoskeleton rearrangement and thus participates in pericyte migration and recruitment onto the blood vessel. Alternatively, the Phosphatidylinositol-3-OH kinase (Pi3K) binds other PDGFR $\beta$  phosphorylated tyrosine residues, leading to Akt pathway activation, contributing to pericyte survival [97]. Similar to that of *Pdgf-b* deficient mice, *Pdgfrb* knockout is lethal, with the mouse embryos developing microhemorrhages [70].

#### *TGF $\beta$ /TGF $\beta$ R pathway*

Transforming growth factor beta (TGF $\beta$ ) binds to TGF $\beta$  receptor (TGF $\beta$ R), which further phosphorylates the Activin receptor-like kinase (Alk) proteins. Interestingly, an *in vitro* study emphasized the need for pericyte-EC interaction for TGF $\beta$  signaling pathway activation by both cell types [98]. In pericytes, Alk5 activation participates in pericyte maturation [98] and reinforces EC-pericyte attachment through upregulation of the adherens junction protein N-cadherin [99]. Therefore, while the PDGF-BB/PDGFR $\beta$  signaling pathway recruits pericytes on EC, TGF $\beta$ /TGF $\beta$ R mainly strengthens the attachment between both cell types.

#### *VEGF-A/VEGFR2 pathway*

VEGF-A is secreted by EC and binds to the VEGF receptor 2 (VEGFR2) expressed by both EC and pericytes, playing a role in pericyte detachment during angiogenesis. Moreover, VEGF-A does not only act as an angiogenic factor [29], but also promotes EC and pericyte survival, through the expression of several anti-apoptotic proteins [100].

### *Angiopoietins/Tie2 pathway*

Angiopoietin (Ang) 1 and 2 have opposite roles in EC. Pericyte secretion of Ang1 binds to the Tie2 receptor on EC, which in turn support the BBB integrity [101]. Contrarily, EC secretes also Ang2, an antagonist of Ang1, interfering with the Tie2 receptor, and promoting EC proliferation and migration, as well as BBB permeability [101].

### *Other pericyte regulators*

Numerous other signaling pathways are involved in pericyte homeostasis in the brain. For instance, the Notch signaling pathway contributes to pericyte coverage and BBB integrity [102], and the EphrinB2/EphB4 pathway activation participates in pericyte coverage onto the blood vessels [103].

## **Pericyte changes in neurodegenerative diseases**

Extensive studies have investigated the changes in pericytes in AD, reporting (i) pericyte dysfunction [104], (ii) pericyte degeneration, induced by the progressive accumulation of  $\beta$ -amyloid [105] and (iii) faster disease progression in a pericyte-deficient mouse model of AD [106].

On the contrary, scarcely any data are available regarding brain pericytes in Parkinson's disease (PD). One previous study showed no degenerative pericytes in PD patient brain tissue [107], and a recent report described the pericyte capacity to communicate through nanotunnels with neighboring cells to capture alpha-synuclein ( $\alpha$ -syn) *in vitro* [108]. In the Huntington's disease (HD) brain, pericyte coverage was shown to be dramatically decreased, although another study reported no change [20, 109]. However, in both diseases, vascular changes were reported (see below).

Since brain pericytes contribute to the progression of the pathology in AD, understanding their roles in PD and HD may unravel other pathological mechanisms implicated in disease progression. Moreover, due to their multiple roles in the brain, pericytes may constitute an interesting target cell for regenerative therapies in these disorders.

## **Parkinson's and Huntington's disease**

Among neurodegenerative disorders, the incidence for PD and HD is approximately 260 and 1 new case(s) per 100000 people per year, respectively [110, 111], with PD being the second most common neurodegenerative disease in man.

While the disease mechanisms may differ among neurodegenerative diseases, they do however share some similar characteristics [112]. Firstly, one of the common hallmark is the progressive accumulation and aggregation of disease-specific proteins in the form of dense inclusions, i.e. Lewy bodies in PD and Huntingtin (HTT) inclusions in HD [113, 114]. Secondly, certain neuronal subtypes are vulnerable to each specific aggregate, resulting in a variety of motor, cognitive and/or psychiatric symptoms. Thirdly, several risk factors influence disease onset and progression, such as genetic mutations, environmental factors and aging [112].

While symptomatic medications exist for PD and HD [115, 116], no disease-modifying treatment is currently available to slow down or stop the disease progression, nor to restore the neuronal cell loss occurring in these brain disorders. To manage the larger number of patients and the connected health costs, brain research will have to direct a sharp focus towards elucidating disease mechanisms and developing neurorestorative and neuroprotective treatments.

## **Parkinson's disease**

PD was first described 200 years ago by James Parkinson, reporting cases of patients presenting what he defined as “shaking palsy” [117]. To date, it is one of the most common neurodegenerative disease, affecting 1% of the population above 60 years of age worldwide [118], a number expected to increase with an aging population.

### *Pathophysiology*

PD is characterized by motor symptoms such as rigidity, resting tremor and bradykinesia [119]. Specifically, a progressive loss of dopaminergic neurons occurs in the substantia nigra pars compacta (SNpc) [120], a brain structure that belongs to the basal ganglia circuitry and responsible for the execution of voluntary movement [121].

In the healthy brain, SN dopaminergic neurons innervate the striatum and release dopamine (DA) [122]. This neurotransmitter binds to DA receptors on medium spiny neurons (MSN), divided into two types: MSN of the direct pathway become activated, while MSN of the indirect pathway are inhibited. These differential neuronal inputs are then eventually transmitted to the cortex to initiate a coordinated voluntary movement. In PD, the death of SNpc dopaminergic neurons leads to a decrease in DA release in the striatum. Therefore an imbalance between the direct and indirect pathway activation occurs, which causes the cortex to receive insufficient neuronal input [123].

In PD patients, motor symptoms usually occur when at least 50% of the striatal dopaminergic fibres are lost [124]. However, already before motor impairment, some non-motor features can sometimes be identified, including sleep disorder and

loss of smell [125]. In PD, motor symptoms worsen overtime and can be associated with cognitive dysfunction [126].

### *Etiology*

Most PD cases (over 90%) are sporadic, and numerous factors have been linked to an increased risk of developing the disease [127], rendering disease mechanisms difficult to entangle. Aging is an important factor in PD, as the incidence is more than 70 times higher in men over 80 years of age than in men in their 40s [110]. Aging may contribute to some cellular dysfunctions thought to contribute to PD development, such as mitochondrial dysfunction [128, 129]. Certain environmental toxins (pesticides) were also discovered to increase the risk of developing PD [127, 130]. Moreover, inflammation may play a role in PD pathology, as certain anti-inflammatory drugs were shown to decrease the risk of developing the disease [131]. Recently, an interest arose in the brain vasculature, as (i) several vascular alterations were described in PD patients (see below) and (ii) inducing vascular changes led to dopaminergic cell loss in the brain, suggesting that the brain vasculature is an important player in neurodegeneration, particularly in PD [132].

The remaining 5-10% of PD cases can be attributed to genetic mutations. In 1997, a study demonstrated the link between the PD pathology and the  $\alpha$ -syn protein, where an A53T mutation in the  $\alpha$ -syn gene was observed in a familial form of PD [133].

The term “synuclein” was proposed in reference to the location of the protein, first found at the nuclear envelope, and later on detected in the presynaptic compartment in neurons [134, 135]. The physiological role of  $\alpha$ -syn remains poorly understood, although a possible implication in synaptic plasticity has been suggested [136]. In PD,  $\alpha$ -syn constitutes the main component of Lewy bodies [114], a structure first identified in PD in the early 1900’s, and considered now the main hallmark of the pathology [137].

Therefore, the cause for developing PD seems to be a complex interplay between genetic, aging, environmental and possibly vascular risks factors.

### *Growth factors as a therapy for Parkinson’s disease*

Presently, only symptomatic treatments are available for PD patients, mainly pharmacological treatments aiming for DA replacement, e.g the DA substrate L-3,4-dihydroxyphenylalanine (L-DOPA), and deep brain stimulation [138, 139]. However, pharmacological treatment is of limited use, as its prolonged administration causes patients to develop abnormal involuntary movements (dyskinesias) and other side effects [140]. Moreover, none of the currently

clinically available treatments modifies disease progression or restores the nigrostriatal dopaminergic system.

Growth factors have been investigated in the hope to stop the cell loss or even restore dopaminergic cells and fibres, which should in turn alleviate motor impairment [141]. The most extensively studied growth factor for PD therapy is GDNF, known to increase dopaminergic cell survival and improve synaptic activity [142-144]. In most rodent models of PD, GDNF treatment showed neuroprotection and neurorestoration of the nigrostriatal dopaminergic pathway, associated with an improvement of behavioural deficits [145]. However, the neurorestorative properties of GDNF treatment in PD patients are so far inconsistent among different clinical studies [146, 147].

PDGF-BB has been shown to stimulate dopaminergic neurite outgrowth *in vitro* [148]. Further, *in vivo* studies demonstrated that intracerebroventricular (i.c.v.) infusion of PDGF-BB induced neurorestoration of the nigrostriatal dopaminergic pathway in several rodent models of PD, associated with behavioural recovery [149]. These findings led to a first clinical trial, where i.c.v. infusion of PDGF-BB resulted in an increase in the DA transporter (DAT) binding in the putamen of PD patients who had received the highest dose (5µg per day for 12 days) of PDGF-BB [150]. Therefore, although the mechanism of these beneficial effects remains unclear, PDGF-BB treatment shows promise as a novel neurorestorative treatment for PD patients.

Further investigation of disease-modifying therapies in PD, in particular growth factor treatments, is still required in order to discern their neurorestorative potential.

### *Modelling Parkinson's disease*

In order to investigate the mechanisms underlying PD pathology, as well as to study potential neurorestorative therapies, the necessity for animal models is in no doubt. Two major approaches have been extensively used to model PD in animals, based on the induction of nigrostriatal dopaminergic cell loss.

Toxin-induced models of PD utilize compounds that specifically target the dopaminergic cells in the SN [151]. The main toxin used is 6-hydroxydopamine (6-OHDA), which can be stereotactically injected into the striatum, SN or in the medial forebrain bundle (MFB), causing a dose-dependent lesion of the nigrostriatal dopaminergic pathway [152]. Specifically, 6-OHDA enters dopaminergic cells through DAT, where 6-OHDA oxidation generates reactive oxygen species, leading to oxidative stress-induced cell death [153].

When injected in the striatum, 6-OHDA is retrogradely transported by dopaminergic fibres, producing progressive terminal fibre loss prior to SNpc cell



death, which takes place over several weeks [152, 154]. Moreover, striatal 6-OHDA injections can be used to produce a more partial lesion, where a certain amount of dopaminergic fibres remains in the striatum [152, 154]. Partial 6-OHDA lesions are advantageous over a complete lesion to (i) reproduce earlier stages of PD and (ii) investigate certain neuroprotective/neurorestorative therapies where beneficial effects depend on remaining striatal dopaminergic fibres [149, 151]. On the contrary, 6-OHDA injection in the SN induces a faster depletion of dopaminergic cells compared to striatal injection, with an almost complete striatal dopaminergic fibre loss over few days [155].

However, both 6-OHDA targets (striatum and SN) also represent possible neurorestorative sites of investigation for potential therapies, where the 6-OHDA toxin may be a confounding factor. Therefore, in several studies, 6-OHDA was instead injected into the MFB in mice [156, 157], leading to a complete nigrostriatal lesion [157].

6-OHDA lesion models offer the benefit to induce a specific unilateral lesion, using the contralateral brain hemisphere as internal control. Moreover, behavioural impairment in unilateral 6-OHDA PD models can be well identified using a range of behavioural tests, such as spontaneous or drug-induced rotation assessments [158], which are necessary to evaluate potential benefits of neurorestorative therapies.

The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxin also induces dopaminergic cell loss, and is mainly used in mice and monkeys [159]. MPTP is injected in the periphery and converted in the brain into the 1-methyl-4-phenylpyridinium ion (MPP<sup>+</sup>) [160], a toxic metabolite taken up by dopaminergic cells. In turn, MPP<sup>+</sup> induces impairments in the mitochondrial respiration chain, leading to dopaminergic cell death [159].

To a lesser extent, the pesticide rotenone has been utilized. In this model, the reproducibility of the location and extent of the lesion are variable depending on the administration route, in addition to peripheral problems in the heart and liver [161]. Chronic rotenone infusion in the rat blood circulation presents, however, the advantages of inducing selective nigrostriatal dopaminergic cell loss, forming  $\alpha$ -syn inclusions and mimicking the environmental etiology of PD [162].

In most toxin-induced lesion models of PD, the age-dependent and slowly progressive nature of the pathology is not reproduced [151]. To circumvent the latter drawback, several transgenic animals have been generated [163]. Transgenic mice overexpressing wild-type (WT) or mutant  $\alpha$ -syn develop a progressive behavioural impairment and DA homeostasis abnormalities in the striatum [164-166]. Moreover, they present the advantage to also reproduce some of the non-motor symptoms of PD, such as loss of smell [164, 165]. However, while

progressive, these models do not present a significant dopaminergic cell loss. Similarly, mutations of other genes discovered to increase the risk factor for PD pathology, e.g. *Leucine rich repeat kinase 2 (LRRK2)*, also display alterations of the striatal dopamine cycle, associated with behavioural deficits, but only have a modest reduction in the number of SN dopaminergic cells [167].

Therefore, to this date, no animal model can altogether (i) reproduce the age-related progressive nature of the disease, (ii) cause a significant dopaminergic cell loss associated with behavioural impairment (iii) induce  $\alpha$ -syn-containing Lewy bodies and (iv) resemble clinical symptoms of PD [168].

## **Huntington's disease**

Huntington's disease was first identified by George Huntington in 1872, already suggesting it to be a hereditary disorder, "confined to certain and fortunately few families" [169]. However, the gene mutation causing HD disease onset was only discovered 120 years later [170].

### *Pathophysiology*

Huntington's disease pathology results from a specific mutation of the *HTT* gene, where additional cytosine-adenosine-guanine (CAG) repeats are inserted in the exon one [170]. In humans, a minimum of 40 CAG repeats are necessary to develop full disease penetrance, while between 36 and 39 CAG repeats, an incomplete penetrance is observed [171].

This leads to the production of a mutant form of the HTT protein, which misfolds and forms aggregates [113]. In the brain, striatal MSN are the most sensitive neuronal cell type to these aggregates, and the progressive loss of MSN leads to involuntary movements known as chorea (from the Greek word "*χορεία*": to dance) [172]. Rigidity and bradykinesia can also appear in HD patients, coupled with cognitive and psychiatric symptoms. As the disease progresses, other brain regions become affected, including the cortex, the hippocampus and the cerebellum, resulting in a more general brain atrophy in HD.

### *Modelling Huntington's disease*

Since the discovery of the *HTT* gene mutation responsible for the disease development, toxin-induced models of HD, such as the 3-nitropropionic acid (3-NPA) model, were gradually abandoned in favor of transgenic animal models [173].

The most widely characterized transgenic HD model is the R6/2 mouse, where the first exon of the human *HTT* gene carrying 150 CAG repeats is incorporated into the mouse genome [174]. R6/2 mice display weight loss and fast-progressive

behavioural impairment, with a lifespan usually not exceeding 13 weeks of age [174]. However, the CAG extension is not stable and increases generation after generation, which surprisingly delays the onset of the disease in the R6/2 mouse [175].

Other transgenic mouse models include the expression of the entire human mutant *HTT* gene (YAC128 and BACHD mouse models), or knock-in models where additional CAG repeats are integrated into the mouse *Htt* gene (HdhQ150 mouse model) [173].

### **Vascular changes in Parkinson's and Huntington's disease**

An interest in the brain vasculature is currently growing in the field of neurodegenerative diseases. Indeed, vascular alterations were hypothesized to contribute to disease progression, or even being potential key factors for neurodegeneration [106, 132]. However, studies in PD and HD are sparse and differ depending on the species investigated, the animal models applied, and the markers used to explore the vascular changes.

In PD (Table 1), the first studies demonstrated an increase in EC and vessel number, associated with a higher VEGF expression, in the SN of PD patients and a monkey PD model, but not in the striatum [176-178]. A higher VEGF concentration was also reported in the cerebrospinal fluid of PD patients [179]. However more recent reports suggested no change in vessel number and density in both the SN and the striatum, although an increase in the number of  $\alpha v\beta 3^+$  angiogenic vessels was observed [180, 181]. Even further, a decrease in vessel length and branching point number and an increase in vessel diameter have been recently described in the SN of PD patients [182]. The CBF in PD brain striatum has also been shown to be reduced [183].

Moreover a disruption of the BBB has been detected in animal models of PD as well as in PD patients. Specifically, BBB leakage was observed, associated with a decrease in TJ proteins and an increase in MMP secretion [21, 180, 184-186]. Finally, an increase in string vessel number and density was recently reported, indicating a potential vascular regression in PD brain [185].

Based on these studies, the microvasculature in the PD brain seems to undergo both an angiogenic and pruning vascular response, but the exact mechanisms and consequences underlying the vascular remodeling in PD remain elusive.

**Table 1. Summary of the vascular alterations occurring in the PD brain**

Blue: angiogenesis; Red: BBB; Green: CBF; Grey: other. Markers used for the different vessel assessments are indicated in brackets. Ret: Reticulin; Lam: Laminin; Coll-IV: Collagen-IV; Hb: Hemoglobin; Fb: Fibrin; Fbg: Fibrinogen; FITC: Fluorescein isothiocyanate; Alb: Albumine; EB: Evans Blue.

Vascular Change	Substantia Nigra	Striatum	Ref
<b>EC number</b>	Increase in PD patient (EC nucleus morphology)		[177]
<b>Vessel number</b>	No change in PD patient ( $\alpha\text{v}\beta\text{3}$ ) Increase in MPTP monkeys (Ret)	No change in PD patient ( $\alpha\text{v}\beta\text{3}$ ) Increase in 6-OHDA rat (Lam)	[181] [176] [178]
<b>VEGF expression</b>	Increase in PD patient Increase in MPTP monkey	No change in PD patient No change in MPTP mice	[187] [176] [188]
<b>Vessel density</b>	No change in PD patient (Coll-IV)	No change in PD patient (Coll-IV)	[185]
<b>Angiogenic vessel</b>	Increase in PD patient ( $\alpha\text{v}\beta\text{3}$ ) Increase in 6-OHDA rats ( $\alpha\text{v}\beta\text{3}$ )	Increase in PD patient ( $\alpha\text{v}\beta\text{3}$ )	[181] [180]
<b>Vessel length</b>	Decrease in PD patient (Factor VIII)	No change in PD patient (Factor VIII)	[182]
<b>Vessel branches</b>	Decrease in PD patient (Factor VIII)	No change in PD patient (Factor VIII)	[182]
<b>Vessel diameter</b>	Increase in PD patient (Factor VIII)	No change in PD patient (Factor VIII)	[182]
<b>CBF</b>		Decrease in PD patients	[183]
<b>BBB leakage</b>	Increase in 6-OHDA rats (FITC-Alb)	Increase in PD patient (Hb, Fb and Fbg) Increase in 6-OHDA rats (FITC-Alb) Increase in MPTP mice (FITC-Alb; EB)	[21, 185] [180] [184, 186]
<b>Tight junction</b>		Decrease ZO-1 expression in MPTP mice	[184]
<b>MMP</b>		Increase MMP9 expression in MPTP mice	[184]
<b>Other</b>	Increase string vessel number and density in PD patient	Increase string vessel number and density in PD patient as a trend	[185]

In HD (Table 2), an increase in the blood vessel density has been reported by several independent research groups, both in the cortex and striatum of R6/2 mice and HD post-mortem patient brain [20, 189], as well as in other HD mouse models [109, 190]. In this line, the number of vessels as well as branching points were also higher in HD [20], which may be a consequence of the increased VEGF expression in the HD brain [20, 109]. Moreover, the vessel length was increased in the striatum of the YAC128 mouse model of HD, while the vessel diameter was not changed in the HD patient brain, but decreased in R6/2 mice [20, 190]. In parallel, an elevated CBF was described in the human HD brain and in R6/2 mice [20, 191], although the vascular reactivity to a carbogen challenge was less intense [191].

The BBB is also affected in HD, with an increase in BBB leakage in the HD post-mortem brain, but with contradictory results in rodent models of HD [20, 189, 190, 192]. A drastic loss in TJ proteins was also reported in both human HD brain and rodent HD models [20, 192]. Finally, mutant HTT aggregates were found in several compartments of the vasculature, such as EC, basal lamina and pericytes [20].

**Table 2. Summary of the vascular alterations occurring in the HD brain**

Blue: angiogenesis; Red: BBB; Green: cerebral blood flow; Grey: other. Markers used for the different vessel assessments are indicated in brackets. Coll-IV: Collagen-IV; EM: electron microscopy; Fb; Fibrin; Alb; Albumin; EB: Evans blue;

Vascular Change	Striatum	Cortex	Ref
<b>Vessel density</b>	Increase in HD patient (Coll-IV) Increase in R6/2 mice (Coll-IV) Increase in YAC128 mice (Coll-IV) Increase in Hdh150Q mice (Coll-IV)	Increase in HD patient (Coll-IV) Increase in R6/2 mice (Coll-IV)	[20, 189] [20, 189] [20, 109, 189] [190] [109]
<b>Vessel number</b>	Increase in HD patient (Coll-IV)		[20]
<b>Vessel branches</b>	Increase in R6/2 mice (EM)		[20]
<b>VEGF expression</b>	Increase in HD patient as a trend Increase in astrocytes of R6/2 mice	Increase in astrocytes of R6/2 mice and HD patient	[20] [109]
<b>Vessel length</b>	Increase in YAC128 mice (Coll-IV)		[190]
<b>Vessel diameter</b>	No change in HD patient (Coll-IV) Decrease in R6/2 mice (Coll-IV)		[20]
<b>CBF</b>	Increase in R6/2 mice	Increase in R6/2 mice Increase in PD patient	[191] [20]
<b>Vascular reactivity</b>	Decrease in R6/2 mice	Decrease in R6/2 mice	[191]
<b>BBB leakage</b>	Increase in HD patients (Fb) Increase in R6/2 mice (Alb) No change in R6/2 mice (EB) No change in YAC128 mice (EB) Increase in 3-NPA rats (EB)		[20] [20] [189] [190] [192]
<b>Tight junction</b>	Decrease ZO-1 and Claudin 5 expression in R6/2 mice and HD patients Decrease ZO-1 expression in 3-NPA mice		[20] [192]
<b>MMP</b>	Increase MMP9 activity around blood vessel in 3-NPA rats		[193]
<b>Other</b>	mHTT aggregates in different vascular compartments in R6/2 mice		[20]

An angiogenic response occurs in the HD brain, associated with a BBB disruption and an increased cerebral blood flow. Because of these vascular changes, the brain vasculature may play a key role in disease progression in HD, but whether these changes are compensatory or contribute to the disease progression remains unknown.



# Aims of the thesis

The overall goal of this thesis was to examine pericyte changes in PD and HD, and how pericytes are altered upon neurorestorative treatment with PDGF-BB in PD. Specifically, we aimed at:

- Establishing a new partial 6-OHDA lesion mouse model of PD to study neurorestorative therapies (**Paper I**)
- Examining the pericyte changes in a partial 6-OHDA MFB lesion PD mouse model and their alterations upon PDGF-BB treatment (**Paper II**)
- Understanding the secretive properties of pericytes upon PDGF-BB stimulation *in vitro* (**Paper III**)
- Investigating the pericyte alterations in a progressive  $\alpha$ -syn-overexpressing mouse model of PD (**Paper IV**)
- Investigating how pericytes are altered in both the R6/2 mouse model of HD and in the HD patient post-mortem brain (**Paper V**)





# Material and methods

All animal experiments were conducted in accordance with ethical guidelines and approved by the ethical committee of Lund University (Sweden).

## Transgenic mouse for pericyte identification

In **Papers I, II and V**, we used heterozygote knock-in transgenic mice where the *Green fluorescent protein (GFP)* gene is expressed under the *Rgs5* promoter, so as to specifically track RGS5<sup>+</sup> pericytes, as described previously [194]. Briefly, GFP expression was identified in PDGFRβ<sup>+</sup> pericytes and not in CD31<sup>+</sup> EC, confirming the specificity of the *Rgs5*<sup>GFP/+</sup> transgenic mouse at the vascular level. Pericyte coverage of the blood vessels remained unchanged between transgenic and non-transgenic animals. Finally, *Rgs5* transgenic animals were viable and did not display behavioural deficits.

## Animal models of PD

### Partial 6-OHDA MFB lesion mouse model of PD

To induce a unilateral dopaminergic lesion (**Papers I and II**), *Rgs5*<sup>GFP/+</sup> mice were anaesthetized using 4% isoflurane (IsoFlo vet; Apoteksbolaget, Sweden), placed on the stereotaxic frame, and kept in 2% Isoflurane until the end of surgery. A glass capillary (outer tip: 50μm) was attached to a 10μL-Hamilton syringe and placed at the following coordinates to target the MFB (relative to bregma): anterior-posterior (A/P) = -1.2; medio-lateral (M/L) = -1.3 and dorso-ventral (D/V) = -4.75 (from the dura mater). The syringe was left in place for 2 min before injection (1μL) of different 6-OHDA doses (0.3, 0.7, 1.0 or 3.6μg/μL), dissolved in 0.9% NaCl supplemented with 0.02% ascorbic acid, at the rate of 0.5μL/min. The capillary was left in place for an additional 3 min before careful removal, and the skin was sutured. To reduce the post-operative mortality rate, animals were monitored daily with special care, as described previously [195]. Sham-lesioned surgery was performed similarly, with the animals receiving 1μL of 0.9% NaCl supplemented with 0.02% ascorbic acid, and were used as controls.

## Transgenic mouse model of PD

In **Paper IV**, we used homozygote mice for the bacterial artificial chromosome (BAC)- $\alpha$ -syn-GFP construct (referred to as “TG”), where the human  $\alpha$ -syn gene was fused to the *GFP* gene and overexpressed under the mouse  $\alpha$ -syn promoter, as described previously [164]. Typically,  $\alpha$ -syn-GFP expression was observed in the dopaminergic cells of the SN and in striatal terminals. Moreover, the DA homeostasis in the striatum of TG animals is reduced already from 3 months of age, although no significant SN dopaminergic cell death occurs in this PD mouse model [164]. Finally, TG animals exhibited progressive behavioural impairment, with defects in (i) amphetamine (AMPH)-induced locomotor activity from 7 months of age and (ii) the rotarod test from 12 months of age. Animals were kept until 3, 8 or 13 months of age, and homozygote *Wt/Wt* (referred to as “WT”) were used as controls.

## Mouse model of HD

In **Paper V**, female *Rgs5<sup>GFP/GFP</sup>* homozygote mice were crossed with male R6/2 mice, resulting in offspring being either *Rgs5<sup>GFP/+</sup>* (referred to as “control”) or *Rgs5<sup>GFP/+</sup>* R6/2 (referred to as “R6/2”). In this study, R6/2 mouse CAG repeat length comprised between 253 and 287, compared to the 150 CAG repeat in the mouse-generating study [174]. Consequently, a delay in the phenotype onset occurs in these animals, as described elsewhere [175]. Animals were kept until 12 or 18 weeks of age.

## Human post-mortem tissue

Human control and HD brain samples of the superior frontal gyrus (**Paper V**) were obtained from the Neurological Foundation of New Zealand Human Brain Bank (Department of Anatomy with Radiology, University of Auckland, New Zealand). The sample collection was approved by the University of Auckland Human Subjects Ethics Committee [196]. Control subjects did not show any sign of neurological disease. The evaluation of disease stage for HD patients was based on the Vonsatell scale, including five grades respective to the progressive severity of the pathological changes of the HD brain [197]. After autopsy, brain fixation was performed with formalin perfusion (15% v/v). The brains were block-sectioned and post-fixed similarly. Blocks were sectioned at 40 $\mu$ m thickness using a cryostat and kept in an anti-freeze solution at -20°C until immunohistochemistry was performed.

# Human brain pericytes

## Cell culture

Human brain pericytes (**Paper III**) were isolated and characterized as previously described [44], upon written consent by the donor patient, and approved by the Scania University Hospital (Lund, Sweden). Pericytes were maintained in Stemline Medium (Sigma-Aldrich) supplemented with 2% fetal bovine serum (Invitrogen), 1% Penicillin/Streptomycin (Gibco) and basic fibroblast growth factor (bFGF; 20ng/mL; Invitrogen), at 37°C in 5% CO<sub>2</sub>.

To assess the effect of PDGF-BB, pericytes were seeded in 6-well plates (100 000 cells/well) in the same culture condition with the addition of PDGF-BB (20ng/mL; R&D System) for 72 hours, before supernatant collection and storage at -80°C. For analysis of the growth factor released in the medium, the PDGFR $\beta$  inhibitor Sunitinib (2 $\mu$ M; Selleck Chemicals) was added one hour prior to PDGF-BB treatment. Untreated human brain pericytes were used as controls.

## Microvesicle extraction

MV were isolated by centrifugations of the supernatant, first at 1500g for 5 min for debris removal, and then at 16 000g for 45 min to collect MV. MV were washed with 0.9% NaCl and centrifuged at 16 000g for 45 min, successively, three times. MV were quantified by flow cytometry (BD Biosciences), using flow count beads (Beckman Coulter), and identified according to their diameter (0,1-1 $\mu$ m).

## Growth factor analysis

For human BDNF, bFGF,  $\beta$ NGF and VEGF concentrations, pericyte medium or pericyte-derived MV were analyzed using a human multiplex sandwich enzyme-linked immunosorbent assay (ELISA) (Meso Scale Discovery), following the manufacturer instructions.

## PDGF-BB treatment *in vivo*

PDGF-BB was infused into the brain lateral ventricle (**Paper II**). For this, anesthetic and surgical procedures were performed similarly as described above, and a catheter was implanted at the following coordinates (relative to bregma): A/P = -0.5; M/L = -1 and D/V = -2 relative to the dura mater. The catheter was linked to a mini-osmotic pump (Alzet) containing human recombinant PDGF-BB (R&D System) diluted in artificial cerebrospinal fluid (148 mM NaCl, 3 mM KCl, 1.4 mM anhydrous CaCl<sub>2</sub>, 0.8 mM MgCl<sub>2</sub>, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.2 mM NaH<sub>2</sub>PO<sub>4</sub>),

supplemented with gentamicin (50g/mL; Life Technologies), and inserted subcutaneously on the back of the animals. PDGF-BB (36ng/day) was continuously infused into the lateral ventricle at a rate of 0.11 $\mu$ L/hour, for 14 days. The miniosmotic pump was finally removed from a small skin incision on the back of the animal before suturing. Vehicle-treated animals underwent the exact same surgical procedure and were infused with artificial cerebrospinal fluid without PDGF-BB.

## Behavioural tests

For all behavioural tests, animals were habituated to the room at least 2 hours prior to testing (**Papers I, II, IV and V**).

### Stepping test

To test for forelimb akinesia, mice were gently lifted by the tail, with only the forelimbs still apposed to the substrate, and pulled backwards in a 10cm-wide, 50cm-long corridor, in 10 sec. The test was repeated 3 to 5 times, video recorded, and the number of steps were analyzed in slow motion. Data are expressed as percentage of contralateral steps.

### Corridor test

To assess for lateralized sensorimotor integration, mice were subjected to the corridor test [198]. For this, animals were food-restricted, with the weight monitored not to be less than 85% of the initial weight of the animals during the test period (7 days). A prior habituation of the corridor (length: 60cm; width: 4cm; height: 15cm) was performed, with 10 min per day for the two first days. For the next 5 consecutive days, animals were habituated to an empty corridor for 5 min, and then transferred to the test corridor presenting 10 pots on each side, each spaced by a distance of 5 cm and containing sugar pellets. The number of retrievals were counted over 5 minutes, or until 20 retrievals occurred. Data are expressed as percentage of contralateral retrieval.

### Cylinder test

For spontaneous forelimb lateralization, mice were placed in a glass cylinder (diameter: 19cm; height: 20cm), surrounded by 2 mirrors to allow for 360° vision, and left to explore for 3 min. The experiment was recorded and analyzed in slow motion using a video player software (VLC software). Data are expressed as percentage of contralateral paw touches.

## **Drug-induced rotation tests**

Animals were placed in a glass sphere (50cm diameter), attached to a harness linked to a Rotometer apparatus (Omnitech Electronics), and habituated for 15 min. Animals were then injected either intraperitoneally (i.p.) with 5mg/kg AMPH (Sigma-Aldrich) or sub-cutaneously with 0.1mg/kg Apomorphine (Sigma), and the rotations were recorded for 40 min.

## **Rotarod test**

To assess general motor impairment, mice were first habituated to the Rotarod apparatus (Rotamex 4/8; Columbus instruments), with the rod turning at 4rpm for 30 sec. Following this, mice were placed on the rod with increasing rotation speed (4 to 40 rpm over 5 min), and latency to fall was recorded for 3 trials, spaced by 15 min in between each trial.

## **Open Field test**

General locomotor activity was assessed with the mice placed in an open field arena, for 1 hour in the dark, and recorded with the ANYmaze software. Two to three days later, animals were injected i.p. with AMPH (5mg/kg diluted in 0.9% sterile saline) and recorded similarly.

## **Tissue processing**

A lethal dose of pentobarbital (Apoteksbolaget) was injected i.p., and anaesthetized animals were transcardially perfused initially with 0.9% NaCl for 5 min, and then with 4% cold paraformaldehyde for 2 min. Brains were extracted and kept overnight in 4% paraformaldehyde, before transferring them into 30% sucrose diluted in phosphate buffer saline (PBS). Coronal sections (30-40 $\mu$ m thickness) were cut on a frozen microtome and stored in anti-freeze solution at 4°C until immunohistochemistry was performed.

## **Immunohistochemistry**

Sections were washed 3 x 10 min with PBS and incubated with a blocking solution containing 5% normal goat or donkey serum, 0.25-1% TritonX-100 or 1% Tween-20, diluted in PBS, for 30 min at room temperature (RT). For PDGFR $\beta$ , a heat-induced epitope retrieval was performed before the blocking step by incubating the sections in citrate buffer (pH = 6), at 80°C for 30 min. Sections were then

incubated with primary antibodies (Table 3) diluted in the blocking solution containing 3% serum (antibody solution), over night at RT.

**Table 3. Summary of the primary antibodies used in this thesis**

PDGFR $\beta$ : Platelet-derived growth factor receptor  $\beta$ ; GLUT1: Glucose transporter 1; PDCLX: Podocalyxin; EC: Endothelial cell; TH: Tyrosine hydroxylase; HTT: Huntingtin; IHC: Immunohistochemistry; WB: Western Blot; Conc: concentration

Antibody	Marker	Conc	Reactivity specie	Application	Company	Paper
GFP	Pericyte	1:5000	mouse	IHC and WB	Abcam	II, V
PDGFR $\beta$	Pericyte	1:200	mouse human	IHC	Cell Signalling	II, IV, V
NG2	Pericyte	1:200 1:2000 1:1000	mouse human mouse	IHC IHC WB	Millipore	II, V
CD13	Pericyte	1:100	mouse	IHC	AbD Serotec	II, V
GLUT1	EC	1:400	mouse	IHC	Abcam	II
PDCLX	EC	1:400	mouse	IHC	R&D System	IV, V
CD31	EC	1:400	mouse	IHC	BD Pharmingen	V
TH	Dopaminergic cell	1:1000	mouse	IHC and WB	Chemicon	I, II
NeuN	Neuron	1:200	mouse	IHC	Millipore	V
EM48	Mutant HTT aggregate	1:400 1:1000	mouse human	IHC	Millipore	V

For immunofluorescence, sections were washed as above and incubated with corresponding fluorophore-tagged secondary antibodies (1:500; Jackson ImmunoResearch) diluted in the antibody solution, for 1 hour at RT in darkness, and washed similarly before mounting. Specifically for GFP staining, sections were first incubated with a biotinylated goat anti-chicken secondary antibody (1:200; Vector Laboratories), 2 hours at RT, and the signal was amplified with 488-conjugated Streptavidin (1:2000; Thermo Fischer), with a 1 hour incubation at RT.

For brightfield stainings, and prior to the blocking step, sections were transferred to a peroxidase quenching solution (3% H<sub>2</sub>O<sub>2</sub>, 10% methanol, diluted in PBS), 15 min at RT and then washed with PBS. After the primary antibody incubation step, sections were rinsed and incubated in the antibody solution containing the corresponding biotinylated secondary antibodies (1:200; Vector Laboratories), for 2 hours at RT. After washing, the signal was enhanced using an avidin-biotin kit (Vectastain Elite ABC kit, Vectore Laboratories) and revealed using the chromogen 3,3-diaminobenzidine (DAB Peroxidase Substrate Kit; Vector Laboratories). Sections were mounted, dehydrated in gradual ethanol concentrations, incubated in Xylene baths for 2 x 5 min, and coverslipped.

For human sections, a similar approach was used except that a heat-induced antigen retrieval was systematically performed for all stainings. Autofluorescence

was quenched using 0.1% Sudan Black B diluted in 70% ethanol, 5 min at RT, after applying the secondary antibodies, before thorough washing and mounting.

## Cell quantification

### Dopaminergic cells

Tyrosine hydroxylase (TH)-expressing dopaminergic cells were quantified using unbiased stereology (optical fractionator probe; Stereo Investigator software; MBF Bioscience) (**Papers I and II**). Six to 8 sections were used in a 1:5 series. The SNpc was delineated at 5X magnification, using a Nikon 80i microscope (Leica), and cells were counted at 100X magnification. The number of counted cells was adjusted so as to achieve a Gundersen coefficient <10%. The cell population (N) was then estimated using the formula:

$$N = Q \frac{1}{asf} \frac{1}{ssf} \frac{1}{hsf}$$

where Q represented the counted cell number, *asf* the area sampling fraction, *ssf* the section sampling fraction and *hsf* the height sampling fraction [199].

Striatal densitometry was determined after picture acquisition of four TH-immunoreactive sections of the striatum (10X), using a brightfield microscope. For each picture, the striatum was delineated and the optical density was measured using the Image J software (National Health Institute, USA). The optical density of the corpus callosum was used as a control.

### Pericytes

For PDGFR $\beta^+$  pericytes (**Papers II, IV and V**), one picture of the dorso-lateral striatum (**Paper II**) and four pictures representing the entire striatum (two pictures of dorsal and ventral striatum) (**Papers IV and V**) were taken at 20X magnification using a brightfield microscope (Olympus) coupled with the CellSens software, for two to three consecutive sections. Cells were then counted using the Image J software. For GFP $^+$  and/or NG2 $^+$  cell counts (**Papers II and V**), as well as CD13-expressing PDGFR $\beta^+$  pericyte number quantification (**Paper V**), four pictures of the dorso-lateral striatum (**Paper II**) and four pictures of the entire striatum (two pictures of dorsal and ventral striatum; **Paper V**) were taken using a Zeiss LSM510 confocal microscope (Zeiss), for two to three consecutive sections.



# Blood vessel analysis

## Density

For Glucose transporter 1 (GLUT1)-positive blood vessel quantification (**Paper II**), confocal pictures of the dorso-lateral striatum were taken at 20X magnification, for two to three consecutive sections. For Podocalyxin (PDCLX)-positive blood vessel quantification (**Paper IV**), four confocal pictures of the striatum were taken at 40X for two consecutive sections. Images were then processed with Image J software, where a threshold was applied similarly for each picture to create a binary image, and the area was calculated.

## Number, branching points and length

CD31<sup>+</sup> vessel length, number and branching points were estimated by taking four 40X pictures per region of interest, for two to three consecutive sections, and analyzed using the image J software.

## Pericyte coverage

For CD13<sup>+</sup> pericyte coverage onto GLUT1<sup>+</sup> blood vessels (**Paper II**), confocal pictures of the dorsolateral striatum were taken at 20X for both markers. For CD13<sup>+</sup> pericyte coverage onto PDCLX<sup>+</sup> blood vessels (**Papers IV and V**), four confocal pictures of the striatum were taken at 20X for both markers. Channels were separated and CD13<sup>+</sup>, GLUT1<sup>+</sup> or PDCLX<sup>+</sup> respective blood vessel areas were measured as above, and the CD13<sup>+</sup> area was expressed as percentage of the GLUT1<sup>+</sup> or PDCLX<sup>+</sup> blood vessel area.

## EM48<sup>+</sup> mutant HTT and $\alpha$ -syn-GFP inclusions

GFP<sup>+</sup> pericytes in R6/2 mice (**Paper V**) were thoroughly examined for any EM48<sup>+</sup> mutant HTT aggregates, taking two confocal pictures of the striatum (40X magnification) for 6 consecutive sections. PDGFR $\beta$ <sup>+</sup> and NG2<sup>+</sup> pericytes in HD post-mortem brain were examined similarly (**Paper V**). PDGFR $\beta$ <sup>+</sup> pericytes in the striatum of TG mice (**Paper IV**) were also examined for  $\alpha$ -syn-GFP<sup>+</sup> inclusions using confocal microscopy on several sections.

## Statistics

For two-group comparison, a two-tailed unpaired (**Papers III, IV and V**) or paired (**Paper II**) student t-test was used. For multiple group comparison, a one-way analysis of variance (ANOVA) followed by a Tukey post-hoc was used (**Papers I, II and III**).

To discriminate partial-lesioned animals from sham- or complete-lesioned mice (**Paper I**), a diagnostic test was run for each behavioural test. The sensitivity and specificity for several cut-off values was calculated, creating a receiver operating characteristic (ROC) curve with the associated area under the curve (AUC).

For human pericyte quantifications (**Paper V**), a one-way analysis of covariance (ANCOVA) was performed, using age as a covariate, followed by a Tukey post-hoc on the adjusted means.

Differences were considered significant at  $p < 0.05$ .



# Results

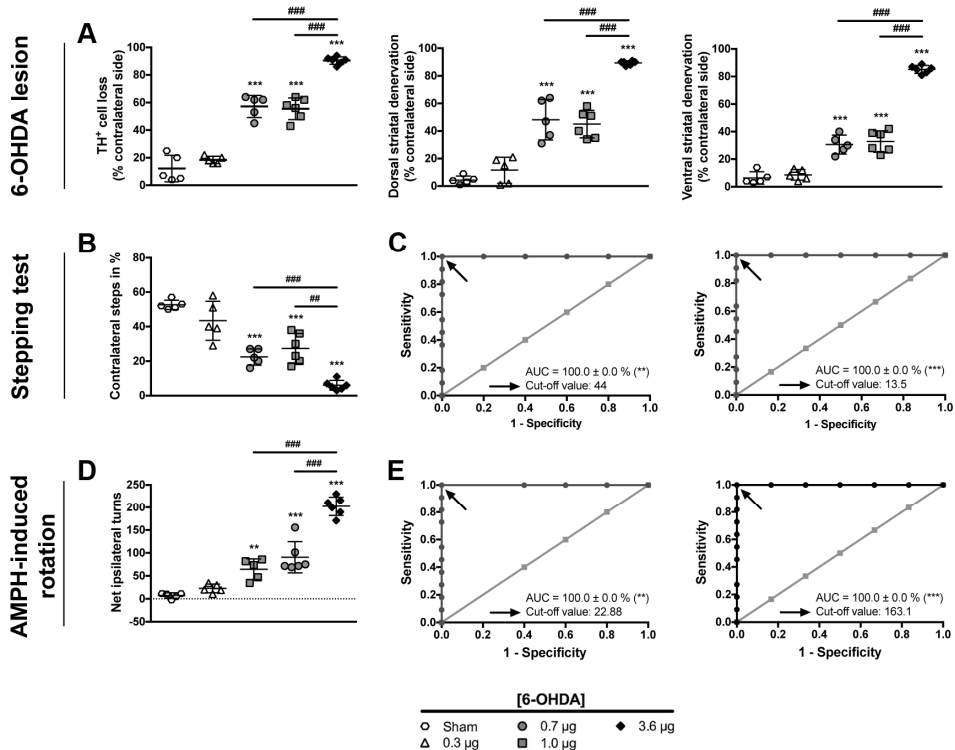
## Vascular changes in mouse models of PD and effect of PDGF-BB treatment

### **Characterization of a 6-OHDA partial MFB lesion mouse model of PD**

Neurorestorative or neuroprotective therapies for PD are usually more effective when some dopaminergic cells remain in the SN [149]. To achieve a partial versus a complete lesion of the dopaminergic pathway, the 6-OHDA toxin has been commonly injected in either the striatum or the SN [151]. However, because the toxin targets represent also the possible neurorestorative sites of the investigated therapies, we sought to establish a new partial 6-OHDA lesion mouse model, using different 6-OHDA doses injected into the MFB.

Using the TH marker for dopaminergic cell, we observed that a 6-OHDA dose of 3.6 $\mu$ g led to a complete TH<sup>+</sup> cell loss (> 80% cell loss), accompanied by a severe depletion of the TH<sup>+</sup> striatal fibres (Figure 4A). On the contrary, the 0.3 $\mu$ g 6-OHDA dose group did not show any significant nigrostriatal TH<sup>+</sup> cell or fibre loss. Interestingly, a 6-OHDA dose of 0.7 or 1.0 $\mu$ g resulted in a moderate nigrostriatal lesion with both the TH<sup>+</sup> cell and fibre loss percentages significantly higher than that in control mice, but lower compared to the 3.6 $\mu$ g 6-OHDA dose group.

The partial TH<sup>+</sup> dopaminergic cell loss in the 0.7 $\mu$ g and 1 $\mu$ g 6-OHDA dose groups were accompanied by a behavioural impairment in several behavioural tests, which was not the case for the 0.3 $\mu$ g dose group. Stepping test scores in the 0.7 and 1.0 $\mu$ g 6-OHDA dose groups were significantly lower than sham-lesioned controls, but higher than the 3.6 $\mu$ g 6-OHDA dose group (Figure 4B).



**Figure 4. Injection of 0.7 or 1.0µg 6-OHDA doses into the MFB results in a partial dopaminergic loss associated with a moderate behavioural impairment**  
**(A)** TH<sup>+</sup> dopaminergic cell loss in the SN and associated TH<sup>+</sup> fibre denervation in the dorsal and ventral striatum. **(B)** Stepping test scores for each group and **(C)** ROC curves (black lines) for comparison between partial 6-OHDA lesion mice and (i) sham-lesioned animals (left panel) and (ii) complete-lesioned animals (right panel). **(D)** Amphetamine-induced rotation test scores for each group and **(E)** ROC curves for comparison between partial 6-OHDA lesion mice and (i) sham-lesioned animals (left panel) and (ii) complete-lesioned animals (right panel). **(A, B and D)** One-way ANOVA with Tukey post-hoc: \*\*p<0.01 and \*\*\*p< 0.001 compared to Sham-lesioned group; ## p<0.01 and ### p<0.001 compared to 0.7 and 1.0µg 6-OHDA groups. **(C and E)** \*\*p<0.01 and \*\*\*p<0.001 indicate whether the AUC is significantly different from 50% (grey lines) 6-OHDA: 6-hydroxydopamine; AMPH: amphetamine; AUC: area under the curve.

A diagnostic test, with a ROC curve, demonstrated that together, the 0.7 and 1.0µg 6-OHDA dose groups were distinguishable from control mice with the stepping test score < 44%, and from the 3.6µg 6-OHDA dose mice with score > 13.5%, with a maximal AUC, reflecting the specificity and sensitivity of the behavioural test (Figure 4C). Similarly, both the 0.7 and 1.0µg 6-OHDA dose groups showed a distinct moderate impairment on the AMPH-induced rotation test. In this test, the 0,7 and 1,0µg 6-OHDA dose groups were discerned from the control and 3.6µg 6-OHDA groups, with scores > 22.88 and < 163.1 net ipsilateral turns, respectively, and with a maximal AUC (Figure 4D and E).

Other behavioural assessments, including the corridor test and the cylinder test also discriminated the partial 6-OHDA lesion groups from the sham or complete lesion groups, albeit with a lower AUC for at least one comparison, while the apomorphine-induced rotation test could only discriminate the partial 6-OHDA lesion groups from the sham-vehicle group (**Paper I**).

These results indicate that 0.7 and 1.0 $\mu$ g 6-OHDA doses in the MFB induce a partial nigrostriatal lesion with a moderate behavioural impairment, with the stepping and AMPH-induced rotation tests best capable of distinguishing them from sham or complete lesion mice.

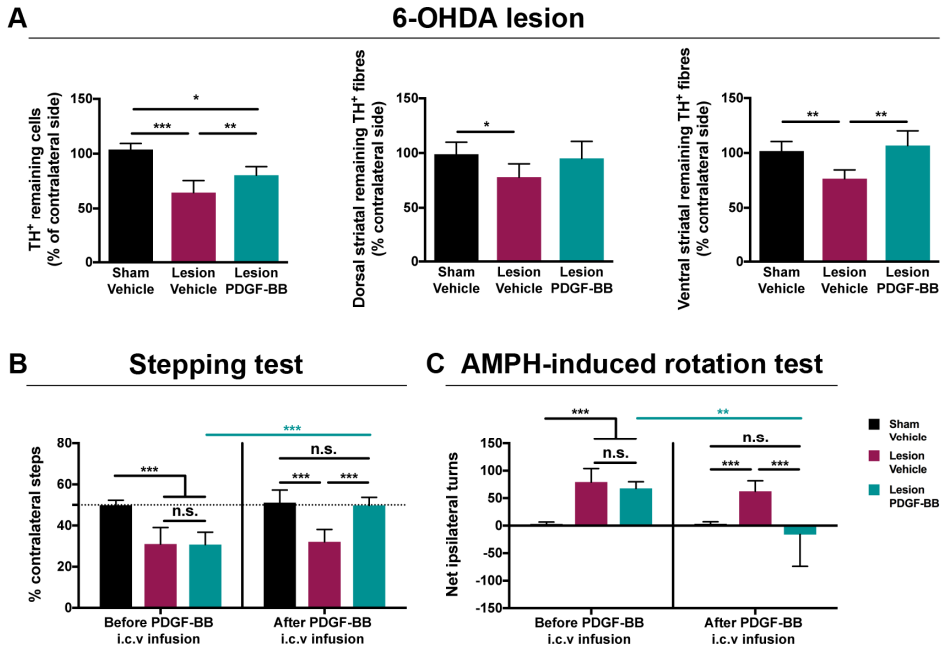
### **PDGF-BB treatment normalizes the pericyte activation in the striatum in a partial 6-OHDA MFB lesion mouse model of PD**

PDGF-BB treatment has shown promise for PD therapy. In rodent PD models, PDGF-BB induced a neurorestoration of the nigrostriatal pathway [149]. The first clinical trial involving PD patient further confirmed the beneficial effect of PDGF-BB, showing an increase in DAT binding in the putamen of PD patients receiving the highest dose [150]. However the mechanism behind the PDGF-BB-mediated neurorestorative effect remains unknown. In the brain, the PDGF-BB receptor is expressed by pericytes (PDGFR $\beta$ ), promoting their recruitment onto immature blood vessels during angiogenesis [200].

We first confirmed the neurorestorative effect of PDGF-BB in the established partial 6-OHDA MFB lesion mouse model of PD, eight weeks after the end of treatment. The number of remaining TH<sup>+</sup> cells of the SNpc was decreased in the vehicle-treated, 6-OHDA-lesioned mice, which was associated with a decrease in dorsal and ventral striatal TH<sup>+</sup> fibres, compared to control mice (Figure 5A).

The number of SN TH<sup>+</sup> cells was significantly increased in PDGF-BB-treated, 6-OHDA-lesioned mice compared to 6-OHDA-lesioned animals under vehicle treatment. This was, however, lower than that in the control group. Similarly, the striatal TH<sup>+</sup> fibre density was increased in 6-OHDA-lesioned animals treated with PDGF-BB, as a trend for the dorsal striatum and significantly for the ventral striatum.

This partial nigrostriatal dopaminergic restoration mediated by PDGF-BB was accompanied by a strong behavioural recovery. Indeed, before treatment, both vehicle- and PDGF-BB-treated 6-OHDA-lesioned groups showed the same extent of moderate behavioural impairment in the stepping test and the AMPH-induced rotation test (Figure 5B and C). However, six to eight weeks after treatment, while the 6-OHDA lesioned-mice treated with vehicle still showed behavioural deficits on the same tests, PDGF-BB-treated 6-OHDA-lesioned mice showed significant behavioural recovery, with a behavioural score similar to that of control mice.



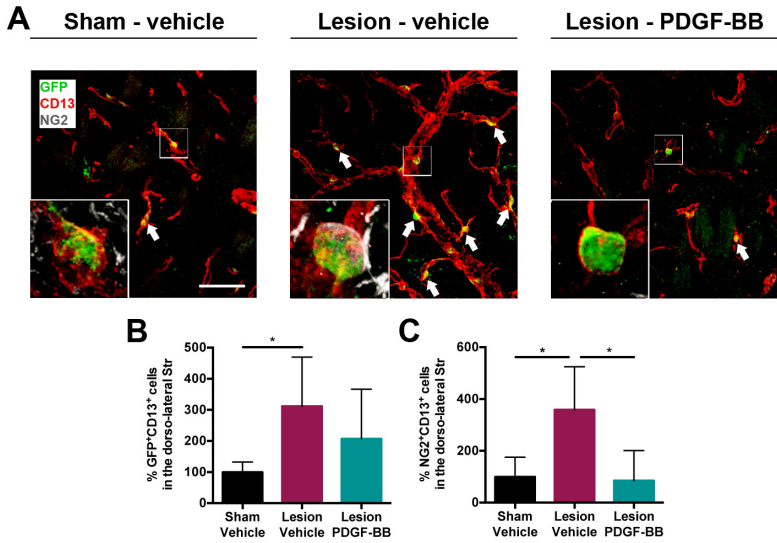
**Figure 5. PDGF-BB induced a partial restoration of the nigrostriatal dopaminergic pathway, associated with a complete behavioural recovery in a partial 6-OHDA MFB lesion mouse model of PD** (A) TH<sup>+</sup> dopaminergic cell counts in the SN and striatal TH<sup>+</sup> fibre density evaluation in sham- or 6-OHDA-lesioned animals, with or without PDGF-BB treatment. (B and C) Behavioural scores for the stepping (B) and the AMPH-induced rotation (C) tests, before or after mini-osmotic pump implantation, in the different groups. One-way ANOVA with Tukey post-hoc (black asterisks): \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ ; Two-tailed paired student t-test (blue asterisks): \*\* $p < 0.01$  \*\*\* $p < 0.001$ . AMPH: Amphetamine; i.c.v.: intracerebroventricular.

Behavioural recovery was also observed for the corridor, the cylinder and the apomorphine-induced rotation tests (**Paper II**).

Altogether, these results confirmed that PDGF-BB promotes a partial neurorestoration of the nigrostriatal dopaminergic pathway, associated with a complete behavioural recovery in a panel of different behavioural tests.

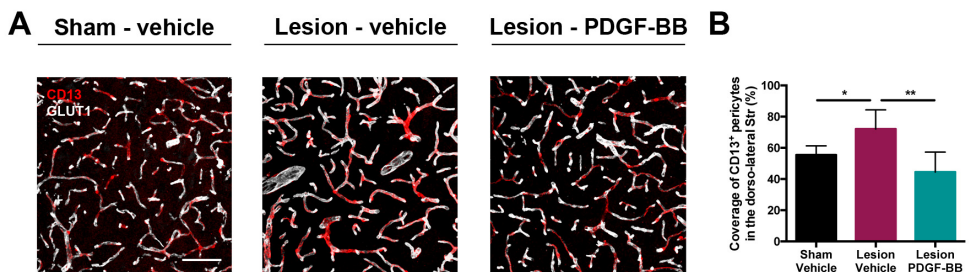
We then examined pericyte changes in the ipsilateral striatum, using RGS5 (GFP) and NG2, together with the pericyte marker CD13. We found that eight weeks after treatment, the percentages of CD13<sup>+</sup> pericytes expressing GFP or NG2 were significantly higher than in vehicle-treated sham-lesioned mice (Figure 6A-C).

Upon PDGF-BB treatment, the percentage of CD13<sup>+</sup> pericytes expressing activation markers were normalized to vehicle-treated sham-lesioned group, as a trend for GFP, and significantly for NG2.



**Figure 6.** The increased expression of the activation markers RGS5 (GFP) and NG2 in pericytes is normalized upon PDGF-BB treatment, in the dorso-lateral striatum of a partial 6-OHDA MFB lesion mouse model of PD (A) Representative pictures of CD13<sup>+</sup> pericytes expressing GFP and NG2 in the ipsilateral striatum. (B and C) Quantifications of (B) GFP<sup>+</sup>CD13<sup>+</sup> pericytes and (C) NG2<sup>+</sup>CD13<sup>+</sup> pericytes. One-way ANOVA with Tukey post-hoc: \*p<0.05. Scale bar: 50µm.

Moreover, the CD13<sup>+</sup> pericyte coverage onto the GLUT1<sup>+</sup> blood vessels was increased in the 6-OHDA-lesioned mice treated with vehicle only (Figure 7A and B). This was reduced to control levels upon PDGF-BB treatment.



**Figure 7.** CD13<sup>+</sup> pericyte coverage is normalized upon PDGF-BB treatment in the dorso-lateral striatum of a partial 6-OHDA MFB lesion mouse model of PD. (A) Representative pictures of CD13<sup>+</sup> pericyte coverage onto GLUT1<sup>+</sup> blood vessel in the striatum, with (B) quantification. One-way ANOVA with Tukey post-hoc: \*p<0.05 and \*\*p<0.01. Scale bar: 100µm.

These results indicate that, in the striatum of a partial 6-OHDA MFB lesion mouse model of PD, pericytes become activated with (i) increased expression of the activation markers, RGS5 and NG2, and (ii) increased pericyte coverage. Pericyte

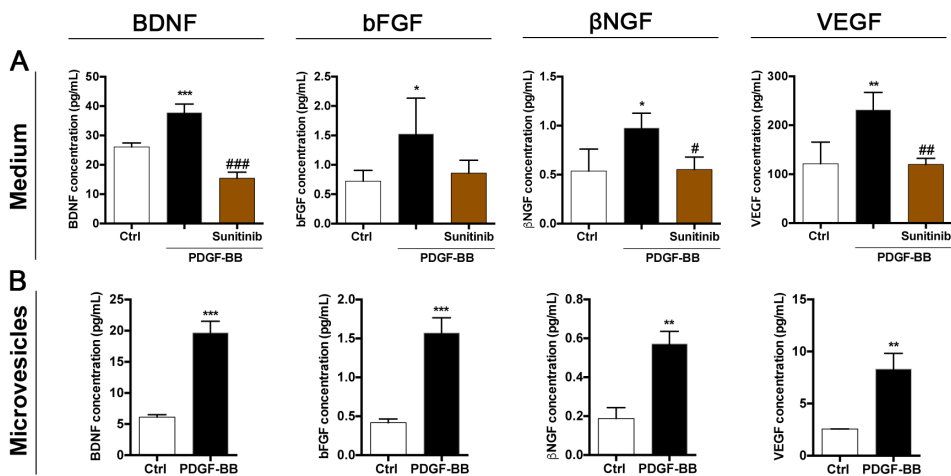


activation was, however, normalized to values of sham-lesioned mice long-term after PDGF-BB treatment

### PDGF-BB-stimulated pericytes release growth factors *in vitro*

PDGF-BB treatment has a long-term effect on nigrostriatal restoration as well as on pericyte activation in the striatum of a partial 6-OHDA MFB lesion mouse model of PD (**Paper II**). The observed neurorestoration could be due to an indirect effect of PDGF-BB on pericytes, stimulating the secretion of restorative molecules. We thus further examined the pericyte response to PDGF-BB regarding their neurotrophic and angiogenic growth factor secretion *in vitro*.

PDGF-BB treatment increased the pericyte release of BDNF, bFGF,  $\beta$ NGF and VEGF in the cell medium (Figure 8A), which was reduced to control values using sunitinib, a PDGFR $\beta$  inhibitor. More specifically, PDGF-BB stimulated the pericyte release of MV (**Paper III**), with a higher proportion of MV containing BDNF, bFGF,  $\beta$ NGF and VEGF, compared to controls (Figure 8B).



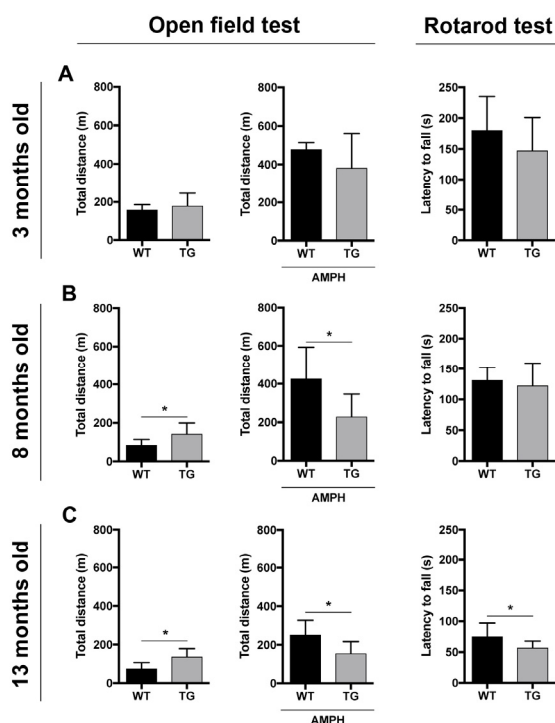
**Figure 8. Pericytes secrete growth factor-containing MV upon PDGF-BB stimulation *in vitro***  
 (A) BDNF, bFGF,  $\beta$ NGF and VEGF concentrations in pericyte medium in control and PDGF-BB stimulated pericytes, with or without sunitinib. One-way ANOVA with Tukey post-hoc: \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  compared to control (Ctrl) group, and # $p < 0.05$ , ## $p < 0.01$  and ### $p < 0.001$  compared to PDGF-BB-treated group. (B) BDNF, bFGF,  $\beta$ NGF and VEGF concentrations in pericyte-released MV in control and PDGF-BB-stimulated pericytes. Two-tailed unpaired student t-test: \*\* $p < 0.01$  and \*\*\* $p < 0.001$ .

These results indicate that PDGF-BB-stimulated pericytes secrete higher concentrations of different growth factors in the medium and in MV, which is specific to the PDGFR $\beta$  signaling pathway.

## Vascular changes in the striatum of an $\alpha$ -syn mouse model of PD

Although partial 6-OHDA lesion models of PD reflect an earlier stage of the disease, toxic PD models cannot reproduce the age-dependent progression of the PD pathology as well as the  $\alpha$ -syn accumulation hallmark of the disease. We therefore investigated the vascular changes occurring in the TG mouse model of PD, overexpressing the  $\alpha$ -syn protein fused with GFP at 3, 8 and 13 months of age.

At 3 months of age, no behavioural impairment was observed in the different behavioural tests, compared to control animals (Figure 9A). At 8 months of age, spontaneous locomotor activity was increased in TG in the open field test, which was, however, lower than WT mice upon AMPH stimulation (Figure 9B). 13-month-old TG animals displayed also a reduced AMPH-induced locomotor activity in comparison to control mice, and also showed impairment on the rotarod test (Figure 9C).

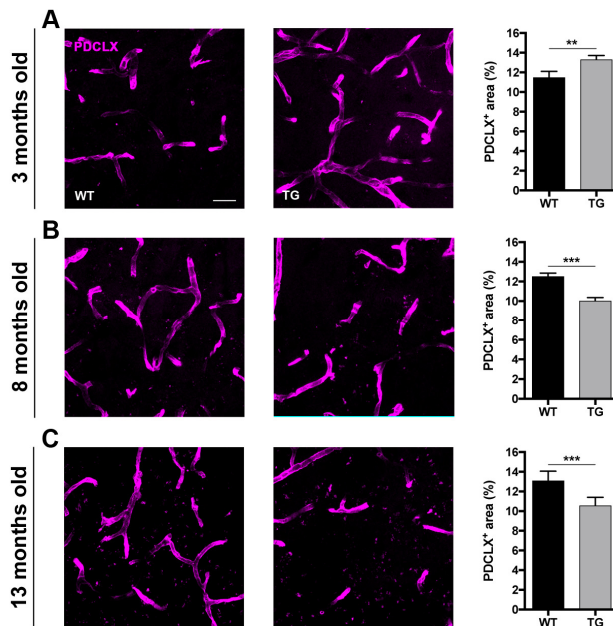


**Figure 9. Behavioural impairment is observed at 8 and 13 months of age in TG mice**

(A) No behavioural impairment observed at 3 months of age in TG animals. (B) Behavioural changes in the open field test were identified in 8-month-old TG animals, compared to WT. (C) Behavioural deficits in the open field test were also observed in TG at 13 months of age, in addition to impairment in the rotarod test. Two-tailed unpaired student t-test: \* $p < 0.05$ . AMPH: Amphetamine.

These results confirmed a behavioural impairment in TG animals, with no impairment (3 months old), mild impairment (8 months old) and severe impairment (13 months old).

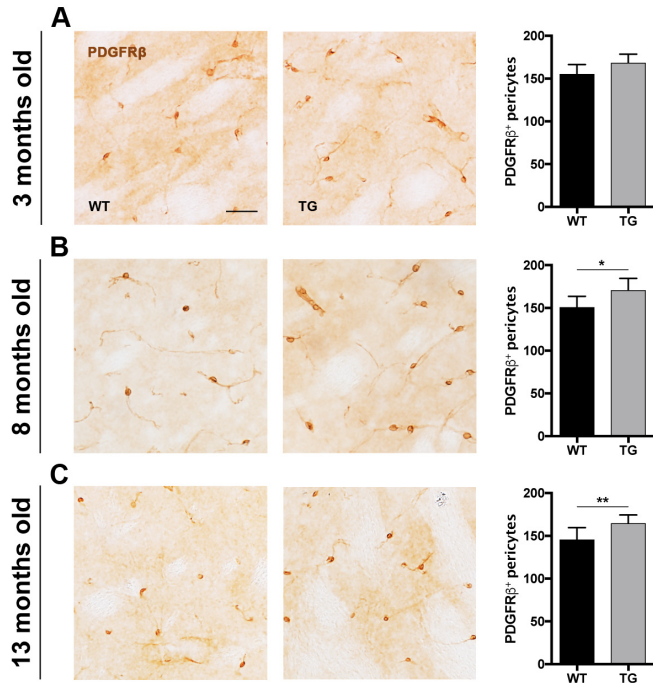
Next, we investigated the vascular response in TG animals at different time-points. At 3 months of age, the PDCLX<sup>+</sup> blood vessel area in TG mice was greater in comparison to WT animals (Figure 10A). However at 8 and 13 months of age, the PDCLX<sup>+</sup> blood vessel area significantly decreased in the TG group compared to controls (Figure 10B and C).



**Figure 10. PDCLX<sup>+</sup> blood vessel area increases at 3 months of age before decreasing from 8 months of age in TG mice**

(A-C) Representative pictures of PDCLX<sup>+</sup> blood vessel in the striatum at 3 (A), 8 (B) and 13 (C) months of age with their respective area quantifications. Two-tailed unpaired student t-test: \*\*p<0.01 and \*\*\*p<0.001. Scale bars: (A-C) 20µm.

We then examined the pericyte response in  $\alpha$ -syn transgenic mice. We found that the number of PDGFR $\beta$ <sup>+</sup> pericytes increased only at 8 and 13 months of age, compared to controls (Figure 11A-C).



**Figure 11. PDGFR $\beta$ <sup>+</sup> pericyte number increases at 8 and 13 months of age in TG mice**

(A-C) Representative pictures of PDGFR $\beta$ <sup>+</sup> pericytes in the striatum at 3 (A), 8 (B) and 13 (C) months of age with their respective quantifications. Two-tailed unpaired student t-test: \*\*p<0.01 and \*\*\*p<0.001. Scale bars: (A-C) 20 $\mu$ m.

Moreover, no  $\alpha$ -syn-GFP inclusions were not identified in PDGFR $\beta$ <sup>+</sup> pericytes, but observed in PDCLX<sup>+</sup> blood vessels (**Paper IV**).

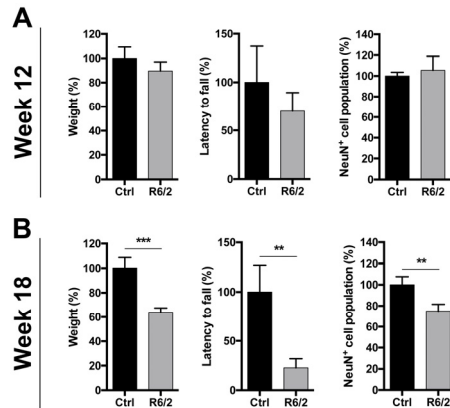
These results suggest that first, blood vessel density increases at 3 months of age. However, later, a decreased blood vessel area is found at 8 and 13 months of age. This was associated with an increase in PDGFR $\beta$ <sup>+</sup> pericyte number in the striatum from 8 months of age, at the same time when behavioural impairment is observed.

## Vascular changes in HD

Several studies have now shown vascular alterations in the HD brain, including blood vessel sprouting, BBB disruption and increased CBF [20, 109, 189]. We thus aimed at elucidating the pericyte response in the R6/2 mouse model of HD.

For this, we used the R6/2 mouse model of HD, at two different time-points. While 12-week-old R6/2 mice did not show any sign of weight loss, behavioural

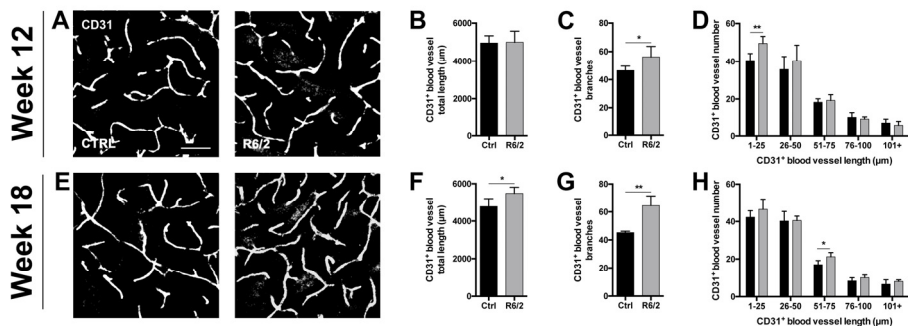
impairment or striatal neuronal cell loss, these features were present at 18 weeks of age (Figure 12A and B).



**Figure 12. R6/2 mice display weight loss, behavioural impairment and neuronal cell loss at 18 weeks of age**

(A) At 12 weeks of age, R6/2 mice did not show any weight loss, behavioural impairment nor striatal neuronal cell loss, compared to control (Ctrl) mice. (B) At 18 weeks of age however, R6/2 mice displayed weight loss, behavioural impairment and striatal neuronal cell loss, compared to Ctrl mice. Two-tailed unpaired student t-test: \*\* $p < 0.01$  and \*\*\* $p < 0.001$ .

First, we investigated the vascular changes in the R6/2 mouse brain using the EC marker CD31. At 12 weeks of age, no change in the CD31<sup>+</sup> blood vessel total length was observed in the striatum of R6/2 mice, compared to control animals (Figure 13A and B). However, the number of branching points was already increased, associated with an increase in short (1 to 25 $\mu$ m-long) blood vessels (Figure 13C and D).



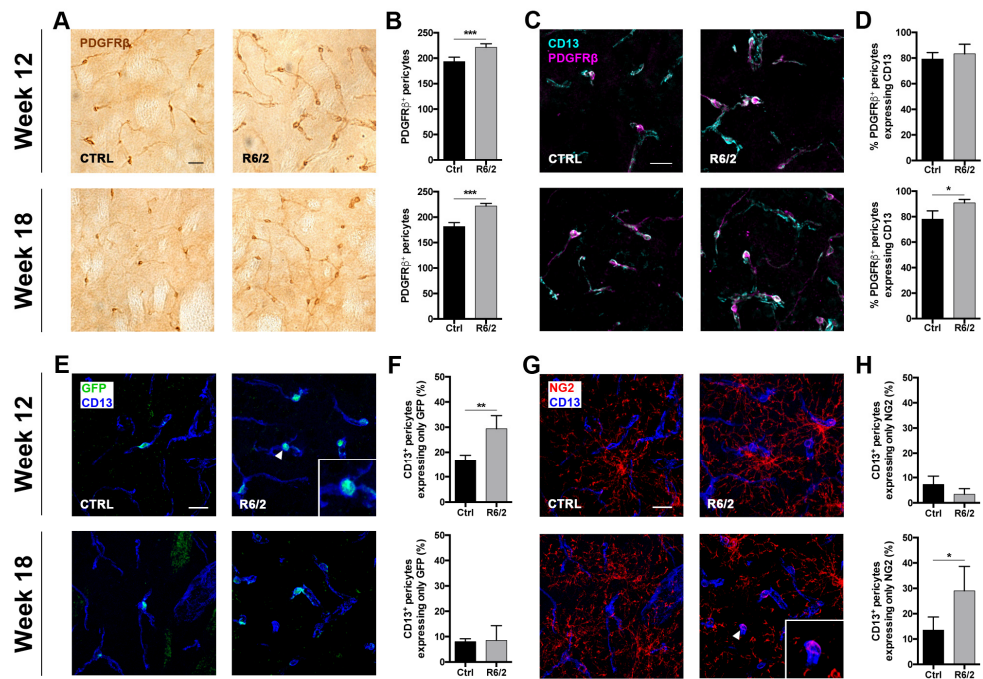
**Figure 13. Blood vessel alteration in the R6/2 mouse striatum**

(A) Representative pictures of CD31<sup>+</sup> blood vessels at 12 weeks of age, with quantification of (B) blood vessel total length, (C) the number of blood vessel branching points and (D) the number of blood vessel per length. (E) Representative pictures of CD31<sup>+</sup> blood vessels at 18 weeks of age, with quantification of (F) blood vessel total length, (G) the number of blood vessel branching points and (H) the number of blood vessel per length. Two-tailed unpaired student t-test: \* $p < 0.05$  and \*\* $p < 0.01$  compared to control mice (Ctrl). Scale bar: 25 $\mu$ m.

At 18 weeks of age, the total length of the blood vessel was increased, together with the branching point number as well as the number of medium length blood vessel, in R6/2 mouse striatum, compared to controls (51 to 75 $\mu$ m-long)(Figure 13E-H). In the cortex, blood vessel length, number and branching points were solely increased at 18 weeks of age (**Paper V**).

These results indicate an increase in blood vessel branching points and short blood vessels already from 12 weeks of age, when no significant behavioural impairment nor neuronal cell loss were observed, only in the striatum, which further persisted with an increase in blood vessel total length at 18 weeks of age.

The number of PDGFR $\beta^+$  pericytes was increased already from 12 weeks of age in the R6/2 striatum, compared to control mice, and remained higher at 18 weeks of age (Figure 14A and B).



**Figure 14. Increased numbers of PDGFR $\beta^+$  pericytes and percentages of CD13 $^+$ , GFP $^+$  and NG2 $^+$  pericytes in the striatum of R6/2 mice**

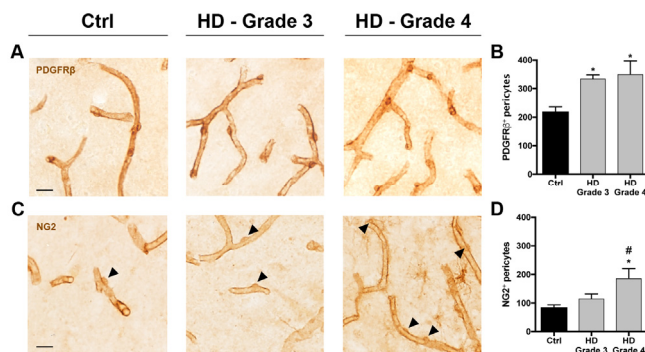
Representative pictures of (A) PDGFR $\beta^+$  pericytes at week 12 and 18 with (B) their respective quantifications, (C) PDGFR $\beta^+$ CD13 $^+$  pericyte percentage at week 12 and 18 with (D) their respective quantifications, (E) GFP $^+$ CD13 $^+$  pericyte percentage at week 12 and 18 with (F) their respective quantifications and (G) NG2 $^+$ CD13 $^+$  pericyte percentage at week 12 and 18 with (H) their respective quantifications. Two-tailed unpaired student t-test: \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  compared to control mice (Ctrl). Scale bar: 20 $\mu$ m.

Among the PDGFR $\beta$ <sup>+</sup> pericyte population, a higher percentage of cells expressed CD13 in R6/2 mice compared to controls, solely at 18 weeks of age (Figure 14C and D). Similar findings were found in the R6/2 mouse cortex (**Paper V**).

When investigating the pericyte activation state using RGS5 (GFP) and NG2, we found a sequential activation of pericytes in the striatum. First, the percentage of CD13<sup>+</sup> pericytes expressing GFP was higher in 12-week-old R6/2 mice, compared to control animals, but not at 18 weeks of age (Figure 14E and F). Conversely, the percentage of CD13<sup>+</sup> pericytes expressing NG2 was increased only at 18 weeks of age in R6/2 mice, compared to control animals (Figure 14G and H). Similar findings were made in the R6/2 mouse cortex (**Paper V**). Finally, no EM48<sup>+</sup> mutant HTT aggregates were observed in GFP<sup>+</sup> pericytes (**Paper V**).

Taken together, these results indicate that the number of pericytes increases in the R6/2 mouse striatum already when no behavioural impairment. Moreover, this is coupled with more pericytes expressing markers of activation, with first in RGS5 when no motor deficits are apparent, and later with NG2.

Finally, we investigated whether our findings in the R6/2 mice were recapitulative of that which occurs in the human HD brain. Analyses of PDGFR $\beta$ <sup>+</sup> cells revealed an increased number in the human HD brain, at grades 3 and 4, when compared to control brains (Figure 15A and B).



**Figure 15. Pericyte alterations in the HD human brain**

(A) Representative pictures of PDGFR $\beta$ <sup>+</sup> pericytes in control, grade 3 and grade 4 HD brains with (B) their respective quantifications. (C) Representative pictures of NG2<sup>+</sup> pericytes in control, grade 3 and grade 4 HD brains with (D) their respective quantifications. One-way ANCOVA with Tukey post-hoc on adjusted means: \*p<0.05 compared to control (Ctrl) group. Scale bars: 20 $\mu$ m.

Moreover, the number of NG2<sup>+</sup> pericytes increased specifically at grade 4 in the HD brain, compared to control and grade 3 HD brains (Figure 15C and D). Similar to R6/2 mice, no EM48<sup>+</sup> mutant HTT aggregates were identified in PDGFR $\beta$ <sup>+</sup> and NG2<sup>+</sup> pericytes (**Paper V**).



# Discussion

In this thesis, significant changes are demonstrated in the brain vasculature in PD and HD, with specific emphasis on pericytes.

In PD, pericyte alterations were observed in the striatum of PD models exhibiting motor deficits. Interestingly, the PDGF-BB-mediated neurorestorative effect was accompanied by a normalization of the pericyte activation, indicated by a decrease in RGS5<sup>+</sup> and NG2<sup>+</sup> pericyte numbers in the striatum of the PD mouse model used. Furthermore, PDGF-BB stimulation *in vitro* promoted human pericyte secretion of MV and several growth factors. Blood vessel density increased already when no motor abnormalities were observed, in the overexpressing  $\alpha$ -syn-GFP transgenic mouse model of PD, although a vascular regression occurred later when mice exhibited behavioural impairment.

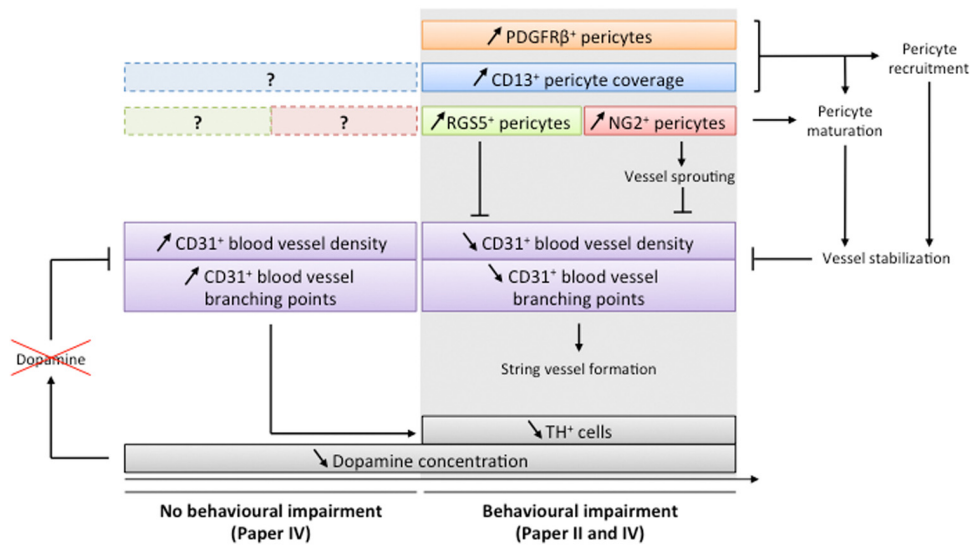
In the R6/2 mouse model of HD, pericyte changes occurred already when no motor impairments were observed, associated with an increase in the number of vessel branching points and sprouts in the striatum. These vascular changes persisted at late stage of the disease, associated with behavioural deficits.

In PD, two different mouse models were used: partial 6-OHDA MFB lesion mice (**Papers I and II**) and an  $\alpha$ -syn-GFP-overexpressing transgenic mouse model (**Paper IV**). Partial 6-OHDA lesion mouse models are useful for neurorestorative therapies, as a battery of different behavioural tests are available to assess for motor recovery [201]. They also recapitulate an early PD stage, with some dopaminergic cells still remaining in the SN. This is of interest for disease-modifying therapies, to assess for neuroprotection and neuroregeneration of the nigrostriatal dopaminergic pathway. Moreover, the 6-OHDA injection into the MFB allows the neurorestorative sites (SN and striatum) to be investigated without the toxin confounding the findings. In contrast, transgenic mouse models mimic the age-dependent and slow progression of the disease. This enables the investigation of disease mechanisms at different time-points during disease progression [164]. However, compared to 6-OHDA lesion models, no dopaminergic cell loss was reported in this transgenic PD mouse model, although the striatal DA homeostasis was disrupted already from 3 months of age [164].

Numerous vascular changes have now been reported in the PD brain, including an increased  $\alpha\beta3^+$  angiogenic vessel number, BBB disruption and decreased CBF. Importantly, most studies examined only the late stage of the disease [21, 177, 180, 181, 183-188]. In this thesis, a vascular response was described for the first



time in the overexpressing  $\alpha$ -syn-GFP mouse model of PD. At the early stage, when no behavioural impairment is observed, an increase in the blood vessel density already occurred in the striatum (**Paper IV**) (Figure 16). This early vascular response may be due to the lower striatal DA level observed already from 3 months of age in this animal model [164]. Indeed, DA was shown to stabilize tumor blood vessels [202] and to negatively regulate the VEGF-mediated angiogenic response *in vivo* [203].



**Figure 16. Possible mechanisms involved in the the vascular changes occurring in PD**

At the early stage of the disease, an increased vascular density occurs in the striatum, which may result from the lower DA concentration observed in the striatum of the  $\alpha$ -syn PD mouse model. Later when mice exhibit behavioural impairment, vascular regression occurs, which could lead to the string vessel formation observed in the human PD brain. PDGFR $\beta$ <sup>+</sup> pericyte number, as well as pericyte coverage, increase at the late stage of the disease, which may be an attempt to stabilize the blood vessels. Moreover, RGS5<sup>+</sup> and NG2<sup>+</sup> pericyte numbers also increase at later stage, perhaps with the aim to inhibit vessel regression and promote vessel sprouting, respectively. However the RGS5 and NG2 expression in pericytes, as well as pericyte coverage, remain to be elucidated at earlier stages of the disease, when no motor abnormalities are present.

However it is worth noting that this primary vascular response may be deleterious, as a study showed that triggering VEGF-induced vascular alterations in the brain led to dopaminergic cell neurodegeneration [132]. Vascular changes may thus be a key component for PD pathology initiation and progression.

At the later stage of the disease, when behavioural deficits were apparent, the vascular density was decreased (**Paper IV**). This is supported by findings by others reporting an increased number of string vessels in PD patient brains [185], i.e. extracellular matrix filaments reminiscent of blood vessels, suggesting vascular regression. However, other studies reported no change in vessel length

and branching points [182], nor vascular density ([185] and **Paper II**) in the PD human brain and mouse model of PD, which may be due to the different vessel markers used in those studies (Collagen-IV, Factor VIII, GLUT1 and PDCLX) ([182, 185], **Papers II and IV**). Moreover, an increase in  $\alpha\beta3^+$  angiogenic vessel density was still observed in PD pathology [180, 181]. This implies that the vascular angiogenic response may be coupled with a vascular pruning.

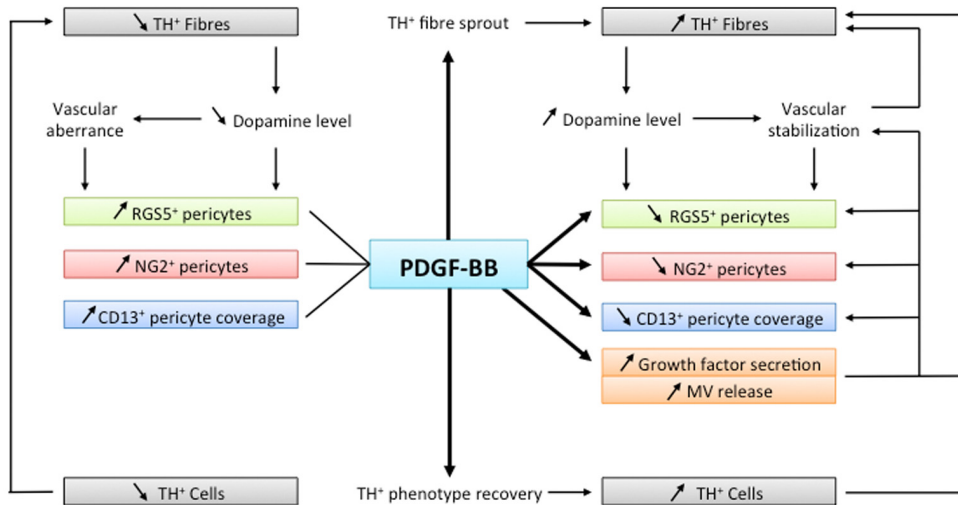
The number of PDGFR $\beta^+$  pericytes was increased in the striatum of both PD models, at late disease stage when behavioural impairment was observed (**Papers II and IV**). PDGFR $\beta$  signaling is involved in pericyte recruitment onto the blood vessel for vascular stabilization [95]. It is thus conceivable that PDGFR $\beta^+$  pericytes would act as a compensatory mechanism in the PD brain, participating in the stabilization of the vasculature. Pericyte recruitment may, however, not succeed in preventing vascular regression at the late stage of the disease, as observed in the overexpressing  $\alpha$ -syn-GFP mouse model of PD.

Pericytes have been shown to upregulate RGS5 and NG2 during vascular remodeling and in certain brain pathologies, reflecting a more activated state [56, 68, 75, 78]. It is thus not surprising that the number of RGS5 $^+$  and NG2 $^+$  pericytes increased in the striatum of the partial 6-OHDA lesion mouse model of PD (**Paper II**). RGS5 $^+$  pericytes have been described to (i) participate in the pericyte coverage on the blood vessels and (ii) inhibit vessel regression [73, 74]. NG2 $^+$  pericytes may play a role not only in the angiogenic response in the PD brain, but also in pericyte maturation [76, 79]. An increase in the CD13 $^+$  pericyte coverage of the blood vessels was also observed in the PD striatum (**Paper II**). CD13 expression was shown to increase during angiogenesis, which may explain the increased CD13 $^+$  pericyte coverage on the PD vasculature [86]. This increased coverage may also be an attempt to maintain the blood vessels, as pericyte recruitment supports vessel stabilization [33].

Neurorestorative therapies are urgently needed for PD, as only symptomatic treatments are currently available [138, 139]. PDGF-BB treatment for PD has shown beneficial effects, inducing neurorestoration of the nigrostriatal dopaminergic pathway, associated with behavioural recovery in animals models of PD [149], which led to the first clinical trial [150]. In this thesis, PDGF-BB treatment partially restored the nigrostriatal pathway and promoted a complete behavioural recovery (**Paper II**). The increase in SN TH $^+$  cell number upon PDGF-BB treatment may be attributed to the recovery of the dopaminergic phenotype, which has been suggested to be lost prior to cell death in PD [124] (Figure 17).

PDGF-BB has been shown to have a high binding affinity to PDGFR $\beta$  [93]. PDGFR $\beta^+$  pericyte numbers remained, however, unchanged with or without PDGF-BB treatment in the striatum in 6-OHDA-lesioned mice. This may indicate

that exogenous PDGF-BB infusion supports pericyte differentiation instead of recruitment and/or proliferation [204].



**Figure 17. Possible mechanisms involved in PDGF-BB-mediated neurorestoration in PD**

In PD, TH<sup>+</sup> dopaminergic cell loss is associated with decreased striatal TH<sup>+</sup> fibre density. The resulting diminished striatal DA level may promote vascular alterations which, in turn, could contribute to the increased in RGS5<sup>+</sup> and NG2<sup>+</sup> pericyte numbers and pericyte coverage. PDGF-BB treatment in PD could act in two ways. First, a direct effect of PDGF-BB on the nigrostriatal pathway could promote TH<sup>+</sup> cells to regain their dopaminergic phenotype, and/or induce TH<sup>+</sup> fibre sprout in the striatum. This would lead to increase in DA level in the striatum, which would thus stabilize the blood vessels and, in turn, decrease RGS5 and NG2 expression in pericytes and pericyte coverage. Alternatively, PDGF-BB could directly reduce pericyte expression of RGS5 and NG2, whereby switching to more mature pericytes. This would be associated with a decrease in pericyte coverage, which altogether would lead to vascular stabilization, and possible recovery of the striatal TH<sup>+</sup> fibre density. PDGF-BB-stimulated pericytes could also induce pericyte secretion of several neurotrophic factors, partly through MV release, which would also give trophic support and stimulate TH<sup>+</sup> fibre sprout.

Surprisingly, the numbers of RGS5- and NG2-expressing pericytes were normalized to control values in the striatum of 6-OHDA-lesioned mice treated with PDGF-BB. Two explanations can be drawn from this: first, a direct effect of PDGF-BB would switch pericyte phenotype from an angiogenic to a more vessel-stabilizing state. This is supported by an *in vitro* study demonstrating PDGF-BB-induced inhibition of RGS5 expression [205]. Second, the restoration of the TH<sup>+</sup> dopaminergic cell phenotype in the nigrostriatal dopaminergic system would normalize the DA concentration in the striatum. As DA participates in blood vessel stabilization [202, 203], DA may reduce vascular remodeling, as observed by the downregulation of RGS5 and NG2 expression in pericytes. This further indicates a possible role of PDGF-BB for pericyte maturation, whether direct or indirect.

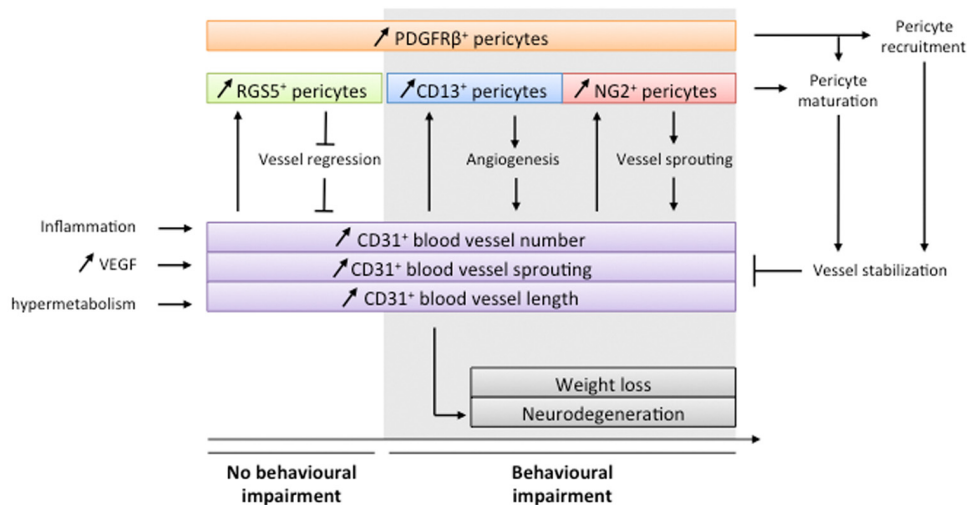
Of note, because PDGF-BB promotes neurorestoration together with vascular normalization in a PD mouse model, this reinforces the idea that vascular changes observed in PD may not be beneficial and could contribute to PD pathology.

To further understand whether pericytes could possess an active role in the PDGF-BB-mediated neurorestorative effect observed in the partial 6-OHDA mouse model of PD, the pericyte secretome was analyzed in response to PDGF-BB *in vitro* (**Paper III**). Upon PDGF-BB stimulation, pericytes secrete higher quantities of certain trophic factors, including BDNF,  $\beta$ NGF, bFGF and VEGF. BDNF,  $\beta$ NGF and VEGF were shown to promote dopaminergic cell survival, neurite outgrowth and DA release [178, 206, 207]. A bFGF-mediated neurorestorative effect was also demonstrated in a PD mouse model [208]. Furthermore, PDGF-BB triggered pericyte secretion of MV, a characteristic previously described in MSC [49], with a higher quantity of secreted MV containing the similar growth factors.

The pericyte secretome may contribute to the PDGF-BB-mediated dopaminergic cell restoration observed in PD, and MV secretion might serve as a more long distance communication in the brain. Alternatively, as GDNF supports BBB integrity [40], it is conceivable that these neurotrophic factors also act on the blood vessel homeostasis. For instance, BDNF promotes EC survival [209],  $\beta$ NGF induces EC migration [210] and bFGF promotes VEGF-mediated EC survival [211, 212].

The data provided in this thesis cannot conclude whether PDGF-BB-induced pericyte changes actively participate in the neurorestorative effect of this treatment *in vivo* in PD. However, as (i) BBB disruption seems to have an impact on neurodegeneration in PD [132], (ii) pericyte activation is normalized upon PDGF-BB and (iii) PDGF-BB stimulated pericytes secrete several neurotrophic factors, brain pericytes may constitute an interesting cell target for therapies in PD.

In HD, several vascular changes were recently reported, including increased blood vessel density and sprouting, as well as BBB disruption and elevated CBF ([20, 109, 189-191] and **Paper V**) (Figure 18). The vascular angiogenic response may be related to the elevated VEGF concentration [20, 109] or the higher brain metabolic demands [213] in the HD brain. Specifically, an increase in branching points and vessel sprouts was found already at early disease stage in the R6/2 mouse striatum, which is in concordance with Cepeda-Prado and colleagues, who reported increased CBF at the early disease stage in R6/2 mice [191]. Furthermore, these vascular alterations were observed in the striatum prior to the cortex (**Paper V**). Therefore, vascular changes may parallel the progressive neuronal cell loss in the HD brain, occurring with first striatal neuronal cell loss, before cortical cell death at the later stage of the disease.



**Figure 18. Possible mechanisms involved in the vascular changes occurring in HD**

In the R6/2 striatum, an increase in blood vessel number, sprouting and length are observed already when no behavioural impairment can be visualised. This may result from (i) the progressive inflammation occurring in the HD brain, (ii) the increased VEGF level or (iii) brain hypermetabolism. This was associated with an increase in PDGFRβ<sup>+</sup> pericyte number, which persists at the later stages of the disease, when motor deficits are present. Similar to PD, PDGFRβ<sup>+</sup> pericytes may be involved in pericyte recruitment and maturation, in an attempt to stabilize the brain vasculature in HD. RGS5<sup>+</sup> pericyte number increases solely at the early disease stage, to perhaps contribute to vascular sprouting. On the contrary, CD13<sup>+</sup> pericyte number increases at the late disease stage, which could be in relation to the neoangiogenesis and inflammation occurring in the HD brain. NG2<sup>+</sup> pericyte number also increases at the late disease stage, which indicates either a role in (i) vascular sprouting in the HD brain, or (ii) pericyte maturation in order to stabilize the vasculature in the HD striatum.

As vascular remodeling occurs, pericyte changes were expected in the HD brain. However, the number of PDGFRβ<sup>+</sup> pericytes was already increased at the early stage in the R6/2 striatum and cortex, when no behavioural impairment was observed. This indicates that PDGFRβ<sup>+</sup> pericytes not only respond before significant behavioural deficits in the R6/2 mice, but also prior to significant vascular changes in the cortex (**Paper V**). PDGFRβ<sup>+</sup> pericytes may possess mechanisms to already sense and compensate the forthcoming vascular changes occurring during HD progression. Similar to PD, PDGFRβ<sup>+</sup> pericytes might promote blood vessel stabilization. Alternatively, the origin of brain pericytes differs depending on their location in the brain with (i) a neuroectodermal origin for forebrain pericytes and (ii) a mesodermal origin for pericytes of the mid- and hindbrain [58]. This may entail different responses of cortical and striatal pericytes in the HD brain.

Pericytes changed their marker expression in HD in a time-dependent manner. At the early disease stage, the number of RGS5<sup>+</sup> pericytes increased, which could be attributed to their role during pericyte covering the blood vessels and inhibition of vessel regression [73]. At the later stage of the disease, the number of NG2<sup>+</sup>

pericytes increased in both R6/2 mice and human HD brain. NG2 expression in pericytes may be a consequence of the vascular remodeling occurring in the HD brain, but could also contribute to pericyte maturation [76, 78]. Moreover, the number of PDGFR $\beta$ <sup>+</sup> pericytes expressing CD13 increased at the late stage in the R6/2 brain. This could be a consequence of the progressive inflammation in addition to the angiogenic response occurring in the HD brain [20, 214], as both stimuli trigger CD13 expression [86].

In the HD brain, the pericyte response may occur as a first step prior to neuronal cell death and even before significant vascular changes occur. Further studies are required to demonstrate whether pericyte changes (i) initiate or react to more subtle vascular alterations, (ii) stabilize or amplify the aberrant vasculature (iii) contribute or halt HD pathology progression and (iv) are overall beneficial, deleterious or both in the HD brain, depending on their protein expression and function.



# Conclusions and future perspectives

The brain microvasculature has been shown to be an important component in neurodegenerative diseases, possibly influencing disease onset and progression. Particularly, pericytes are cells enwrapping the microvessels that express a broad range of markers and possess a variety of different functions in the brain. This thesis emphasizes pericyte alterations in PD and HD.

Several neurodegenerative diseases have been described to correlate with pericyte loss. Instead, pericyte numbers were increased in PD and HD brains, and acquired features of activation, indicating vascular remodeling.

In PD, these features were evident at early disease stage, when behavioural impairment was observed, the only time-point investigated for pericyte changes. PDGF-BB treatment not only promoted neurorestoration, but also decreased the observed pericyte activation in the established PD mouse model. Moreover, PDGF-BB stimulated pericyte secretion of several neurotrophic factors *in vitro*. This strongly supports the hypothesis that the brain vasculature may (i) influence neurodegeneration, and (ii) be a target for neurorestorative therapies.

In HD, increased pericyte number and activation were observed already at the early disease stage, together with vascular changes. This further suggests that the vasculature possesses an active and early role in neurodegeneration.

Therefore, it would be relevant to address the following questions regarding pericyte changes in PD and HD brains: is the pericyte protein expression profile changed at earlier stages of PD? Are pericyte changes observed already after birth, or even during embryonic development in HD? Do pericyte changes facilitate or counteract PD and HD progression? Of interest, elucidating (i) how vascular changes impact neurodegeneration and (ii) how pericytes could hinder this feature would unravel new mechanisms for possible neurorestorative therapies in neurodegenerative diseases.

Moreover, PDGF-BB treatment in a viable pericyte-deficient mouse model of PD would give more clues regarding the contribution of pericytes in the PDGF-BB-mediated neurorestorative effect. Investigating the signaling pathways involved in the growth factor secretion by PDGF-BB-stimulated pericytes would also provide additional information regarding their possible implication in neurorestorative therapies. It would also be relevant to test whether the observed PDGF-BB-mediated neurorestorative effect would be applicable to the R6/2 mouse model of HD.



Thanks to their dynamic, diversity and numerous roles in the brain, targeting brain pericytes could be of clinical relevance for future therapies in neurodegenerative diseases.

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