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Battistella, Roberta

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ROBERTA BATTISTELLA
DEPT OF EXPERIMENTAL MEDICAL SCIENCE | FACULTY OF MEDICINE | LUND UNIVERSITY

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Roberta Battistella

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Abstract

Over the past decade, our understanding of brain-waste clearance underwent a revolution after the discovery of the so-called glymphatic system. The glia-lymphatic (abbreviated, glymphatic) system was first described in 2012 as a paravascular macrosystem involved in the distribution of nutrients and clearance of metabolic waste from the brain parenchyma. The main actor of the system is the cerebrospinal fluid (CSF), which from the subarachnoid space (SAS) flow into the perivascular space (PVS) of brain penetrating arteries, and from there into the brain parenchyma, facilitated by aquaporin-4 (AQP4) water channels on astrocitic endfeet surrounding blood vessels. Intriguingly, once in the parenchyma, the CSF mixes with the interstitial fluid (ISF), releasing nutrients and collecting metabolic wastes and toxic proteins, including Aβ and Tau, two proteins involved in the pathogenesis of Alzheimer´s disease (AD).

Importantly, recent studies have shown that the glymphatic system, and therefore its function in "cleaning" the brain from toxic wastes and proteins, is impaired during aging. Age is the main risk factor for developing neurodegenerative disease caused by accumulation of toxic proteins, e.g. AD or Parkinson´s diseases (PD). Therefore, the understanding of how glymphatic system functions is of utmost importance.

Because of the novelty of this field, it is needed to find consensus on the techniques and methodology to study glymphatic system, as well as interpretation of the results.

The aim of this thesis was then to describe methodologies useful for glymphatic studies in rodents, and to apply these methods to study CSF movement in the brain both in physiological and pathological settings (i.e., hypothermia and PD).

Specifically, in Paper I we described the CM injection method to study glymphatic function in the rodents´ brain. In Paper II, we quantitatively compare the efficiency of different lectins and staining methods to label vasculature in rodents, as an important step for AQP4 polarisation studies. In Paper III, we investigate how AQP4 is affected by antisense oligonucleotides (ASO) targeting *Aqp4* mRNA. In Paper IV, we investigated glymphatic system function in two different mouse models of PD, and test whether glymphatic system is involved in the clearance of α-syn (a protein involved in PD pathogenesis) from the brain parenchyma. Finally, in Paper V we explored glymphatic function and AQP4 in hypothermia, a condition often related to anaesthesia.

Overall, this thesis helped the field of brain fluid dynamics investigation by providing a description of the techniques that can be used in pre-clinical research to investigate glymphatic function on a macroscopic level and dissect the microscopic players of the system. The methods described in this thesis can be adapted to the investigation of glymphatic function in different physiological and pathological settings, as well as different pre-clinical models. Advancing pre-clinical research with reproducible and standardised methods is fundamental for following translational applications.

Key words Glymphatic system, Aquaporin-4, Lectins, Parkinson´d disease, α-synuclein, hypothermia

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Ai miei nonni, alla mia famiglia

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List of original papers and manuscripts

This thesis is based on the following papers and manuscripts:

Paper I:

Cisterna Magna Injection in Rats to Study Glymphatic Function.

Ramos, M., Burdon Bechet, N., **Battistella, R**., Pavan, C., Xavier, A., Nedergaard, M., & Lundgaard, I.

Methods in molecular biology (Clifton, N.J.) (2019).

Paper II:

Not All Lectins Are Equally Suitable for Labeling Rodent Vasculature.

Battistella, R., Kritsilis, M., Matuskova, H., Haswell, D., Cheng, A. X., Meissner, A., Nedergaard, M., & Lundgaard, I.

International journal of molecular sciences (2021).

Paper III:

Effects of ASO-mediated knock down on AQP4 isoforms in the murine brain.

Roberta Battistella, Hani N. Alsafadi, Berit Powers, Linda Decker, Darcy Wagner, Frank Rigo, Iben Lundgaard.

Manuscript (2022).

Paper IV:

AQP4 polarisation impairment in Parkinson´s Disease mouse models and its relationship to glymphatic system.

Roberta Battistella, Arpine Sokratian, Chenchen Liu, Tekla Kylkilahti, Alessandro Gans, M. Angela Cenci, Andrew West, Iben Lundgaard.

Manuscript (2022).

Paper V:

Effect of systemic hypothermia on glymphatic function.

Chenchen Liu, Na Liu, Nagesh C. Shanbhag, Marios Kritsilis, Nicholas Burdon Bèchet, **Roberta Battistella**, Iben Lundgaard.

Manuscript, submitted to Journal of Cerebral Blood Flow and Metabolism (2022).

List of papers and manuscripts not included in the thesis

Activity-Induced Amyloid-β Oligomers Drive Compensatory Synaptic Rearrangements in Brain Circuits Controlling Memory of Presymptomatic Alzheimer's Disease Mice.

Pignataro, A., Meli, G., Pagano, R., Fontebasso, V., **Battistella, R.**, Conforto, G., Ammassari-Teule, M., & Middei, S.

Biological psychiatry (2019).

Spinal nerve meninges host the early invasion of T lymphocytes in the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis

Ramos-Vega M., Pavan C., Mori Y., Pla-Requena V., Peng W., Liu N., **Battistella R.**, Liu G., Goldman S., Lundgaard I & Nedergaard M.

Manuscript in preparation (2022).

Popular summary

The brain is the most complex organ in our body, ensuring that all the other parts work in concert to carry out all the needed functions for us to be alive. Compared to the other organs in our body and despite its small dimensions, the brain has a magnificently high metabolism. This comes with a cost of producing a lot of "waste" that needs to find a way out, to ensure that it will still function appropriately. A fun fact about the brain is that this organ is devoid of the so-called lymphatic vessels, structures similar to the blood vessels, although they do not transport blood. Instead recollect fluids from different parts of the body and whatever "waste" they may contain, to redirect them into the blood circulation for disposal. Then, questioning "How does the brain clean itself?" may not appear that obvious.

Surprisingly, in 2012, the discovery of a new brain-wide clearance system active during sleep, named "glymphatic system", provided a new key to answer this question. Moreover, it provided new insights into understanding the role of physiological sleep, which was long considered detrimental from an evolutionary point of view, although fundamental for the maintenance of cognitive health. During sleep, indeed, the glymphatic system allows for the movement of a special fluid surrounding the brain, called the cerebrospinal fluid (CSF), into the brain itself, helped by special channels permeable to water (aquaporin 4, AQP4) localised on star-shaped cells in the brain, named "astrocytes". Once inside the brain, the CSF releases nutrients and, most importantly, collects the wastes produced by the brain, thus serving a "cleansing" role. Importantly, it has recently been demonstrated how age or neurodegenerative disorders can affect the system, and therefore its function in "cleaning" the brain, leading to accumulation of products, including amyloid-β, that are detrimental for cognitive health. Therefore, understanding how glymphatic system functions is of utmost importance.

Because of the novelty of this field, it is needed to find consensus on the techniques and methodology to study glymphatic system, as well as interpretation of the results. Therefore, the present thesis aimed to describe different methods that can be used to investigate glymphatic function in physiological and pathological settings.

Specifically, in Paper I we described a method to study glymphatic function in the rodents´ brain, which then we applied in Paper IV and Paper V to investigate the function of glymphatic system in mouse models of Parkinson´s disease (PD) and in a mouse model of anaesthesia-induced hypothermia, respectively. In Paper II, we

showed that in studies where the visualisation of the cerebral vasculature is needed, i.e. to study AQP4 localisation in astrocytes, lectins can be used with similar efficacies to other markers of blood vessels. In Paper III, we showed that antisense oligonucleotides (ASO) targeting AQP4 mRNA can be used to decrease the quantity of *Aqp4* mRNA in the brain, but not the localisation of AQP4 on the astrocytes, which is important for glymphatic function. In Papers IV and V, applying the methods described in Papers I-III, we showed that AQP4 localisation in the astrocytes was altered when PD pathology or anaesthesia-induced hypothermia occur. Moreover, we showed that glymphatic system does not work properly in a subgroup of mice characterised by more aggressive PD pathology.

Overall, this thesis helped the field of brain fluid dynamics investigation by providing a description of the techniques that can be used in pre-clinical research to investigate glymphatic function on a macroscopic level and dissect the microscopic players of the system. The methods described in this thesis can be adapted to the investigation of glymphatic function in different physiological and pathological settings, as well as different pre-clinical models. Advancing pre-clinical research with reproducible and standardised methods is fundamental for following translational applications.

Riassunto

Il cervello è l'organo più complesso nel nostro organismo, e fa si che tutti gli altri organi lavorino in concerto per permettere le azioni necessarie alla sopravvivenza. Se messo a confronto con gli altri organi nel nostro organismo, e nonostante le sue relativamente piccole dimensioni, il cervello ha un'attività elevata. Ciò significa che i molti scarti prodotti durante tale attività, devono trovare una via d'uscita, per far si che il cervello continui a funzionare correttamente. Un fatto interessante riguardo il cervello, è che quest'organo manca dei cosiddetti vasi linfatici, strutture simili ai vasi sanguigni, con la differenza che non trasportano sangue. Al contrario, questi vasi raccolgono i fluidi presenti nel resto nel nostro organismo e qualsiasi sostanza di scarto essi possano contenere, per portarli nel circolo sanguigno ed eliminarli. Quindi, la risposta alla domanda "Come fa il cervello a liberarsi dai rifiuti?" potrebbe non sembrare così ovvia.

Nel 2012, la scoperta di un sistema coinvolto nello smistamento dei rifiuti nel cervello e attivo durante il sonno, ha fornito una nuova chiave di lettura per rispondere a questo quesito. Inoltre, ha fornito una possibile spiegazione per la funzione di un fenomeno, quello del sonno, svantaggioso da un punto di vista evoluzionistico, ma fondamentale per il mantenimento di una buona salute mentale. Infatti, durante il sonno, il sistema glinfatico si assicura che uno speciale liquido che si trova attorno al cervello, chiamato liquido cerebrospinale (CSF) entri invece al suo interno. Questo movimento è facilitato da speciali canali che lasciano passare acqua, chiamati Aquaporina-4 (AQP4), che si trovano su cellule nel cervello che hanno una forma stellata, dette astrociti. Una volta all'interno del cervello, il CSF rilascia nutrienti e, di maggior importanza, raccoglie gli scarti prodotti dalle cellule durante il giorno, svolgendo così una funzione di pulizia del cervello. Recentemente è stato dimostrato come l'invecchiamento o le malattie neurodegenerative possano diminuire la funzionalità del sistema, determinando l'accumulo nel cervello di rifiuti e proteine tossiche, come beta-amiloide, che possono comprometterne la corretta funzionalità. Appare evidente, quindi, che capire come funziona il sistema glinfatico è di fondamentale importanza.

Data la novità di questo campo di ricerca, è necessario trovare un accordo sulle tecniche e la metodologia per studiare il sistema glinfatico, così come per interpretarne i risultati. Di conseguenza, lo scopo di questa tesi è quello di descrivere diversi metodi che possono essere utilizzati per studiare la funzionalità del sistema glinfatico in condizioni fisiologiche e patologiche.

Più nel dettaglio, nell'articolo I abbiamo descritto un metodo per studiare il funzionamento del sistema glinfatico nel cervello di roditori. Questo metodo è stato successivamente applicato in Paper IV e Paper V per studiarne la funzionalità in modelli murini di malattia di Parkinson e in un modello murino di ipotermia indotta da anestesia. Con l'articolo II, abbiamo dimostrato che, in studi dove sia necessario visualizzare i vasi sanguigni nel cervello, ad esempio per studiare la localizzazione di AQP4 negli astrociti, le lectine possono essere utilizzate, avendo esse un'efficienza simile agli altri marcatori di vasi sanguigni. Nell'articolo III, abbiamo dimostrato che molecole che si legano all'RNA messaggero di *Aqp4*, chiamate oligonucleotidi anti-senso (ASO), possono essere utilizzate per ridurre la quantità *Aqp4* nel cervello a livello molecolare, ma non sono in grado di modificarne la localizzazione negli astrociti, un fattore importante per la funzionalità del sistema glinfatico. Negli articoli IV e V, applicando i metodi descritti negli articoli I e II, abbiamo dimostrato che la localizzazione di AQP4 negli astrociti è compromessa in presenza di malattia di Parkinson o di ipotermia indotta da anestesia. Inoltre, abbiamo anche dimostrato che la funzionalità del sistema glinfatico è alterata in un sottogruppo di animali che presenta segni di una malattia di Parkinson più aggressiva.

In conclusione, questa tesi ha avvantaggiato lo studio del sistema glinfatico, fornendo una descrizione delle tecniche che possono essere utilizzate in ricerca preclinica a livello macroscopico, ma offrono anche la possibilità di dissezionare le componenti microscopiche del sistema. I metodi descritti in questa tesi possono essere adattati a diversi scenari fisiologici e patologici, e a diversi modelli per la ricerca preclinica. Seguire metodi standardizzati e riproducibili è fondamentale per portare la ricerca preclinica un passo più vicino a future applicazioni terapeutiche.

Populärvetenskaplig sammanfattning

Hjärnan är det mest komplexa organet i vår kropp, vilket säkerställer att alla andra delar samverkar för att utföra alla funktioner som behövs för att vi ska vara vid liv. Jämfört med de andra organen i vår kropp har hjärnan en otroligt hög ämnesomsättning, med tanke på dess mycket begränsade dimensioner. Men en hög ämnesomsättning innebär också att hjärnan under sin aktivitet producerar mycket "avfall" som behöver hitta en väg ut för att garantera att den fortfarande fungerar korrekt. En kuriosa om hjärnan är att detta organ saknar de så kallade lymfkärlen, strukturer som liknar blodkärlen, även om de inte transporterar blod, utan istället hämtar vätskor från olika delar av kroppen och det ”avfall” de kan innehålla, för att omdirigera dem till blodcirkulationen för bortförsel. Med detta i åtanke kan frågan om "Hur rengör hjärnan sig själv?" kanske inte verkar så självklar.

Överraskande nog, 2012 gav upptäckten av ett nytt hjärnomfattande reningssystem aktivt under sömnen, kallat " glymfatiska systemet", en ny nyckel för att svara på denna fråga. Dessutom gav det nya insikter om att förstå rollen av fysiologisk sömn, som länge ansågs vara skadlig ur en evolutionär synvinkel, även om den är grundläggande för att upprätthålla kognitiv hälsa. Under sömnen tillåter det glymfatiska systemet förflyttning av en speciell vätska som omger hjärnan, kallad cerebrospinalvätskan (CSF), in i själva hjärnan, med hjälp av speciella kanaler som är genomsläppliga för vatten (aquaporin 4, AQP4) lokaliserade på stjärnformade celler i hjärnan, kallade "astrocyter". Väl inne i hjärnan frigör CSF näringsämnen och, viktigast av allt, samlar upp det avfall som produceras av hjärnan och fyller därmed en "renande" roll. Viktigt är att nyare studier har visat att detta system, och därför dess funktion för att "städa" hjärnan, inte fungerar effektivt när vi åldras, eller när neurodegenerativa störningar, inklusive Alzheimers sjukdom, uppstår. Detta leder till ackumulering av produkter, inklusive amyloid-β, som är skadliga för kognitiv hälsa. Därför är det ytterst viktigt att förstå hur det glymfatiska systemet fungerar.

På grund av hur nytt detta område är, finns det nödvändigt att finna konsensus om teknikerna och metoderna för att studera det glymfatiska systemet, såväl som tolkningen av resultaten. Därför syftar denna avhandling till att beskriva olika metoder som kan användas för att undersöka glymfatisk funktion i fysiologiska och patologiska miljöer.

Specifikt beskrev vi i Paper I en metod för att studera glymfatisk funktion i gnagares hjärna, som vi sedan använde i Paper IV och Paper V för att undersöka funktionen hos det glymfatiska systemet i musmodeller av Parkinsons sjukdom (PD) och i en musmodell av anestesi-inducerad hypotermi. I Paper II visade vi att i studier där visualisering av hjärnans kärl behövs, det vill säga för att studera AQP4-lokalisering i astrocyter, kan lektiner användas med liknande effektivitet som andra markörer för blodkärl. I Paper III visade vi att antisensoligonukleotider (ASO) riktade mot AQP4-mRNA kan användas för att minska mängden Aqp4-mRNA i hjärnan, men inte lokaliseringen av AQP4 på astrocyterna, vilket är viktigt för glymfatisk funktion. I Paper IV och V, med tillämpning av metoderna som beskrivs i Paper I-III, visade vi att AQP4-lokalisering i astrocyterna förändrades när PD-patologi eller anestesi-inducerad hypotermi inträffar. Dessutom visade vi att det glymfatiska systemet inte fungerar korrekt i en undergrupp av möss som kännetecknas av mer aggressiv PD-patologi.

Sammantaget hjälpte denna avhandling området för hjärnvätskedynamik genom att undersöka och ge en beskrivning av de tekniker som kan användas i preklinisk forskning för att undersöka glymphatisk funktion på en makroskopisk nivå och dissekera de mikroskopiska spelarna i systemet. Metoderna som beskrivs i denna avhandling kan anpassas till undersökning av glymfatisk funktion i olika fysiologiska och patologiska miljöer, såväl som olika prekliniska modeller. Att främja preklinisk forskning med reproducerbara och standardiserade metoder är grundläggande för att följa translationella tillämpningar.

List of abbreviations

α-syn	alpha-synuclein
α-syn PFF	α-syn pre-formed fibrils
6-OHDA	6-hydroxydopamine
Aβ	Amyloid-beta
aCSF	Artificial cerebrospinal fluid
AD	Alzheimer´s disease
AQP4	Aquaporin-4
ASO	Antisense oligonucleotide
BBB	Blood-brain barrier
CBF	Cerebral blood flow
CD-31	Cluster of differentiation 31
CM	Cisterna magna
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTRL	Control
DA	Dopamine
DAPC	Dystrophin associated protein complex
DAT	Dopamine transporter
dCLs	Deep cervical lymph nodes
DLB	Dementia with Lewy Bodies
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GLUT-1	Glucose transporter 1
i.c.v.	Intracerebroventricular

i.p.	Intraperitoneal
IB4	Isolectin B4
ICP	Intracranial pressure
ISF	Interstitial fluid
KD	Knock-down
KO	Knock-out
KX	Ketamine-Xylazine
LB	Lewy bodies
LEA	*Lycopersicum Esculentum Agglutinin*
LN	Lewy neurites
MCA	Medial cerebral artery
MCAO	Medial cerebral artery occlusion
MFB	Medial forebrain bundle
mPFFs	Mouse α-syn PFF
MRI	Magnetic resonance imaging
NE	Norepinephrine
OAPs	Orthogonal arrays of particles
PD	Parkinson´s disease
PFC	Prefrontal cortex
Phospho-α-syn	Phosphorylated α-syn
PVS	Perivascular space
SAS	Subarachnoid space
SNpc	Substantia nigra pars compacta
TBI	Traumatic brain injury
TH	Tyrosine Hydroxylase
tMCAO	Transient MCAO
WB	Western blot
WGA	*Wheat germ agglutinin*
Wt	Wild-type

Introduction

The glymphatic system, an introduction

Over the past decade, our understanding of brain-waste clearance underwent a revolution after the discovery of the so-called glymphatic system (Iliff et al., 2012). The glia-lymphatic (abbreviated, glymphatic) system was first described by the Nedergaard group in 2012 as a perivascular macrosystem involved in the distribution of nutrients and clearance of metabolic waste from the brain parenchyma, or neuropil (Iliff et al., 2012). The nomenclature is based on the combination of "glia", a supporting cell type in the brain that plays a very important role in its function, i.e., astrocytes, also known as glial cells, and "lymphatic", because of the waste clearance function, which resembles the role of the lymphatic system in the rest of our body, i.e., collecting wastes from the tissues and redirecting them in the blood circulation (Iliff et al., 2012). The first description of the glymphatic system was of vital importance as it provided new insights into understanding the role of physiological sleep (Xie et al., 2013), which was long considered detrimental from an evolutionary point of view, although fundamental for the maintenance of cognitive health (Frank & Heller, 2018).

During physiological sleep the glymphatic system allows for the movement of the cerebrospinal fluid (CSF) from the subarachnoid space (SAS) into the perivascular space (PVS), or Virchow-Robin´s space, of the penetrating brain arteries (Xie et al., 2013), driven by arterial pulsations (Mestre, Tithof, et al., 2018). From the PVS, CSF then moves into the neuropil, a process facilitated by water channels expressed selectively on the portion of the astrocytes (endfeet) surrounding blood vessels, known as aquaporin 4 (AQP4) water channels (Mestre, Hablitz, et al., 2018). The movement of the CSF into the neuropil during the sleep state is thought to be driven by hydraulic forces following the expansion of the extracellular space surrounding neurons (Xie et al., 2013). Importantly, once in the neuropil, the CSF mixes with the interstitial fluid (ISF), releasing nutrients and collecting waste derived from neuronal activity and the subsequent high brain metabolism during the day, thus serving a "cleansing" role for the brain (Lundgaard et al., 2017). Finally, according to the original glymphatic hypothesis, the CSF containing brain metabolic waste is recollected in the perivenous space of large calibre veins (Iliff et al., 2012, 2014) (Figure 1), wherefrom it exits the central nervous system (CNS) through various efflux routes that will be described in greater detail in the upcoming paragraphs.

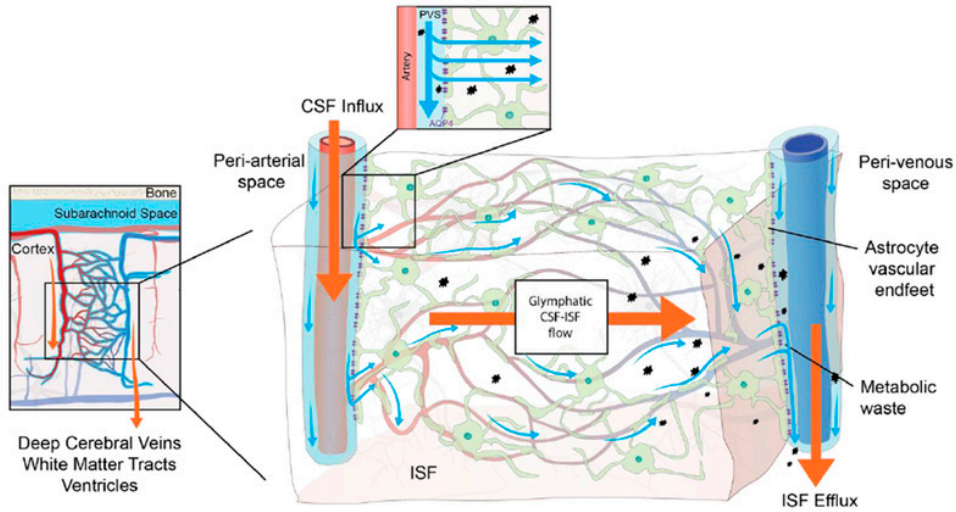

Figure 1: The glymphatic system
Schematic representation of the glymphatic system. The schematic highlights (i) the peri-arterial CSF influx from the SAS into the PVS surrounding penetrating arteries; (ii) the AQP4-dependent movement of the CSF into the parenchyma; (iii) the bulk flow of CSF/ISF in the brain parenchyma and (iv) recollection of CSF/ISF fluid and cleared wastes into the peri-venous space. Reprinted with licensed permission from (Rasmussen et al., 2018).

Main actors in the glymphatic system

In the paragraph above, the readers have been introduced to the fundamentals of the glymphatic system. However, now it is time to dive into the specifics of the main "actors" of the system, and their function.

Cerebrospinal fluid

CSF is a colourless liquid secreted by the epithelium of the choroid plexus, which lines the walls of the cerebral ventricles. It has also been described as a plasma ultra-filtrate as it is derived from across the endothelium of choroidal capillaries (di Terlizzi & Platt, 2006). The analysis of CSF composition revealed the presence of different ions (including sodium, potassium, calcium, magnesium, and chloride), glucose, proteins (i.e. albumin, γ-globulin), as well as some enzymes and neurotransmitters. The overall CSF composition is tightly regulated and remains constant, although some enzyme and neurotransmitter concentrations may vary in the case of certain pathologies (di Terlizzi & Platt, 2006).

The first existing evidence of the presence of CSF inside the skull of humans was reported in the year 1500 BC, in the Egyptian Edwin Smith papyrus (Deisenhammer

et al., 2015; van Middendorp et al., 2010), whilst the first description of the ventricular system dates back to Aristotele´s *Historia Animalium*, although at the time it was thought that the brain ventricles were not filled with liquids, but rather with the gaseous *spiritus animalis* (Deisenhammer et al., 2015; Rasmussen et al., 2022). Only centuries later, Leonardo Da Vinci was able to provide the first anatomical description of the ventricular system, following the injection of hot wax into the ventricular system of an ox (Deisenhammer et al., 2015; Herbowski, 2013). The first evidence that ventricles were not filled with gaseous *spiritus animalis,* but rather a transparent fluid, later called CSF, was provided by Domenico Cotugno´s studies on fresh cadavers (di Ieva & Yaşargil, 2008; Herbowski, 2013).

Later, in 1960 the choroid plexus was proven to be the main production site of CSF (Cserr, 1971; de Rougemont et al., 1960), although studies where the choroid plexus was removed surgically, showed that the choroidal secretion and filtration accounts only for around 80% of CSF production (Hassin GB et al., 1937; Milhorat et al., 1971), whilst the remaining 20% is thought to be of extra choroidal origin (Cserr, 1971; Cserr et al., 1977, 1981; McComb, 1983). Specifically, this portion is thought to reflect the influx of vascular fluids through the blood-brain barrier (BBB) at the level of the microvasculature (Orešković et al., 2016); this fluid is then thought to move towards the ventricles so that its volume sums up to the one produced by choroid plexuses (Rasmussen et al., 2022). However, this hypothesis lacks testing due to unavailability of techniques able to distinguish between CSF from the two sources (Rasmussen et al., 2022). Other sources of CSF production are thought to be in the SAS, and even in the spinal cord (SC). Once produced, CSF moves through a series of ventricles across the neuroaxis, and escapes from the 4th ventricle via the foramen of Magendie and the foramina of Luschka, draining into the SAS surrounding the brain and the SC. Rodents have been shown to lack the foramen of Magendie. Instead, CSF flows into the SAS via the lateral foramina of Luschka (Damkier et al., 2013; Deisenhammer et al., 2015). The above-described movement of the CSF, driven by the pressure derived from active CSF production by the choroid plexuses, was termed "third circulation" by Harvey Cushing, to describe a circulation that was parallel to the bloodstream (Cushing Harvey, 1925; Herbowski, 2013).

Although it was shown in the 1900's that CSF marked with horseradish peroxidase flows from the SAS in the perivascular space (described below) of the brain penetrating arteries in cats and dogs (Rennels et al., 1985), only in 2012 did Nedergaard´s group describe a functional role for this movement with the glymphatic hypothesis (Iliff et al., 2012).

Perivascular spaces

The perivascular space (PVS), or Virchow-Robin space, derived from the name of the two scientists that first described it in the 19th century (Woollam & Millen, 1955), is a fluid-filled space surrounding the arteries and the veins of the brain,

which disappears at the level of capillaries (Hannocks et al., 2018) (Figure 2). In penetrating arteries on the dorsal aspect of the cortex, it is delimited by the astrocytic endfeet and the pial membrane on the brain parenchyma side, and smooth muscle cells on the vascular side (Wardlaw et al., 2020). PVSs of penetrating arteries are in direct contact with the SAS and in this way represent its extension when the brain arteries dive deep into the brain parenchyma (Wardlaw et al., 2020). At the level of the microvasculature, PVS disappears since the pial membrane is replaced by the basal lamina, which is surrounded by astrocytic endfeet. The spaces then reappear at the level of the venous system (Wardlaw et al., 2020).

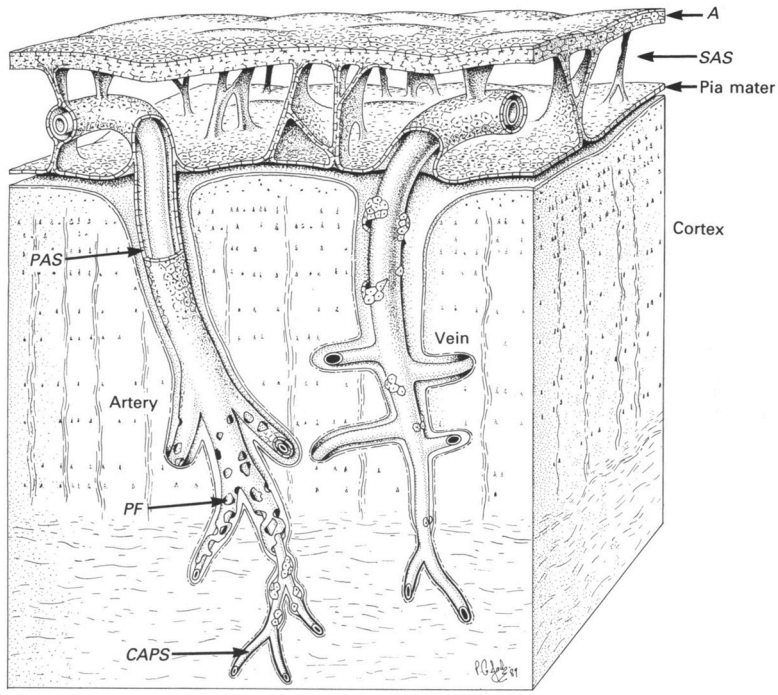

Figure 2: Anatomy of PVS.
Schematic drawing of the anatomy of PVS, A: arachnoid; SAS: subarachnoid space; PAS: periarterial space; PF: perforated layer of pial cells, when the penetrating artery starts branchyíng; CAPS: capillary. Reprinted from (Zhang et al., 1990).

PVSs are an important compartment for the glymphatic system, since it is from these anatomical locations that the CSF can move through the brain parenchyma, via astrocytic endfeet surrounding the arterial vasculature. This movement is facilitated by the AQP4 channels selectively expressed in this location (see below) (Mestre, Hablitz, et al., 2018). However, according to the original glymphatic hypothesis, PVSs also play a role in the re-collection and drainage of the CSF after it exchanges solutes with the ISF, at the level of venous vasculature (Iliff et al., 2012). It has been

proposed that the volume of perivascular spaces can vary depending on vasoconstriction and vasodilation dynamics of the cerebral vasculature, but also on astrocytic swelling (Hablitz & Nedergaard, 2021). Intriguingly, it has been shown that substantial rearrangement of PVSs happens in different pathologies, i.e. PVSs collapse in cerebral amyloid angiopathy disease (Attems et al., 2010; Gatti et al., 2020) and are enlarged in cerebral small vessels disease (Mestre et al., 2017).

Astrocytes and Aquaporin-4 water channels

Astrocytes functions in the brain

Astrocytes are among the prominent cell types that make up the neuroglia. It has been calculated that astrocytes represent between 20 and over 50% of the total number of cells in the brain, depending on the species of study (Hasel & Liddelow, 2021). The name depicts the shape of these cells, whose morphology is star-like (from αστον, Astron = star in Ancient Greek). Astrocytes in the brain exert important physiological functions: their proximity to cerebral vasculature allows them to collect glucose from the blood, and store it as glycogen reserve, or convert it to lactate for neuronal function; they also control the availability of neurotransmitters in the neuropil and take part in synapse formation and elimination, suggesting an active role in the learning and memory processes (Hasel & Liddelow, 2021). However, astrocytes are also among the first responders to pathological insults in the brain including infections, traumatic injuries, or chronic diseases accompanied by neuroinflammation, e.g. Alzheimer´s disease (AD) or Parkinson´s disease (PD) (Hasel & Liddelow, 2021). The astrocyte response to these events has been shown to vary between three main states, that can be described as (i) normal physiological, (ii) pathological trophic, (iii) pathological toxic. It has been shown that, after LPS administration, activated microglia release pro-inflammatory cytokines, including interleukin 1α (IL-1α), tumour necrosis factor α (TNF-α) and complement 1q (C1q) that are able to induce a reactive neurotoxic phenotype in astrocytes, supporting a detrimental function (Liddelow et al., 2017). Similarly, blocking the formation of the reactive neurotoxic phenotype in astrocytes in a transgenic mouse model of PD, where mutant alpha-synuclein (α-syn) was genetically overexpressed, was proven to be neuroprotective (Yun et al., 2018). However, astrocytic activation does not always generate unfavourable consequences, and it has been shown that the formation of the glial scar after ischemic stroke helps to delimit the affected area in the brain and protect the surroundings (Choudhury & Ding, 2016). When coping with an insult, astrocytes undergo a massive structural re-organisation known as gliosis, and their morphology changes from star-shaped cells with thin long processes, to enlarged cells protruding thick processes from the cell bodies, and they overexpress proteins like glial fibrillary acidic protein (GFAP) (Escartin et al., 2021). Astrocytes are fundamental to brain physiology, in particular to maintain neuropil homeostasis; indeed,

astrocytes project their endfeet towards the cerebral vasculature, contributing in this way to the so-called neuro-vascular unit, together with endothelial cells of the BBB, smooth muscle cells, pericytes, microglia, neurons and extracellular matrix (Kugler et al., 2021). Notably, astrocytes have also been recognized to exhibit a fundamental role in the physiology of the glymphatic system, which is the main object of study in this thesis. Astrocytes are highly polarized cells, meaning that the protein expression varies greatly between the cellular body and their processes. This is particularly relevant for AQP4, a water-permeable channel protein selectively expressed (or polarized) at the astrocytic endfeet that surrounds blood vessels (Eidsvaag et al., 2017). Here AQP4 facilitates the movement of CSF from the perivascular space of penetrating brain arteries into the neuropil (Mestre, Hablitz, et al., 2018). Intriguingly, it has been reported that gliosis correlates with rearrangements in protein localisation, in particular concerning AQP4 (Hablitz & Nedergaard, 2021).

Aquaporin-4 water channels in the brain

AQP4 is part of a large family of proteins that are selectively permeable to water and allow its bilateral transport across biological membranes. AQP4 cDNA was first found in the rodent brain in 1994 by the independent work of Agre´s and Verkman´s groups (Hasegawa et al., 1994; Jung et al., 1994), which described its highly polarized association with astrocytic membranes abutting blood vessels and facing the pial membrane (S. Nielsen et al., 1997). AQP4 polarisation in the endfeet of astrocytes is the result of its interactions with parts of the dystrophin associated protein complex (DAPC) (Hablitz et al., 2020b; Nicchia et al., 2008) (Figure 3, A). The knock-out (KO) of alpha-syntrophin (α-syntrophin), a protein of the DAPC, has been shown to reduce AQP4 polarisation, without impacting AQP4 total expression (Amiry-Moghaddam et al., 2003).

Several isoforms of AQP4 have been described in the rodent brain (Jorgačevski et al., 2020) with M1 and M23 being the most abundant (Jung et al., 1994; Lu et al., 1996), with a ratio of 1:3 (Palazzo et al., 2019). Interestingly, M23 has been shown to be translated from the same mRNA as M1, through a leaky scanning mechanism, with translation starting at Met23, instead of Met1 (A. Rossi et al., 2010). Recently, De Bellis et al. showed the existence, *in vivo,* of additional isoforms of AQP4, characterized by an extended C-terminal and generated via a translational readthrough (TRT) mechanism of the canonical AQP4 isoforms mRNA (de Bellis et al., 2017). Those extended isoforms of AQP4 (AQP4ex) represent ~ 10% of all AQP4 isoforms. The function of the extended C-terminus of AQP4ex isoforms has been implicated in water permeability, cellular localisation through interactions with α-syntrophin and, most importantly, in the organisation and size of OAPs (see below) (de Bellis et al., 2017).

In the plasmalemma, four AQP4 monomers assemble to form tetramers (Neely et al., 1999) (Figure 3, E). Each AQP4 monomer has a water channel. The specificity

towards water molecules is due to three different specialisations along the channel itself (Tani et al., 2009):

I. size of the pore of ~2.8 Å, which excludes molecules bigger than water from passage.

II. presence of an arginine residue in the pore, which prevents the passage of protonated water and other cations.

III. presence of positively charged dipoles in the pore, that exclude cations from passage and induce re-orientation of water molecules while passing through the channel.

When assembled in tetramers, AQP4 monomers form a central pore that has been implicated in the transport of gas and ions. Tetramers of AQP4 are able to assemble into supra-molecular organised structures, known as orthogonal arrays of particles (OAPs) (Crane & Verkman, 2009; Verbavatz et al., 1997) (Figure 3, B-D).

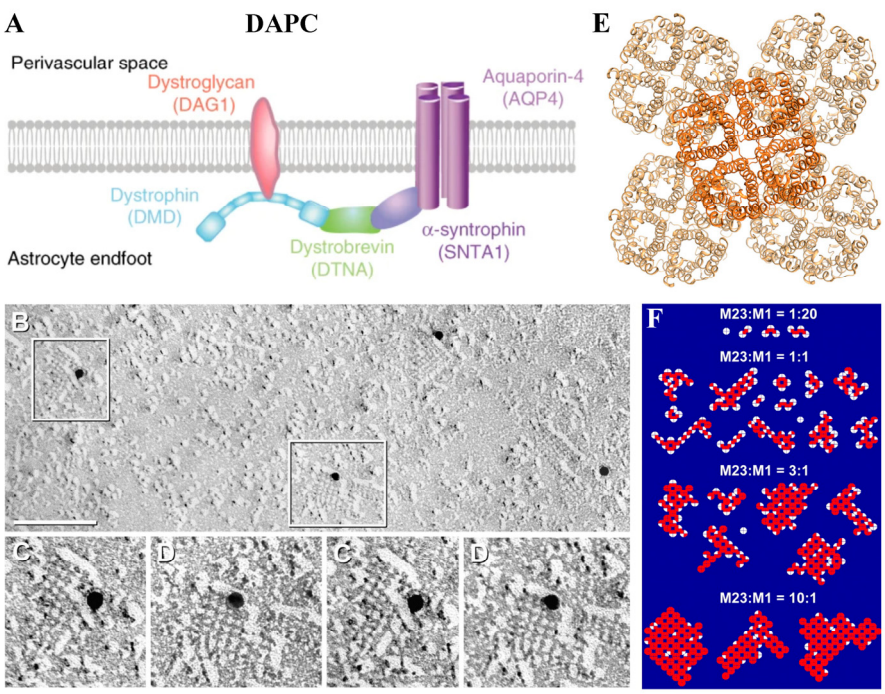

Figure 3: AQP4 and its assemblies.
A) Schematic representation of the DAPC anchoring AQP4 to the astrocytic endfeet membrane surrounding blood vessels. B-D) AQP4 square arrays, also known as OAPs, as seen after freeze-fracture and immunogold analysis. E) Reconstruction of molecular structure of AQP4, seen perpendicularly to the plasmalemma. In the schematic, it is possible to appreciate AQP4 associated in tetramers, and 5 tetramers interacting with each other in the plasmalemma. F) Schematic of possible conformations of OAPs at different M23:m1 ratio obtained with mathematical modeling. Adapeted from (Hablitz et al., 2020; Jin et al., 2011; Rash et al., 2004; Roche & Törnroth-Horsefield, 2017). B, C, D and F were reproduced under licensed permission.

Specifically, in OAPs M1 and M23 isoforms of AQP4 assemble in heterotetramers, where a M23 core is surrounded by M1 (Bellis et al., 2021) (Figure 3, F). It has been shown that M23 alone can form OAPs; however, those structures are bigger than heterotetrametric OAPs and seem to exert a cell adhesion function (Ciappelloni et al., 2019; Furman et al., 2003; Smith et al., 2014). On the other hand, M1 alone, has been proven unable to form OAPs, because the extended N-terminus appears to inhibit the formation of functional OAPs; however, M1 can aggregate in small homotetramers that are highly mobile in the cellular membrane, and support cellular migration functions (Ciappelloni et al., 2019; Furman et al., 2003; Smith et al., 2014).

As described previously, AQP4 has been shown to play a relevant role in the physiology of glymphatic system. The KO of AQP4 in transgenic mice significantly decreases the influx of tracers injected in the CSF in the perivascular space of brain penetrating arteries, and subsequently into the neuropil, but also the clearance of injected radiolabelled amyloid-beta (Aβ), which is retained in the parenchyma (Iliff et al., 2012). AQP4 involvement in glymphatic influx has been questioned by a study which found no differences in cisterna magna (CM) injected tracer influx in the brain parenchyma between $AQP4^{-/-}$ mice and their littermates (Smith et al., 2017). However, those results were contested in a following study where AQP4-dependent CSF movement into the brain was confirmed independently by 5 groups using different transgenic AQP4 KO mouse lines (Mestre, Hablitz, et al., 2018). Interestingly, the same study also showed that the lack of AQP4 polarisation at the astrocytic endfeet, can disrupt glymphatic influx in the brain, as seen in α-syntrophin KO transgenic mice (Mestre, Hablitz, et al., 2018). This notion is relevant to the results presented in this thesis, since it has been shown that AQP4 polarisation is negatively impacted by aging and neurodegenerative diseases, e.g. Alzheimer´s disease (Zeppenfeld et al., 2017), and is coupled to impaired glymphatic influx in the brain (Kress et al., 2014).

How can we study AQP4 polarisation?

The reader may have already understood the great importance of AQP4 to the physiology of glymphatic system in the paragraphs above. AQP4 polarisation is the molecular readout of glymphatic function, prompting the need for developing of reliable and unbiased methods to study AQP4 and its polarisation. The easiest way to investigate AQP4 polarisation is by utilising immunofluorescence imaging protocols. This method has the advantage that the brain tissue processed for glymphatic studies, i.e. the analysis of the tracer penetrance in the brain parenchyma, can be used not only for AQP4 staining, but also to check several other proteins and markers of interest, maximizing the use of the same tissue and contributing to a reduction in the number of experimental subjects needed in the study.

It has already been mentioned that AQP4 is a highly polarized protein, meaning that its maximal expression in astrocytes happens in the astrocytic endfeet abutting blood vessels, rather than in the astrocytic soma (Eidsvaag et al., 2017; S. Nielsen et al., 1997). Therefore, to better quantify AQP4 polarisation in brain specimens, AQP4 visualisation through immunofluorescence protocols is advantageously carried out in presence of other markers for blood vessels. Historically, blood vessels in brain specimens have been labelled *ex vivo* with endothelial cells markers, e.g. cluster of differentiation (CD) 31 (Bell et al., 2010; Koonce et al., 2017; Müller et al., 2002), or basement membrane markers, e.g. laminin (Eriksdotter-Nilsson et al., 1986; Wälchli et al., 2015). Glucose transporter-1 (GLUT-1), a protein expressed by either endothelial cells or astrocytes at the BBB, has also been widely used to specifically label the cerebral vasculature (Leino et al., 1997). Lectins, which are glycoproteins with a carbohydrate recognition domain, bind selectively to carbohydrates on the luminal aspect of the endothelium (Simionescu et al., 1982), and have been widely used to label the vasculature either by *ex vivo* immunohistochemical approaches (Mazzetti et al., 2009; Santulli et al., 2011; Sorriento et al., 2009), or by direct delivery into the bloodstream as an intermediate step during transcardial perfusion (Bryson et al., 2011; Robertson et al., 2015; Wälchli et al., 2015).

AQP4 polarisation can be then analysed using e.g. confocal images of the labelled tissue. Many studies have used a thresholding approach to quantify overall AQP4 polarisation in big portions of tissue, but a more sophisticated and detailed analysis has been described by Munk et al. in 2019 (Munk et al., 2019). Briefly, AQP4 polarisation is analysed in individual vessels imaged at very high magnification and calculated as the ratio between the signal at the blood vessel wall, and the signal in the surrounding tissue (signal-to-noise ratio) (Munk et al., 2019). For the specifics of this approach, I refer the readers to the "Materials and Methods" section of this thesis.

Recently, Nedergaard´s group has described an alternative method to study AQP4 polarisation based on the ratio of M1 and M23 isoforms (Hablitz et al., 2020). Since there are no available antibodies that can selectively label the two isoforms, this method requires a western blot (WB) approach, where M1 and M23 isoforms can be visualised as separate bands with different molecular weights and quantified. Therefore, this method requires that brain tissue is processed differently for the purpose, not allowing for other imaging studies of glymphatic function.

In the paragraph above, the importance of the tetramerization of M1 and M23 to form OAPs with different functions has been described (Crane & Verkman, 2009; Neely et al., 1999; Verbavatz et al., 1997), and therefore a combination of the two different approaches, i.e. immunolabelling of brain sections and WB, could be useful in AQP4 polarisation studies.

Investigation of AQP4 function with KO mouse models

To study the water transport function of AQP4 in the brain, several AQP4 KO mouse models have been developed over the years. The pioneer work of Verkman´s group in 1997 (T. Ma et al., 1997) lead to the development of a transgenic null mouse model deficient in the AQP4 water channel through replacement of the wild-type (wt) Aqp4 gene with a vector containing only exons 1-3 and part of exon 4 in embryonic stem cells (ES). The characterisation of those mice revealed no differences, compared to wt littermates, in several readouts (e.g. pre/peri-natal lethality, weight, brain function and behaviour, organs´ morphology and urine osmolarity). Furthermore, no compensation mechanisms, e.g. increase of transcript levels of other AQPs, were detected. Other AQP4 KO mouse models were developed through replacement of 250 nucleotides in the exon 1 of the wild type gene with an enhanced green fluorescent protein (eGFP) cassette (Ikeshima-Kataoka et al., 2013), or through depletion of exons 1–3 to avoid the expression of any translational splicing form (Thrane et al., 2011). Moreover, mouse models specifically targeting the AQP4 pool localized at the astrocytic endfeet were also developed, in particular by depleting proteins that are important for AQP4 anchoring, e.g. dystrophin (Vajda et al., 2002) and α-syntrophin (Amiry-Moghaddam et al., 2003). Additionally, conditional AQP4 KO mouse models have been developed, targeting the pool of AQP4 residing in astrocytes and without affecting AQP4 pools in other organs, e.g. in the kidney or in the muscle (Haj-Yasein et al., 2011). More recently, a KO mouse model targeting AQP4 specific isoforms M23 (Bellis et al., 2021; Pisani et al., 2021) and AQP4ex (Palazzo et al., 2019) has been developed, substantially advancing the study of their specific functions. Relatively to the glymphatic system, such abundance of AQP4 KO mouse models has finally proven that CSF movement in the brain parenchyma is AQP4-dependent (Mestre, Hablitz, et al., 2018).

However, the use of congenital AQP4 KO mouse models do not enable studies where a spatially and temporally controlled removal of AQP4 is of interest, paving the way for alternative approaches, e.g. regional restricted AQP4 knock-down (KD) after injection in the rodent brain of short interference RNA (siRNA) targeting AQP4 (Badaut et al., 2011; Fukuda et al., 2013). These studies are important to understand whether a local impairment of the molecular machinery supporting glymphatic function, which may occur following a brain insult, would have an impact on the global CSF movement in the neuropil.

CSF efflux routes

As previously described, after it exchanges solutes with the ISF in the brain parenchyma, CSF is recollected in the perivenous space of large draining veins of the brain (Iliff et al., 2012), in the space between fibers in the white matter, and around the ependymal membrane surrounding the lateral ventricles (Cserr et al.,

1977; Rasmussen et al., 2022). However, it appears obvious that, together with new fluid production by the choroid plexuses in the ventricles, an equal amount of fluid must be drained to maintain the homeostasis in the brain. Historically, the main extra-cranial site of CSF efflux has been described as the arachnoid granulations in the superior sagittal sinus (Upton & Weller, 1985). However, a lack of *in vivo* evidence for this route has led the scientific community nowadays to question egress of CSF through arachnoid granulations, and further study the routes of CSF efflux (Proulx, 2021) (Figure 4). In keeping with this, the following pathways appear to support a higher degree of efflux:

I. Olfactory route, along the *fila olfactoria* of olfactory neurons crossing the cribriform plate to reach the nasal submucosa (Bradbury & Westrop, 1983; Brierley & Field, 1948; Gomez et al., 1985); this route has been suggested to support the highest degree of CSF efflux, and it appears to be evolutionary conserved (Bradbury & Westrop, 1983; de Leon et al., 2017; Johnston et al., 2004; Norwood et al., 2019).

II. Perineuronal space of cranial (Gomez et al., 1988; Proulx, 2021; Rasmussen et al., 2022; Wang et al., 2020) and spinal nerves (Brierley, 1950; Brierley & Field, 1948; Q. Ma, Decker, et al., 2019), where it has been histologically shown that the arachnoid and endothelial layers are lacking, allowing for a potential route for bulk flow; moreover, lymphatic vessels have been identified in the perineuronal sheet of facial nerves in rodents (Furukawa et al., 2008).

III. Lymphatic vessels in the dura mater, re-described in 2015 by two independent groups (Aspelund et al., 2015; Louveau et al., 2015); ablation of these vessels with photoconversion of Visudyne, has been shown to correlate with altered CSF clearance and exacerbation of pathology in mouse models of neurodegenerative diseases and cognitive impairment (da Mesquita et al., 2018).

IV. Ventral aspect of the skull, specifically at the level of the circle of Willis and carotid cistern (Decker et al., 2022). Interestingly, it has also been shown that lymphatic vessels exist in the dura in this location (Ahn et al., 2019).

V. Dural space surrounding the superior sagittal sinus, as seen in humans after intrathecal administration of gadobutrol (Ringstad & Eide, 2020).

VI. External layer of cerebral vasculature (adventitia).

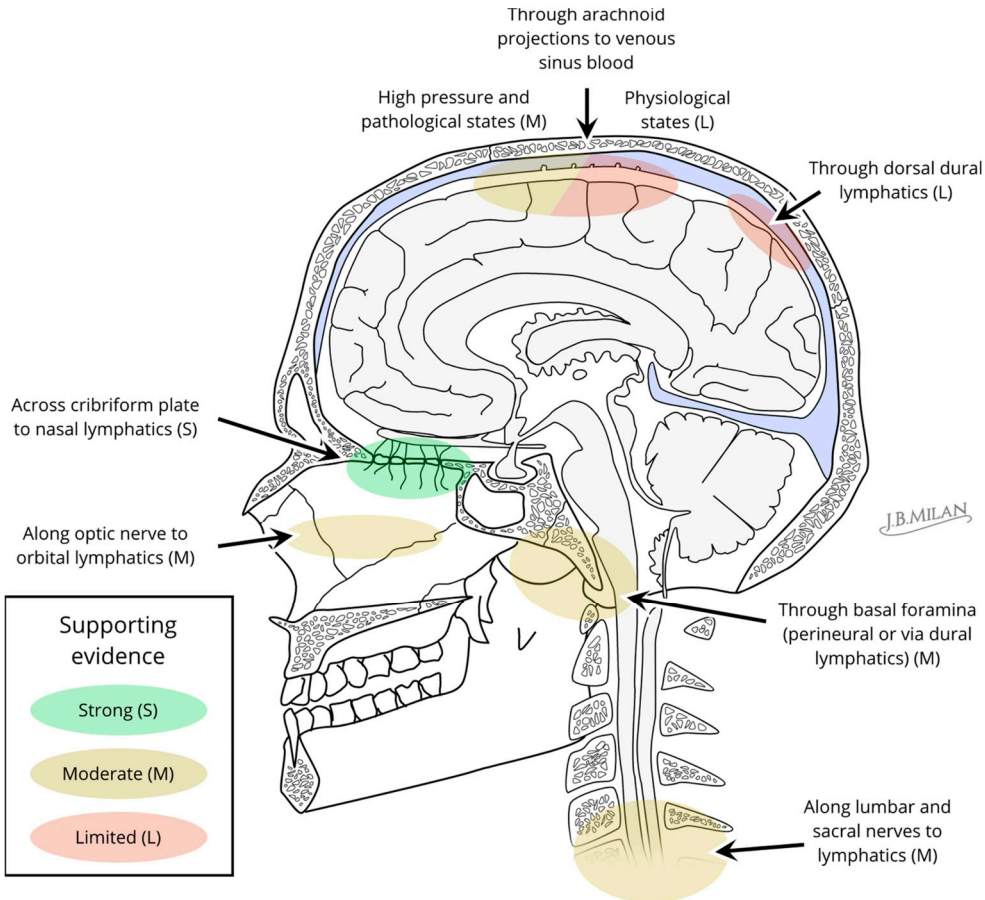

Figure 4: Schematic representation of the CSF extra-cranial efflux pathways.
Schematic drawing representing the pathways that are thought to sustain extracranial CSF efflux, labelled with green, yellow or red colors based on the related supporting evidences. Reprinted from (Proulx, 2021).

Even though the mechanism has not yet been fully elucidated, and the contribution of each of these pathways is still a matter of debate in the scientific community, from these pathways the fluid is then drained into the cervical lymphatic system, and back to the blood circulation in approximately equal quantities (Boulton et al., 1998).

Methods to study glymphatic system

Ex vivo approaches

The golden standard method to study glymphatic system and its function is the injection of fluorescent tracers in the CSF compartment (Bèchet et al., 2021a; Xavier et al., 2018). In this way, CSF will appear fluorescent when a light with suitable wavelength is applied, revealing the position and distribution of a fluid that would otherwise be transparent. Since it is important to maintain the homeostasis in the brain tissue, intraventricular (i.c.v.) injections of tracers are discouraged, because of the parenchymal and dural damage that occurs after needle penetration in the lateral ventricle (Mestre, Hablitz, et al., 2018). Instead, the cisterna magna (CM), the largest cistern around the brain, is a suitable location for such an injection. The CM injection technique permits to get access to the CSF without damaging the brain or the dura mater on the dorsal aspect of the skull (Xavier et al., 2018); a detailed description of the procedure in rodents can be found in the Methods chapter of this thesis.

The main pitfall of this approach is that the fluorescence analysis in the brain or other organs of interest must be performed *ex vivo*, upon organs collection and fixation. This is a limitation of this approach, since it has been shown that PFA fixation during transcardial perfusion alters significantly the morphology of PVS, the main conduit for CSF penetration in the brain parenchyma (Mestre, Tithof, et al., 2018).

In vivo approaches

The first visualisation of the glymphatic transport in the brain *in vivo* was provided by Iliff et al. in 2013 (Iliff, Wang, et al., 2013). They used a two-photon microscopy approach to image the movement of a CM-injected fluorescent tracer in the PVS of cerebral arteries. However, two-photon imaging does not allow for visualisation of tracer penetrance in deeper regions of the brain, producing a resolution that can cover only few microns of depth into the cortical surface. Moreover, the field-of-view that can be imaged at one given time is limited, permitting the imaging of only one or two vessels at the time, and limiting therefore the application of this approach (Iliff, Wang, et al., 2013).

Other *in vivo* approaches to study glymphatic system are based on the possibility to image contrast agents injected in the CM through magnetic resonance imaging (MRI) (Iliff, Lee, et al., 2013). This approach has the advantage that the dynamics of CSF movement can be imaged in real time in the whole brain, and it has been shown to detect slower CSF flow dynamics that happen after subarachnoid haemorrhage (Goulay et al., 2017) and ischemic stroke (Gaberel et al., 2014; Mestre et al., 2020). Intriguingly, using an intrathecally injected contrast agent and

following MRI in patients affected by normal pressure hydrocephalus, Eide and Ringstad were the first to demonstrate the existence of glymphatic pathways in humans (Ringstad et al., 2018).

Recently, Keil et al. described an approach based on imaging of infrared (IR) CSF tracers in the dorsal cortex through the intact skull, using an IR imaging system available for rodents (Keil et al., 2022). However, the resolution and the depth of the imaging field that can be obtained with such imaging approach, makes it necessary to pair this *in vivo* approach with an *ex vivo* analysis of fluorescent tracers injected in conjunction in the CM.

Regulation of glymphatic function

Since its first description in 2012, great effort has been put into understanding how the glymphatic system is regulated, and how to manipulate the system. Several physiological drivers have been found (e.g., arterial pulsations and sleep, discussed below), but researchers have also shown that the system can be positively regulated by a myriad of factors, e.g. low doses of alcohol (Lundgaard et al., 2018), agonists of AQP4 (Huber et al., 2018), voluntary exercise (He et al., 2017; von Holstein-Rathlou et al., 2018).

Arterial pulsations

Since the CSF moves from the SAS into the brain parenchyma via PVS surrounding the penetrating arteries, it has been long hypothesized that the vascular compartment could have influenced this movement (Iliff, Wang, et al., 2013).

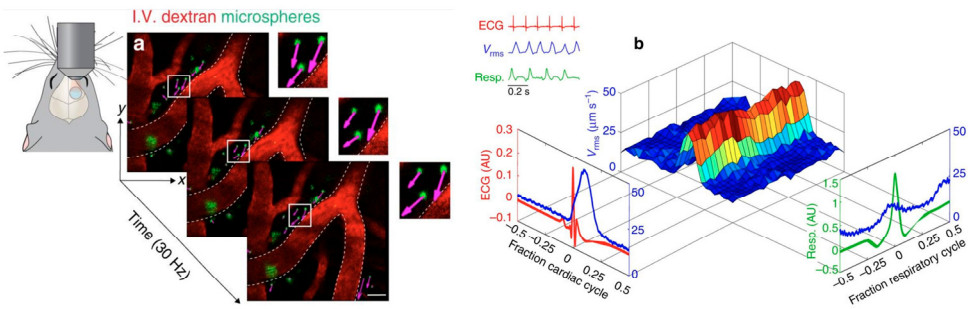

Figure 5: Arterial pulsations guide CSF flow in the perarterial space.
A) *In vivo* 2-photon imaging of a cortical artery via a skull window. The intravenous injected tracer, to allow visualization of the vasculature, is represented in red. In green, instead, the microspheres injected in the CM. B) Respresentation of the microspheres velocity in fuction of electroencephalogram and respirations signal. Adapted from (Mestre, Tithof, et al., 2018).

In 2018, Mestre et al., through particle tracking in the PVS after injection in the CM, showed quantitatively that CSF glymphatic flow is driven by arterial pulsations

(Mestre, Tithof, et al., 2018) (Figure 5). Moreover, the increase of blood pressure in a mouse model of hypertension showed an altered pulsatility of the arterial wall, reflected by a reduced flow in the PVS (Mestre, Tithof, et al., 2018).

Sleep and anaesthesia

Since the first publication in 2012, it was known that CSF influx in the brain parenchyma was connected to sleep, and it appeared abolished during the awake state (Iliff et al., 2012). In 2013, Xie et al. showed that CM-injected tracers penetrated deeper along PVS of penetrating brain arteries when the mice were voluntarily sleeping or were anesthetized with a mix of Ketamine and Xylazine (KX) (Xie et al., 2013). These changes in CSF flow dynamics were accompanied by an increase in the extracellular space in the sleeping or KX-anesthetized mice, compared to the awake ones (Xie et al., 2013). Moreover, in the same study, they showed that the inhibition of the noradrenergic (NE) system has similar effects to physiological sleep and KX anaesthesia on glymphatic system (Xie et al., 2013).

It must be noted that not all the anaesthetics have the same effect on glymphatic function. A study published in 2019 showed that many of the most common used anaesthetics (e.g. isoflurane, pentobarbital, avertin) do not facilitate glymphatic influx in the brain, probably because of the different power spectra of slow waves delta oscillation under different-anaesthetics regimes (Hablitz et al., 2019). High influx of CSF in the brain correlates with the power of the slow waves delta oscillations, and KX anaesthesia appears to induce a pattern of slow waves delta oscillations most similar to those seen during physiological sleep (Hablitz et al., 2019).

Interestingly, a recent study showed that it is not sleep *per se* to induce glymphatic function, but that the process is rather controlled by an endogenous circadian rhythm (Hablitz et al., 2020). CSF influx in the brain peaks in the middle of the resting phase in mice, regardless of the anaesthetic used. This process also appears to regulate AQP4, whose polarisation undergoes circadian variations. Intriguingly, CSF influx in AQP4$^{-/-}$ mice was not affected by circadian rhythm (Hablitz et al., 2020b).

However, it must be mentioned that core body temperature decreases rapidly following anaesthesia, a phenomenon known as anaesthesia-induced hypothermia (Buggy & Crossley, 2000). Anaesthesia-induced hypothermia has been shown to correlate with increased risk of developing AD and cognitive dysfunction (Almeida & Carrettiero, 2018; Buggy & Crossley, 2000), and pre-clinical studies in aged mice showed that anaesthesia-induced hypothermia caused increased Tau hyperphosphorylation and cognitive decline (Xiao et al., 2013). Despite KX anaesthesia has been shown to induce glymphatic function when maintaining the animals´ temperature at 37 ºC with heating carpets (Hablitz et al., 2019; Xie et al., 2013), the effect of KX anaesthesia-induced hypothermia on glymphatic system had

not been investigated by other groups when this thesis was written, and our results concerning this topic will be discussed in Paper V.

Aging effect on glymphatic system

In a study published in 2014, Kress et al. showed that the distribution of CSF tracers injected in the CM is impaired in middle-age and old mice, compared to young ones (Kress et al., 2014). These changes in CSF flow dynamics appear to be coupled to reduced pulsatility of arteries penetrating deep in the brain parenchyma. Moreover, loss of perivascular AQP4 polarisation was found in old mice compared to young ones, particularly at the level of the cortex, hippocampus and striatum, which were also the most affected regions by altered CSF influx (Kress et al., 2014). Moreover, old mice retained more an inert tracer exogenously injected in the brain parenchyma compared to young mice, suggesting that also CSF efflux and clearance of wastes is also impaired in the aging brain (Kress et al., 2014).

A follow-up study showed that similar pathways can be found in the aging human brain, where AQP4 expression is altered and its polarisation correlates positively with cognitive performance and negatively with amyloid burden (Zeppenfeld et al., 2017).

Interestingly, aging, which appears to have a detrimental effect on glymphatic function, has been also shown to be one major risk factors for developing neurodegenerative diseases, e.g. Alzheimer´s disease (AD) or Parkinson´s disease (PD) (Hou et al., 2019). The latter will be extensively discussed in the following paragraphs.

Parkinson´s disease

A brief description

Parkinson´s disease (PD) is the second most common neurodegenerative disorder worldwide. The first description of the disease can be found in a publication, entitled "*Essay on the Shaking Palsy*", written in 1817 by Dr. James Parkinson (Parkinson, 2002). He described for the first time, six cases of patients, joined by "*paralysis agitans*", an unusual motor behaviour characterized by bradykinesia, rigidity, tremors at rest and postural instability. These motor symptoms are still used for the diagnosis of the disease that was later named PD after him (Fahn, 2006).Yet, since this time much more knowledge has been accrued surrounding the pathophysiology of PD.

Many years before motor symptoms occur, different pre-motor symptoms can appear in PD patients, i.e. anosmia, constipation, depression and REM sleep disturbances. Other non-motor symptoms, i.e. cognitive decline and psychiatric symptoms, are associated instead with the latest stages of the disease (Simon et al., 2020; Sung & Nicholas, 2013).

PD is a multifactorial disease, and 85-90% of the total cases are considered sporadic, despite some genetic risk factors have been identified; however, only a 10-15% of the total cases shows an aetiology directly related to a genetic mutation (Simon et al., 2020).

Neuropathological hallmarks

From a histopathological point of view, PD is characterized by the progressive degeneration of Tyrosine Hydroxylase-positive (TH^+) Dopamine-releasing (DA) neurons (Figure 6), whose cell bodies lay in the substantia nigra pars compacta (SNpc) in the midbrain, and whose projections reach the basal ganglia, where they control movements through activation of the direct and indirect pathways (Poewe et al., 2017; Simon et al., 2020). It has been long thought that the onset of motor symptoms would reflect a neurodegeneration extent in the SNpc of 50-70% (Fearnley & Lees, 1991); however, recent studies suggest that motor symptoms correlate with degeneration of the DA projections in the basal ganglia, rather than SNpc DA neurodegeneration (Marras et al., 2018).

In all PD cases, except some rare genetic inherited forms (Schneider & Alcalay, 2017), DA neurodegeneration in the SNpc is accompanied by the presence of the so-called Lewy bodies (LB) and Lewy neurites (LN) (Poewe et al., 2017). The presence along all the brain regions of patients affected by "*paralysis agitans*" of intraneuronal globular or serpentine-like inclusions, named "*corpora amylacea*" and stained in bright red by Mann´s technique, was reported for the first time by Fritz Jacob Heinrich Lewy in 1912 (Engelhardt & Gomes, 2017). A later analysis of the components of LB and LN showed that ubiquitin was largely expressed in those inclusions, and therefore used as a main marker for their detection in brain tissue. Later in 1998, Maria Grazia Spillantini showed for the first time that alpha-synuclein (α-syn) was instead the major proteinaceous component of LB and LN (Spillantini et al., 1997) (Figure 6). Due to the nature of the LB and LN, PD is now referred as a "synucleopathy", together with other diseases like Dementia with Lewy Bodies (DLB), or Multiple System Atrophy (MSA), all characterized by the presence of α-syn inclusions (Takeda et al., 2006). Intriguingly, in 2003, based on post-mortem analysis of LB and LN in PD patients' brain specimens, Braak and colleagues proposed Lewy pathology in PD to be characterized by 6 stages (Braak et al., 2003). The so-called Braak´s hypothesis arises from the assumption that sporadic forms of PD may be caused by a double attack of unknown pathogens

against the olfactory neurons in the nasal cavity, and the enteric neurons of the enteric nervous system (ENS) in the gut (Braak et al., 2003; Rietdijk et al., 2017). From these locations, the Lewy pathology is supposed to spread towards the CNS via the olfactory nerves and the vagus nerve, with a very specific pattern. During stages I-II, the pathology is limited to the brainstem, in particular to the medulla, other than in the olfactory bulb; this stage is also known as prodromal, or pre-symptomatic, since PD-like motor symptoms do not appear yet, and usually only pre-motor symptoms, e.g. hyposmia/anosmia or constipation, may appear. During stages III-IV, the Lewy pathology extends to the pons, and from there to the midbrain and forebrain, and it is during these stages that motor symptoms and sleep disturbances start to occur. The latest stages of Lewy pathology (V-VI) are characterized by extensive inclusions in the neocortex, and it is during this stage that cognitive impairment and psychiatric symptoms start to affect PD patients (Braak et al., 2003; Goedert, 2015).

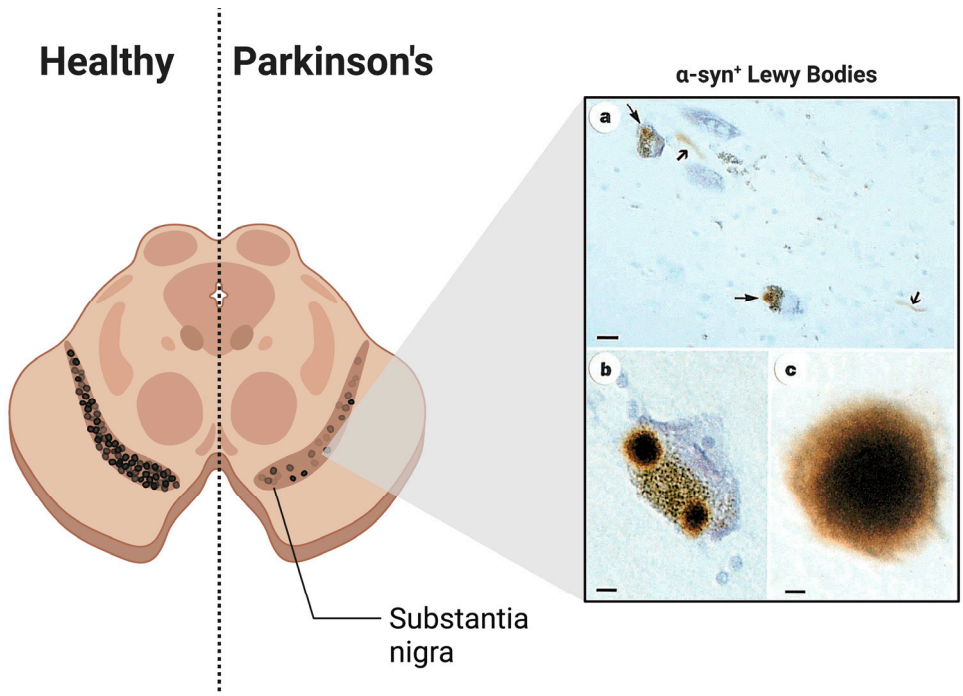

Figure 6: Neuropathological hallmarks of PD.
Schemating illustrating the SNpc of an healthy human and a patient affected by PD. The SNpc in PD patients is also charachterised by α-syn⁺ inclusions known as LB Thin arrown in the inset) and LN (thick arrow in the inset). Created with BioRender. Adapted from (Spillantini et al., 1997) under licensed permission.

α-synuclein involvement in PD

α-synuclein biology

α-synuclein (α-syn) is a 140-aminoacid soluble protein, encoded by the *SNCA* gene. α-syn is primarily expressed in neurons, where it is enriched at the synapses, but it is also found in the liver, in the muscle, in lymphocytes and red blood cells (Burré et al., 2018). The physiological functions of α-syn are unknown, but its cellular localisation and KO effects suggest that it is involved in regulation of neurotransmitters release, synaptic function, and plasticity (Lashuel et al., 2013). It has been shown that α-syn directly interacts with synaptic vesicles, controlling their trafficking from the synapsis reserve pool to the active zone. Moreover, it was found that α-syn directly interacts with proteins of the SNARE complex, which is involved in the release of neurotransmitters, DA included (Burré et al., 2010).

Structurally, α-syn is organised in three domains (Lashuel et al., 2013) (Figure 7):

I. amphipathic N-terminus domain (aa 1-64), involved in the interactions with biological membranes.

II. central region (aa 65-90), known as the non-amyloid-β domain (NAC), characterised by highly hydrophobic sequence that drives the aggregation of α-syn.

III. disordered C-terminus tail (aa 91-140), involved in the regulation of nuclear localisation and interactions with other proteins and intracellular solutes.

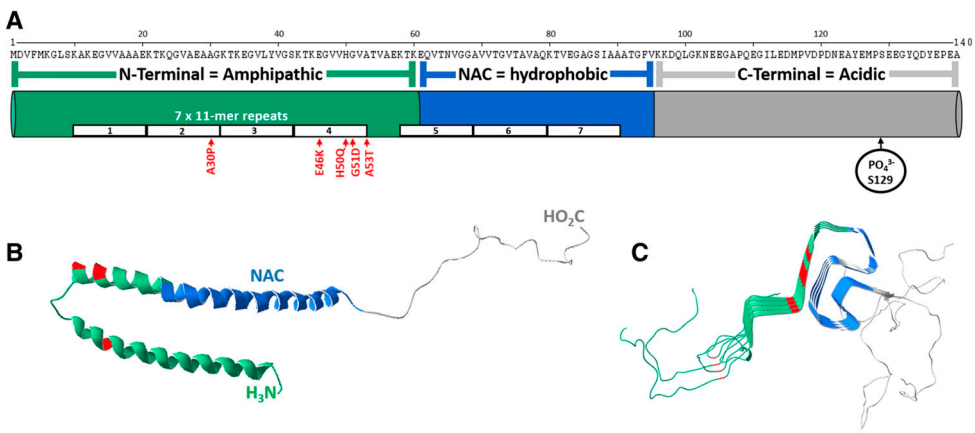

Figure 7: Structure of α-syn.
A) Aminoacidic sequence of α-syn, with the three domains in different colors. B) Molecular model of human α-syn bound to micelle. C) Molecular structure of a α-syn fibril formad by 4 monomors, obtained with cyo-electron mycroscopy. Reprinted from (Twohig & Nielsen, 2019).

From a conformational point of view, α-syn exists in the cells mainly as a soluble unfolded monomer (Eliezer et al., 2001); however, it shows an incredibly "conformational flexibility" (Ullman et al., 2011), meaning that it can rapidly change its conformation upon binding or interaction with biological membranes and other proteins (Ramakrishnan et al., 2006), and indeed it has been shown that, when bound to synthetic or biological membranes *in vitro*, α-syn adopts an α-helix structure (Chandra et al., 2003). The preponderant view at present is that, *in vivo*, native α-syn does exist in an equilibrium between different conformational configurations, and that specific stressors can induce conditions in the cells that are suitable for α-syn to adopt a conformation that would favour oligomerisation processes through formation of β-sheet structures (Villar-Piqué et al., 2016). Oligomers are considered the seeds for the formation of α-syn fibrillar structures that are insoluble (Conway et al., 2000, 2006), and therefore accumulate in the cells, forming the previously mentioned LB and LN. The specific mechanism through which α-syn takes part in PD pathogenesis will be discussed in the following paragraphs.

α-synuclein in PD pathogenesis

The first evidence that α-syn was involved in PD pathogenesis was provided in 1997, when Polymeropoulus et al. identified a mutation in the *SNCA* gene that was associated with familiar autosomal dominant PD (Polymeropoulos et al., 1997). Moreover, duplications or triplication of the *SNCA* gene are associated with higher levels of α-syn, contributing to PD pathogenesis (Singleton et al., 2003). Other common mutations in the *SNCA* gene, e.g. A53T, have been shown to reduce the affinity of monomeric α-syn to the plasmalemma, which therefore increases the concentration of available monomeric α-syn in the cytosol, pushing the equilibrium towards the formation of oligomers (Ramakrishnan et al., 2006). Mutations that affect post-translational modification, e.g. inducing increased phosphorylation of S129, promote fibrillization processes and enhances α-syn toxicity (Ma et al., 2016). Neurons have developed sophisticated pathways to manage this process, meaning that in cells, monomeric α-syn and its aggregates can be actively eliminated through proteolysis, interaction with molecular chaperones, proteasome and lysosomal-mediated autophagy (Cuervo et al., 2004; Klucken et al., 2004; Webb et al., 2003). However, many of these pathways appear to fail in PD, leading to the accumulation of insoluble intracellular inclusions of α-syn (Crews et al., 2010).

α-synuclein transmission and propagation

The failure of the cellular mechanisms for α-syn removal appears to promote a pathological release of α-syn from the so-called "donor cell" through different pathways, involving vesicles exocytosis, exosomal release, or trans-synaptic

delivery (Alvarez-Erviti et al., 2011; Emmanouilidou et al., 2010; Jang et al., 2010). Interestingly, a mechanism of passive leakage through damaged membrane has been reported to be exacerbated in presence of fibrillar α-syn, determining α-syn release in the extracellular space (Chaudhary et al., 2014; Volles & Lansbury, 2002).

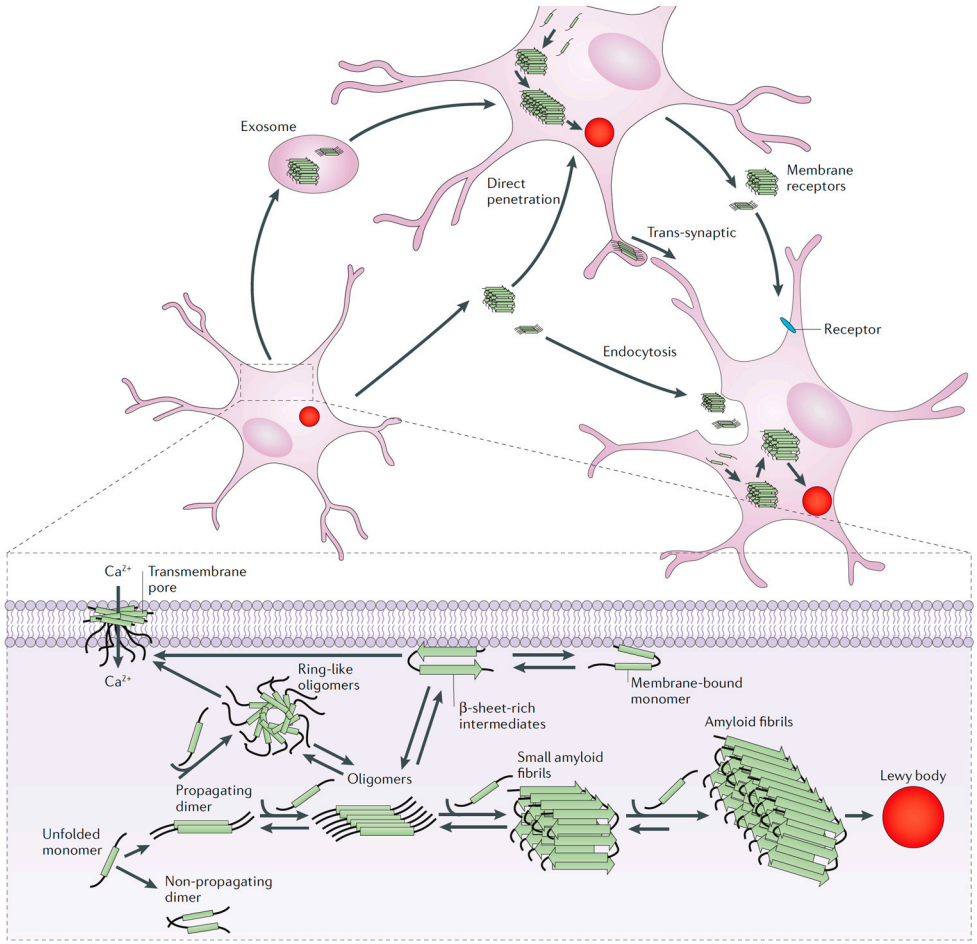

Figure 8: Mechanisms of α-syn toxicity and propagation.
Schematic showing the toxicity mechanisms of α-syn in the cell, that leads to the formation of LB. The schematic also shows the most common mechanisms of toxic α-syn propagation between cells. Reprinted with licensed permission from (Lashuel et al., 2013).

The toxic α-syn oligomers enter the "recipient cell" though receptor-mediated endocytosis, or direct penetration (Lashuel et al., 2013) (Figure 8). In every case, the result of this process is that the internalised α-syn oligomer is capable of starting a nucleation phase in the recipient cells, recruiting endogenous monomeric α-syn in the cytosol to be added to the forming fibrillar structure (Lashuel et al., 2013; Luk

et al., 2009) (Figure 8). It is proposed that this mechanism contribute to the propagation of α-syn pathology in the brain, and it seems to be in accordance with the Braak hypothesis, which suggests a "prion-like" transmission of α-syn pathology in the brain. The evidence that α-syn inclusions were found in fetal neurons grafted in the brain of PD patients during autopsies after several years from grafting (Chu & Kordower, 2010), and the evidence that LB and LN pathology, accompanied by PD motor symptoms, arises in the rodent brain after injection of α-syn fibrils (Abdelmotilib et al., 2017; Luk et al., 2012), further corroborates this mechanism as one of the main processes occurring in the pathogenesis of PD.

Mouse models to study PD

Mouse models of PD are extremely useful to mimic PD pathology in pre-clinical research, and therefore represent a useful tool for translational studies. However, it must be noted that the available mouse models show different pathological phenotypes, and usually do not recapitulate all the major hallmarks of the disease. In the following paragraphs, a description of the characteristics of the most used rodent models of PD in preclinical research will be provided (Figure 9).

Pharmacological neurotoxic models

This category includes mouse models where SNpc degeneration is achieved by injection of toxic compounds. Usually, these toxins act either through inhibition of the mitochondrial complex I, or through increase of intracellular reactive oxygen species (ROS). The result, in both cases is oxidative stress and mitochondrial dysfunction, ultimately leading to neuroinflammation, toxicity and neurodegeneration (Cenci & Björklund, 2020; Raza et al., 2019). Interestingly, it has been shown that DA neurons in the SNpc are particularly susceptible to this cascade (Dias et al., 2013).

The most common toxins used in these models are:

I. 1-methyl-4-phenyl-1,2,3,6-tetrahydroptridine (MPTP): this compound is able to cross the BBB and can be administered systemically (Langston J W et al., 1984). It is converted in the active compound MPP^+ that is selectively taken up by DA neurons through DA transporter (DAT). Within the cell it induces ROS formation and oxidative stress, and degeneration (Desai et al., 1996). Intriguingly, one study showed that mice implanted with osmotic mini-pumps for MPTP treatment can develop α-syn inclusion in the remaining DA neurons (Fornai et al., 2005), despite a failure to replicate these findings in a follow-up study (Alvarez-Fischer et al., 2008).

II. Rotenone: this compound can be administered orally, subcutaneously, intravenously, or intraperitoneally and exert its toxic effect on DA neurons though inhibition of the mitochondrial complex I. Also in this case, α-syn inclusion in the brain have been reported after rotenone administration (Betarbet et al., 2000; Raza et al., 2019).

III. 6-hydroxydopamine (6-OHDA): this compound is an analogue of DA (Jellinger et al., 1995). It is selectively taken up by DAT in the pre-synaptic terminal, where it is oxidized in ROS and induces mitochondrial dysfunction and oxidative stress (Kupsch et al., 2014; Rotman & Creveling, 1976). 6-OHDA, in comparison to the other toxins, does not cross the BBB, and therefore it needs to be directly injected in the brain through stereotaxic approaches to exert its toxic effect (Cenci & Björklund, 2020). This procedure allows for the possibility to choose between a model with an almost complete DA depletion, resembling the latest stages of PD in patients, or a less dramatic DA depletion, based on the injection site (SNpc and medial forebrain bundle-MFB, or striatum, respectively) (Francardo et al., 2011). However, no studies have reported the presence of α-syn inclusions in this model of PD (Cenci & Björklund, 2020). The 6-OHDA model was one of the two mouse models chosen in Paper IV of this thesis.

α-synuclein models

Whilst pharmacological toxic models are usually characterized by acute neurodegeneration, α-syn models have the advantage of allowing one to model progressive PD pathogenesis in the rodent brain, mimicking the steps of protein misfolding, aggregation and transmission. To achieve this, several approaches have been developed in the years:

I. Adeno-associated virus (AAV)-induced α-syn overexpression: in this model, α-syn overexpression induces progressive degenerative changes in the midbrain, and a strong Lewy-like pathology characterised by the presence of phosphorylated α-syn inclusions at Ser129 (Phospho-α-syn), a post-translational modification found also in LB and LN of PD brains (Volpicelli-Daley et al., 2016). This approach allows one to distinguish between a pre-symptomatic stage, and a symptomatic stage during which motor phenotype starts to occur, following DA degeneration (Decressac et al., 2012; Volpicelli-Daley et al., 2016).

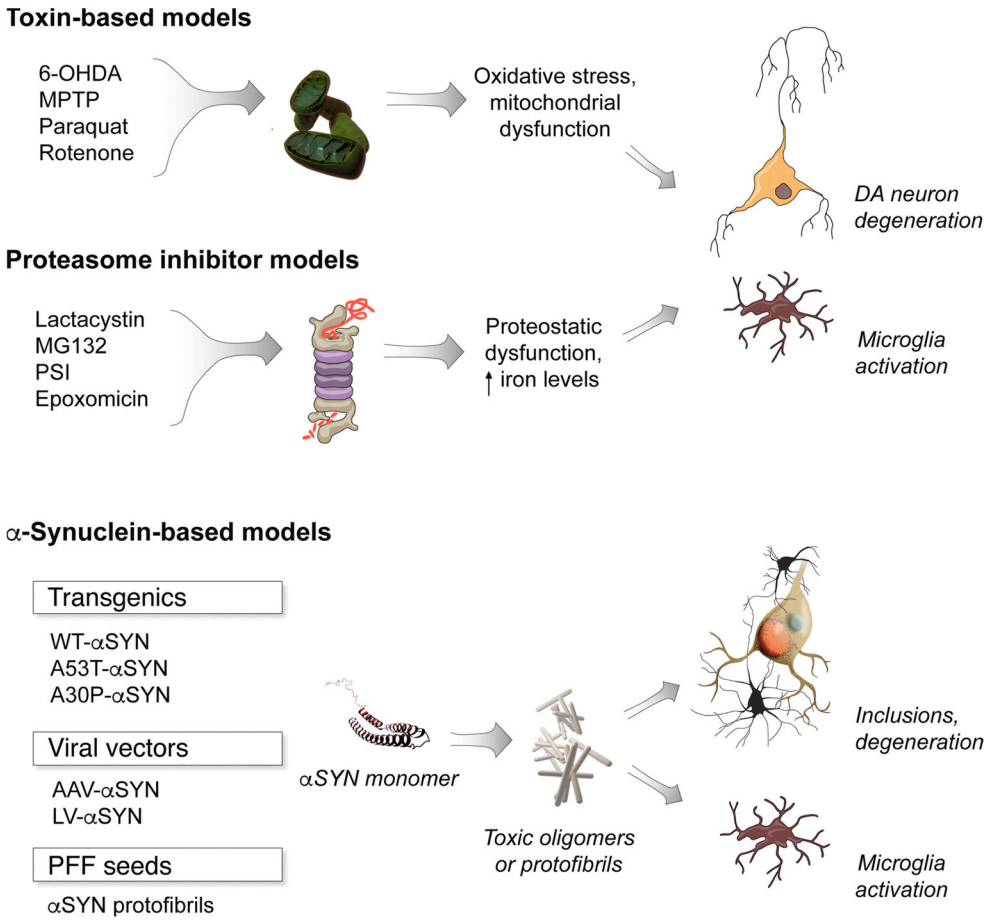

Figure 9: Mouse models of PD.
Schematic showing the mechanisms underlying PD pathology in the most common models of PD. Reprinted with licensed permission from (Cenci & Björklund, 2020).

II. Transgenic α-syn overexpressing mice: in this model, rodents (typically mice) are genetically modified to overexpress human α-syn, in wt or mutated forms. Depending on the promoter that drives the expression, α-syn transgenic mice can develop different phenotypes and the progression of the PD pathology can vary (Cenci & Björklund, 2020). A widely used α-syn transgenic model is the A53T line, where human α-syn carrying the A53T mutation is overexpressed in the mouse brain. A53T mutation in the *SNCA* gene encoding for α-syn in humans has been linked to early-onset familiar forms of PD, and it causes a faster fibrillization of oligomeric α-syn (L. Chen et al., 2015).

III. α-syn pre-formed fibrils (PFF) injection: in this model, α-syn exogenously treated to form fibrillary structures is injected in the brain (typically in the striatum or SNpc) where it acts as a seed for aggregation of endogenous α-syn once it is taken up by neurons in the surrounding area (Abdelmotilib et al., 2017; Luk et al., 2012). It has been shown that the exogenous α-syn must be of the same species of the endogenous one, to be able to act as a seed and induce the formation of α-syn inclusions that are phosphorylated at Ser129 (Luk et al., 2012). In this model, the inclusions start forming in brain regions functionally connected to injection site, suggesting that PFF are transported retrogradely, at least immediately after the injection, and then cell-to-cell transmission may happen at a later stage (Cenci & Björklund, 2020). Indeed, the injection of PFF in the striatum induces the formation of phosphorylated inclusions not only in the striatum, but also in the SNpc, amygdala, and neocortex, all regions synaptically connected to the striatum (Abdelmotilib et al., 2017). Compared to the AAV or the transgenic approaches, in this model the time required to develop phosphorylated inclusions, DA and motor deficits, is in the range of many months (Abdelmotilib et al., 2017; Luk et al., 2012), and therefore many researchers tend to adopt in their model a combination of AAV-induced α-syn expression and PFF inoculation or PFF inoculation in transgenic mice (Thakur et al., 2017). In Paper IV of this thesis, a simple inoculation of PFF in the striatum was chosen to obtain a α-syn mouse model.

What is glymphatic system contribution to brain diseases?

The “cleansing” function ascribed to the glymphatic system in the brain, and the evidence that aging has a detrimental effect on such function, have raised the hypothesis that glymphatic system may play a pivotal role in the pathogenesis of brain diseases, both acute and chronic neurodegenerative. Therefore, great effort has been made to elucidate the role of glymphatic system in pathological situations.

Acute brain conditions

Haemorrhagic and ischemic stroke

Different studies showed that CSF glymphatic influx in the brain is reduced in subarachnoid haemorrhage (SAH) in rats (Gaberel et al., 2014; Golanov et al., 2018) and non-human primates (Goulay et al., 2017), and it has been proposed that this impairment can reflect astrogliosis and loss of perivascular AQP4 localisation,

accompanied by increased Tau deposition and T-cells infiltration in the brain parenchyma (Pu et al., 2019). In a mouse model of acute ischemic stroke following middle cerebral artery occlusion (MCAO), it has been shown that minutes after the insult, the swelling of the tissue in the ischemic hemisphere is driven by rapid influx of CSF in the brain parenchyma, facilitated by the unaltered AQP4 polarisation at the astrocytic endfeet (Mestre et al., 2020). However, at later time point (i.e., 3 hours after MCAO), perivascular CSF influx is reduced, probably as a consequence of brain edema in the ischemic hemisphere (Gaberel et al., 2014).

Traumatic brain injury (TBI)

In pre-clinical studies, it has been shown that mild traumatic brain injury in rats is associated with acute impairment of glymphatic function, seen as reduced CSF contrast agent influx and efflux via MRI (Christensen et al., 2020). However, in mice it has been reported that CSF influx impairment lasts up to 28 days after the TBI procedure, and that efflux of radio-labelled solutes from the controlateral cortex is reduced 7 days after TBI (Iliff et al., 2014).

Specifically, TBI induces a rearrangement of perivascular AQP4 localisation, with consequent loss of polarisation. Moreover, increased phosphorylation of Tau is detected after TBI, and it is exacerbated in presence of AQP4 KO (Iliff et al., 2012, 2014). Intriguingly, it has been shown that after TBI, several biomarkers of injury show increased concentration in the serum (Plog et al., 2015). However, genetic, pharmacological, or mechanical disruption of glymphatic system, significantly reduces the clearance of these markers in the blood, suggesting that their transport in the blood happens through glymphatic pathways (Plog et al., 2015).

Neurodegenerative diseases

Alzheimer´s disease (AD)

Alzheimer´s disease is the most prominent neurodegenerative dementia, and it is characterized by neurodegeneration and cognitive decline, accompanied by extracellular amyloid-beta (Aβ) plaques deposition and intracellular hyperphosphorylated Tau inclusions, known as neurofibrillary tangles (NFTs) (Soria Lopez et al., 2019). In 2012, Iliff et al. showed that Aβ can be removed from the neuropil through the glymphatic system as the protein was retained in the brain parenchyma after injection in mice lacking AQP4 (Iliff et al., 2012). Since then, several pre-clinical studies have confirmed the relationship between glymphatic system and Aβ deposition in AD, adding evidence for a role of AQP4 in the process. The influx and clearance of radiolabelled Aβ is impaired in the APP/PS1 mouse model of AD (Peng et al., 2016). Another study showed that, in the same mouse model of AD, genetic AQP4 KO resulted in an increased load of Aβ plaques in the brain (Xu et al., 2015). However, Aβ is not the only pathological hallmark of AD,

although its extracellular presence as a soluble protein (before aggregating in Aβ plaques) made the hypothesis of its relationship to glymphatic system more intuitive. As stated above, AD is also characterized by the presence of hyperphosphorylated Tau in intracellular inclusions named NFTs (Long & Holtzman, 2019).

It has been proposed that intracellular Tau, in particular conditions, can assume a fibrillary structure rich in β-sheets, prone to aggregation in insoluble intracellular inclusions, i.e., NFTs (Guo et al., 2016; Long & Holtzman, 2019). The β-sheets-rich fibrillary Tau can spread in the brain with a pattern reminiscent of that already discussed for α-syn, based on the cell-to-cell transmission of Tau fibrillary seeds from a "donor" cell to a "recipient" cell (Braak & Braak, 1991; Vogels et al., 2020), where it will act as a template for nucleation of endogenous soluble Tau in insoluble fibrils.

Intriguingly, Harrison et al. showed that in the rTg4510 mouse model of Tau deposition, the caudal cortex was particularly impacted by reduced CSF-ISF exchange, compared to wt mice (Harrison et al., 2020). Congruently, injection of brain homogenates containing Tau either in the rostral or caudal cortex of rTg4510 mice resulted in higher levels of Tau in the CSF upon injection in the rostral cortex, compared to non-injected mice (Harrison et al., 2020). The clearance of Tau from the brain parenchyma was AQP4-dependent, demonstrated by the evidence that treatment with TGN-020 (a pharmacological antagonist of AQP4) decreased the concentration of total and phospho-Tau in the CSF. Moreover, only the caudal cortex, but not the rostral one, was impacted by loss of AQP4 polarisation at the astrocytic endfeet (Harrison et al., 2020).

This was the first study on its own to show that an intracellular protein like Tau could be cleared by glymphatic pathways, and most importantly could affect glymphatic function, pointing at the possibility that similar patterns could affect α-syn pathology in PD, a topic discussed in the paragraph below.

Parkinson´s disease (PD)

As previously stated, PD is the most prominent neurodegenerative motor disorder, accompanied in the latest stages of the pathology by cognitive decline, when the α-syn pathology reaches the neocortex (Braak et al., 2003). α-syn accumulates in the cell bodies and neurites of neurons in insoluble inclusions (Spillantini et al., 1997). Similarly to Tau, it has been shown that α-syn injected in the parenchyma is retained at the injection site in AQP4$^{-/-}$ (Zou et al., 2019) and AQP4$^{+/-}$ mice (Cui et al., 2021), suggesting that a partial decrease of AQP4 also has an effect on clearance of α-syn occurring though glymphatic pathways. Moreover, partial AQP4 KD in AQP4$^{+/-}$ mice worsened α-syn inclusion deposition pattern in various brain regions, increased the phosphorylated insoluble fraction of α-syn in the cortex and in the striatum, and exaggerated TH^{+} cells degeneration in the SNpc (Cui et al., 2021). The

study published by Zou et al. indirectly showed that the glymphatic system is impaired in PD, via ligation of deep cervical lymph nodes (dCLs) in the A53T transgenic mouse model of PD, where a mutant form of α-syn is overexpressed, causing an increased fibrillization (Zou et al., 2019). The authors showed that the meningeal pathway of CSF efflux was blocked upon ligation of dCLs, causing impaired drainage of CM-injected tracers in the dCLs and in the midbrain. Moreover, all α-syn forms (monomeric and oligomeric) were increased in the midbrain of A53T mice after dCLs ligation, as well as soluble and insoluble α-syn fractions (Zou et al., 2019). This phenotype consequently produced extensive TH^+ cells degeneration, motor impairment, GFAP and Iba1 immunoreactivity, and AQP4 mis-localisation. Interestingly, the same study showed that α-syn tended to accumulate in the PVS of brain vessels in the SNpc, suggesting that this pathway for CSF flow and glymphatic function may be disrupted in PD (Zou et al., 2019). This evidence was corroborated by human MRI studies showing that the index indicating the diffusion in PVSs is lower in PD patients compared to healthy controls (Chen et al., 2021). The evidence that CSF efflux though meningeal lymphatics is impaired in PD has been further demonstrated by Ding et al., showing with human MRI studies that patients with idiopathic PD exhibit impaired meningeal lymphatic drainage compared to cognitively normal controls (Ding et al., 2021). Moreover, they further confirmed Zou´s findings, showing that mice bilaterally injected in the striatum with α-syn PFFs exhibited decreased CSF drainage to dCLs, suggesting impaired meningeal lymphatic efflux, possibly related to the disruption of tight junctions in the lymphatic endothelial cells (Ding et al., 2021).

The association of glymphatic function with slow-wave sleep (Hablitz et al., 2019), and the evidence that sleep disturbances are often reported by patients affected by neurodegenerative disease, PD included (Sung & Nicholas, 2013), have been previously discussed. Interestingly, Morawska et al. showed that chronic treatment with a compound able to improve slow-waves sleep in the vesicular monoamine transporter 2 (VMAT2)-deficient (LO) mouse model of synucleopathy, ameliorated α-syn deposition in the prefrontal cortex (PFC), although this intervention did not have any positive effect on the motor behaviour (Morawska et al., 2021). Moreover, this treatment was also associated with increased perivascular localisation of AQP4 in the PFC of LO mice. A sleep deprivation regimen had instead the opposite effect. These results were further confirmed in the A53T transgenic mouse model of PD (Morawska et al., 2021).

Despite all these studies pointing to a link between α-syn deposition in PD, glymphatic function and AQP4, a direct proof that glymphatic system is impaired in PD has yet to be established, and this topic will be extensively investigated and discussed in Paper IV of this thesis.

Aims of the thesis

The aim of this thesis is to describe methodologies useful for glymphatic studies in rodents, and to apply these methods to study CSF movement in the brain both in physiological and pathological settings, i.e., hypothermia and PD.

The specific aims of the papers and manuscripts included in this thesis are:

Paper I

Describe the CM injection method to study glymphatic function in the rodents´ brain.

Paper II

Quantitatively compare the efficiency of different lectins and staining methods to label vasculature in rodents; this step is important for AQP4 polarisation studies.

Paper III

Investigate how AQP4 is affected by antisense oligonucleotides (ASO) targeting *Aqp4* mRNA.

Paper IV

Investigate glymphatic system function in different mouse models of PD, and test whether glymphatic system is involved in the clearance of α-syn from the brain parenchyma.

Paper V

Explore glymphatic function and AQP4 in hypothermia, a condition often related to anaesthesia.

Methods

In this chapter, a summary of the methods used in Papers I-V will be described. However, for the specific methods of each paper and manuscript, the reader is highly recommended to refer to the final version attached at the end of this thesis.

Animals

Paper I describes the CM procedure in adult Sprague Dawley rats, aged 10 weeks. In **Papers II-V**, C57BL/6 adult mice (ranging between 12 and 16 weeks of age) were used. C57BL/6 mice were purchased either by Charles River (in Papers II-V) or Janvier (in Paper II). In Paper II, transgenic mice expressing eGFP under control of Tie-2 promotor were purchased from The Jackson Laboratory (stock No. 003658)

All mice were kept in standard laboratory conditions, with a 12 hour dark-light cycle and *ad libitum* access to water and food. The experiments were conducted according to the ethical permits: M47-16 (Paper IV); 5.8.18-08269/2019 and updated version 5.8.18-20240/2021 (Papers II-V) approved by the Malmö-Lund ethical committee on animal research, Jordbruksverket.

Transient MCAO (tMCAO) experiments in Paper II were conducted according ethical permit no 81-02.04.2019.A214/01 approved by Landesamt für Natur-, Umwelt- und Verbraucherschutz, whilst experiments with Tie-2-eGFP transgenic mice in Paper II were approved by the University of Rochester Medical Center Committee on Animal Resources.

Stereotaxic surgeries

In **Papers III** and **IV**, stereotaxic surgery procedures were adopted to inject different substances in specific brain regions. In this section, it will first be described the common procedure, and then the details of the injections in each paper.

Briefly, mice were anesthetized via 2.5% isoflurane in atmospheric air for anaesthesia induction and, once their reflexes ceased, they were fixed on a stereotaxic frame adapted for mice surgery. 1.5-2% isoflurane in atmospheric air

was used for anaesthesia maintenance and infused through a nosecone positioned on the nose of the mouse. The body temperature was kept stable using a heating pad to avoid anaesthesia-induced hypothermia. The skin of the head covering the skull was sterilised using Chlorhexidine before an incision was made with a scalpel along the antero-posterior axis of the skull. After exposing the skull, mediolateral and antero-posterior coordinates were checked in comparison to the Bregma coordinates, to assure that the skull was flat. Then, the stereotaxic apparatus was used to move the glass capillary mounted on a Hamilton syringe at coordinates in correspondence of the injection site, where a craniotomy was made using a dental drill, paying attention to not damage the dura mater. Solutions were injected through the glass capillary connected to a Hamilton syringe. To avoid backflow, the capillary was left in place for 2 min before and 4 min after the injection. After the surgical procedure was completed, the skin on the head was sutured applying tissue glue (Histoacryl®) and a local analgesic treatment was injected subcutaneously in the wound area (Marcain, bupivacaine, 2.5 mg/ml). Mice were allowed to recover in a heated cage until they woke up from the anaesthesia, and then were returned to their home-cage.

In **Paper III**, 10 µl of AQP4-targeting ASO and control CTRL ASO (scrambled sequence) were injected i.c.v. into the right lateral ventricle at the following coordinates: AP = 0.3; ML = -1 (right); DV = -3 from the skull surface, according to the manufacturer's instructions (Ionis Pharmaceuticals). Wt group did not undergo any surgical procedure and was used as a second control group.

In **Paper IV**, to study the effect of DA signalling depletion on glymphatic function, 1 µl of 6-OHDA toxin (concentration: 3.5 µg/µl in 0.02% ascorbic acid) was injected unilaterally in the MFB at the following coordinates: AP= -0,7; ML= 1,2; DV= -4,7 (from the dura mater). The injection rate was 0.2 µl/min. SHAM mice underwent the same procedure, with the exception that no solution was injected in the MFB, and the needle was left in place for the same amount of time. Importantly, due to the high mortality following 6-OHDA lesion, mice injected with 6-OHDA underwent a post-operative care regime up to two weeks after the surgery, meaning that they were kept in a heating cabinet to avoid hypothermia, received injections of 0.9% NaCl, and were fed with a high calorie diet (Dietgel).

To study the involvement of glymphatic system in the progression of Lewy pathology in PD, 10 µg of mPFFs were injected bilaterally in the dorsal striatum at the following coordinates: AP= 1.0; ML= ±1.85; DV= -3.0 (from the skull surface). The injection rate was 0.2 µl/min. The control group underwent the same procedure, but the corresponding volume of artificial CSF (aCSF) was injected in the dorsal striatum instead of mPFFs.

To study the involvement of glymphatic system in the removal of α-syn from the brain, 10 µg of ATTO-647 labelled human PFFs (ATTO-647 hPFFs) were injected unilaterally in the SNpc at the following coordinates: AP= -3.1; ML= 1.2; DV= -3.8

(from the dura surface), or in the dorsal striatum (see coordinates above). The injection rate was 0.2 µl/min. The control group underwent the same procedure, with the exception that 10 µg of ATTO-647 labelled BSA-PFFs (ATTO-647 BSA-PFFs) were injected.

Behavioural assessment

Cylinder test

In **Paper IV**, to assess DA denervation in 6-OHDA lesioned mice, 1 or 5 weeks after the lesion, mice were placed in a clear glass cylinder (∅ 10 cm, height 14cm) to assess their forelimb asymmetry. The cylinder test allows to test the spontaneous limb use during vertical exploratory behaviour in rodents, as previously described (Andreoli et al., 2021; Francardo et al., 2011). Mice were recorded for 3 to 10 minutes, in order to score at least 10 total contacts between both the forelimbs (ipsilateral and contralateral) and the wall of the glass cylinder. The use of the paw contralateral to the lesion side (left paw) was expressed as a % of the total number of contacts. Exclusion criteria was contralateral forelimb use > 30%, to ensure that only animals with a nearly complete DA denervation of motor striatal regions were included in the study. All mice exhibiting contralateral forelimb use > 30% were excluded from the study (Andreoli et al., 2021).

Rotarod

In **Paper IV**, mPFFs or aCSF were injected bilaterally in the striata of wt mice. Therefore, behavioural tests to assess forelimb asymmetry, i.e. cylinder test, could not be used in these mice. We evaluated then motor coordination and motor learning in mPFFs and aCSF injected mice, implementing an accelerating rotarod behavioural paradigm, modified from Sebastianutto et al. (Sebastianutto et al., 2017). The protocol consisted in accelerating the rotating rod from 4 rotation per minute (rpm) to 40 rpm, with constant acceleration, over a period of 300 seconds. Mice were trained on day 1 (training day) and then tested for 3 consecutive days (test day 1-3), and underwent 3 trials per day, 15 minutes apart each. The very first trial on the training day was used as habituation and therefore not included in the analysis.

Hypothermia treatment

In **Paper V**, wt mice underwent different hypothermia regimes to test whether anaesthesia-induced hypothermia could affect glymphatic function. To ensure an activation of the glymphatic system, mice were anaesthetised with an i.p. injection of a mixture of ketamine (Ketaminol, 100mg/kg) and xylazine (Rompun, 20mg/kg) solved in 0.9% sodium chloride. Indeed, as previously discussed, KX anaesthetic appears to induce a pattern of slow waves delta oscillations in EEG most similar to those seen during physiological sleep, which is a physiological booster of glymphatic function.

After anaesthesia was induced and mice reflexes ceased, they were placed on a physiological monitoring apparatus connected to a mouse adaptor for head fixation, equipped with a rectal thermometer system for body temperature maintenance. This allowed to record hearth and respiration rate during the entire duration of the experiment. The temperatures of the physiological system during the experiment were set as follows for the different experimental groups: normothermia = 37°C; mild hypothermia = 33°C; moderate hypothermia = 30°C. In the acute cohort, the normothermia or hypothermia regimes were kept for 60 minutes, before the CM injection of CSF tracer was done (see below). In the repeated cohort, the normothermia or hypothermia regimes were induced in mice every day for 4 consecutive days, and on Day 5 they underwent CM injection (see below).

CM injections

CM injection was described in detail in **Paper I** and was subsequently used in **Paper IV** and **Paper V** to test glymphatic function in PD mouse models and in a model of anaesthesia-induced hypothermia, respectively.

The procedure was carried similarly to as described in (Xavier et al., 2018). Mice were weight and were injected with 10 µl/g of body weight with the KX anaesthetic mix, prepared as described above. When the reflexes ceased, mice were mounted on a stereotaxic frame or on a physiological monitoring apparatus connected to a mouse adaptor for head fixation, and the head was positioned in a way that the nose was pointing slightly downwards. After locating the occipital skull crest, an incision was made on the skin in its proximity, exposing the muscles of the neck. The CM can be exposed by pulling apart the neck muscles along the midline and can be seen as a darker triangle pointing down, covered by the dura membrane. The CSF tracer (total volume = 10 µl) is injected through a dental needle connected to a PE tube attached to a Hamilton syringe, at a constant rate of 1 µl/min, after punching of the dura mater covering the CM. It must be noted that the approved ethical permit for animal experiments did not allow recovery after CM injection, therefore this procedure has

always been terminal in our experiments. Animals' euthanasia at the end of the CM injection has been performed as describe below.

The fluorescent CSF tracers used in Papers IV and V was Bovine serum albumin (BSA) conjugated to the fluorophore AlexaFluor647, (BSA-647, 65 kDa).

Euthanasia and sample collection

Decapitation

In **Paper IV** and **Paper V** mice were terminated after CSF tracer circulation (30 minutes after the end of the tracer injection in the CM) by decapitation while they were still under KX anaesthesia. Decapitation was chosen over transcardial perfusion since studies showed that infusion of fixative agents, e.g. PFA, cause collapse of PVSs, affecting CSF tracer distribution as well (Mestre, Tithof, et al., 2018). Organs of interest, including brain, CL and vertebral column were collected directly after and post-fixed by overnight immersion in PFA 4%. Spinal cords were dissected from the vertebral columns after overnight post-fixation in PFA 4%.

In **Paper III**, mice were anesthetised with KX and then decapitated to collect the brain. The cerebellums were separated and snap-frozen for mRNA and protein extraction (see below for details). The rest of the brain was post-fixed by overnight immersion in PFA 4% for sectioning and immunofluorescence stainings (see below for further details).

Perfusion

In **Paper II**, to perform lectins labelling of blood vessels, wt mice were anesthetised with KX and then perfused via transcardial injection of 1x phosphate-buffered saline (1x PBS) into the bloodstream to remove any trace of blood from the tissue. Fixation of the tissues occurred following transcardial perfusion of PFA 4%. Then, the organs of interest were collected and post-fixed by overnight immersion in PFA 4% before sectioning for immunofluorescence stainings (see below for further details).

For the comparison of the two methods for labelling blood vessels with lectins, i.e. immunohistochemical approach and injection of lectins directly in the bloodstream, lyophilised WGA lectin was solved in PBS 1x at different concentrations, and injected as an intermediate step during transcardial perfusion. Specifically, after transcardial injection of 1x PBS, 5ml of WGA lectin solution at the desired concentration were injected and allowed to bind to blood vessels for 2 minutes, before 4% PFA was infused for tissue fixation.

In **Paper IV** and **Paper V**, transcardial perfusion with 1x PBS followed by 4% PFA was used when the samples were taken for iDISCO+ tissue optical clearing and light sheet imaging (see below for more details).

Tissue processing

Vibratome sectioning and immunohistochemistry

After overnight post-fixation in PFA 4%, brains were washed in 1x PBS before being glued to an appropriate holder for vibratome sectioning. 100 µm thick sections were collected in 24-well plates and stored until used for experiments. All brains in **Papers II-V** were sectioned following this same procedure.

In **Paper IV** and **Paper V**, brain sections at coordinates AP from Bregma -2, -1, 0, 1, 2 (plus 5 sections along the SNpc in Paper IV) were mounted onto a Superfrost glass slide and coverslipped with Fluoromont mounting media, before being imaged with a fluorescent microscope (see below) to assess fluorescent CSF tracer penetrance in the brain parenchyma.

Immunofluorescence

Immunohistochemistry was performed through an immunofluorescent staining approach of free-floating sections in **Papers II-V**.

Different markers were used, i.e. AQP4, Lectins, GLUT-1, Iba-1, TH, phospho-Ser139 α-syn. For the specific protocols the reader is suggested to refer to the methods section of the Papers attached at the end of this thesis.

First, an antigen retrieval step, e.g. incubation in Sodium Citrate Buffer pH 6.00, for 30 min at 37 ºC, was carried out; this step, although facultative, is necessary to break the bonds formed during fixation of the tissue, that may cause inaccessibility of the epitope recognised by the primary antibody. The first step was followed by a blocking and permeabilization step, e.g. incubation with Normal Donkey Serum or BSA in Triton X, 1 hour at room temperature under gentle shacking. This second step is necessary to permeabilise the cellular membranes and allow the antibodies to bind to their epitope, but also to block any unspecific binding of the antibodies that will result in background signal during imaging sessions. Then, an incubation with the desired primary antibody/ies, specifically designed to bound to an epitope expressed on the marker of interest, is performed. This step is usually carried out overnight at 4 ºC under gentle shacking, but in some cases a longer incubation may be required. The primary antibody incubation is followed by washes with PBS 1x to remove the excess of non-bound primary antibody/ies. To ensure signal enhancement, an incubation with secondary antibody/ies conjugated to a fluorescent

fluorophore and directed against the primary antibodies, follows. This step is usually carried out at room temperature for 1.5-2 hours under gentle shacking. Then, the removal of non-bound secondary antibody/ies is achieved with PBS 1x washes. If Lectin is used as a marker of blood vessels, an incubation with the desired lectin solution is carried out for 1 hour at room temperature under gentle shacking, before a further incubation with 4′,6-diamidin-2-fenilindolo (DAPI) solution to visualise cellular nuclei. After PBS 1x washes to remove the excess of Lectin and DAPI, the tissue is mounted on Superfrost glass slide and coverslipped with Fluoromont mounting media.

mRNA and protein extraction

In **Paper III**, RNA and protein isolation from mouse cerebellums was performed as previously described with a few modifications (Stegmayr et al., 2021).

To extract the mRNA, the tissue was homogenised in TRIzol with the help of a Tissue Lyzer machine. Then, the samples were incubated for 10 minutes on ice. Phase separation was achieved upon addition of chloroform at 20% of TRIzol volume, followed by incubation for 15 minutes at room temperature. Then, the samples were centrifuged at 12000 g for 15 min at 4°C and transferred to an RNA binding column, after mixing the clear aqueous phase with equal volume of 70% ethanol. The remaining phases were stored at -80°C for protein isolation (see below). Finally, the RNA was purified and eluted with 50 µl RNase/DNase free water.

To carry our protein extraction, the interphase and phenol phase from the RNA isolation were thawed on ice and any residual aqueous phase was removed. Then the DNA was precipitated upon addition of 100% ethanol, and proteins were precipitated upon addition of 150% volume of isopropanol, followed by incubation at room temperature for 10 minutes and centrifugation at 12,000xg for 10 minutes at 4°C. Protein pellets were washed with guanidine-ethanol solution (x3) followed by 100% ethanol, and then were air-dried, before they were broken in 50ul urea/DTT using a needle. 450ul urea/DTT was added and incubated at room temperature for 2 hours, and then the samples were incubated at 95°C for 3 minutes before immediately placing them on ice. A sonication was carried out using short bursts for 3 minutes, and then the samples were vortexed until the pellets were completely dissolved. Supernatants were collected after centrifugation at 10,000xg at room temperature, and Bradford assay was used to determine protein concentrations.

qPCR

After the purification step, NanoDrop was used to assess RNA concentration. 1 µg of RNA was used in the RT reaction using a transcription kit available for purchase. SYBR Green supermix was used for the qPCR, with an annealing temperature of 56 °C.

The primers used in **Paper III** were:

- Aqp4 total, forward: 5'-CTGGAGCCAGCATGAATCCAG-3', reverse: 5'-TTCTTCTCTTCTCCACGGTCA-3';
- Aqp4 M1 isoform, forward: 5'-CCCGTAATCTGACTCCCAGTG-3', reverse: 5'-GGAATGTCCACACTTACCCCA-3';
- Aqp4 M23 isoform, forward: 5'-TTATGGTTCACGGGTTTGGATG-3', reverse: 5'-TGGTGACTCCCAATCCTCCAAC-3';
- Hprt, forward: 5'- CCTAAGATGAGCGCAAGTTGA-3', reverse: 5'-CCACAGGACTAGAACACCTGCTAA-3'.

ΔCt was calculated as: $Ct_{Hprt} - Ct_{Aqp4}$.

WB

In **Paper III**, gel electrophoresis was performed using 12% Tris-HCl gels, loaded with 15µg protein/sample. To ensure the separation of AQP4 isoforms, the gels were run at 200V until the loading buffer dye escaped the gel. Then, nitrocellulose membranes with 0.2µM pore size were used for wet transfer at 100V for 1 hour on ice. The blots were incubated with blocking solution (5% Bovine Serum Albumin in TBST), followed by incubation with AQP4 antibody and β-tubulin (for normalisation). The blots were imaged after development of the signal with ECL kit. Finally, the bands of interest were quantified using ImageLab software.

iDISCO$^+$ tissue clearing

In **Paper IV** and **Paper V**, upon completion of the transcardial perfusion, the samples planned for tissue clearing were extracted and post-fixed overnight in 4% PFA. Then, an overnight incubation in EDTA solution was used for decalcification, if bone was present. Finally, the iDISCO+ (immunolabeling-enabled three-dimensional imaging of solvent-cleared organs) protocol was carried out as previously described (Bèchet et al., 2020). Briefly, tissues were dehydrated in increasing methanol /H2O series (20%, 40%, 60%, 80%, 100%, 100%, 1 hour each), delipidated with methanol/dichloromethane (33%/66% for 3 hours), and pure dichloromethane (2 times, 15 min each), and optically cleared with Ethyl Cinnamate (ECi) for at least 1 week prior to imaging with light sheet microscopy (see below).

Imaging and analysis

In vivo imaging and fluorescent microscopy

At the end of the behavioural assessment of mPFFs and aCSF mice in **Paper IV**, and at the end of the hypothermia treatment in **Paper V**, mice underwent a session of *in vivo* imaging of the CSF fluorescent tracer injected in the CM through the intact skull. The skin on the skull surface was removed, and after CM cannulation, mice were moved under a SMZ25 Nikon fluorescent Stereo Microscope for imaging every minute starting at T_0 (beginning of intracisternal infusion) until the end of the circulation time (30 minutes after the end of CSF tracer infusion). At the end of the tracer circulation time, the skin covering the neck region was removed, and the sCL exposed, allowing *in vivo* imaging of the tracer.

The CSF tracer distribution in the whole brain, or in other organs of interests, was assessed via imaging of the whole samples with a SMZ25 Nikon fluorescent Stereo Microscope.

The CSF tracer distribution in the brain parenchyma was assessed via imaging of brain sections obtained with vibratome with a Nikon Ti2 Eclipse fluorescent microscope.

Images of the brain *in vivo* and *ex vivo*, or of other organs of interest were analysed using Fiji software, drawing a region of interest (ROI) around the area were information on the CSF tracer intensity was needed, and the mean intensity was calculated automatically by the software. The mean intensity of the CSF tracer in brain sections images was analysed using an automatized software developed as a Macro for Fiji.

Confocal microscopy

Immunofluorescent stainings in **Papers II-V** were imaged using a Nikon A1RHD confocal scanning microscope. The acquired images were analysed using Fiji software, as follows:

- AQP4 polarisation and lectins labelling efficacy were calculated as the ratio between the AQP4/Lectin signal at the vessel walls and the AQP4/Lectin background signal obtained drawing a cross-section of the vessels using the line-plot tool of Fiji, as described previously (Manouchehrian et al., 2021; Munk et al., 2019).
- The colocalization of LEA lectin signal with GLUT-1 signal was conducted identifying $Tie2^+$ vessels and assessing the labelling with the other two

markers, and points were assigned for each LEA and GLUT-1 labelling: 1 = stained; 0 = non-stained .

- The analysis of LEA lectin specificity in labelling blood vessels was performed identifying the number of GLUT-1^+ blood vessels and subtracting this number to the number of total elements labelled by LEA lectin .
- TH+ cells were manually counted in acquired images, after maximum intensity projection (MaxIP) processing.
- phospho-Ser139 α-syn^+ inclusions were counted in acquired images, after MaxIP processing, automatically through the Analyse Particle function.

Light-sheet microscopy

In Paper IV and Paper V, the samples treated with the iDISCO+ tissue clearing protocol were imaged with light-sheet imaging using LaVision-Miltenyi Biotec Blaze microscope.

In **Paper IV**, optically cleared tissues (head-neck region) of mice injected either with ATTO-647 hPFFs or ATTO-647 BSA-PFFs, were imaged with light-sheet microscope at 1x magnification, step size of 4µm, and mosaic acquisition setting to obtain consecutive z-stacks with 10% overlap. The mosaic acquisition was stitched manually in Arivis Vision4D to allow 3D reconstruction and visualisation of the raw data.

In **Paper V**, optically cleared brains of mice treated with normothermia, mild hypothermia and moderate hypothermia regimes were imaged as a single z-stack with the 1.1x objective and a step size of 2µm. 3D reconstructions were done in Arivis Vision4D and were analysed with the Intensity Threshold Segmenter tool, which automatically calculated the sum voxel intensity and total volume of all voxels with an intensity value above the threshold. The analysis of PVSs was done as previously described (Bèchet et al., 2021b). Briefly, orthogonal views of the inner cortical surface were generated to obtain a cross-sectional view of the PVSs, that were counted to obtain the density and cortical area coverage.

Statistical analysis

All statistical analysis in this thesis was performed using GraphPad Prism software. Normality of the data was tested. If the data were normally distributed, the following statistical tests were applied: student t-test was used to compare two groups (paired t-test for dependent groups, and unpaired t-test for independent groups). When

comparing more than two groups, One-Way ANOVA followed by Tukey´s post-hoc comparison was used. If more than one variable wanted to be assessed, Two-Way ANOVA followed by Tukey´s post-hoc comparison was used. If the data failed to show normal distribution, non-parametric tests were used: Mann–Whitney test for non-paired data, Wilcoxon test for paired data, and Kruskal–Wallis test for comparisons between more than two groups. All values are expressed as the mean ± SEM, and $p < 0.05$ was accepted as statistically significant.

Key results

In this chapter, a summary of the results of the papers and manuscripts included in this thesis will be presented. However, the reader is highly recommended to refer to the final version of the papers and manuscripts attached at the end of the thesis for further details.

Paper I: CM injection of fluorescent tracers is a valuable method to label CSF and study glymphatic function.

In **Paper I**, we report a detailed description on how to perform a CM injection to study glymphatic system in rats with a standardised and reproducible procedure (Figure 10). This procedure can be easily adapted to studies in other animal models, e.g. mice (Xavier et al., 2018) or even pigs (Bèchet et al., 2021a). Moreover, we provide an extensive list of the needed equipment, and tips for a successful injection.

Here there is a brief description of the procedure. Rats were weight and were injected with 10 µl/g of body weight with the KX anaesthetic mix (Ketamine 100mg/kg, Xylazine 20mg/kg). When the reflexes ceased, rats were shaved around the neck, in proximity of the end of the skull, before placing them on a stereotaxic frame. The head was positioned in a way that the nose was pointing slightly downwards. After locating the occipital skull crest, an incision was made on the skin in its proximity, exposing the muscles of the neck. The CM can be exposed by pulling apart the neck muscles along the midline and can be seen as a darker triangle pointing down, covered by the dura membrane. The CSF tracer (total volume = 10/20 µl) is injected through a dental needle connected to a PE tube attached to a Hamilton syringe, at a constant rate of 1 µl/min, after punching of the dura mater covering the CM.

Notably, the same procedure can be used for injection of tracers other than fluorescent ones, e.g. contrast agents for *in vivo* MRI detection of CSF flow dynamics (Iliff, Lee, et al., 2013).

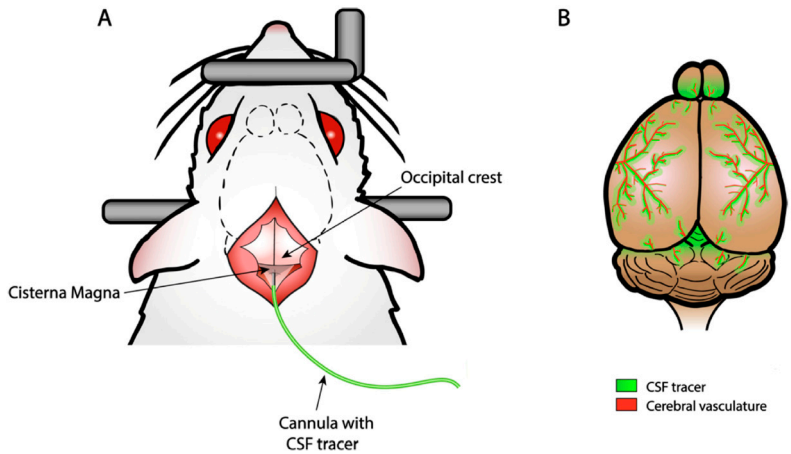

Figure 10: Schematic representation of the CM injection procedure in rats
A) Schematic representation of the surgical procedure for cannula insertion in the CM of rats. B) Schematic of a rat brain with highlighted CSF influx (green) in the PVSs surrounding brain vasculature (red), 30 minutes after the end of the CM injection.

Paper II: Lectins efficacy in labelling blood vessels depends on the carbohydrates binding affinities and labelling method.

In **Paper II**, we aimed to test three of the most common lectins used in literature to label blood vessels (*Lycopersicum Esculentum Agglutinin,* LEA; *Griffonia Simplicifolia* isolectin B4, IB4; *Wheat Germ Agglutinin*, WGA) since the lack of a systematic comparison of their labelling efficacy in literature has been hampering the possibility to compare results in different studies (Figure 11).

We showed that LEA had the best labelling efficacy in labelling cerebral blood vessels, seen as the quantification of the signal-to-noise ratio at the blood vessel wall in brain sections after histological staining (Figure 11, A-D). Moreover, we showed that the injection of WGA lectin in the bloodstream during terminal transcardial perfusion ameliorated the labelling efficacy of the lectin that had the worst readout in histological staining of brain sections. The signal-to-noise ratio of the highest concentration of WGA used was similar to the one of the best lectin staining in histology (Figure 11, E-I). This suggested that the method used to label blood vessel can impact the labelling efficacy.

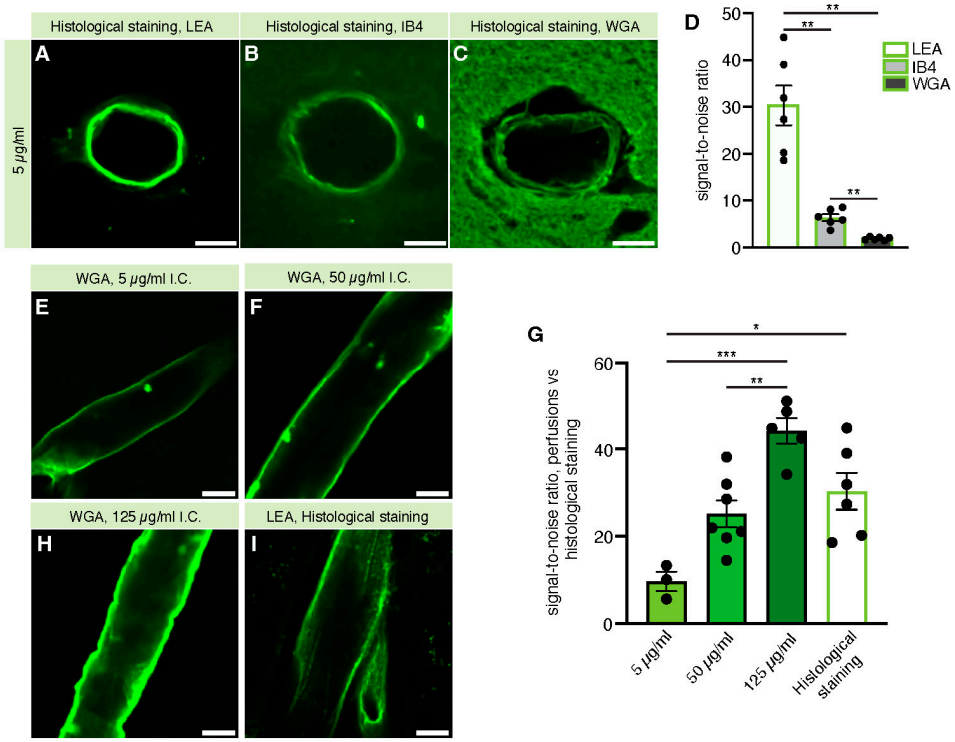

Figure 11: Labeling efficacy of different lectins and staining methods.
A-C) Representative confocal images of coronal blood vessels stained with three different lectins: LEA, *Lycopersicum Esculentum Agglutinin*; IB4, *Griffonia Simplicifolia* isolectin B4; WGA, *Wheat Germ Agglutinin*. Scale bars: 10 µm. D) Quantification of the signal-to-noise ratio of the three lectin labeling cerebral blood vessels. E-I) Comparison of labeling efficacy of histological staining with LEA vs transcardial perfusion of increasing concentrations of WGA lectin in the bloodstream. Scale bars: 10 µm.

Moreover, we showed that lectin labelling of blood vessels after infusion in the bloodstream, is overlapping with Tie-2 signal in transgenic mice, and with GLUT-1 histological staining, suggesting that lectin specificity towards blood vessels is comparable to other commonly used markers for cerebral vasculature (Figure 12, A-B). Intriguingly, we found that in the infarcted hemisphere of mice affected by ischemic stroke after transient MCAO (tMCAO), the specificity of LEA lectin for blood vessels was decreased (Figure 12, C-F). Using ionized calcium binding adaptor molecule 1 (Iba1) as a marker for microglia, we showed that LEA signal overlapped with Iba1 signal in the infarcted hemisphere, suggesting that lectin started labelling microglia. We hypothesised that in the infarcted hemisphere, microglia start expressing new carbohydrates residues on the plasmalemma, that can be recognised and bound by LEA lectin, therefore decreasing the specificity for blood vessels.

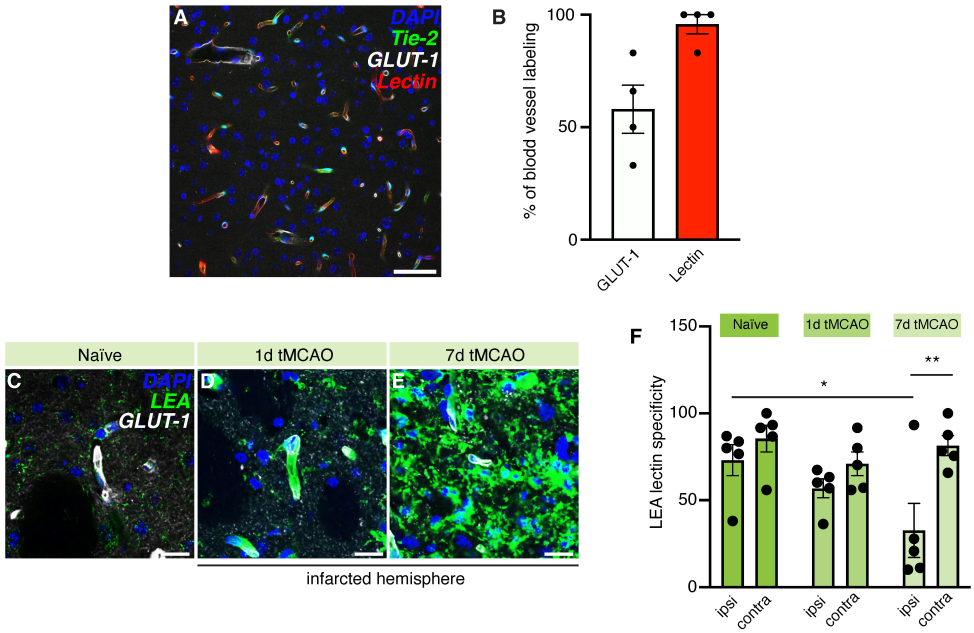

Figure 12: Analysis of lectin specificity.
A-B) Analysis of confocal images of the brain of Tie-2 reporter mice i.v. injected with biotinilated lectin and stained with Cy3-streptavidin and Glut-1 antibody, showed that lectin binding is specific for blood vessels. Scale bar: 50 µm. C-F) Specificity of LEA lectin towards blood vessels decreases as a result of ischemic stroke in the infarcted hemisphere. Scale bars: 20 µm.

Altogether, our results showed that different lectins have different efficacies in labelling cerebral blood vessels. Moreover, the choice of the method used for labelling can improve the efficacy. In particular, the injection of lectins in the bloodstream as an intermediate step during terminal transcardial perfusion significantly decreases the signal background. Therefore, lectins can be considered a useful marker to label the cerebral vasculature and can therefore be used in AQP4 polarization studies to determine the localisation of AQP4 at the astrocytic endfeet surrounding blood vessels. However, our results suggest that in particular circumstances that involve microglia activation, lectin may lose specificity towards blood vessels and label other structures in the brain that start expressing specific carbohydrates residues (i.e. microglia), hampering therefore their use in pathological settings.

Paper III: AQP4 ASOs injected i.c.v. in mice induce a long-term KD of *Aqp4* mRNA and protein reduction, without affecting the polarization at astrocytic endfeet.

In **Paper III**, we aimed to investigate the effects of antisense oligonucleotides (ASO) targeting *Aqp4* mRNA on AQP4 isoforms in the mouse brain. ASOs are single-stranded sequences of DNA, composed by 8 to 50 nucleotides that are able to bind with high specificity the complementary RNA through a Watson-Crick base pairing. They enable a sustained KD of the targeted mRNA through different mechanisms of action, involving recruitment of ribonuclease (RNase) H for degradation of the heteroduplex RNA-DNA(ASO), translational inhibition or modifications of the targeted RNA that decreases its stability (Schoch & Miller, 2017).

We showed that AQP4 ASO i.c.v. injection in wt mice induced a reduction of the total *Aqp4* mRNA lasting up to 4 weeks after the injection (Figure 13, A). Moreover, designing specific primers for M1 and M23 isoforms, we were able to show that at the same time point, the mRNA of both isoforms is downregulated, suggesting a pan-*Aqp4* mRNA reduction (Figure 13, B-C).

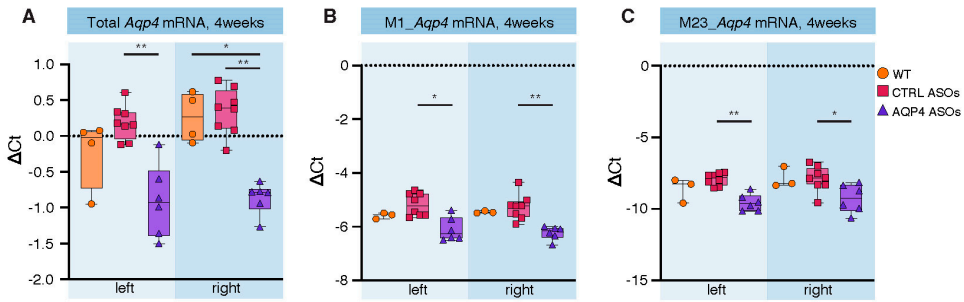

Figure 13: Effect of i.c.v. injected ASOs on *Aqp4* mRNA.
A-C) Quantification of total *Aqp4* (A), *M1* (B) and *M23* (C) mRNA 4 weeks after ASOs i.c.v. injection in mice.

We were also interested in finding out whether the mRNA KD had a functional effect on AQP4 protein, and therefore we performed a WB to quantify the gel bands corresponding to M1 and M23 isoforms (Figure 14, A). We found that M23, but not M1, was significantly reduced 4 weeks after AQP4 ASO i.c.v. injection, suggesting that AQP4 ASO has an effect not only at a transcriptional, but also at a translational level *in vivo* (Figure 14, B-C). Since we know that the presence of both M1 and M23 isoforms is important to form functional OAPs localised at the astrocytic endfeet, we analysed AQP4 polarization in brain sections at the level of the cortex and hippocampus (Figure 14, D-I). Our results (Figure 14, J) showed that AQP4 polarization is comparable between the groups in both regions analysed, suggesting

that, despite a downregulation of M23 protein, AQP4 can still correctly localise at the astrocytic endfeet, where it exerts its function.

Taken together, our results suggest that ASO targeting *Aqp4* mRNA can be used to achieve a long-term transcriptional KD, lasting at least 4 weeks. The mRNA KD reflects a downregulation on the protein level too, despite it does not appear to functionally affect AQP4, which still localizes at the astrocytic endfeet.

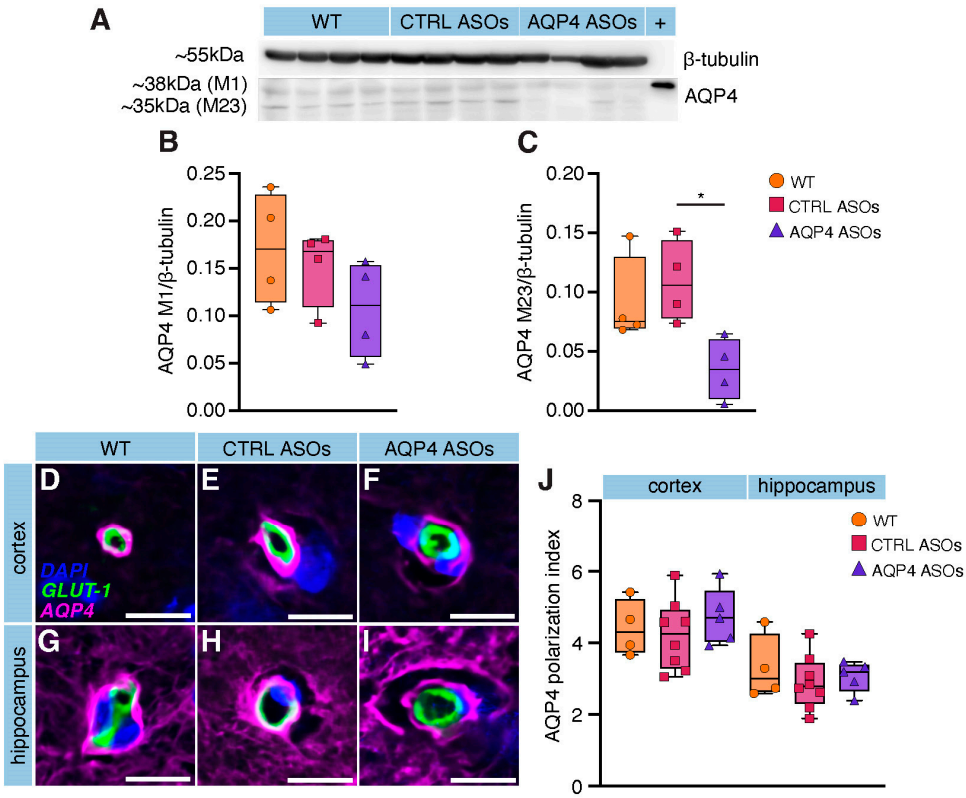

Figure 14: Effect of i.c.v. injected ASOs on AQP4 protein content and polarization.
A-C) Quantification of M1 (B) and M23 (C) bands in WB gel (A). D-J) Analysis of AQP4 polarization (J) in confocal images of cortex and hippocampus labelled with AQP4 (pink) around GLUT-1-stained blood vessels (green). Scale bar: 10 µm.

Paper IV: AQP4 polarization is impaired in PD mouse models, but it does not affect glymphatic function.

In **Paper IV**, we aimed to investigate CSF distribution and flow dynamics in the brain of two different mouse models of PD, the 6-OHDA lesion and the PFFs injected one. Despite the function of glymphatic system in AD has been extensively investigated (Harrison et al., 2020; Iliff et al., 2012; Peng et al., 2016; Xu et al., 2015), and some studies pointed to a link between glymphatic system, AQP4 and α-syn pathology in PD (Cui et al., 2021; Ding et al., 2021; Zou et al., 2019), the role played by glymphatic system in the pathogenesis of PD and clearance of α-syn has yet to be fully elucidated.

In the 6-OHDA mice. the lesion of the nigro-striatal pathway upon 6-OHDA injection in the MFB (Figure 15, A) results in a consistent decrease of the use of the paw contralateral to the lesioned hemisphere (Figure 15, B). The analysis of glymphatic function was carried out upon injection of a fluorescent tracer in the CM of lesioned mice and controls (CTRL and SHAM-lesioned mice) at two different time points (1- and 5-weeks post-lesion, Figure 15, A). The quantification of the tracer distribution in the dorsal cortex (Figure 15, C-G, M) did not show any difference between the groups, despite we found that AQP4 polarization at the astrocytic endfeet in the SNpc was impaired 5 weeks after the lesion of the nigrostriatal pathway (Figure 15, H-L, N). These results suggest that a rearrangement of AQP4 subcellular localization happens in the SNpc 5 week after the lesion of the nigro-striatal pathway, impacting AQP4 polarization. However, the impairment of the molecular machinery supporting glymphatic function does not seem to correlate with altered global glymphatic functionality, since the tracer distribution in the dorsal cortex appears comparable between the groups.

Since we sought to investigate glymphatic system involvement in the progression of LB pathology, we injected mouse PFFs (mPFFs) bilaterally in the striata of wt mice (Figure 16, A) to obtain a model of LB pathology (Abdelmotilib et al., 2017).

mPFFs injected mice are affected by altered motor coordination and motor learning, as shown by their worst performance on the accelerating rotarod paradigm 6 months after mPFFs injection (Figure 16, B). Moreover, at the same time point, we found that the number of TH^+ cells was significantly lower compared to aCSF injected mice (Figure 16, C-E), and positively correlated with the performance on the rotarod. Furthermore, the injection of mPFFs, but not aCSF, in the striata, induced the formation of phosphorylated α-syn inclusions not only in the injected area, but also in the prefrontal cortex (PFC) and SNpc (Figure 16, F-N). The characterization of the bilateral mPFFs injected mice showed that this model mimics several features of PD, including DA neurons degeneration, motor deficits and LB-like pathology, and can therefore be used to assess the involvement of glymphatic function in PD pathogenesis and LB pathology progression.

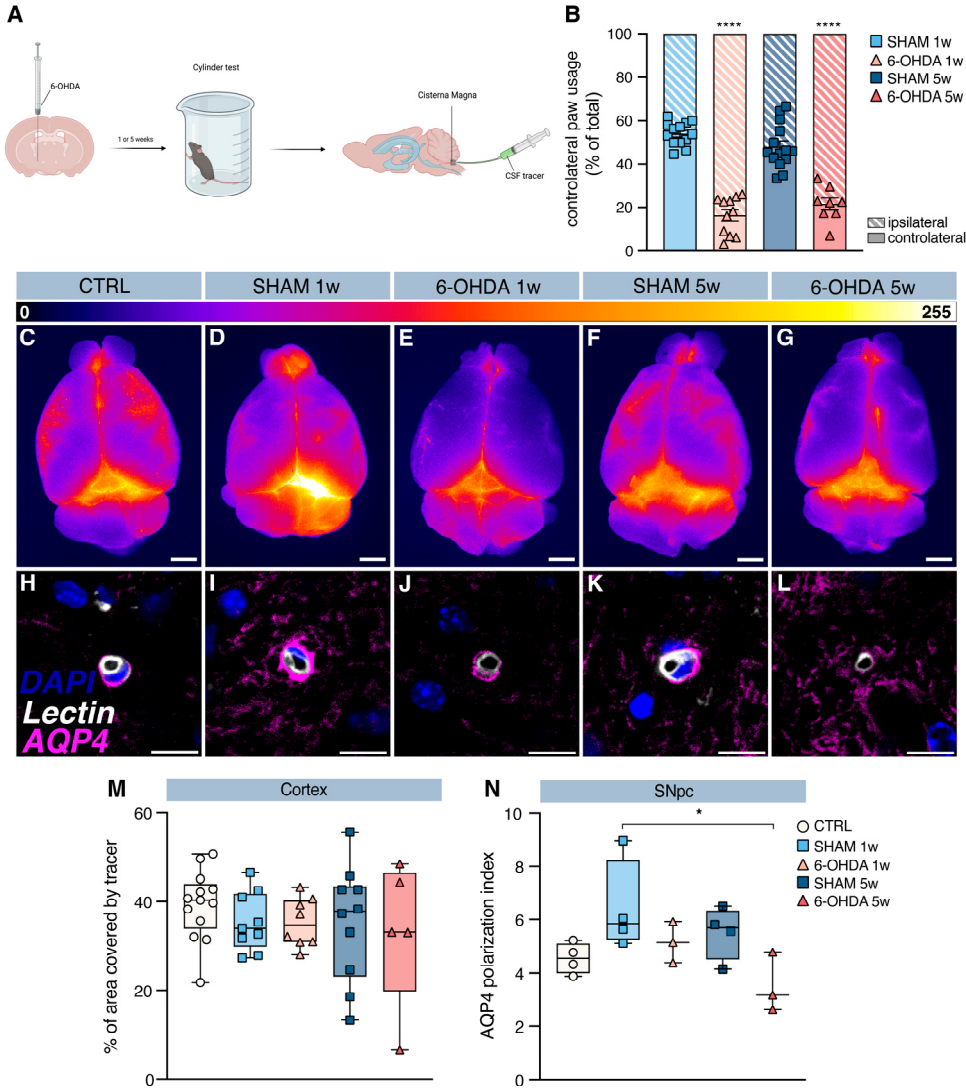

Figure 15: Glymphatic function in 6-OHDA lesioned mice.
A) Cartoon depicting the experimental plan to investigate glymphatic function in 6-OHDA lesioned mice. B) Graph showing the behavior of 6-OHDA- and SHAM-lesioned mice in the cylinder test. C-G) Representative images of the dorsal view of the brains injected with a fluorescent tracer in the CM of CTRL, Sham- and 6-OHDA-lesioned mice, 1 or 5 weeks post-lesion. Scale bars: 2mm. H-L) Representative confocal images of AQP4 staining around Lectin-labelled blood vessels in the SNpc. Counterstain: DAPI. Scale bars: 10 μm. M) Graph showing the quantification of the CM-injected tracer in the dorsal cortex of CTRL, SHAM- and 6-OHDA-lesioned mice, 1 and 5 weeks after the lesion, seen as the % of cortical area covered by tracer. N) Graph showing the quantification of AQP4 polarization in the SNpc of CTRL, SHAM- and 6-OHDA-lesioned mice, 1 and 5 weeks after the lesion.

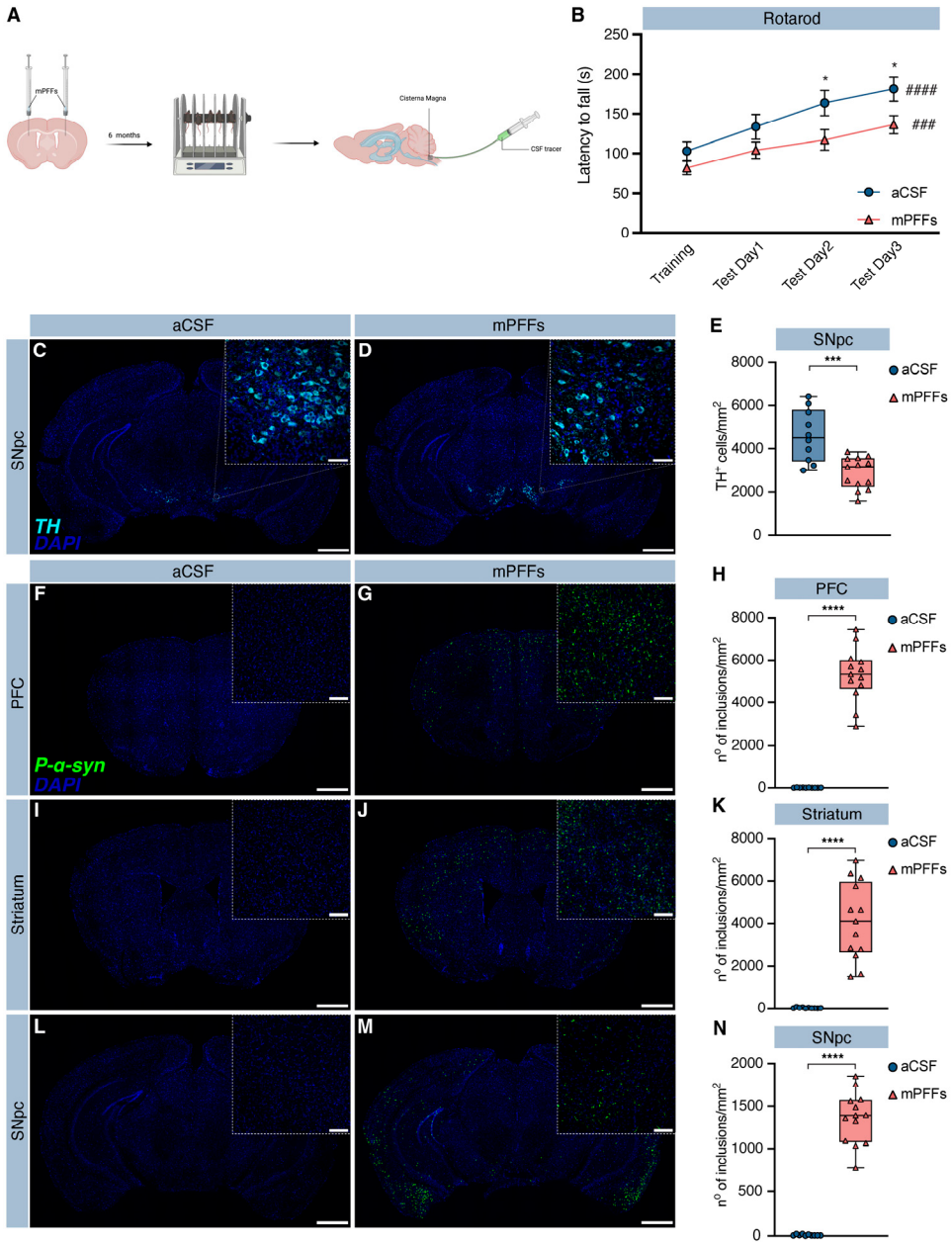

Figure 16: Characterisation of the mPFFs mouse model of PD.

A) Cartoon depicting the experimental time-line to assess motor behaviour deficits and CSF flow dynamics in mPFFs and aCSF control mice. B) Graph showing the latency to fall from an accelerating rotarod of aCSF and mPFFs mice. C-E) Confocal images of TH$^+$ neurons in the SNpc of aCSF and mPFFs mice and quantification (E) of the number of TH$^+$ neurons. Scale bars: 1mm for low magnification images, and 50 μm for insets. (F-N) mPFFs mice, but not aCSF ones, develop phosphorilated α-syn inclusion in different brain regions: PFC, respresentative images in F-G, and quantification in H; striatum, respresentative images in I-J, and quantification in K; SNpc, respresentative images in L-M, and quantification in N. Scale bars: 1mm for low magnification images, and 100 μm for insets.

To this end, we injected a fluorescent tracer in the CM of aCSF and mPFFs at the end of the behavioural assessment and we imaged its movement *in vivo* through the intact skull (Figure 17, A-I). The quantification of the tracer mean fluorescence intensity *in vivo* over 40 minutes revealed no difference between the two groups (Figure 17, E), as well as the analysis *ex vivo* of the distribution of the tracer in the brain parenchyma (Figure 17, J-L), suggesting that glymphatic system is still functional in mPFFs mice, despite DA neurodegeneration, motor deficits and wide presence of insoluble inclusions in the brain. Interestingly, we found that, despite global glymphatic function was comparable to the control group, in mPFFs mice AQP4 lost its perivascular localization in the striatum and in the SNpc (Figure 17, M-P), two of the most affected brain regions in PD and specifically in this mouse model, suggesting that a local disruption of the molecular machinery involved in CSF movement does not affect globally the system.

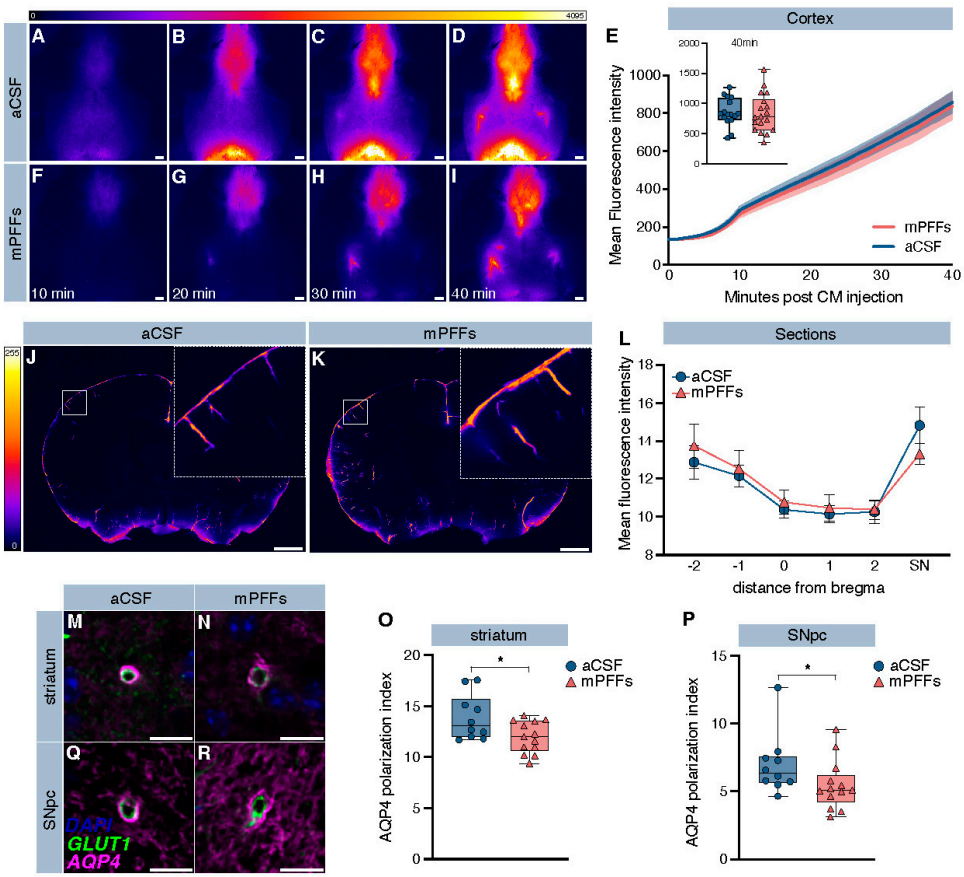

Figure 17: Assessment of glymphatic function in aCSF and mPFFs injected mice.
A-I) Assessment of glymphatic function in the mPFFs model of PD. Representative images of the CSF tracer distribution *in vivo* in aCSF (A-D) and mPFFs (F-J) mice, and relative quantification (E). Scale bars: 1mm. J-L) Representative images of CSF tracer distribution in brain sections of aCSF and mPFFs mice and relative quantification (L). Scale bars: 1mm. M-R) Analysis of AQP4 polarization around GLUT-1$^+$ blood vessels in the striatum (M-N) and SNpc (Q-R) of aCSF and mPFFs mice and relative quantification (O and P, respectively). Scale bars: 10 μm.

Surprisingly, the total count of phosphorylated inclusions in the PFC, striatum and SNpc, allowed for the discrimination of two different subgroups of mPFFs injected mice, based on the median: mPFFs^{low}, n° of inclusions < median, and mPFFs^{high}, n° of inclusions ≥ median (Figure 18, A).

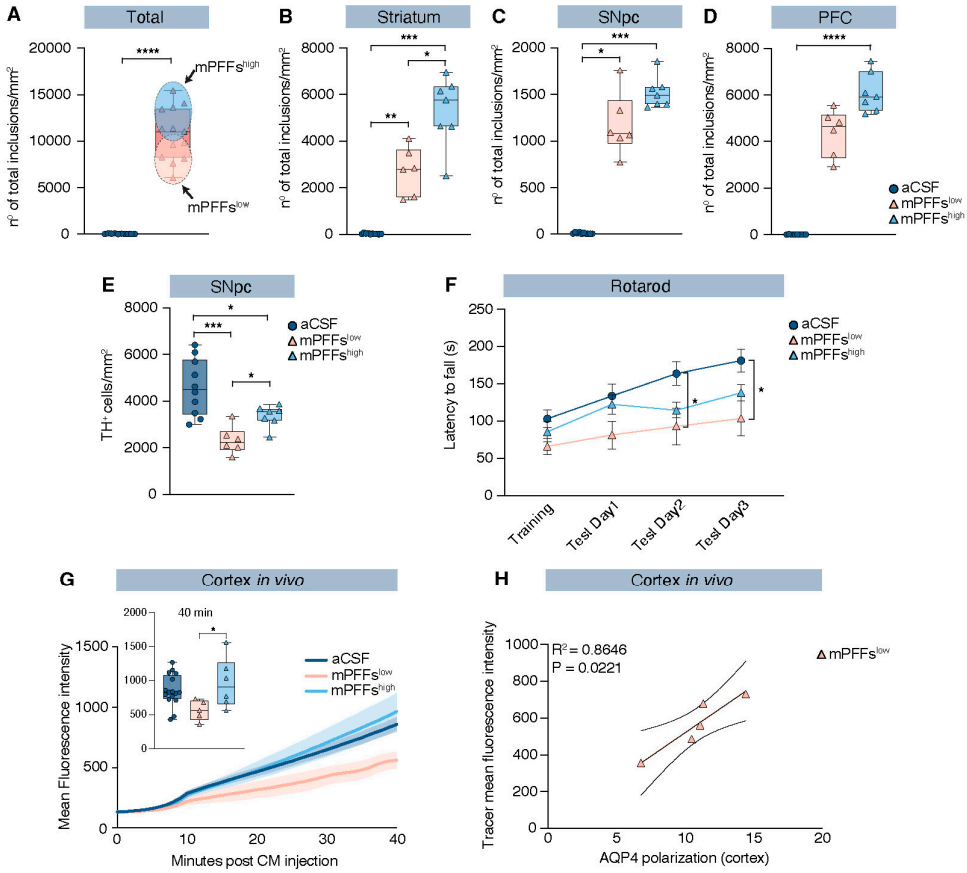

Figure 18: Discrimination of a subgroup of mPFFs injected mice with worsened phenotype and altered glymphatic dynamics.

A) Quantification of the total number of phosphorilated α-syn inclusions in the PFC, Striatum and SNpc allows for the discrimination of two distinct subgroups of mPFFs injected mice, based on the median: mPFFs^{low}, n° of inclusions < median, and mPFFs^{high}, n° of inclusions ≥ median. B-D) Graphs showing consistenly lower n° of inclusions in the 3 brain regions analysed in the mPFFs^{low} group compared to the mPFFs^{high} group. E) Quantification of the number of TH^{+} DA neurons is lower in the SNpc of the mPFFs^{low} group. F) Graph showing the worsened performance of mPFFs^{low} mice on the accelerationg rotarod. G) The quantification of the CSF tracer mean intensity in the cortex *in vivo* shows altered flow dynamics in the mPFFs^{low} group. H) Graph showing the exhisting correlation between AQP4 polarization in the cortex and CSF tracer mean intensity in the cortex in the mPFFs^{low} group.

The characterization of the mPFFs^{low} subgroup revealed that these mice consistently show less inclusions in the regions analysed compared to the mPFFs^{high} group (Figure 18, B-D), but they are interested by a worst neurodegeneration in the SNpc (Figure 18, E) and worst motor performance on the rotarod (Figure 18, F).

Moreover, we showed that CSF flow dynamics *in vivo* are impaired in the mPFFs^{low} mice (Figure 18, G), and in this group AQP4 polarization in the dorsal cortex positively correlates with CSF tracer mean fluorescence intensity in the cortical surface *in vivo* (Figure 18, H). These results suggest an interindividual variability in the response to mPFFs, highlighting the presence of a subgroup of mPFFs injected mice exhibiting a worsened PD phenotype and altered glymphatic function. These mice appear to be more susceptible to mPFFs and possibly show an accelerated progression of the PD pathology.

Overall, our results suggest that lack of DA signalling in the brain following lesion of the nigro-striatal pathway with 6-OHDA is accompanied by a reorganization of AQP4 subcellular localization, which in the SNpc is not retained any longer at the astrocytic endfeet where it promotes the movement of CSF from the perivascular space of brain penetrating arteries to the brain parenchyma. A similar pattern was seen also in the brain of mice developing phosphorylated α-syn inclusions upon intra-striatal injection of mPFFs, interested by loss of AQP4 polarization in the striatum and in the SNpc. However, either lack of DA signalling or development of Lewy pathology did not associate with altered glymphatic functionality, despite α-syn fibrils appeared to be cleared from the brain parenchyma through glymphatic pathways. This suggests the possibility that it is not PD pathology *per se* to cause a decline in glymphatic function, but rather that a decline in CSF circulation in the brain, caused e.g. by physiological aging or other mechanisms that are still unknown, can worsen PD pathology and its progression. Our results highlighting the presence of a subgroup of mPFFs injected mice exhibiting a worsened progression of PD pathology and impaired CSF flow dynamics, are in line with this hypothesis, suggesting that a sustained glymphatic function in the earlier stages of the disease, may contribute to the obstruction of the machinery involved in glymphatic clearance of toxic α-syn, and therefore to a decreased functionality of the system.

Paper V: Moderate hypothermia impairs CSF flow dynamics and AQP4 polarization.

In **Paper V**, we aimed to investigate the direct effect of different hypothermia regimes on glymphatic system.

In our experimental paradigm, acute moderate hypothermia (body temperature: 30 ^{o}C) induced a decrease in heart rate and cerebral blood flow (CBF) compared to normothermia, accompanied by reduced CM-injected CSF tracer flow in the perivascular space of the medial cerebral artery (MCA) region *in vivo* (Figure 19, A-B). These results were confirmed by an *ex vivo* approach using light sheet

imaging of iDISCO+ cleared brains (Figure 19, C-G), suggesting that glymphatic function is impaired when body temperature decreases below a physiological level.

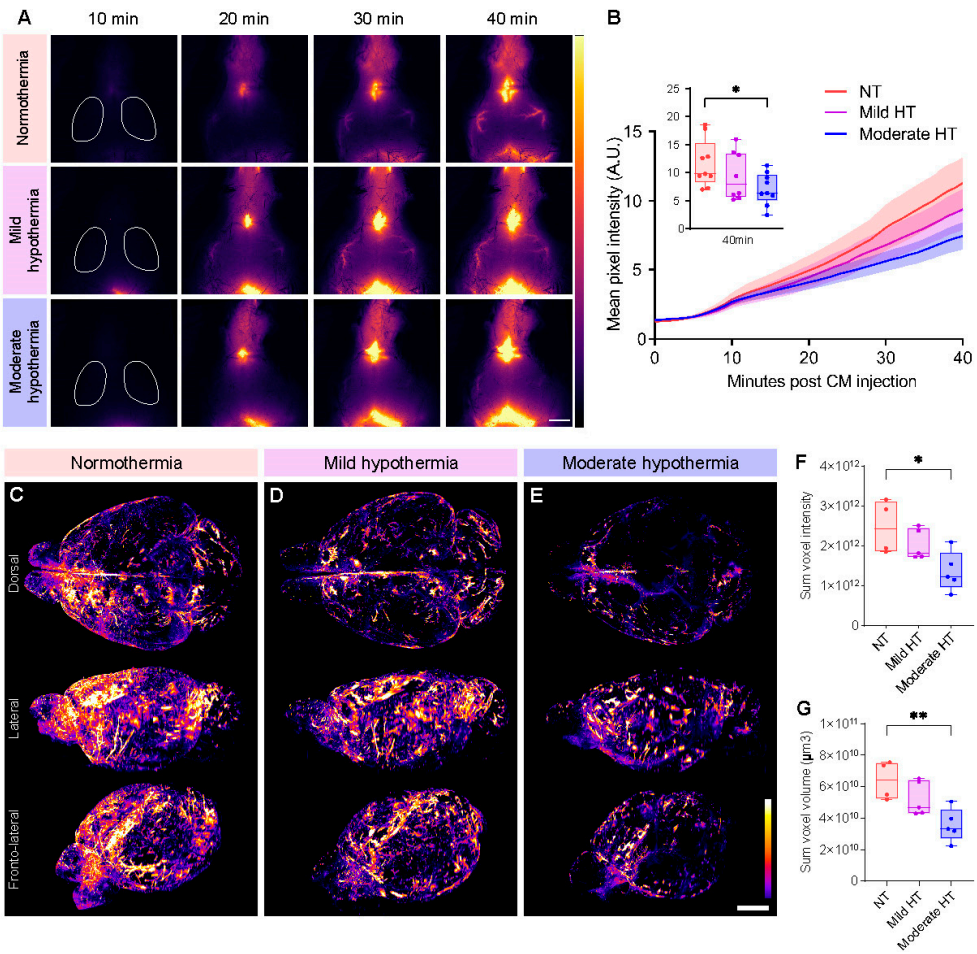

Figure 19: Glymphatic influx in the brain after induction of hypothermia.
A) Representative images of *in vivo* CSF tracer dynamics over 40 minutes in mice undergoing normothermia, mild hypothermia or moderate hypothermia regimes. The dotted line in the10 min panel indicates the ROI quantified in B. Scale bar: 2.5 mm. B) Quantification of CSF tracer mean intensity in the MCA region over 40 minutes *in vivo*. C-E) Representative images of dorsal, lateral and fronto-lateral 3D reconstructions of CSF tracer in cleared brains of normothermia, mild hypothermia or moderate hypothermia mice after light -sheet imaging. Scale bar: 2mm. F-G) Quantification of the voxel intensity and volume in 3D reconstructed whole brains from mice undergoing normothermia, mild hypothermia or moderate hypothermia regimes.

Moreover, the depth of CSF tracer penetrance in the brain parenchyma at the level of the dorso-lateral cortex along PVSs of brain penetrating arteries was reduced in mice treated with acute moderate hypothermia (Figure 20, A-C, J). Light sheet imaging of cleared brains (Figure 20, D-I) showed also that the nº of PVSs filled

with CSF tracer was reduced in mice treated with moderate hypothermia regime (Figure 20, K), as well as the averaged area covered by the CSF tracer per influx site (Figure 20, L), suggesting that CSF tracer penetration via PVSs is impaired under moderate hypothermia conditions.

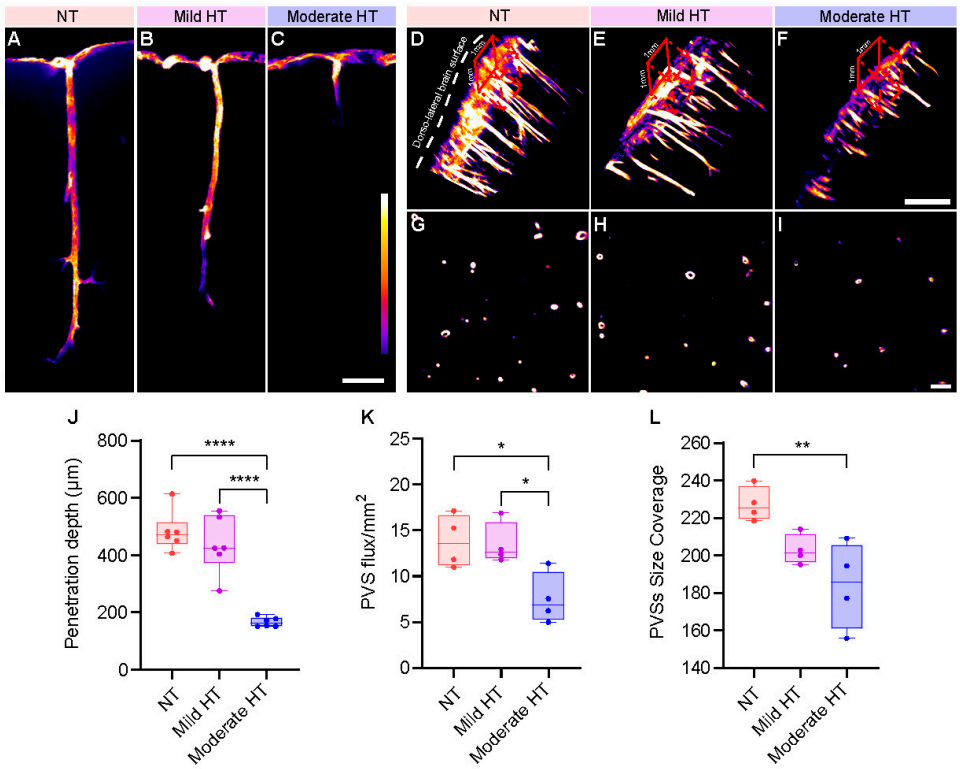

Figure 20: Distribution of perivascular CSF tracer after induction of hypothermia.
A) Representative confocal images of PVSs filled with CSF tracer in the dorso-lateral cortex of mice treated with normothermia, mild hypothermia or moderate hypothermia regimes. Scale bar: 100 µm. D-E) 3D reconstruction of tracer penetrance along PVSs from the dorso-lateral surface of the brain of normothermia, mild hypothermia or moderate hypothermia treated mice, cleared and imaged with light-sheet microscope. Scale bar: 500 µm. G-I) Representative images of PVS influx sites in (D-F) seen from the surface. Scale bar: 100 µm. J) Quantification of the CSF tracer penetration depth along PVSs. K) Quantification of the n° of PVSs filled with CSF tracer in the cortical surface. L) Quantification of the area in the cortex positive to CSF tracer in PVSs.

Moreover, when moderate hypothermia is induced repeatedly, not only CSF flow dynamics *in vivo* are affected (Figure 21, A-B), but also AQP4 polarization at the astrocytic endfeet is lost in the dorsal and lateral cortices (Figure 21, C-D). The existing positive correlation between AQP4 polarization in the cortex and the CSF tracer mean intensity in the cortex (Figure 21, E), suggests that reduced glymphatic function under repeated hypothermia regime may be caused by an altered sub-cellular localization of AQP4.

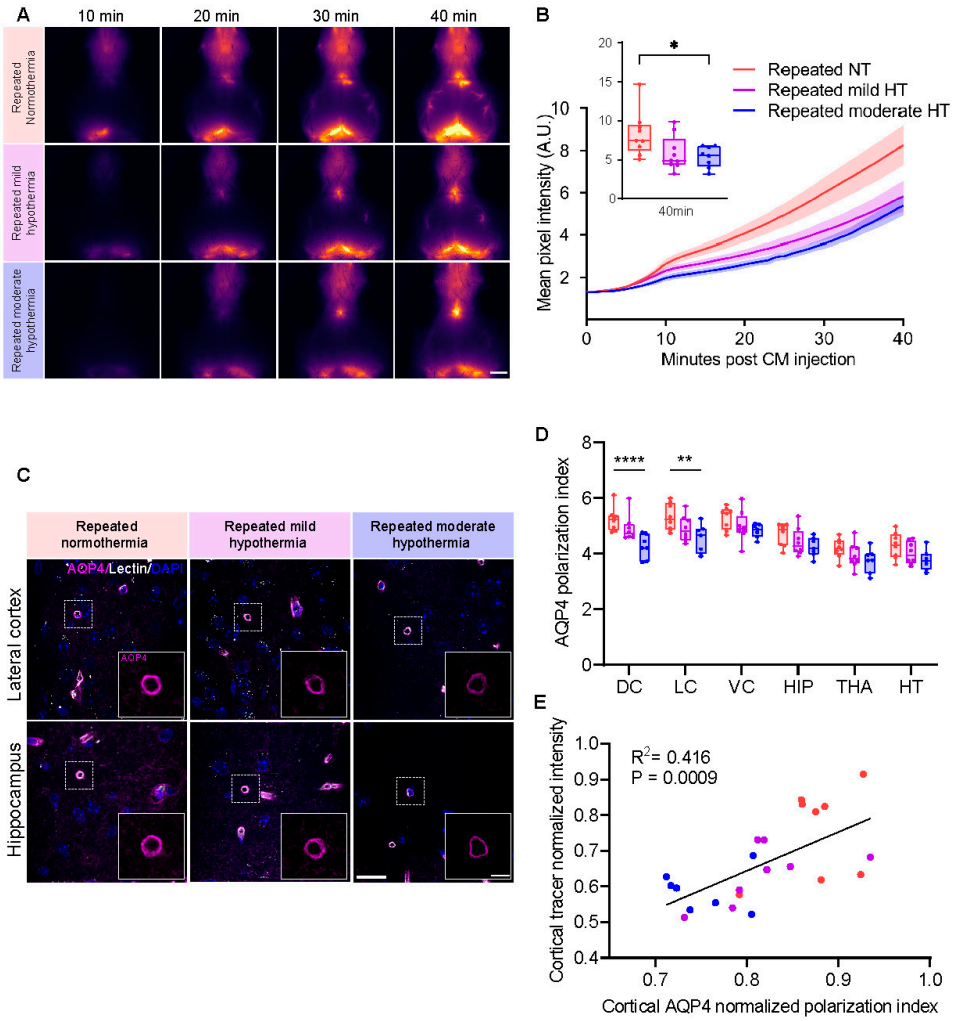

Figure 21: Effect of repeated hypothermia on glymphatic function and AQP4 polarization.
A) Representative images of *in vivo* CSF tracer dynamics over 40 minutes in mice treated with repeated normothermia, repeated mild hypothermia or repeated moderate hypothermia. Scale bar: 2.5 mm. B) Quantification of CSF tracer mean intensity in the MCA region over 40 minutes *in vivo*. C) Representative confocal images of AQP4 staining surrounding Lectin⁺ blood vessels in the lateral cortex and hippocampus of mice treated with repeated normothermia, repeated mild hypothermia or repeated moderate hypothermia. Scale bars: 20 μm and 5 μm for insets. D) Quantification of the AQP4 polarization index in the dorsal cortex (DC), lateral cortex (LC), ventral cortex (VC), hippocampus (HIP), thalamus (THA) and hypothalamus (HT). E) Graph showing the existing correlation between AQP4 polarization in the cortex and CSF tracer mean intensity in the cortex.

Taken together, these results suggest that moderate hypothermia has a detrimental effect on glymphatic system, disrupting two of the main mechanisms involved in CSF movement into the brain parenchyma, namely the flow along PVSs and AQP4 polarization.

Discussion and concluding remarks

Glymphatic system has been discovered as recently as 10 years ago (Iliff et al., 2012). This brain waste clearance system has similar function to the lymphatic system, hence the nomenclature. Its functioning is based on the movement of the CSF, produced by the choroid plexuses in the lateral and third ventricles, which then in turn flows into the SAS via the CM. From the SAS, CSF enters the brain parenchyma via PVSs of brain penetrating arteries, facilitated by AQP4 localized at the astrocytic endfeet abutting blood vessels (Nedergaard, 2013).

Because of the novelty of the field, it is needed to find consensus on the techniques and methodology to study glymphatic system, as well as interpretation of the results. Considering this, the present thesis aimed to describe different methods that can be used to investigate glymphatic function on a macroscopic level, and dissect the microscopic players of the system, with the final goal to apply such methods for glymphatic studies in physiological and pathological settings. Advancing pre-clinical research with reproducible and standardised methods is fundamental for following translational applications.

Paper I

Being a transparent fluid, CSF must be labelled in some way to be detected and track its localization in the CNS. Classical approaches of accessing the CSF via intraventricular (i.c.v.) injections have the cons to require craniotomy, disruption of the dura mater and trauma to the brain parenchyma upon needle insertion in the brain, affecting the system (Mestre, Hablitz, et al., 2018). Injection of tracers in the CM has previously been proven a valid method for getting access to the CSF with a minimally invasive procedure for the brain, and with minimal variation of intracranial pressure (ICP) after injection of small quantities of volume (Jessen et al., 2015).

In Paper I, we described a detailed method to perform a CM injection in rodents to study the glymphatic system with a standardised procedure. Indeed, the investigation of glymphatic function with classical i.c.v. injections for accessing the intraventricular CSF and use of gaseous anaesthetics like isoflurane during the surgery, have added confounding factors to data interpretation, inducing several scientists to reject the existence of a brain-wide clearance system. In fact, the choice

of an invasive procedure for the brain, i.e. i.c.v. injection, which requires craniotomy, dura disruption and brain parenchyma damage upon needle insertion, or the use of anaesthetics, i.e. isoflurane, which induce an EEG spectra different from the one seen in physiological sleep, are all procedures that cause a decline in CSF movement in the brain, and therefore should be avoided when the investigation of glymphatic function is of interest (Hablitz et al., 2019; Mestre, Hablitz, et al., 2018). However, the feasibility of the CM injection technique has been questioned in the past, based on the argument that the high volumes of CSF tracers injected in the CM would definitely increase the ICP *in vivo* and therefore alter CSF flow dynamics. A recent study where a double cannulation of the CM allowed for the simultaneous injection of tracer and withdrawal of the same amount of fluid, showed no increase of total CSF volume or ICP (Raghunandan et al., 2021), solving these criticisms and confirming that, at the moment, CM injection of CSF tracers is the best method to study glymphatic system.

Then, the detailed procedure for CM injections described in Paper I aims to provide a standardised guide for researchers that are approaching the glymphatic field for the first time, with detailed description of the equipment needed, anaesthesia and useful tips with the goal to help set-up the surgical method and avoid lack of reproducibility and biased interpretation of the results. Moreover, this procedure has been shown to be adaptable to other animal models, particularly in pigs (Bèchet et al., 2021a), and for *in vivo* studies (Iliff, Lee, et al., 2013; Keil et al., 2022).

Despite it is the best option to study glymphatic function at the moment, the CM procedure results very invasive for the animal itself, and due to ethical concerns, in most of the cases the CM injection is a terminal procedure. On the other hand, even in the occurrence of ethical approval for non-terminal CM cannulation, e.g. for studies where glymphatic function needs to be investigated in awake mice, mice have to be house-caged singularly, but this has shown to increase stress and raises ethical concerns. Since stress has been shown to decrease glymphatic function as well (Wei et al., 2019), this procedure could add confounding factors and variability to the data, biasing the interpretation of the results. These limitations related to the CM injection method, hamper the possibility to perform longitudinal pre-clinical studies, which would be of great relevance especially when investigating glymphatic function in pre-clinical models of neurodegenerative diseases. Indeed, the possibility to perform CM injections to study glymphatic function in the same animal at different stages of the disease of interest, would help to answer one of the main “the chicken or the egg” question in the field: whether neurodegenerative diseases cause a malfunction of the glymphatic system, or whether a decline in glymphatic function, e.g. due to aging, happens before the onset and just accelerates the progression of the disease (Nedergaard & Goldman, 2020; Rasmussen et al., 2018).

Paper II

The vascular compartment is important for the physiology of glymphatic system, primarily because the bulk flow of CSF in the PVSs is driven by arterial pulsations (Mestre, Tithof, et al., 2018). However, the vascular compartment represents also one of the physical boundaries of the glymphatic system, keeping the CSF flowing in the PVSs separated from the bloodstream, whilst on the other side AQP4 localised at the astrocytic endfeet allows for a sustained influx of CSF in the brain parenchyma (Wardlaw et al., 2020). In the Introduction chapter of this thesis, the importance of the sub-cellular AQP4 localization, polarised at the astrocytic endfeet abutting blood vessels, has been extensively discussed (Zeppenfeld et al., 2017), as well as the methodology to investigate AQP4 polarization (Hablitz et al., 2020; Munk et al., 2019), highlighting the importance to visualise blood vessels in such studies. Historically, blood vessels in the brain have been labelled in histological specimens using different markers, e.g. CD-31 (Bell et al., 2010; Koonce et al., 2017; Müller et al., 2002), laminin (Eriksdotter-Nilsson et al., 1986; Wälchli et al., 2015) or GLUT-1 (Leino et al., 1997). Since 1982, lectins have been used to label the vasculature either by immunohistochemical approaches (Mazzetti et al., 2009; Santulli et al., 2011; Sorriento et al., 2009), or direct delivery into the bloodstream (i.e. as an intermediate step during terminal transcardial perfusion) (Bryson et al., 2011; Robertson et al., 2015; Wälchli et al., 2015), upon discovery of their binding capacity to the luminal aspect of blood vessels, via specific carbohydrates interactions (Simionescu et al., 1982).

In **Paper II**, we aimed to provide a quantitative analysis of the labelling efficacy of different lectins and different methods for blood vessels staining. Indeed, different lectins and applications have been used to label blood vessels in different studies, but a systematic comparison of the labelling efficacies is still lacking, making it difficult to compare results in different studies and choose the most suitable lectin for a specific experimental set-up. In our study, among the three lectins of choice (LEA, IB4 and WGA), LEA showed the best labelling efficacy in immunohistochemistry conditions of brain sections. We also showed that the labelling method, i.e. direct injection of lectins in the bloodstream during terminal transcardial perfusion, can significantly improve the labelling efficacy, decreasing the signal background. The systematic comparison of (i) different lectins labelling efficacy and (ii) different labelling methods using lectins in Paper II, provided a detailed description of how lectins can be used to label vasculature in the brain and other organs, depending on the experimental setting. Specifically, for AQP4 polarization studies in the brain, both methods, i.e. histological staining with LEA vs direct injection of WGA in the bloodstream during terminal perfusion, may be used.

However, we showed that LEA lectin specificity for blood vessels is decreased in pathological settings, e.g. stroke, due to microglia labelling. We hypothesised that

in the infarcted hemisphere, microglia start expressing new carbohydrates residues on the plasmalemma, that can be recognised and bound by LEA lectin, therefore decreasing the specificity for blood vessels. It is known that, in the initial stages of ischemic stroke, microglia assume a neuroprotective phenotype, and only later on switch towards a neurotoxic phenotype in the peri-infarct region, upregulating a different set of molecules (Hu et al., 2012). We hypothesised that a reactive microglia phenotype may be the necessary condition for LEA lectin to label microglia in the brain. However, the evidence that lectins can stain microglia is not new, since it has been shown that lectins can label macrophages (Düllmann et al., 2002) and indeed *Ricinus communis* agglutinin 1 (RCA-1) has been widely used in rodent (Hauke & Korr, 1993) and human tissue (Andjelkovic et al., 1998) to this purpose. Another study showed that RCA-1 failed to label microglia in absence of a strong inflammatory response (Hirko et al., 2008), evidence that supports our hypothesis that a reactive microglia phenotype may be the necessary condition for lectins to label microglia in the brain. However, it is not clear whether microglia upregulate the same setting of molecules in response to insults of different origins, therefore future research should verify how the binding patterns of LEA and other lectins with different carbohydrates specificity vary in different pathological settings. Considering also that microglia assume a neuroprotective phenotype, and only later on switch towards a neurotoxic phenotype in the peri-infarct region (Hu et al., 2012), it should also be addressed whether LEA shows a variable time-course in labelling of the microglia. Notably, reactive microglia with neurotoxic phenotype induced by LPS exposure, have also been shown to induce *in vitro* and *in vivo* a neurotoxic reactive phenotype in astrocytes (Liddelow et al., 2017). Whilst the time-course of the molecular expression for both cellular types has been established in a model of acute neuroinflammation upon i.p. lipopolysaccharide (LPS) injection, little is known regarding this phenomenon, that may also impact lectin specificity for blood vessels, in stroke mouse models. Moreover, the fact that a glial scar forms after ischemic stroke to help delimiting the affected area in the brain and protecting the surroundings (Choudhury & Ding, 2016), suggests that more than one population (e.g. neurotoxic and neuroprotective) of microglia and astrocytes can be found in the same affected area, and this may affect lectin labelling of blood vessels as well. Intriguingly, acute inflammation upon injection of LPS, other than inducing a reactive neurotoxic phenotype in astrocytes following microglia activation (Liddelow et al., 2017), has been shown to impair glymphatic system, without affecting AQP4 polarization (Manouchehrian et al., 2021). This evidence suggests that a neurotoxic microglia and astrocytes phenotype may be involved in CSF flow dynamics impairment, although this is a mere speculation, and this hypothesis has not been directly tested by our or other groups. However, if this hypothesis will be confirmed in the LPS model, the switch of microglia/astrocytes phenotype may represent the mechanism underlying glymphatic impairment in the aftermath of stroke in mouse models as well (Gaberel et al., 2014; Mestre et al., 2020).

Then, if the investigation of AQP4 polarization has to be carried out in pathological settings with known or suspected inflammation, it has to be taken into account that the specificity of LEA towards blood vessels may decrease, and an approach of lectins injection directly in the bloodstream would be preferred, because of the decreased background signal. However, this method is discouraged for *in vivo* imaging of the vasculature, due to the capability of lectins to cause coagulation of red blood cells, with potentially fatal consequences (Zubcevic et al., 2016). Instead, infusion can be performed in conjunction with transcardial perfusion.

Lastly, it must be noted that adhesion molecules on the luminal surface of the endothelium, i.e. ICAM-1, VCAM-1 and PECAM-1, have been shown to undergo increased expression after TBI induction (Dell'Aquila et al., 2021). Bearing in mind that lectins are glycoproteins with a carbohydrate-binding domain binding to the luminal site of blood vessels(Simionescu et al., 1982), it should be tested whether their binding pattern changes accordingly despite injection directly in the bloodstream.

Paper III

In the Introduction chapter of this thesis, the relevance of AQP4 in brain fluid movements and homeostasis has been extensively discussed (S. Nielsen et al., 1997). The central role of AQP4 in the physiology of glymphatic system has also been discussed (Jessen et al., 2015). The investigation of AQP4 function in the brain has greatly benefit from studies in animal models lacking AQP4, and such models have been fundamental also to confirm the pivotal role of AQP4 in glymphatic system (Mestre, Hablitz, et al., 2018).

KO mouse models of AQP4 are genetically modified mice where AQP4 expression is prevented by different approaches, e.g. replacement of the wild-type (wt) *Aqp4* gene with modified vectors lacking exons of the wt gene, or depletion of exons in the wt gene (Ikeshima-Kataoka et al., 2013; T. Ma et al., 1997; Thrane et al., 2011). However, in genetically modified mouse models, AQP4 KO is congenital, meaning that the lack of AQP4 is extended to the whole brain, and other organs (e.g. skeletal muscle and kidney), and lasts throughout the entire animal lifespan, possibly allowing for compensatory mechanisms to occur. Moreover, it is difficult to study the function of specific isoforms in these models since they are characterized by lack of the total *Aqp4* mRNA. Several studies have recently pointed out that the two main isoforms of AQP4 in the brain, M1 and M23, may assemble differently in the plasmalemma, and therefore play different roles in water flux (Ciappelloni et al., 2019; Smith et al., 2014). In particular, it appears that to exert water channel's function, M1 and M23 have to assemble in OAPs in heterotetramers where a M23 core is surrounded by M1 (Pisani et al., 2021). Hence, the ratio of M1 and M23 can be used as a readout of AQP4 polarization (Hablitz et al., 2020), an important

hallmark for glymphatic function, as widely described in the Introduction chapter of this thesis.

An alternative approach to the use of congenital AQP4 KO mouse models is the injection in the brain of short interference RNA (siRNA) targeting AQP4 (Badaut et al., 2011; Fukuda et al., 2013). However, despite the double-strand nature of short interference siRNA makes them a robust tool to induce mRNA KD in cell cultures, they show only a transient effect *in vivo*. On the other hand, ASO have been shown to have a sustained effect *in vivo* (Schoch & Miller, 2017).

In **Paper III**, we aimed therefore to further investigate AQP4 physiology *in vivo*. As mentioned before, AQP4 KO models have been greatly useful to advance the knowledge of AQP4 functions, especially in relation to the glymphatic system (Mestre, Hablitz, et al., 2018). However, AQP4 KO models are usually congenital, hampering the possibility to investigate the effects of a spatially and temporally controlled removal of AQP4, or of its isoforms. Therefore, we sought to investigate the effects of AQP4-targeting ASO injected *in vivo* on a transcriptional and translational level, with particular focus to AQP4 isoforms. In our study, we showed that i.c.v. injection of AQP4 ASO *in vivo* induced a significant reduction of the total *Aqp4* mRNA after 4 weeks. At the same time point, both *M1* and *M23* mRNAs of *Aqp4* isoforms were reduced, but the AQP4 ASO effect at the transcriptional level was noticeable only with regards to the M23 isoform of the protein, despite no effect was detected in AQP4 polarization at the astrocytic endfeet. This is surprising, since the ratio of M1 and M23 isoforms of AQP4 has been shown to be a measurement of AQP4 polarization (Hablitz et al., 2020). However, bearing in mind that in physiological conditions M1/M23 ratio has been calculated as 1:3 (Palazzo et al., 2019) and that to form OAPs, M1 and M23 aggregates in heterotetramers, a mathematical modelling of all the possible conformations of the heterotetramers showed that higher M23:M1 ratio results in bigger size OAPs (Jin et al., 2011), which have been shown to mediate mostly cell-cell-adhesion (Ciappelloni et al., 2019; Furman et al., 2003; Smith et al., 2014). Lower M23:M1 ratio, around 1:1, results in the formation of smaller OAPs, whilst OAPs do not form at even lower M23:M1 ratio (Jin et al., 2011). In our study we did not calculate the M23:M1 ratio, but bearing in mind that the relative M23:M1 ratio has been shown to affect AQP4 localization in the cell as well (Smith et al., 2014), we can hypothesise that the M23 decrease affected the M23:M1 ratio to a level that functional OAPs could still form and localise at the astrocytic endfeet (Figure 3, F)

AQP4 polarization does not necessarily correlate with AQP4 protein content, as shown by studies where the KO of proteins part of the DAPC, important to localise AQP4 at the astrocytic endfeet, e.g. α-syntrophin or dystrophin, did not alter total AQP4 content (Amiry-Moghaddam et al., 2003). This evidence suggests that the AQP4 KD on the mRNA and protein level does not necessarily affect the protein localization and supposedly its function, since it is at the level of the astrocytic

endfeet that AQP4 regulates the water movement (S. Nielsen et al., 1997). Therefore, for a functional effect, it may be much more of interest to affect AQP4 localization in the astrocytes rather than the mRNA and protein content. Interestingly, a recent study, showed that CNS edema in rodents can be attenuated by blocking the calmodulin signal pathway, involved in the localization of AQP4 at the blood spinal cord barrier (Kitchen et al., 2020), suggesting that it is possible to modify AQP4 polarization targeting the molecular mechanisms involved in its trafficking *in vivo.* However, the sustained polarization at the astrocytic endfeet despite a partial KD, may reflect a slow protein turnover as well. *In vitro* studies showed that AQP4 half-life is around 24 hours (de Bellis et al., 2014; Neely et al., 2001), but a quantification *in vivo* is still lacking, and future studies should address this point. Indeed, it may be possible that AQP4 half-life *in vivo* is longer, determining a low protein turnover, leading in turn to an unaffected protein polarization at the astrocytic endfeet. The knowledge of AQP4 half-life *in vivo* will be important to design experimental plans that will allow for acute and chronic functional studies of AQP4 and its isoforms *in vivo*, considering also that ASOs are stable for long periods of time *in vivo*. Future studies should also address the effect of AQP4 ASOs locally injected in different brain regions. This knowledge will be important to understand whether a local impairment of the molecular machinery supporting glymphatic function, that can happen as a result to an insult in the brain, would impact CSF movement in the neuropil globally.

Paper IV

PD is the second most common neurodegenerative disease in the world after AD, and the most prominent motor disorder. The motor symptoms that characterise PD, including bradykinesia, rigidity, tremors at rest and postural instability, arise as a consequence of the degeneration of TH^+ DA neurons in the SNpc that control movements via activation of the direct or indirect pathways in the striatum, where they project their axons (Fahn, 2006; Simon et al., 2020). However, PD is also characterised by inclusions, known as LB and LN, in the cell bodies and neurites, respectively, of the remaining neurons (Poewe et al., 2017). α-syn is the major component of such inclusions (Spillantini et al., 1997), and it has been hypothesised that Lewy pathology associated to α-syn can transmit to synaptically connected brain regions, giving rise to the so-called “non-motor symptoms” of PD, which happen before motor symptoms occur, or at the latest stages of the pathology, according to the Braak hypothesis (Braak et al., 2003). Moreover, recent studies have shown that pathological α-syn fibrils with seeding activity can be found in the CSF in patients with early stages LB pathology (de Luca et al., 2019; Groveman et al., 2018; Han et al., 2020; M. Rossi et al., 2020), possibly suggesting that, when toxic α-syn is released by affected neurons in the extracellular space, part of it is

flushed out via glymphatic pathways, before being directly taken up by neighbour cells.

Despite the function of glymphatic system in AD has been extensively investigated (Harrison et al., 2020; Iliff et al., 2012; Peng et al., 2016; Xu et al., 2015), and some studies pointed to a link between glymphatic system, AQP4 and α-syn pathology in PD (Cui et al., 2021; Ding et al., 2021; Zou et al., 2019), the role played by glymphatic system in the pathogenesis of PD and clearance of α-syn has yet to be fully elucidated. In **Paper IV**, we therefore aimed to investigate CSF distribution and flow dynamics in the brain of two different mouse models of PD.

Our first choice was the 6-OHDA lesion mouse model. This is a pharmacological neurotoxic model of PD which mimics the latest stages of PD, since the toxin analogue of DA exerts its effect by being selectively taken-up by DA neurons though DAT and causes their degeneration (Cenci & Björklund, 2020). We showed that glymphatic function is unaltered in the unilateral 6-OHDA lesion model, despite AQP4 is mis-localised in the SNpc 5 weeks after the lesion. It has to be noted that when released from SNpc neurons in the striatum, DA acts at the same time on two different receptors, namely D1 and D2, to control the direct and indirect motor pathways. In supplementary figure 1 of Paper IV, we also showed that acute whole brain blockage of DA signalling injecting DA antagonists against D1 or D2 receptors intra-peritoneally, does not alter glymphatic function. However, the conditions used in our study do not completely resemble how DA signalling is affected in the brain of PD patients, where DA loss interests both hemispheres and is chronic. Therefore, future research should address this limitation in our study, expanding the investigation of DA signalling loss effects on glymphatic function in a chronic setting, and accounting for a synergistic effect of DA on both receptor types.

However, despite the 6-OHDA lesion model is very useful to recapitulate the latest stages of PD, since it is interested by a very dramatic DA neurodegeneration in the SNpc, it does not induce spontaneous development of insoluble inclusions made of α-syn (Cenci & Björklund, 2020). Therefore, to study the involvement of glymphatic system in Lewy pathology progression and α-syn removal from the brain parenchyma, we bilaterally injected wt mice with mPFFs of α-syn to induce LB pathology in the brain (Abdelmotilib et al., 2017). With this approach, we showed that AQP4 is not polarised any longer at the astrocytic endfeet in the SNpc and striatum of mPFFs injected mice. This result is in line with a previous study, where AQP4 was mis-localized in A53T transgenic mice, and α-syn tended to aggregate in the perivascular space of blood vessels in the SNpc (Zou et al., 2019). However, we did not detect any impairment in CSF flow dynamics in the brain of mPFFs injected mice in our study. A previous study showed that, blocking the meningeal lymphatic vessels in mice overexpressing mutated α-syn upon ligation of dCL, reduces glymphatic influx in the SN, aggravates α-syn deposition in the

brain and reduces glymphatic efflux to the dCL through the meningeal lymphatic pathway (Zou et al., 2019). Another study, confirmed that injection of α-syn PFFs in the striata of hemizygous AQP4$^{+/-}$ mice induces a worsened α-syn pathology in the brain, DA neurodegeneration in the SNpc and behavioural performance than α-syn PFFs injection in wt mice (Cui et al., 2021). The discrepancies in the results may be due to a different experimental set-up, that in the Zou and Cui studies aimed to worsen glymphatic function via impairment of meningeal lymphatic efflux upon dCL ligation, or with decreased AQP4 expression, respectively. Therefore, the above-mentioned studies may represent a proof-of-concept that glymphatic system is involved in the clearance of α-syn from the brain and therefore in the progression of PD pathology, rather than showing that the glymphatic system is impaired in PD.

We hypothesised that it is not PD pathology *per se* to cause an impairment in glymphatic function, but rather the reverse. However, the hypothesis that an impairment in glymphatic function, e.g. due to physiological aging (Kress et al., 2014), can aggravate PD pathology should be further addressed. The evidence that AQP4-dependent glymphatic clearance seems to be involved in the removal of α-syn from the brain in our study and in previous ones (Cui et al., 2021; Nimmo et al., 2020; Zou et al., 2019), suggests that this may be the case. Moreover, our results highlighting the presence of a subgroup of mPFFs injected mice exhibiting a worsened parkinsonian phenotype and impaired CSF flow dynamics, suggests that other failing mechanisms in PD (Crews et al., 2010) can lead to an accelerated PD pathology. We hypothesised that the intracellular systems for α-syn removal, e.g. the ubiquitin-proteasome system or the autophagy-lysosomal pathway involved in α-syn degradation (Cuervo et al., 2004; Klucken et al., 2004; Webb et al., 2003), may fail and contribute to accumulation of toxic forms of α-syn, and consequent obstruction of the machinery involved in glymphatic clearance of wastes, leading to a decreased functionality of the system. However, this is a limitation of our study since this hypothesis has not been directly tested. Moreover, it has still to be elucidated at which stage of the pathology a disfunction in glymphatic system may happen and lead to the exacerbation of Lewy pathology.

A further limitation is the lack of assessment of astrocytes and microglia reactivity, hampering the investigation of the involvement of neuroinflammation dynamics in the mPFFs-injected subgroup where the progression of PD pathology was more aggressive. It is known that neuroinflammation plays a role in many neurodegenerative diseases, PD included (Booth et al., 2017; Kam et al., 2020; Marogianni et al., 2020). A recent study showed that preventing reactive astrocytic A1 phenotype, by blocking microglia from releasing neurotoxic factors, is protective in PD mouse models (Yun et al., 2018). However, despite it is known that gliosis impairs AQP4 polarization (Yun et al., 2018), whether the conversion of astrocytes from neuroprotective to neurotoxic phenotypes can impact AQP4 polarization, and therefore glymphatic function, has still to be elucidated.

Therefore, future research should aim to the identification of critical time-points of α-syn glymphatic clearance, that could be targeted for disease-modifying interventions. Indeed, nowadays PD diagnosis is still based on motor symptoms, which happens when patients are already interested by a widespread DA neurodegeneration in the SNpc (Fearnley & Lees, 1991; Marras et al., 2018). Current therapies for PD are based on symptoms treatment though replacement of DA lost upon neurodegeneration in the SNpc, via administration of a DA precursor, e.g. Levodopa (Kobylecki, 2020). However, the treatment of patients for several years with Levodopa has been shown to induce side effects, including Levodopa-induced dyskinesia, which lower the quality of life of PD patients even more (Cenci et al., 2020). Pioneer cell replacement approaches, useful for replacement of degenerated DA cells, have been carried out in the ´80 in Lund University (Lindvall et al., 1989), where the body of work collected in this thesis has been conducted. For several years such approaches have been considered the new frontier for PD treatment, considering that transplantation of human ventral midbrain tissue in the striatum of PD patients showed beneficial effects on clinical symptomatology in some of the patients (Lindvall et al., 1989). However, follow-up studies on brain specimens after natural death of the first transplanted patients, showed that transplanted cells could develop α-syn inclusions, visible years after the transplantation (Chu & Kordower, 2010), evidence that suggested that pathological α-syn could transfer in a cell-to-cell manner and induce fibrillization of endogenous non-pathological α-syn (Lashuel et al., 2013), hampering as well the applicability of stem cells-based or *in vivo* reprogramming approached to bypass the transplantation step (Parmar, 2018). Being PD a progressive neurodegenerative motor disorder, disease-modifying interventions able to slow down the progression of the disease are the missing link to meet the patients' needs. However, the reality is that, despite some of such therapies are already under clinical trials, to date there is no cure for PD, but only therapies that can ameliorate symptoms (Fox et al., 2018). If glymphatic system will be proven to affect PD pathology progression, it may represent a potential target for disease-modifying interventions. Interestingly, the evidence in pre-clinical models that: (i) sleep is a driver of glymphatic function (Xie et al., 2013); (ii) glymphatic function is increased following exercise (He et al., 2017; von Holstein-Rathlou et al., 2018); (iii) glymphatic system follows a circadian rhythm (Hablitz et al., 2020b); (iv) low alcohol doses improve glymphatic function, but high doses impair CSF-ISF glymphatic exchange (Lundgaard et al., 2018); (v) stress is detrimental for glymphatic system (Wei et al., 2019), all together suggests that a healthy lifestyle, including enough exercise and sleep during the night time, and low stress and alcohol consumption, may be a solution to slow down the progression of the disease, at least in the initial stages of PD (Marras et al., 2019; Reichmann et al., 2022). On the other end, the development of drugs that can boost glymphatic function, although well far away from immediate applicability, would greatly benefit from the development of CSF or blood biomarkers assays to predict onset of PD in asymptomatic patients. Some biomarkers are under investigation at

the moment, but still far from applicability in clinical context (Kwon et al., 2022). Moreover, human MRI studies showing that patients with idiopathic PD exhibit impaired meningeal lymphatic drainage compared to cognitively normal controls (Ding et al., 2021), suggests that also CSF efflux pathways may be altered in PD. Then, it will also be important to clarify first whether an increased glymphatic removal of α-syn is feasible in the long run without negative effects on other clearance mechanisms, that can in turn lead to proteins and wastes accumulation and possibly induce an obstruction of the structures supporting CSF glymphatic movement in the brain, with consequent loss of function of the entire system.

Paper V

Physiological sleep and KX anaesthesia increase CSF movement from the PVSs into the brain parenchyma (Hablitz et al., 2019; Xie et al., 2013). Interestingly, core body temperature exhibits variations of 1-3 ºC during the sleep-wake cycle (Guisle et al., 2020), and even larger fluctuations, in the range of 3-10 ºC, happen over a period of 1 hour after isoflurane anaesthesia induction, with a dose-dependent pattern (Liu et al., 2017). Moreover, anaesthesia-induced hypothermia has been shown to affect parameters known to influence CSF movement in the PVSs (Bertalan et al., 2019). Intriguingly, anaesthesia-induced hypothermia has been shown to correlate with increased risk of developing AD and cognitive dysfunction (Almeida & Carrettiero, 2018; Buggy & Crossley, 2000), and pre-clinical studies in aged mice showed that the anaesthesia-induced hypothermia caused increased Tau hyperphosphorylation and cognitive decline (Xiao et al., 2013). However, the mechanisms underlying these phenomena are still unknown.

We therefore aimed to elucidate whether hypothermia negative effects could depend on altered glymphatic function, since it occurs rapidly during sleep and after induction of anaesthesia. In **Paper V**, we showed that induction of hypothermia induces decreased CBF and heart rate, as previously shown (Alva et al., 2006; Bertalan et al., 2019). When we assessed CSF flow dynamics *in vivo*, we found that glymphatic function in moderate hypothermia was impaired, possibly via disruption of two mechanisms involved in AQP4-dependent CSF movement, i.e. CSF flow in the PVSs and AQP4 polarization at the astrocytic endfeet. This result is in contrast with a previous study showing that glymphatic influx under anaesthesia negatively correlates with heart rate (Hablitz et al., 2019). However, in the Hablitz study, mice temperature was kept under normothermia conditions, and this may be the limiting factor for the correlation to exist. It has been shown that body temperature in mice positively correlates with brain water perfusion coefficient and CBF, and negatively with a coefficient of stiffness. These changes were visible especially in the cortex (Bertalan et al., 2019). The decreased CSF flow movement in the brain under moderate hypothermia condition may then be explained by a decreased water perfusion coefficient upon induction of hypothermia. Intriguingly, despite the mild

hypothermia group showed as well decreased heart rate and CBF, in this group glymphatic function was sustained, suggesting that there may be a temperature threshold, above which variation of the water perfusion coefficient do not impact glymphatic function. However, these are just speculations, since the brain water perfusion coefficient was not measured in our cohort, and future studies should verify this hypothesis.

Intriguingly, in some cases, anaesthesia has been associated with the post-operative cognitive decline (POCD) syndrome in patients undergoing surgeries (Fodale et al., 2010), and it has been linked to an increased risk of developing AD (Almeida & Carrettiero, 2018). In line with this, studies in mice have shown that anaesthesia can induce hyper-phosphorylation of Tau, although this process seems associated to anaesthesia-induced hypothermia rather than to the anaesthetic *per se* (Xiao et al., 2013). We hypothesised that the missing link between POCD, increased risk of developing AD and anaesthesia could indeed be the impaired glymphatic function following anaesthesia-induced hypothermia. Since glymphatic system has been implicated in the removal from the brain of Tau (Harrison et al., 2020) and Aβ (Iliff et al., 2012), two proteins involved in the pathogenesis of AD, it appears plausible that a decreased glymphatic clearance of those proteins from the brain can lead to their accumulation and exacerbate cognitive dysfunction. However, this hypothesis was not directly tested in our study, and future research should address this point. Lastly, it must be noted that the disruption of glymphatic system did not occur in mice treated with a mild hypothermia regime, which instead had a similar readout as normothermia-treated mice. Whilst the evidence concerning moderate hypothermia treatment in neuroprotection is controversial, mild hypothermia is recommended by most clinical trials and guidelines as a therapeutic treatment after acute ischemic stroke (Wu & Grotta, 2013) and cardiac arrest (N. Nielsen et al., 2013). Possibly, the beneficial effects of mild hypothermia rely, at least in part, on sustained glymphatic function in the brain.

Concluding remarks

This thesis addressed different methods to study glymphatic system on a macroscopic and microscopic level in rodents. The methods to study glymphatic system here presented can be easily adapted to different experimental set-up and research questions, but also highlighted the current limitations in the field, opening to new questions to be answered in the future.

As previously stated, despite CM injection is the best technique at the moment to get access to CSF in the SAS without affecting the brain (Jessen et al., 2015; Raghunandan et al., 2021), it is a quite invasive procedure for the animals (Bèchet et al., 2021a; Xavier et al., 2018), and due to ethical concerns, it is often allowed only as a terminal procedure, hampering the possibility to carry out longitudinal pre-clinical studies. It has been mentioned that the CM injection technique described in

rodents can be easily adapted to *in vivo* set-ups, e.g. through injection of contrast agents for MRI detection (Iliff, Lee, et al., 2013). Whilst this approach revealed to be very useful for brain-wide quantification of CSF tracer influx in the brain and investigation of the CSF efflux routes with *in vivo* dynamics, it is unfortunately lacking the spatial resolution to dissect the PVSs influx of CSF. To overcome this spatial resolution limitation, it would be plausible to co-inject fluorescent tracers and contrast agents in the CM, to visualise both CSF tracer dynamics *in vivo* and microscopic structures via fluorescent imaging. However, this approach would still allow only an *ex vivo* visualization of PVSs. Therefore, if possible, future research should investigate new methods to study CSF flow dynamics *in vivo* at the level of PVSs with enough spatial resolution.

Moreover, despite the fact that the influx dynamics of CSF tracers are quite well characterised and accepted by the scientific community, the efflux routes of CSF from the brain parenchyma are still controversial (Jessen et al., 2015; Nedergaard, 2013). The beginning glymphatic hypothesis suggested that the CSF circulation in the brain followed 5 consecutive steps: (i) CSF influx from the SAS on the brain surface in PVSs surrounding the arteries; (ii) movement of the CSF from the periarterial space in the brain parenchyma, facilitated by AQP4; (iii) CSF convective movement through the brain parenchyma, mixing with ISF and collection of wastes; (iv) AQP4-facilitated movement of CSF/ISF in the PVSs surrounding veins; (v) outflow back to the SAS or directed to the lymphatic system (Jessen et al., 2015; Nedergaard, 2013).

Bearing this in mind, there are some points of this hypothesis that still need to be elucidated, and it has been argued that proofs for CSF outflow along PVSs surrounding veins are little (Hladky & Barrand, 2022). On the other hand, some studies showed that solutes injected in the parenchyma are found in the wall of arteries, despite post-fixation artifacts may have caused redistribution of the tracer in compartments other than the collapsed PVSs (Bradbury et al., 1981; Dienel & Cruz, 2008; Nimmo et al., 2020). However, a more recent study, showed that cortical injected tracer accumulates rapidly along the wall of arteries, but not of veins, with two-photon imaging *in vivo* (Arbel-Ornath et al., 2013). Therefore, further investigation of the paths followed by CSF/ISF when exiting the brain parenchyma should be done, and it cannot be excluded that parameters like intraparenchymal tracer injection rate or injection site may contribute to different patterns of CSF/ISF outflow from the brain parenchyma. Moreover, it must be considered that stereotaxic surgery approaches have been shown to acutely impair glymphatic system (Mestre, Hablitz, et al., 2018), possibly allowing for the intraparenchymal-injected tracer to use different non-canonical pathways to exit the brain. Therefore, there is need to find and implement a reproducible methodology to study intraparenchymal solutes' efflux in a more physiological setting, avoiding stereotaxic injection of solute in the brain.

Regardless of the specific route, another point that still needs to be elucidated is whether CSF/ISF recirculation in the SAS happens after outflow from the brain parenchyma, or whether it is simply redirected to the cervical lymphatic system. A recent review suggested that around 80% of the CSF/ISF is directed to the lymphatic system, without mixing with SAS or i.c.v. fresh CSF, supposedly avoiding the recirculation of wastes previously cleared from the brain parenchyma (Rasmussen et al., 2022). However, the evidence that ablation of meningeal lymphatic vessels does not alter ICP or brain water content (Aspelund et al., 2015), seems to argue against it. Moreover, assuming that the calculated percentage is correct, it cannot be excluded that this percentage may decrease as a consequence of age, brain insults or diseases, possibly provoking an obstruction of the system by wastes previously cleared from the brain parenchyma.

Moreover, the contribution of each of the pathways shown to be involved in CSF extra-cranial efflux (for details, see the Introduction chapter) is still a matter of debate. To make the picture even more complicated, there is the possibility that only a percentage of CSF in the SAS will get access to the parenchyma. An MRI study quantified this quantity as around 20% (Lee et al., 2018), but it must also be noted that CSF movement is affected by anaesthetics. A recent study indeed showed that CSF tracer injected i.c.v. was rapidly eliminated via the olfactory route and the cribriform plate in awake mice, completely bypassing the dorsal SAS and supposedly the parenchyma, whilst the CSF tracer slowly accumulated in the dorsal SAS in anaesthetised mice (Q. Ma, Ries, et al., 2019). Therefore, more studies should investigate and quantify not only the percentage of CSF/ISF direct outflow to the lymphatic system or other pathways, but also the percentage of SAS CSF entering brain parenchyma, taking into account that the quantity may vary based on the sleep-wakefulness cycle and anaesthetic regime.

In conclusion, this thesis confirms that AQP4 correlates with glymphatic function but with some exceptions, that need to be further investigated. Moreover, a combination of the methods presented in Paper I and Paper II was successfully implemented in Paper IV and Paper V to address glymphatic function in the pathophysiological setting of PD pathogenesis and anaesthesia-induced hypothermia. On the other hand, Paper III proposed an alternative approach for AQP4 studies, avoiding the use of transgenic mouse models, that may be useful depending on the research question that needs to be addressed.

Given the high interest in the glymphatic system, its potential involvement in disease progression, and the need to develop disease-modifying interventions for the most common neurological disorders, this field of research will most likely rapidly develop in the future, and possibly provide diagnostic tests for glymphatic function, that can predict a disease years before its onset, with the chance of early interventions for treatment or prevention.

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About the author

Roberta Battistella developed a fascination for the field of neuroscience during her bachelor's degree in Biology. Her interest in the brain brought her to complete a master's degree in Neurobiology at Sapienza University of Rome in 2017. Her master´s thesis work on compensatory mechanisms supporting memory function in pre-symptomatic Alzheimer´s disease, motivated her to move to Lund, Sweden, pursuing studies on the newly discovered "cleansing system" of the brain, the glymphatic system. She was enrolled as a Human Biology student and as a PhD student thereafter, under the supervision of Dr. Iben Lundgaard. During her time in Lund, Roberta sought to investigate glymphatic function in physiological and pathological settings, with particular regard to the role played by this system in Parkinson´s disease pathogenesis.

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