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Ramos, Marta

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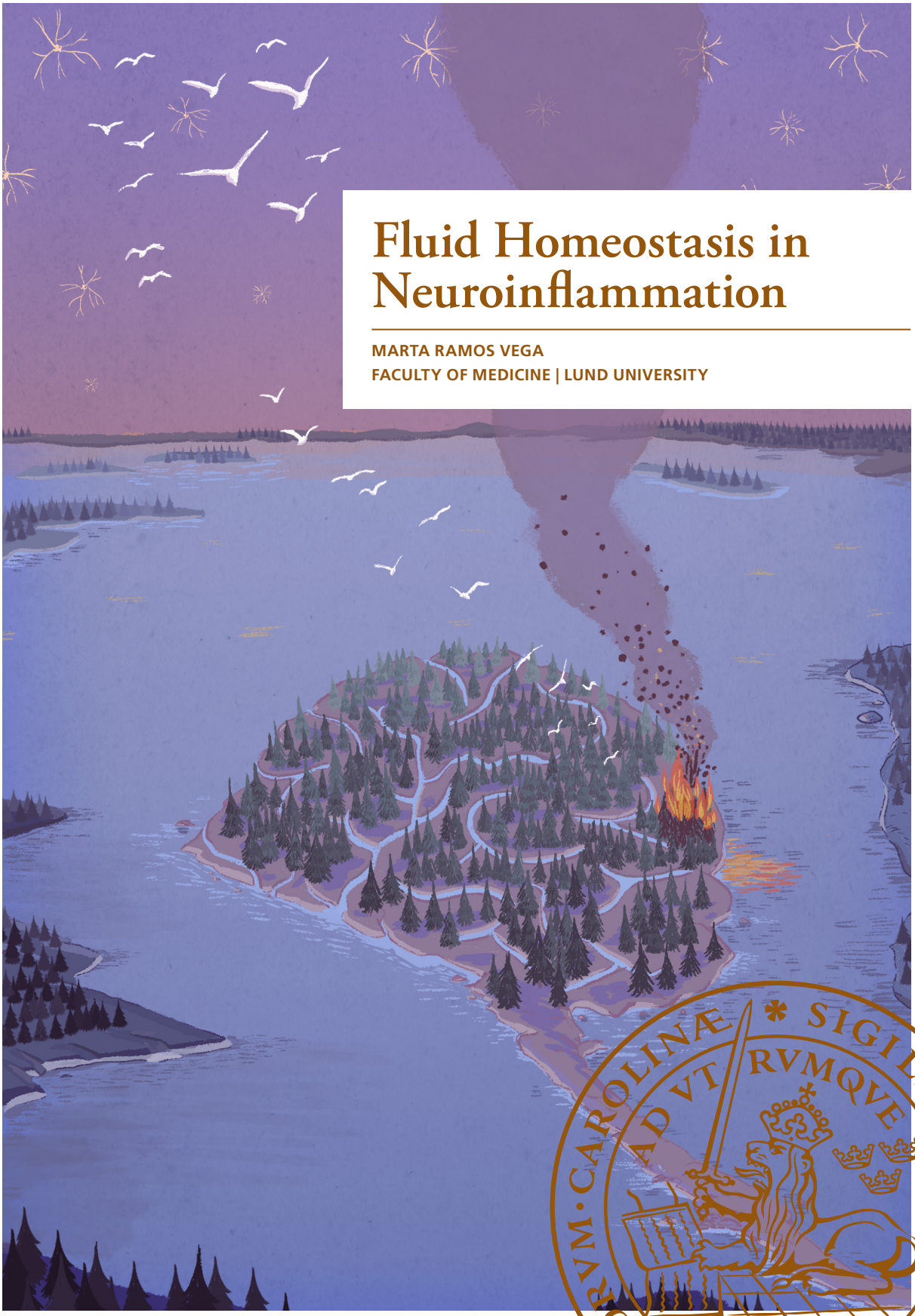

Fluid Homeostasis in Neuroinflammation

MARTA RAMOS VEGA
FACULTY OF MEDICINE | LUND UNIVERSITY

Fluid Homeostasis in Neuroinflammation

Fluid Homeostasis in Neuroinflammation

Marta Ramos Vega

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Abstract

Maintaining fluid homeostasis is very important for brain health, partly because the cerebrospinal fluid, which washes in and out, cleans the brain from accumulated waste. This waste management fluid system that helps maintain the brain clean and healthy, named “glymphatic system”, is dependent on sleep and relies on the “helper cells” of the brain known as astrocytes. During inflammation, the body sends immune cells to help protect an organ against infection or to heal an injury, which, of course, is a good thing. The brain also counts on immune cells that play housekeeping roles, but eventually, immune cells can take on more sinister personalities. Sometimes, when inflammation becomes too much or dysregulated, it can cause complications. Inflammation in the brain (neuroinflammation) has been implicated in cognitive decline and neurodegenerative disorders and is directly responsible for damage in diseases such as multiple sclerosis and meningitis.

The purpose of this thesis was to investigate how neuroinflammation affects the fluid circulation in the brain, but also to decipher how the fluid systems react to help the brain when equilibrium is lost because of inflammation. In paper I, we explored the effects of LPS-induced systemic inflammation on perivascular CSF distribution and its relationship to neuroinflammation. In Paper II, we aimed to determine whether acute bacterial meningitis affects CSF transport in the brain and the role of neutrophil extracellular traps (NETs) for glymphatic function and CSF drainage to the periphery. In papers III and IV, we studied a rodent model of multiple sclerosis, which is a typical inflammatory disease where the insulating covers of nerve cells are damaged. First, we explored the relationship between meningeal inflammation, spinal nerve swelling, and CSF flow disturbances in the spinal cord of EAE mice. In the last paper we tried to elucidate the effects of neuroinflammation in the spinal cord on oxygenation and vascular perfusion in the EAE model.

All in all, the projects in this thesis contribute to the scientific understanding of how neuroinflammation interacts with brain fluid transport. This knowledge can aid us in developing new therapeutic targets to combat inflammation, which has implications for many disorders with an inflammatory component, such as neurodegenerative and neuroinflammatory diseases.

Key words Neuroinflammation, cerebrospinal fluid, brain fluids, inflammation, multiple sclerosis, meningitis

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Marta Ramos Vega

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To my parents

The life so short, the craft so long to learn

- Hippocrates of Kos

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List of original papers and manuscripts

This thesis is based on the following papers:

I. **Acute systemic LPS-exposure impairs perivascular CSF distribution in mice.**
Manouchehrian O., **Ramos M.**, Bachiller S., Lundgaard I. & Deierborg T.
Journal of Neuroinflammation, 2021 Jan 29;18(1):34. doi: 10.1186/s12974-021-02082-6.

II. **DNase treatment prevents cerebrospinal fluid block in early experimental pneumococcal meningitis.**
Pavan C.*, L. R. Xavier A.*, **Ramos M.**, Fisher J., Kritsilis M., Linder A., Bentzer P., Nedergaard M. & Lundgaard I.
Annals of Neurology, 2021 Oct;90(4):653-669. doi: 10.1002/ana.26186.

III. **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., Hauglund N.L, Lundgaard I., Nedergaard M.
Manuscript in preparation

IV. **Mapping of neuroinflammation-induced hypoxia in the spinal cord using optoacoustic imaging.**
Ramos-Vega M., Kjellman P., Todorov M. I., Kylkilahti T., Bäckström B. T., Ertürk A., Madsen C. D., & Lundgaard I.
Acta Neuropathologica Communications, 2022 Apr 11;10(1):51. doi: 10.1186/s40478-022-01337-4.

*Equal contribution

List of papers not included in the thesis

Achieving brain clearance and preventing neurodegenerative diseases—A glymphatic perspective

Kylkilahti, T. M., Berends, E., **Ramos, M.**, Shanbhag, N. C., Töger, J., Bloch, K. M. & Lundgaard, I., *Journal of cerebral blood flow and metabolism*, 2021.

Cisterna magna injection in rats to study glymphatic function.

Ramos, M., Burdon Bechet, N., Battistella, R., Pavan, C., Xavier, A. L. R., Nedergaard, M. & Lundgaard, I., *Methods in molecular biology*, 2019.

Lorcaserin bidirectionally regulates dopaminergic function site-dependently and disrupts dopamine brain area correlations in rats

De Deurwaerdère, P., **Ramos, M.**, Bharatiya, R., Puginier, E., Chagraoui, A., Manem, J., Cuboni, E., Pierucci, M., Deidda, G., Casarrubea, M. & Di Giovanni, G., *Neuropharmacology*, 2020

Lorcaserin alters serotonin and noradrenaline tissue content and their interaction with dopamine in the rat brain

Di Giovanni, G., Bharatiya, R., Puginier, E., **Ramos, M.**, De Deurwaerdère, S., Chagraoui, A. & De Deurwaerdère, P., *Frontiers in Pharmacology*, 2020

Lay summary

In the same way that natural ecosystems consist of lands, trees, animals, rivers, clouds, and so on, the "ecosystem" of the human body consists of organs, fluids, and energy. Just as there are complex relationships in nature between its elements – for example, when the rainwater bathes the soil, the land is replenished and the water is taken up by trees, while rivers transport it back towards open waters; we have similar interactions between the elements in our bodies. And just as the natural ecosystem depends on maintaining an equilibrium, the health of the human body depends on maintaining a balance between and within its organs. This is what we call homeostasis. Particularly, the brain is reminiscent of an island in that it is literally floating in a fluid called cerebrospinal fluid. Maintaining fluid homeostasis is very important for brain health, partly because the cerebrospinal fluid, which washes in and out, cleans the brain from accumulated waste. This waste management fluid system that helps maintain the brain clean and healthy, the so-called "glymphatic system" is dependent on sleep and relies on the "helper cells" of the brain known as astrocytes ("star cells" in Greek).

During inflammation, the body sends immune cells to help protect an organ against infection or to heal an injury, which, of course, is a good thing. The brain also counts on immune cells that play housekeeping roles, but eventually, immune cells can take on more sinister personalities. Sometimes, when inflammation becomes too much or dysregulated, it can cause complications, and just like that, the harmony and homeostasis in the brain are lost. Inflammation in the brain (neuroinflammation) has been implicated in cognitive decline and neurodegenerative disorders and is directly responsible for damage in diseases such as multiple sclerosis and meningitis.

The purpose of this thesis was to investigate how neuroinflammation affects the fluid circulation in the brain, but also to decipher how the fluid systems react to help the brain when equilibrium is lost because of inflammation.

In paper I, we aimed to understand how inflammation initiated in the rest of the body affects the brain and the distribution of cerebrospinal fluid. We injected a bacterial endotoxin in mice and found that the flow of cerebrospinal fluid is disrupted by general inflammation, which was coupled with neuroinflammatory responses in microglial cells.

In paper II, we modeled meningitis (inflammation of the meninges, the layers that surround the brain and spinal cord) in rats. We found that some immune cells called

neutrophils, which are particularly reactive during meningitis can slow down the cerebrospinal fluid by releasing sticky masses called NETs. Targeting those seemed to restore the fluid flow and ameliorate the brain swelling caused by the disease.

In papers III and IV, we studied a rodent model of multiple sclerosis, which is a typical inflammatory disease where the insulating covers of nerve cells are damaged. In the first of these papers, we found that before symptoms appear, swelling of the nerves of the spine can stop the outflow of cerebrospinal fluid from the nervous system, and immune cells can more easily invade the meninges around those nerves. In the latter paper, we studied how the swelling of the spinal cord caused by neuroinflammation affects vascular function and oxygenation of the tissue. Using novel techniques such as optoacoustic imaging, we can detect oxygenation changes in sick mice in vivo, and we show that these changes are related to defects in blood perfusion during neuroinflammation.

All in all, the projects in this thesis contribute to the scientific understanding of how neuroinflammation interacts with brain fluid transport. This knowledge can aid us in developing new therapeutic targets to combat inflammation, which has implications for many disorders with an inflammatory component, such as neurodegenerative and neuroinflammatory diseases.

Resumen en español

Del mismo modo que ciertos ecosistemas naturales se componen de tierra, arboles, animales, ríos, nubes, etcétera; el "ecosistema" del cuerpo humano se compone de órganos, fluidos y energía. Y así como existen complejas interacciones en la naturaleza entre sus elementos (por ejemplo, cuando la lluvia riega los campos, los árboles absorben el agua para nutrirse, el agua es absorbida por los árboles, mientras que los ríos la transportan de regreso a aguas abiertas), tenemos relaciones similares entre los sistemas que componen nuestros cuerpos. De la misma manera que los ecosistemas naturales necesitan estar en equilibrio, nuestro cuerpo y su salud depende de mantener un balance dentro de sus órganos. Esto es lo que llamamos homeostasis. El cerebro en particular recuerda a una isla, ya que está literalmente flotando en un fluido llamado líquido cefalorraquídeo. Mantener homeostasis de los fluidos en el cerebro es muy importante para su salud, en parte porque el líquido cefalorraquídeo, que circula también a través del cerebro, lo limpia de residuos metabólicos acumulados. Este sistema de limpieza a través del fluido, denominado sistema glinfático, está especialmente activo durante el sueño y depende de unas células auxiliares del cerebro con forma de estrella llamadas astrocitos.

Durante los procesos de inflamación, las células del sistema inmune ayudan a proteger los tejidos corporales contra infecciones o daños, lo cual, por supuesto, es bueno. El cerebro también cuenta con células inmunitarias residentes que cuidan del tejido, pero eventualmente las células inmunes pueden cambiar de personalidad. A veces, cuando la inflamación se vuelve excesiva o se desregula, puede causar complicaciones, y así, se pierde la harmonía y homeostasis en el cerebro. La inflamación en el cerebro (neuroinflamación) se ha relacionado con el deterioro cognitivo y los trastornos neurodegenerativos y es directamente responsable del daño en enfermedades como la esclerosis múltiple y la meningitis.

El propósito de esta tesis es investigar cómo la neuroinflamación afecta la circulación de fluidos en el cerebro, pero también descifrar cómo reaccionan estos sistemas de fluidos para ayudar al cerebro cuando se pierde el equilibrio debido a la inflamación.

En el artículo I, nuestro objetivo era comprender cómo la inflamación iniciada en el resto del cuerpo afecta al cerebro y a la distribución del líquido cefalorraquídeo. Inyectamos una endotoxina bacteriana en ratones y descubrimos que el flujo de

líquido cefalorraquídeo se ve interrumpido por la inflamación general, a la vez que activa las células inmunológicas del cerebro, la microglía.

En el artículo II, modelamos la meningitis (inflamación de las meninges) en ratas. Descubrimos que algunas células inmunes llamadas neutrófilos, que son particularmente reactivas durante la meningitis, pueden ralentizar el líquido cefalorraquídeo al liberar una especie de redes (NETs). Dirigirse a ellos pareció restaurar el flujo de fluidos y mejorar la inflamación cerebral causada por la enfermedad. Destruir estas redes mejora el flujo y resuelve el edema (acumulación de fluido) en el cerebro que se produce durante esta enfermedad.

En los artículos III y IV, estudiamos un modelo animal de la esclerosis múltiple, una enfermedad de naturaleza inflamatoria y autoinmunitaria en la que el sistema inmunitario ataca la vaina de mielina que recubre las neuronas. En el primero de estos artículos, encontramos que antes de que los síntomas aparecieran, los nervios espinales estaban hinchados (edema) y no permitían el paso del líquido cefalorraquídeo, y las células inmunes podían invadir así más fácilmente las meninges alrededor de estos nervios. En el otro artículo, estudiamos como el edema en la medula espinal durante la neuro inflamación en el modelo de esclerosis múltiple afecta la función vascular y el oxígeno en el tejido. Utilizando técnicas punteras como la fotoacústica pudimos detectar cambios en oxígeno en los ratones enfermos en tiempo real y mostramos que estos cambios están relacionados con defectos en la perfusión sanguínea durante la neuro inflamación.

En conclusión, los proyectos de esta tesis contribuyen al conocimiento científico de como la neuro inflamación interactúa con el transporte de fluidos en el cerebro. Este conocimiento puede ayudar a que en el futuro desarrollemos nuevas terapias para combatir la inflamación en el cerebro, que tiene implicaciones en muchas enfermedades tanto neuro inflamatoria como neurodegenerativas.

Populärvetenskaplig sammanfattning

På samma sätt som naturliga ekosystem består av landområden, träd, djur, floder, moln och så vidare, består människokroppens "ekosystem" av celler, organ, vätskor och energi. Precis som det finns komplexa förhållanden i naturen mellan dess beståndsdelar – till exempel när regnvatten faller till marken och tas upp av träd, och vattendrag kan transportera det tillbaka mot öppna sjöar och hav; har vi liknande interaktioner mellan beståndddelar i våra kroppar. Och precis som att det naturliga ekosystemet är beroende av att upprätthålla en jämvikt, beror människokroppens hälsa på att upprätthålla en balans mellan och inom dess organ. Detta är vad vi kallar homeostas.

Hjärnan är som en ö, genom att den bokstavligen flyter i en vätska som kallas cerebrospinalvätska. Att upprätthålla homeostas är mycket viktigt för hjärnans hälsa, bland annat eftersom cerebrospinalvätskan, som sköljs in och ut, renar hjärnan från ansamlat avfall. Detta avfallshanteringsvätskesystem som hjälper till att hålla hjärnan ren och frisk sker under sömn och är beroende av hjärnans "hjälparceller" som kallas astrocyter ("stjärnceller" på grekiska).

Vid inflammation skickar kroppen immunceller för att hjälpa till att skydda ett organ mot infektion eller för att läka en skada, vilket naturligtvis är bra. Hjärnan har egna immunceller som är viktiga för homeostas, men dessa celler kan också bidra till sjukdom. När inflammationen blir för kraftig eller blir oreglerad, kan det orsaka komplikationer – som gör att hjärnans harmoni och homeostas går förlorad. Inflammation i hjärnan (neuroinflammation) har kopplats till kognitiva och neurodegenerativa störningar och är direkt ansvarig för skadorna i multipel skleros och hjärnhinneinflammation.

Syftet med detta examensarbete var att undersöka hur neuroinflammation påverkar cerebrospinalvätskan och blodcirkulationen i hjärnan, men också att tyda hur dessa vätskesystem reagerar för att hjälpa hjärnan när jämvikten försvinner på grund av inflammation.

I artikel I syftade vi till att förstå hur inflammation som initieras i resten av kroppen påverkar hjärnan och fördelningen av cerebrospinalvätska. Vi injicerade ett bakteriellt endotoxin i möss och fann att flödet av cerebrospinalvätska störs av allmän inflammation, vilket var kopplat till neuroinflammatoriska svar i mikrogliaceller och sannolikt relaterat till nedgången i hjärtfrekvens.

I artikel II modellerade vi hjärnhinneinflammation (inflammation i hjärnhinnorna - skikten som omger hjärnan och ryggmärgen) hos råttor. Vi fann att vissa immunceller som kallas neutrofiler, som är särskilt reaktiva under hjärnhinneinflammation, kan bromsa cerebrospinalvätskan genom att släppa ut nätliknande strukturer. Att rikta in sig på dessa nät verkade återställa vätskeflödet och lindra hjärnsvullnaden som orsakades av sjukdomen.

I projekt III och IV studerade vi en musmodell med multipel skleros, en typisk inflammatorisk sjukdom där de isolerande höljena på nervceller skadas. I den första av dessa två artiklar fann vi att innan symtom uppträder kunde nervsvullnad i ryggraden stoppa utflödet av cerebrospinalvätska från nervsystemet, och immunceller kunde lättare invadera hjärnhinnorna runt dessa nerver. I den senare artikeln studerade vi hur svullnaden av ryggmärgen under neuroinflammation i multipel sklerosmodellen påverkar kärlfunktionen och syresättningen av vävnaden. Med hjälp av nya tekniker som optoakustisk tomografi kunde vi upptäcka syresättningsförändringar hos sjuka möss in vivo, och vi visade att dessa, åtminstone delvis, är relaterade till defekter i blodgenomströmning under neuroinflammation.

Sammantaget bidrar artiklarna i denna avhandling till den vetenskapliga förståelsen av hur neuroinflammation interagerar med vätsketransport i hjärnan. Denna kunskap kan hjälpa oss att utveckla nya terapeutiska targets för att behandla inflammation, vilket har konsekvenser för många tillstånd i centrala nervssystemet med en inflammatoriska komponenter, såsom neurodegenerativa och neuroinflammatoriska sjukdomar.

Abbreviations

aCSF	Artificial cerebrospinal fluid
AD	Alzheimer's disease
Aβ	Amyloid beta
ANOVA	Analysis of variance
APC	Antigen-presenting cell
AQP4	Aquaporin-4
BBB	Blood-brain barrier
BSCB	Blood-spinal cord barrier
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFA	Complete Freund's adjuvant
CM	Cisterna magna
CNS	Central nervous system
CP	Choroid plexus
CSF	Cerebrospinal fluid
cSVD	Cerebrovascular small vessel disease
DAPI	4′,6-diamidino-2-phenylindole
DBE	Dibenzyl ether
ddH_2O	Double-distilled water
DNase	Deoxyribonuclease
DRG	Dorsal root ganglia
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
EEG	Electroencephalography

ECi	Ethyl cinnamate
Glut1	Glucose transporter 1
GFAP	Glial fibrillary acidic protein
FITC	Fluorescein isothiocyanate
ICP	Intracranial pressure
IFNb	Interferon beta 1
ISF	Interstitial fluid
KO	Knock-out
KX	Ketamine/xylazine
LPS	Lipopolysaccharide
LVs	Lymphatic vessels
Lyve1	Lymphatic vessel endothelial hyaluronan receptor 1
MHCII	Major histocompatibility complex class II
MRI	Magnetic resonance imaging
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
MSOT	Multispectral optoacoustic tomography
NETs	Neutrophil extracellular traps
NKCC1	Na-K-Cl cotransporter 1
OAP	Orthogonal arrays of particles
OE	Oxygen enhancement
PAMP	Pathogen-associated molecular pattern
PD	Parkinson’s disease
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PLP	Proteolipid protein
Prox1	Prospero homeobox protein 1
SEM	Standard error of the mean
SO_2	Oxygen saturation

TLR4	Toll like receptor 4
VesSAP	Vessel segmentation and analysis pipeline
3R	Replacement, reduction, refinement

Introduction

Homeostasis refers to the physiological state of internal, physical, and chemical equilibrium the body needs to maintain for optimal functioning. It requires that many variables are kept within certain limits, such as body temperature and fluid balance (Silverthorn 2019). Maintaining homeostasis in the brain is all the more important because the central nervous system (CNS) controls many of the bodily functions that keep the body in homeostasis.

The brain of mammals is approximately 80% water, distributed in different compartments: the intracellular fluid, the interstitial fluid (ISF), the cerebrospinal fluid (CSF), and the blood (Brinker et al. 2014). Thus, fluid balance and the complex fluid movements between the different compartments are essential for normal brain function. Not only do these fluids help maintain the activity of cells (neurons and glial cells) and clear waste products from the brain, but they are also important for keeping functional interactions between the brain and other organs (Brinker et al. 2014). For example, the CSF aids the distribution of substances and hormonal factors between brain cells, to which minimal changes can result in malfunctioning of the central nervous system and, therefore of, the whole organism.

Many pathologies and disorders are known to be caused or influenced by impairment of brain fluid homeostasis, such as brain edema from stroke or meningitis, hydrocephalous, traumatic brain injury, subarachnoid haemorrhage, and neurodegenerative diseases, among others (Kimelberg 2004).

Fluids and barriers of the CNS

Different interfaces help maintain fluid homeostasis in the CNS while providing protection to the brain and spinal cord from all sorts of potentially harmful substances by regulating their exchange between the blood, the CSF, and the brain parenchyma; most importantly the blood-brain barrier (BBB), the brain-CSF barrier, and the blood–CSF barrier (Miller and Zachary 2017). The largest barrier in the CNS is the BBB (and the blood-spinal cord barrier), which is comprised by the cerebral endothelium. Furthermore, the brain is encased by the meninges and the skull (structural barrier), and thus it is very important to maintain the brain tissue and extracellular fluid volumes stable to avoid an increase in intracranial pressure

(ICP), which can have fatal consequences (Miller and Zachary 2017). Most barriers and mechanisms protecting the CNS are summarized in Table 1.

Defense Mechanisms against Injury and Infectious Microbes in the Central Nervous System
SKIN Structural and functional (secretions) barrier.
CALVARIA, VERTEBRAE Structural barriers
MENINGES, CEREBROSPINAL FLUID Structural and functional (continuous flow of CSF) barrier.
BARRIER SYSTEMS **Blood-brain Barrier** Structural and functional barrier formed by the specialized endothelium and neurovascular unit. **Blood-CSF Barrier** Structural and functional barrier formed by choroid plexus and arachnoid membrane. **Brain-CSF Barrier** Formed by the glia limitans (astrocytic foot processes immediately subjacent to the pia mater) and the ependymal barrier.
MICROGLIA, TRAFFICKING MACROPHAGES Resident and migrating cells that are part of the monocyte-macrophage system.
INMUNOLOGIC RESPONSES Innate and adaptive immunologic responses from the body's immune system.

Table 1. Defense mechanisms of the CNS summarized. Modified from Miller and Zachary (2017).

CNS blood supply

The brain only comprises around 2% of bodyweight but receives about 15% of total cardiac output (Meng et al. 2015), resulting in the brain being one of the most perfused organs of the body. This is related to its high metabolism, particularly oxidative metabolism, which requires constant blood flow. The circulation in the brain is also special due to the rarely prominent role of the main arteries in vascular

resistance, which prevents arterial pressure fluctuations in the microvasculature and provides constant blood flow to the brain (Cipolla 2009).

The cerebral vasculature is organized to supply the brain's metabolic needs (Cipolla 2009). Firstly, there are pial vessels that sit on the surface of the brain within the leptomeninges (meningeal layers that cover the brain) adjacent to the glia limitans (cortex's outermost layer) and are bathed by the CSF (Jones 1970; Mestre et al. 2022). The architecture of pial vasculature forms a collateral network so that if one vessel is occluded the cerebral blood flow, and the delivery of oxygen and nutrients, is not strikingly affected (Nishimura et al. 2007). Eventually pial arteries penetrate the brain and become penetrating arterioles, which are surrounded by the perivascular or Virchow-Robin spaces as a continuation of the subarachnoid space (Cipolla, Li, and Vitullo 2004). Lastly when arterioles dive deeper into the brain tissue, they become parenchymal arterioles and eventually capillaries. The perivascular spaces, arterioles and capillaries are surrounded by the endfeet of astrocytes (Cohen et al. 1996; Rennels and Nelson 1975).

Compared to a large amount of literature available about cerebral vasculature and blood supply, there is limited information about the spinal cord blood circulation (Bartanusz et al. 2011; Joshi, Ornstein, and Young 2010). Briefly, the spinal cord is supplied by three major arteries that run in the subarachnoid space starting from the brain and run along the whole length of the spine (Bosmia et al. 2015). These send branches to the parenchyma of the spinal cord and form anastomoses or connections with segmental medullary arteries which enter the spinal cord alongside nerve roots. Although not as studied as cerebral blood flow, spinal cord blood flow measurements have shown that it mimics cerebral flow considerably (Joshi, Ornstein, and Young 2010).

Despite featuring some different structures, most vessels in the central nervous system have highly specialized endothelium, which constitutes the BBB, with unique barrier properties that regulate the transport of nutrients, solutes and water to and from the brain (Cipolla 2009).

Blood-brain barrier

In this section, I will describe some general properties of the BBB. It should be noted that in the spinal cord, this barrier is called the blood spinal cord barrier (BSCB). It has some distinguishing properties compared to the BBB, such as glycogen deposits (Sharma 2005), increased permeability to certain cytokines (Prockop et al. 1995; Pan, Banks, and Kastin 1997), and decreased expression of specific junction proteins (Ge and Pachter 2006). However, the BSCB shares the same morphological building blocks and most of the proteins with the BBB, as well as the same function; therefore, the BSCB in particular will not be discussed separately in further details.

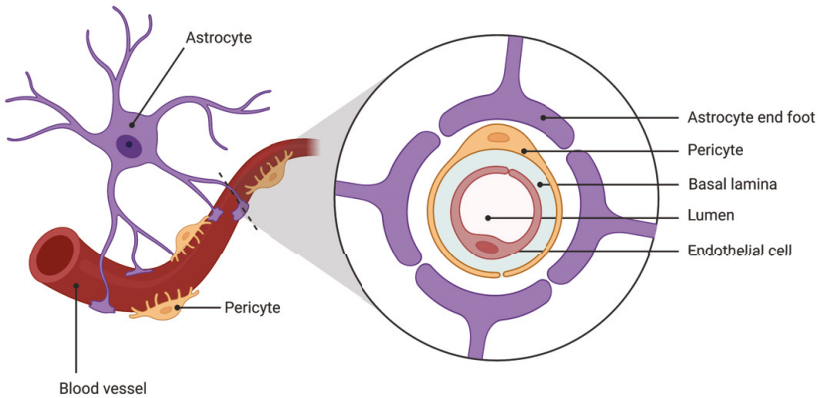

Fig 1. Cellular constituents of the BBB. In addition to specialized endothelial cells, the BBB is comprised of the capillary basal lamina, astrocytic processes, and pericytes. These BBB components together with microglial and neuronal cells complete the structure of the neurovascular unit. Created with Biorender.

The BBB is a selective semipermeable wall of endothelial cells connected by tight junctions, which are comprised of small subunits of transmembrane proteins, such as occludins, claudins, and junction adhesion molecules (Pardridge 2005; Kniesel and Wolburg 2000). These specialized junctions provide a restrictive but controlled barrier by limiting filtration and passive diffusion from the blood into the brain parenchyma but also include transporters that modulate the transport of nutrients into the brain (Brightman and Tao-Cheng 1993). Even though the tight junctions in endothelial cells are what make up the BBB, other components of what is known as the "neurovascular unit", such as astrocytes, pericytes, the basal lamina, and even neurons, also contribute to this barrier (Figure 1) (Muoio, Persson, and Sendeski 2014). Particularly, astrocytes play a vital role in modulating blood flow, upregulating tight junction proteins, and communicating between other components of the BBB and neurons (Rennels and Nelson 1975; Lok et al. 2007; Hamel 2006). Moreover, this interaction is suspected to work both ways; for example, astrocytes express aquaporin-4 (AQP4) channels only in their endfeet that are in direct contact with the vasculature but not in astrocytic membranes that are only interacting with neurons.

Meninges

The meninges are the three membranous layers that cover the brain and spinal cord: the innermost layer is the pia mater, in the middle is the arachnoid mater, a web-like membrane with fluid, and the outermost layer adjacent to the skull is the dura mater

(Figure 2) (Mack, Squier, and Eastman 2009). They perform functions of both vascular support and protection against mechanical damage to the brain, together with the cerebrospinal fluid. Since they are a common site of infection (e.g. meningitis) and intracranial bleedings, they are in many cases involved in cerebral pathologies (Rua and McGavern 2018).

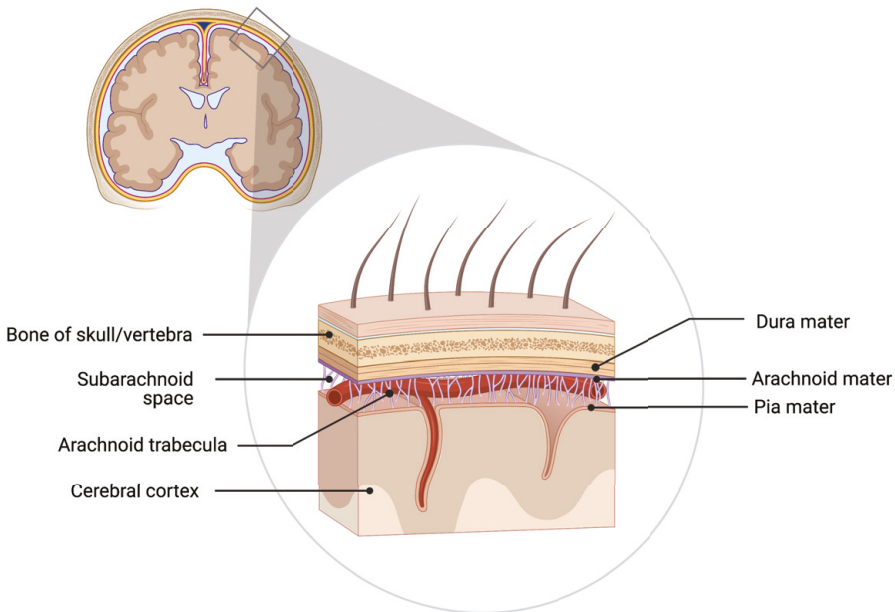

Fig 2. The three layers of the meninges. Meninges are three layers of membranes that cover and protect the brain and spinal cord. Created with Biorender.

The pia mater, which is in direct contact and tightly adhered to the surface of the brain and spinal cord, hosts the pial vessels that supply the brain and is semipermeable to the CSF that flows around the vessels (Jones 1970; Mestre et al. 2022).

The pial layer is connected to the arachnoid mater by collagen bundles and trabeculae (Adeeb et al. 2013; Coles et al. 2017). Both are very thin compared to the dural layer and are jointly referred to as the leptomeninges (Vandenabeele, Creemers, and Lambrichts 1996). The arachnoid layer contains the CSF beneath it, in the subarachnoid space (SAS), and features tight junctions similar to the ones in the BBB to regulate the transport of substances since it is the only barrier that separates the CNS from the most outer and exposed dural layer (Vandenabeele, Creemers, and Lambrichts 1996).

The thicker dura mater is highly vascularized and includes blood vessels and sinuses that drain the dura and cerebral veins (Coles et al. 2017). The dural blood vessels do not present the same junctions as the BBB. They are fenestrated and have the ability to support immune cell trafficking (Rua and McGavern 2018). Notably, the dura has attracted much attention from the scientific community in recent years with the rediscovery of the meningeal lymphatic vessels (LVs). These were first observed by Mascagni in 1787, and later by other two groups (Andres et al. 1987; Mascagni and Santi 1787; Waggener and Beggs 1967). However, their basic biological function was more recently described by the groups of Kipnis and Alitalo, and later discovered in humans by other groups (Louveau et al. 2015; Absinta et al. 2017; Eide et al. 2018; Aspelund et al. 2015). The latest studies showed that meningeal LVs are a network of vessels that express lymphatic endothelial markers such as Prox1, Lyve1 and Podoplanin and run parallel to dural sinuses and arteries in the meninges. The authors proposed that these vessels drain CSF carrying different products into the deep cervical lymph nodes (Louveau et al. 2015; Louveau et al. 2018).

Cerebrospinal fluid

The cerebrospinal fluid or CSF is a clear body liquid that is very similar to blood plasma (although it contains less protein) and occupies the subarachnoid space and brain ventricles. It acts as a mechanical barrier that provides cushioning to the brain, but it also plays an important role in brain homeostasis: modulating acid-base balances, maintaining a correct electrolyte environment, and delivering nutrients such as glucose to the brain (Wood 1980; Wright, Lai, and Sinclair 2012). Even though, over the years, functions and mechanisms of the CSF have been elucidated, many questions remain.

The first written description of the CSF stems from more than 3000 years ago (Blomstedt 2014). It is a text found in an ancient Egypt doctor's journal describing a fluid coming out from a skull fracture. Despite that first recognition and some later descriptions of a fluid in the brain by ancient physicians such as Hippocrates and Galen, subsequent anatomists and physiologists missed the fact that the ventricular system was full of this fluid for more than sixteen centuries (Deisenhammer 2015). It is generally accepted that the author that identified and described the extent and volume of CSF in the ventricles and subarachnoid space for the first time was Domenico Cotugno (Herbowski 2013). In 1828 François Magendie, established the connection and continuity between the ventricles, the subarachnoid space and described that the space surrounded the brain and spinal cord (Magendie 1828; Tubbs et al. 2008). It was Magendie himself who named the liquid "cerebrospinal fluid" for the first time in his study "*Mémoire physiologique sur le cerveau"* (1828) (Deisenhammer 2015; Rasmussen, Mestre, and Nedergaard 2022).

Only a few years later the perivascular spaces or Virchow-Robin spaces, were described by Charles Robin and Rudolf Virchow as channels surrounding the vasculature, separating the blood vessels from the brain and through which molecules and substances could travel (Walter and Scott 2017). The last key discovery in the 18th century came from Key and Retzius, who injected blue gelatin in the CSF compartment and published detailed drawings of where the CSF spreads in the brain (Key and Retzius 1875; Rasmussen, Mestre, and Nedergaard 2022). They discovered that the gelatin traveled to arachnoid granulations and nasal lymphatics, showing for the first time that the CSF exits the brain and goes into the bloodstream through those paths (Figure 3).

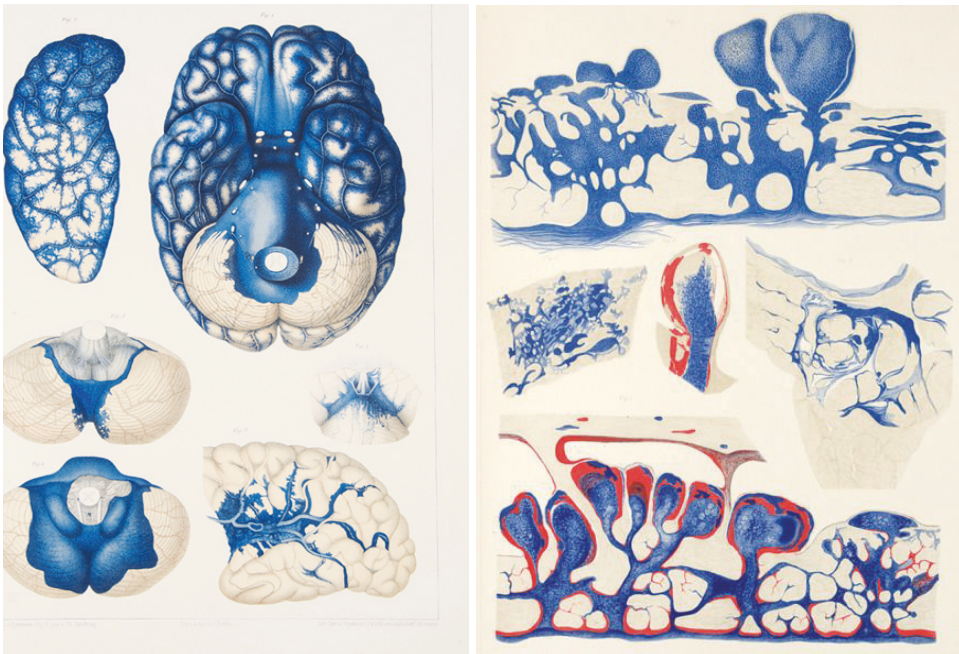

Fig 3. Drawings of CSF distribution by Key A and Retzius G. in *Studien in der anatomie des nervensystems und des bindegewebes* (1875). Detailed illustrations of the CSF distribution along the cerebral superficial subarachnoid space and Virchow-Robin spaces (left) and of CSF being absorbed by the protruding arachnoid granulations (right). Public domain.

Based on these discoveries, in 1926, Harvey Cushing went on to describe the cerebrospinal fluid circulation and proposed his model “the third circulation” (Cushing 1926). His model illustrated that the CSF is produced by the choroid plexus in the ventricles before it leaves the ventricular system through the foramen of Luschka and Magendie into the basal system, to then travel up over the dorsal surface of the brain. From there, it is drained into the arachnoid granulations or

arachnoid villi and is reabsorbed into the blood. For around 400 years, this model was not updated, and it was still taught in medical schools until 10-15 years ago (Rasmussen, Mestre, and Nedergaard 2022).

In the last 15 years intense research in the area has advanced our knowledge and other models have been proposed. Nevertheless, the current view of the production of CSF in the ventricles is not far from what Harvey Cushing described (Rasmussen, Mestre, and Nedergaard 2022). CSF is produced in the cerebral ventricles by the choroid plexus epithelial cells attached to the ventricular walls (Brown et al. 2004; Lun, Monuki, and Lehtinen 2015). However, the contribution of the choroid plexus to CSF formation is still a matter of debate. Some authors believe that the choroid plexus is the sole source or source of the vast majority of CSF formation, while other studies, such as the ones from Bulat and Klarica, proposed that filtration of fluid through the brain capillary membranes substantially contributes to the formation of CSF (Bulat and Klarica 2014; Oresković and Klarica 2010).

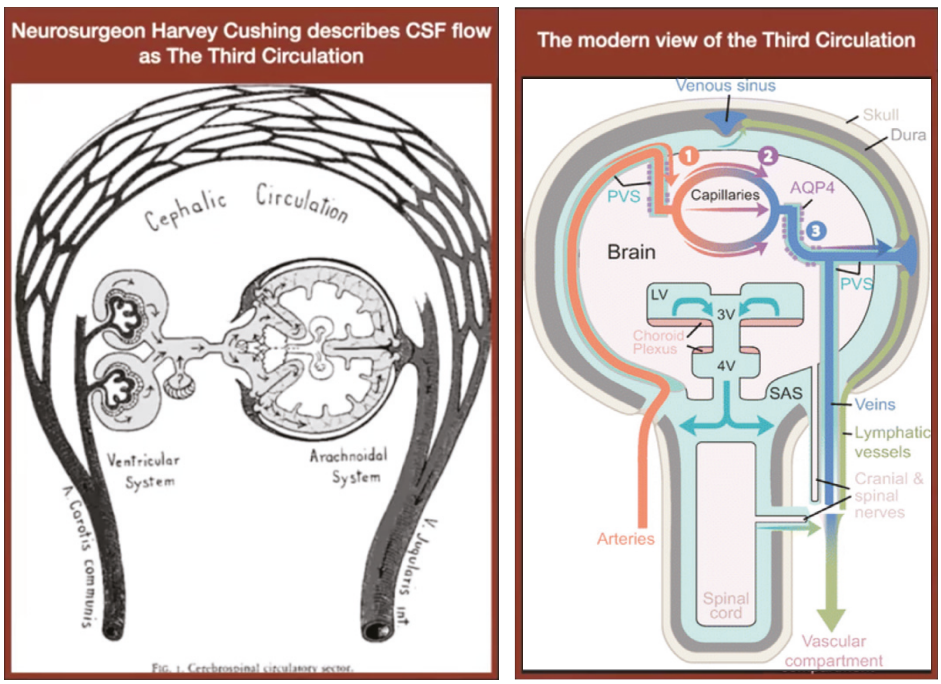

Fig 4. The third circulation by Harvey Cushing vs. the modern view of CSF circulation. Adapted from Rasmussen, Mestre and Nedergaard (2022).

In the current view of CSF circulation, in contrast with the model that Cushing proposed where the CSF was directly absorbed by arachnoid granulations, most scientists now agree that a substantial portion of the CSF is redirected into the brain

(Figure 4) (Rasmussen, Mestre, and Nedergaard 2022). However, there are two different models that try to explain the modern view of the third circulation. The classical model was introduced by Cserr and Bradbury and proposed influx through BBB secretion and movement of solutes by diffusion through the interstitial spaces towards "preferred routes", followed by outflow towards CSF and/or to lymphatics (Hladky and Barrand 2014). The classical hypothesis postulated that there is efflux from the brain parenchyma along both perivenous and periarterial spaces. And some groups still defend the hypothesis that there is CSF efflux along both veins and arteries (Carare et al. 2008; Preston et al. 2003; Schley et al. 2006; Weller et al. 2009). However, there were serious shortcomings of the classical view, such as the lack of data in favor of secretion of fluid across the BBB, and the fact that they did not take the influx of solutes from CSF into the brain into account (Hladky and Barrand 2014), which there was already extensive evidence for (Rennels, Blaumanis, and Grady 1990; Brierley 1950; Stoodley, Jones, and Brown 1996).

From the early research that proved that there is influx of substances from the CSF into the brain, it was Rennels et al. who laid the foundation for the later described new model of CSF circulation, the glymphatic model. Rennels and colleagues injected horseradish peroxidase into the cisterna magna of cats and observed that it moved rapidly along periarterial pathways, suggesting that there is a perivascular fluid circulation in the central nervous system that provides distribution of solutes from the subarachnoid to the brain (Rennels, Blaumanis, and Grady 1990). The glymphatic system was then introduced in 2012 by the group of Maiken Nedergaard and confirmed the initial observations of Rennels and colleagues (Iliff et al. 2012). They demonstrated that the circulation of fluid along perivascular spaces and into the brain parenchyma is critical for the elimination of residual products from the brain, describing one further crucial role for the CSF; the removal of metabolic waste from the brain.

The glymphatic system

The term “glymphatic” originated from the fact that the system relies on water channels on the glial (astrocytic) endfeet in the boundaries of the brain vasculature, and it has been compared in some ways to the lymphatic system present in peripheral tissues (Iliff et al. 2012). In the rest of the body, cells produce metabolic waste that the lymphatic vessels collect from the interstitial space of the tissue and return to the general circulation, aiding waste clearance and immune surveillance (Swartz 2001). The immune system plays a major role in the body's immune function, carrying immune cells that react to antigens and orchestrate the immune response. In the brain there are no lymphatic vessels, and this is one of the reasons as to why it has sometimes been referred to as “immune privileged”. This immune privilege status is likely connected to the neural damage that a strong immune response could cause in the brain (Louveau, Harris, and Kipnis 2015). However, it appears paradoxical not to have a lymphatic system, considering that both the brain and spinal cord are highly energy-demanding organs with a high metabolic waste production rate. In 2012, Iliff and colleagues described the glymphatic system as a model of waste clearance in the brain (Iliff et al. 2012).

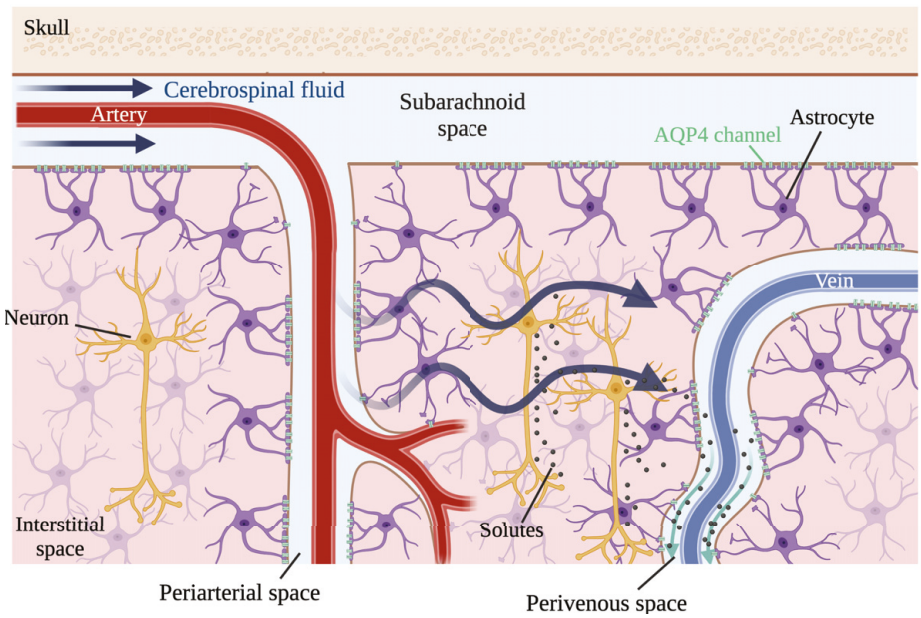

Fig 5. The glymphatic system. The glymphatic theory proposes that from the subarachnoid spaces, CSF flows into the perivascular spaces, where it crosses into the brain parenchyma facilitated by AQP4-channels located on the astrocytic endfeet. This flow helps flush interstitial fluid and waste out of the brain. Created with Biorender.

They labeled the CSF using fluorescent tracers injected in the cisterna magna (CM) and used in vivo two-photon microscopy to show that the CSF is driven from the subarachnoid space into the brain through perivascular spaces along pial arteries by arterial pulsation. They also showed that AQP4 channels in astrocytic endfeet facilitate the influx of CSF into the brain (Iliff et al. 2012; Mestre, Hablitz, et al. 2018). According to this early model, when the CSF enters the brain parenchyma, it drives convective flow of interstitial fluid with waste products out towards perivenous spaces surrounding large veins, from where it is then drained towards the cervical lymphatic system (Figure 5). Over the years, the view that convection drives fluid and waste movement in the interstitial space has changed, and there is a widespread acceptance in the field that the dominant process for transfer of solutes in the brain parenchyma is probably diffusion rather than bulk flow, or a combination of the two (Hladky and Barrand 2014; Rasmussen, Mestre, and Nedergaard 2022). Furthermore, the existence of an efflux path along perivenous spaces is still debated and requires further confirmation. Lastly, the CSF egress paths from the CNS are still the object of intense research and have been updated over the years. It is generally accepted that after exiting the CNS, the fluid and solutes go to peripheral lymph nodes. Wang and collaborators showed that cervical deep lymph node ligation impacts glymphatic function and aggravates Alzheimer's disease (AD)-like pathology (likely because of accumulation of misfolded protein) (Wang et al. 2019). However, which paths the CSF takes to abandon the CNS toward peripheral lymphoid tissues is still debated (Rasmussen, Mestre, and Nedergaard 2022). It is no longer believed that the egress of fluid is only through arachnoid granulations, and there is a lack of in vivo evidence for that route (Hladky and Barrand 2014; Proulx 2021). Other paths have been described (Figure 6): 1) perineural spaces 2) parasagittal spaces in the dura, and 3) meningeal lymphatic vasculature (Rasmussen, Mestre, and Nedergaard 2022; Weller et al. 2018; Brodbelt and Stoodley 2007; Proulx 2021).

1) The outflow through *perineural spaces* is believed to account for most of the efflux of CSF (Proulx 2021). Some studies suggest that around 87% of the CSF that leaves the CNS goes out through olfactory nerves and other cranial nerves (Weller et al. 2018). Another great contributor is believed to be the spinal nerve roots, especially in standing primates (Hladky and Barrand 2014).
2) *Parasagittal dural spaces* are endothelium-lined channels in the dura interposed between CSF and blood compartment. Ringstad and colleagues have shown that the CSF accumulates in these areas by using MRI in humans (Ringstad and Eide 2020). These areas could be drainage sites and bridges between the brain and dural lymphatic vessels (Rasmussen, Mestre, and Nedergaard 2022).

3) The paths that have gathered the most attention in the last years are the *dural lymphatics*, which were described by Mascagni already in 1787 (Mascagni and Santi 1787), but were not investigated further until 2015, when the groups of Kipnis and Alitalo described their functionality, reviving the field's interest in the meningeal lymphatic vessels (Aspelund et al. 2015; Louveau et al. 2015). Kipnis and collagues developed an animal model in which they ablate the meningeal lymphatic vessels (LVs) by injecting the photodynamic drug Visudyne and showed that removing the meningeal LVs decreases CSF circulation in the brain (Da Mesquita et al. 2018).

The relevance and contribution of each of these pathways are still debated and require further investigation.

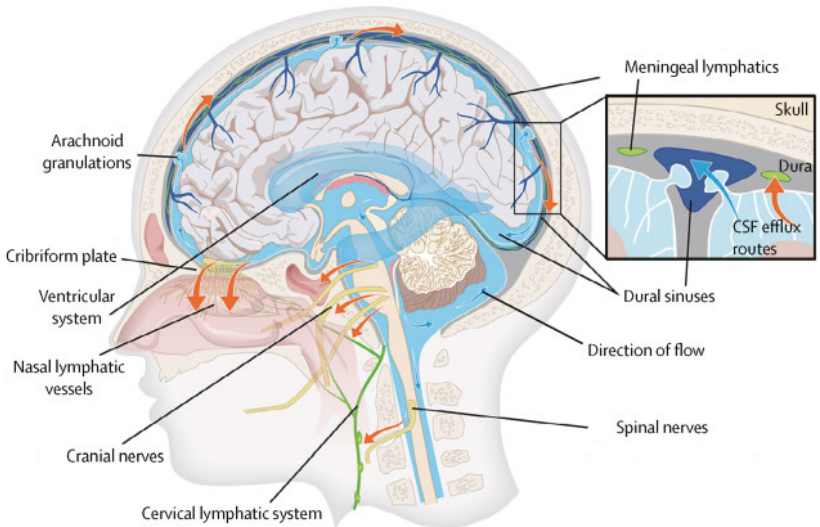

Fig 6. Cerebrospinal fluid efflux in humans. A main perineural egress site is along the olfactory nerve through the cribriform plate, through cranial nerves and spinal nerves. Dural lymphatic vessels have also been shown to carry CSF towards the cervical lymphatic system. Reprint with permission from Rasmussen et al. (2018).

Aquaporin-4 contribution

AQP4 is a transmembrane water channel that dominates in the CNS, presenting a rather interesting localization, mainly perivascular (Nagelhus and Ottersen 2013; Nielsen et al. 1997; Frigeri et al. 1995). This stems from the fact that AQP4 is mainly expressed in astrocytes and is highly polarized towards their endfeet, so it is found in the astrocytic membranes that are contiguous with the cerebral vasculature and adjacent to the borders of the cortex (the glia limitans) (Frigeri et al. 1995; Nielsen et al. 1997). In rodents, the ratio of AQP4 at the astrocytic endfeet vs non-perivascular membranes is around 8:1, and a bit lower in humans (Eidsvaag et al. 2017). AQP4 belongs to a family of integral membrane proteins named aquaporins, which facilitate the flow of water and small uncharged solutes through the cell membrane (King, Kozono, and Agre 2004). The channel is formed by a tetramer composed of four monomers, each of which consists of six transmembrane-spanning domains and two short helical segments with five connecting loops that help form the water pore (Neely et al. 1999). The channel is present in two main isoforms, M1 and M23. The shorter one is M23, which also forms arrays in the astrocyte membrane called OAPs or orthogonal arrays of particles (Suzuki et al. 2008; Strand et al. 2009).

In 2012 the need for AQP4 for adequate CSF flow from perivascular spaces into the brain was established (Iliff et al. 2012). Iliff and colleagues showed that removing AQP4 expression by means of an AQP4 KO model inhibited glymphatic function in the brain. Although a study in 2017 failed to see the same impairment in a different KO model of AQP4 (Smith et al. 2017), starting a debate on the importance of AQP4 for CSF transport, five independent groups have refuted that study and confirmed the initial report about the role of AQP4 (Mestre, Hablitz, et al. 2018). Furthermore, Kress et al. found that the aging brain loses perivascular AQP4 channels at the glial endfeet, with more being found in non-perivascular processes, which is accompanied by a decrease in CSF flow in the brain (Kress et al. 2014). Lastly, loss of perivascular AQP4 also seems to be involved in neurodegeneration, with studies showing that its loss accelerates protein misaggregation (Zeppenfeld et al. 2017; Xu et al. 2015).

Drivers of CSF movement

We know that AQP4 has an essential role in the transport of CSF into the brain, but what drives CSF movement along perivascular spaces? First, most likely, the continuous production of CSF in the ventricles drives the flow in one direction toward the subarachnoid space (Jessen et al. 2015). In addition, respiration is believed to contribute to the movement of fluid along the cerebral aqueduct that connects the ventricles (Klose et al. 2000; Yamada et al. 2013). When it comes to the movement of CSF along perivascular spaces, the first study in 2012 proposed that arterial pulsation created by smooth muscle cells was the driving force, which

was later confirmed in a very elegant study that used particle tracking in CSF (Mestre, Tithof, et al. 2018; Iliff et al. 2012; Iliff et al. 2013). Furthermore, modulating the arterial pulsation by different means, such as injecting dobutamine, an adrenergic agonist that increases pulsatility, or performing internal carotid artery ligation to dampen arterial pulsatility, respectively increased and decreased CSF penetration into the brain (Iliff et al. 2013). These studies further reinforced the idea that arterial pulsatility drives CSF flow in the brain and that it is the arterial and not venous perivascular spaces that are responsible for fluid inflow.

Another important discovery of the first studies on glymphatics was that CSF transport increases during sleep. In 2013, Xie and colleagues imaged asleep mice with a two-photon microscope and reported that sleeping mice had increased CSF influx into the brain (around 90% higher) compared to awake mice (Xie, Kang, Xu, Chen, Liao, Thiyagarajan, O'Donnell, et al. 2013). They also showed that CSF influx during KX anesthesia (a mix of ketamine and xylazine) was comparable to the sleep state. The authors linked this effect to the fact that the volume fraction of interstitial space was about 60% larger in the sleep and anesthetised states. The researchers concluded that the glymphatic system clears the brain of waste most efficiently during sleep. A key regulator of the mechanism behind it was believed to be the neuromodulatory molecule norepinephrine, which is known to drive arousal (Xie, Kang, Xu, Chen, Liao, Thiyagarajan, O'Donnell, et al. 2013; O'Donnell et al. 2012). Administration of norepinephrine receptor antagonists led to increased glymphatic function, while norepinephrine administration resulted in decreased IS volume fraction, similarly to the wake state (Xie, Kang, Xu, Chen, Liao, Thiyagarajan, O'Donnell, et al. 2013). A more recent study showed that not all anesthetics had the same effect on glymphatic function as KX (Hablitz, Vinitsky, Sun, Staeger, et al. 2019). In fact, isoflurane anesthesia inhibited glymphatic transport compared to other types of anesthesia. The differences between anesthetics were linked to the type of EEG activity that they induce: glymphatic function appears to correlate with deep sleep, which is characterized by slow wave EEG activity. More recently, a role of the circadian rhythm in regulating the glymphatic system has also been reported (Hablitz et al. 2020). Considering all known drivers of CSF movement, it becomes evident that lifestyle factors such as adequate sleep and maintenance of vascular health can support adequate CSF transport (Figure 7) (Kylkilahti et al. 2021). Likewise, poor sleep, stress, hypertension and certain diseases could lead to poor glymphatic function.

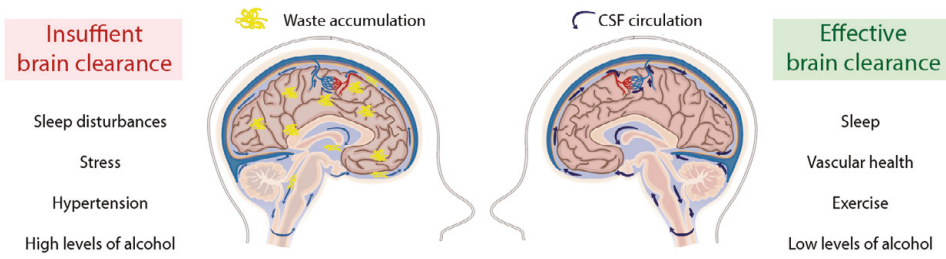

Fig 7. Lifestyle-related factors can modulate brain clearance. Some factors that support good CSF transport and brain clearance include adequate sleep (particularly deep sleep) and maintenance of vascular health through control of blood pressure and exercise. Sleep disturbances, stress, hypertension and high levels of alcohol consumption lead to poor glymphatic dysfunction and may increase the risk for developing or exacerbating neurodegenerative diseases. Figure by Marta Ramos and Tekla Kylkilahti, published in Kylkilahti et al. (2021).

CSF transport in disease

Because the initial glymphatic research showed that the CSF transport is involved in clearing Aβ from the brain, many studies have subsequently focused on understanding the role of the glymphatic system in AD and other neurogenerative diseases. In neurodegenerative diseases such as AD, the brain accumulates misfolded proteins, such as tau in neurofibrillary tangles or Aβ in neuritic plaques (Bossy-Wetzel, Schwarzenbacher, and Lipton 2004). When radiolabeled Aβ was injected into the striatum of AQP4 KO and wild-type mouse clearance of amyloid was impaired by 55% in the KO model (Iliff et al. 2012). Another study found that the clearance of amyloid from the brain doubles during sleep (Xie, Kang, Xu, Chen, Liao, Thiyagarajan, O'Donnell, et al. 2013). It is known that age is the most significant risk factor for developing AD, and interestingly, age has also been shown to have a big impact on glymphatic function (Rasmussen, Mestre, and Nedergaard 2018; Kylkilahti et al. 2021). Brain CSF influx and efflux are strikingly reduced in old mice compared to young mice (Ma et al. 2017). Furthermore, a study showed that aged mice presented an altered distribution of AQP4, reduced arterial pulsatility, and consequently had a reduced amyloid clearance rate from the brain (Kress et al. 2014). Connecting what we know about the effects of sleep and aging on brain clearance, a very recent study found that age-related increases in brain inflammation results in deficits in the brain's capacity to generate certain sleep spindles, which contributes to memory impairment in older adults and may be an early warning sign of AD (Mander et al. 2022).

The glymphatic system has also been implicated in other pathologies in different ways. For example, it has been linked to vascular dementia in connection with the enlargement of perivascular spaces and AQP4 mislocalization (Venkat et al. 2017). In rodent models of stroke, the inflow of CSF was impaired in the ipsilateral cortex

after middle cerebral occlusion (Gaberel et al. 2014). In models of hemorrhagic stroke, impaired CSF flow was attributed to the occlusion of perivascular spaces by deposits of blood factors such as fibrin and fibrinogen (Gaberel et al. 2014; Goulay et al. 2017). The system has also been studied in models of cerebral small vessel disease (cSVD), which is another common cause of vascular dementia. A hallmark of the disease is dilated perivascular spaces and thicker artery walls, likely due to cerebrospinal fluid stagnating along the vasculature (Benveniste and Nedergaard 2022; Ter Telgte et al. 2018; Wardlaw et al. 2020; Benveniste et al. 2020). Another common cause of vascular dementia and cSVD is hypertension and, as expected, CSF flux was also reduced in hypertensive rats (Mortensen et al. 2019).

As shown here, several lines of work have focused on studying the role of CSF dynamics in neurological and neurodegenerative disorders (Rasmussen, Mestre, and Nedergaard 2018; Benveniste et al. 2020). It is worth considering that many neurological and neurodegenerative disorders share inflammatory components, and the glymphatic system, assuming that it has a pseudo-lymphatic function in the central nervous system, is poised to play a role in neuroinflammation too.

Neuroinflammation

Inflammation is a key biological process that occurs in the body tissues as a response to exogenous or endogenous harmful stimuli, such as infectious microbes, mechanical trauma, heat, radiation, and some cancers (Zachary 2017). Usually, inflammatory reactions are initiated in response to injured vascularized tissue and start with vasodilation and increased blood flow to the area. The slowing of the blood flow together with the activation of the vascular endothelium allows for fluid extravasation of plasma and plasma proteins from the blood that enter the tissue through gaps between the endothelial cells (Zachary 2017). With the widening of these gaps, leucocytes, red blood cells, and additional proteins can also enter the tissue. One important protein in these steps is fibrinogen, which forms fibrin in the tissue and has chemotactic properties. Neutrophils and other leukocytes leave the blood and enter the tissue in response to chemoattractants that are released by host cells, immune cells, microbes, or other cells. All of this results in fluid, plasma proteins, and leukocyte accumulation in the tissue and translates into swelling, heat, redness, pain, and loss of function (Zachary 2017). The objective of this response is to dilute, isolate or resolve the source of injury and to repair the tissue. In general, inflammatory responses in the body are beneficial, although in some cases, inflammation can be detrimental and harmful to the body itself.

As discussed in previous sections, the brain is particularly sensitive, and changes that are common in the periphery during inflammation, such as tissue swelling, can have fatal consequences for the brain (Stamatovic et al. 2006). Thus, neuroinflammation or inflammation in the brain is a bit different from the response in the rest of the body because of the BBB and other barriers discussed previously. Despite the BBB, leukocytes such as monocytes and lymphocytes are able to monitor the capillaries and even cross the BBB, into the perivascular spaces in the steady state and patrol the nervous system in a duty of immune surveillance (Engelhardt and Ransohoff 2005; Takeshita and Ransohoff 2012). In addition, one important defense mechanism in the CNS is the presence of resident monocyte-macrophage type cells named microglia. These cells colonize the CNS during embryonal development and early postnatal life (Ginhoux et al. 2013). In the adult brain, they take on roles of immunosurveillance, immunoregulation, and tissue reparation in case of injury. According to the literature, one of the first indications of neuroinflammation is microglia activation (Shabab et al. 2017; Carson et al. 2006). When microglia become activated their shape changes to a more amoeboid conformation and produce cytokines that activate other cells around them, leading to breakdown of the BBB and ultimately to infiltration of leukocytes from the blood (Figure 8) (da Fonseca et al. 2014). As an example, lipopolysaccharide (LPS), which is a bacterial endotoxin found in the membrane of gram-negative bacteria and is known as the prototypical pathogen-associated molecular pattern (PAMP), induces

a systemic inflammatory response but also neuroinflammation by binding to toll-like receptor 4 (TLR4) on microglia. In this case, the activation of microglia leads to NF-κB activation, and to the production of cytokines, chemokines, and pro-inflammatory enzymes, which results in neuroinflammation (Shabab et al. 2017; Kang et al. 2019). Another important form of gliosis (glial cell activation in response to damage) is astrogliosis, which is the most common form of gliosis and involves the proliferation of astrocytes and cellular morphological changes in them such as hypertrophy (Sofroniew 2014). In its most extreme form, astrogliosis leads to the glial scar, which is a way of protecting the injury site and starting the healing of the brain but has been shown to be both beneficial and detrimental in neurological diseases (Liddelow and Barres 2016).

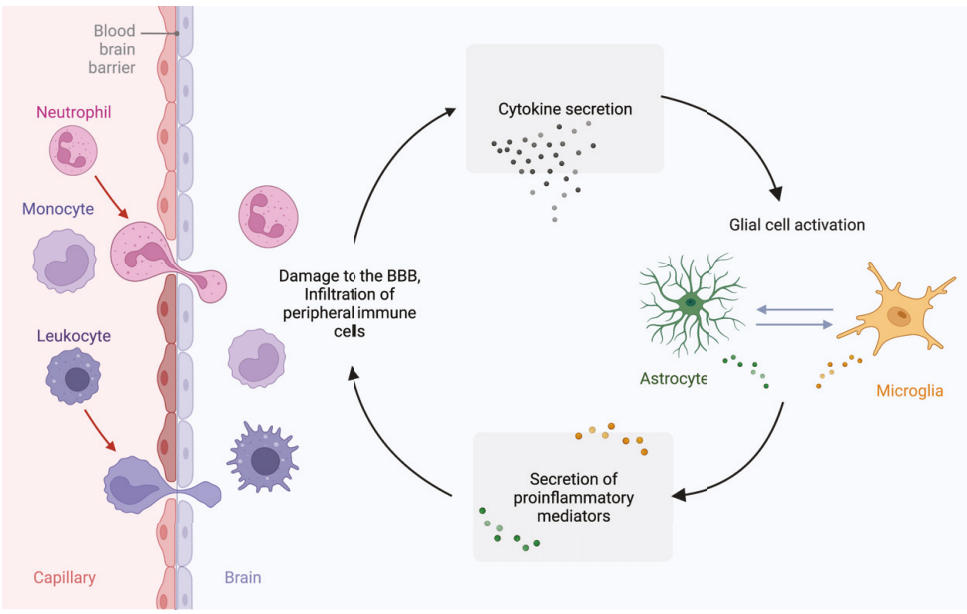

Fig 8. A simplified diagram of neuroinflammation. Neuroinflammation is associated with activation of glial cells with significant cytokine and chemokine production, increased BBB permeability and breakdown and infiltration of peripheral immune cells. Created with Biorender.

Neuroinflammation is an essential but still elusive common feature of many neurological disorders, such as stroke, AD, Parkinson's disease (PD), or traumatic brain injury, where there is leukocyte infiltration into the central nervous system (da Fonseca et al. 2014; Arumugam, Granger, and Mattson 2005; Beuker et al. 2021), and is the main component of neuroinflammatory diseases such as MS and meningitis.

Meningeal immunity

The meninges, some of the physical barriers between the CNS and the periphery, are the point of entry for many pathogens, which arrive through the bloodstream or the CSF but also through the direct contact between the meninges and the nasal cavity (Hoffman and Weber 2009; van Ginkel Frederik et al. 2003). When the meninges become inflamed, this is called meningitis, the most common form being bacterial meningitis. During meningitis, when bacterial components populate and replicate in the subarachnoid space and meninges, a large-scale inflammatory response also occurs in the brain (Hoffman and Weber 2009). The BBB becomes more permeable, leading to excess fluid in the brain, edema, and increased intracranial pressure. This is a major cause of neurological sequela following bacterial meningitis in humans (McGill et al. 2016).

But meningeal immunity does not refer only to meningitis. Recent advances in the field have shown that the meninges play an essential role in facilitating and regulating the immune response in the CNS, and meningeal immunity has been implicated in a number of neurological disorders (Coles et al. 2017; Rua and McGavern 2018; Filiano, Gadani, and Kipnis 2015). In physiological conditions, many different types of immune cells inhabit the meninges, such as macrophages, lymphocytes, mast cells, and neutrophils (Figure 9) (Coles et al. 2017; Filiano, Gadani, and Kipnis 2017; Korin et al. 2017; Nayak et al. 2012; Rua and McGavern 2018). One of these cell types, meningeal and perivascular macrophages, which are resident immune cells, appear to have similar roles to those of microglia in the brain parenchyma, such as immune surveillance, tissue homeostasis and protection from infection (Rua and McGavern 2018; Morse and Low 1972; Schain et al. 2018). In fact, an article published while this thesis was being written showed that the pia matter filters CSF as it enters perivascular spaces, allowing macrophages attached to it to sample its contents (Mestre et al. 2022). The study also provided high-resolution images of the pial structure, showing that it is more of a net than a membrane and that its coverage changes with age and during AD.

Furthermore, T cells can traffic from the blood into the meninges, perhaps in order to perform immune surveillance and then travel from there to cervical lymph nodes (Radjavi et al. 2014). Some authors have proposed that immune responses in the CNS may first develop in the perivascular spaces and meninges before transferring to the brain tissue. In fact, studies have shown that the meninges might play a role in antigen-presenting interactions in CNS inflammatory diseases (Schläger et al. 2016a; Kivisäkk et al. 2009). It is not surprising that the meninges present in many cases with more intense inflammation and at earlier time points than the rest of the CNS does, given the optimal features of the meninges to support robust inflammatory responses. However, inflammation (acute and chronic) of the area can also give rise to detrimental consequences. Investigations of the meninges of patients with MS have shown that T cells can accumulate in ectopic lymphoid

follicles in the meninges, which leads to damage and demyelination around the glia limitans and is related to the severity of symptoms (Russi and Brown 2015; Androdias et al. 2010).

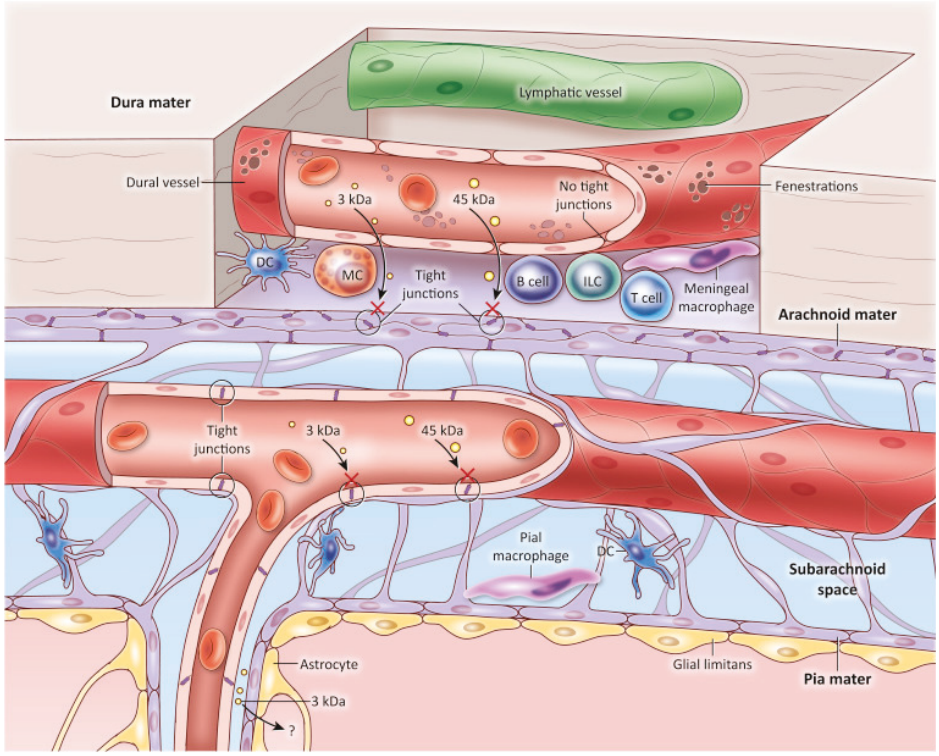

Fig 9. Meningeal immune composition as depicted in *Advances in Meningeal Immunity* (Rua and McGavern 2018). The dura contains many different immune sentinel cells, such as dendritic cells, mast cells, lymphoid cells, lymphocytes, and macrophages. The pia mater also presents some immune cells, such as mast cells, macrophages, and dendritic cells. Licensed reprint from Rua and McGavern (2018).

The immune function of the meninges is still unraveling, and recent exciting discoveries include communicative channels from the calvarial bone marrow to the meninges and an intricate niche for adaptive immune cells in the wall of the superior sagittal sinus (Rustenhoven et al. 2021; Pulous et al. 2022; Mazzitelli et al. 2022; Herisson et al. 2018; Cai et al. 2019). Herisson and colleagues were the first to observe a direct local interaction between the skull bone marrow and the brain through the meninges, which was later confirmed by another study that used whole-body clearing and imaging to reveal short vascular connections between the calvarian marrow and the meninges, which were filled with immune cells during

stroke (Cai et al. 2019; Herisson et al. 2018). More recently, a study showed that the CSF uses this dura-skull channels to access the skull bone marrow and regulate immune responses (Mazzitelli et al. 2022). Almost at the same time, Pulous and collaborators reported that the CSF can exit into the calvarial marrow through those channels during meningitis and that bacteria use this route to invade the skull's hematopoietic niches leading to cranial hematopoiesis (Pulous et al. 2022). As skull channels seem to provide leucocytes to the meninges, the sampling of brain-derived antigens in the CSF likely has broad implications for inflammatory neurological disorders.

Multiple sclerosis and EAE

Multiple sclerosis (MS) is considered by many the prototypical inflammatory and demyelinating disease of the CNS. It is an inflammatory neurodegenerative disease that usually strikes in early adulthood and leads to a wide range of neurological deficits, including physical disability (Compston and Coles 2008). Due to the early onset compared to other neurodegenerative diseases, MS patients experience a significant loss of productivity, which constitutes a big burden on society and the individual. The prevalence of MS has risen 10-fold since 1961 and is expected to continue to increase (Grytten, Torkildsen, and Myhr 2015). In MS, the myelin sheaths that cover neuronal axons are damaged (Compston and Coles 2008). Even though the cause is still unclear, there are some key features that have been established, such as the presence of a strong inflammatory response in the CNS, believed to be the trigger of demyelination. However, evidence suggests that glial damage could also lead to secondary inflammation and loss or harm to the axons (Barnett and Prineas 2004; Popescu, Pirko, and Lucchinetti 2013). In fact, the most common course of disease in MS patients is relapsing-remitting MS, and relapses are usually associated with inflammation and disruption of the BBB, which led scientists to postulate that the relapses correspond with fresh waves of inflammatory mediators and immune cells in the CNS (Barnett and Prineas 2004; Compston and Coles 2008).

Most of the pathological events in MS have been identified or confirmed using the animal model Experimental Autoimmune encephalomyelitis (EAE). EAE is a model of neuroinflammatory disease that resembles and is useful for studying some features and mechanisms of demyelinating diseases (Constantinescu et al. 2011). Depending on which animal species and strain is used to induce EAE (most commonly mice, rats, or rabbits), a different pathology develops, which can be useful to study different disease courses of MS and/or different mechanisms. However, a major difference between MS and EAE is that EAE requires immunization with a myelin protein and adjuvants to be induced, while in humans, the potential autoantigen triggering the inflammatory response is not known (Libbey, Tsunoda, and Fujinami 2010; Gran et al. 2007). It is nevertheless worth

mentioning that multiple reports have suggested that Epstein Barr Virus (EPV) proteins mimic myelin proteins and other CNS proteins, which could be a reason for the autoimmune reaction in MS (Robinson and Steinman 2022). Very recently, Bjornevik and colleagues determined that EPV infection greatly increases the risk of developing MS and precedes disease onset, supporting the role of the virus in its pathogenesis (Bjornevik et al. 2022).

It is believed that MS and EAE are driven by autoimmune targeting of myelin by T lymphocytes, leading to the demyelination of nerve fibers (Fletcher et al. 2010). In the inflamed CNS, T cells in the blood stream are attracted by cytokines to enter across the blood-brain barrier. However, different sites have been proposed as vulnerable to the first wave of T cell entry that initiates this cascade of inflammation and further T cell influx. Several groups have contributed to the conception of a "two-step" model of the pathology of EAE to explain how myelin-specific T cells primed in the periphery can enter the CNS (Bartholomäus et al. 2009; Brown and Sawchenko 2007; Kivisäkk et al. 2009; Reboldi et al. 2009; Ransohoff 2009). In general, the model proposes that T cells transit through the meninges during a "first wave" of T cell infiltration, where they potentially meet APCs, leading to T cell reactivation and to an inflammatory response with a breakdown of BBB and loss of vascular integrity, allowing for lymphocyte migration to the CNS parenchyma in a "second wave". In fact, meningeal reactivation of T cells has been shown to be necessary for immune cells to invade the brain parenchyma (Bartholomäus et al. 2009; Greter et al. 2005; Brown and Sawchenko 2007; Kivisäkk et al. 2009; Ransohoff 2009; Ransohoff, Kivisäkk, and Kidd 2003; Takeshita and Ransohoff 2012). A number of studies in EAE have also detected the presence of T cells and APCs in the dura matter in EAE (Kwong et al. 2017) and leptomeninges (Bartholomäus et al. 2009; Lodygin et al. 2013; Kivisäkk et al. 2009). In addition, the ablation of meningeal LVs appears to diminish EAE pathology and reduces the inflammatory response of T cells, further pointing to the importance of the meninges in the development of EAE (Louveau et al. 2018). Schläger and colleagues also showed that immune infiltration to the spinal meninges occurs before the onset of EAE disease (Schläger et al. 2016b). Interestingly, they also reported that the flow of CSF flushes away T cells from the meninges during EAE, which decreases their level of activation (Schlager et al. 2016).

Glymphatics in neuroinflammation

It is clear from previous sections that the neuroimmune interface between the glymphatic and lymphatic systems allows for immune surveillance in the brain. Yet, little is known about the roles of this interface in the context of neuroinflammation. Findings from the Kipnis group in the EAE model reported an immune-regulating role of the meningeal lymphatics, and more recent findings demonstrate that CNS-

derived antigens accumulate in the dural sinuses and that these sinuses orchestrate T cell trafficking (Louveau et al. 2018; Rustenhoven et al. 2021).

In the pursuit of deciphering the role of CSF transport, many studies have revealed that CSF flow is impaired in chronic neurological disorders of the CNS that have a neuroinflammatory component, such as AD, PD, and chronic models of TBI and stroke (Gaberel et al. 2014; Nedergaard and Goldman 2020; Ren et al. 2013; Ding et al. 2021), while in acute stroke a transient increase in CSF inflow has been reported (Mestre et al. 2020). A recent review highlighted the importance of discerning the roles of CSF movement during neuroinflammation and proposed different potential mechanisms (Mogensen, Delle, and Nedergaard 2021). According to the authors, it is plausible that the upregulation of proinflammatory cytokines induces hypersecretion of CSF and gliosis during the early stages of neuroinflammation. A study showed that TLR4 signaling pathways are crucial for activating the NKCC1 cotransporter, resulting in increased CSF secretion (Karimy et al. 2017). It is clear that changes to CSF production could influence the drainage of fluid from the brain. However, how this happens is still unclear. Astrocytes are also deeply involved in the functioning of the glymphatic clearance, and therefore, astrogliosis resulting from an inflammatory insult could affect brain clearance.In summary, an initial transient overproduction of CSF could lead to more fluid flow with inflammatory molecules towards the periphery, leading to peripheral immune cells migrating to the CNS. However, this could also lead to shunting of the outflow of CSF from the CNS in an attempt to keep brain homeostasis (Mogensen, Delle, and Nedergaard 2021). Furthermore, gliosis with loss of polarized AQP4 could also result in decreased CSF influx into the brain. Thus, glymphatic impairment could lead both to edema formation and to a reduction in clearance of cytokines from the injured area, thus perpetuating neuroinflammation. In fact, a recent study has shown that impairing CSF drainage through meningeal lymphatic vessels in a model of AD leads to microglial activation, deposition of Aβ and neurovascular dysfunction (Da Mesquita et al. 2021).

Despite the suspicions that the glymphatic system plays a role in acute and chronic neuroinflammation, this topic was widely unexplored at the beginning of this Ph.D.

Aims

In this thesis, we aimed to explore how different forms of neuroinflammation affect cerebrospinal fluid flow in the brain and the spinal cord and how it can contribute to disease progression. We also studied how neuroinflammation-derived edema can then affect oxygenation and vascular perfusion in the spinal cord. The specific aims of the papers included in the thesis are:

Paper I

Here we aimed to explore the effects of LPS-induced systemic inflammation on perivascular CSF distribution and its relationship to neuroinflammation.

Paper II

In Paper II, we aimed to determine whether acute bacterial meningitis affects CSF transport in the brain and the role of neutrophil extracellular traps (NETs) for glymphatic function and CSF drainage to the periphery.

Paper III

Here we explore the relationship between meningeal inflammation, spinal nerve swelling, and CSF flow disturbances in the spinal cord of EAE mice.

Paper IV

Our aim was to elucidate the effects of neuroinflammation in the spinal cord on oxygenation and vascular perfusion in the EAE model.

Methods

Key methods used in this thesis are briefly described below, in addition to some methodological considerations. For more details, please see the Materials and Methods section of the attached papers.

Animals and disease models

Animal procedures in this thesis were approved by the Malmö-Lund Ethical Committee for Animal Research in Sweden, the University of Rochester animal care committee and/or the Danish Animal Experiments Inspectorate. In all cases, animals were housed with ad libitum access to food and water and in agreement with existing local guidelines.

In **paper I**, we used adult male C57BL/6 mice from Janvier. In **paper II**, we used adult male Sprague Dawley rats from Taconic. In **paper III,** we used adult female CBL57/Bl mice from Janvier and Prox1-eGFP female mice with a C57BL/6-background. In **paper IV**, we used adult female SJL/J mice from Janvier.

Anesthesia

In **papers I, II, and III,** anesthesia was administered intraperitoneally before surgery or before cisterna magna injections as a mixture of racemic ketamine (Ketaminol®, 100 mg/kg) and xylazine (Rompun®, 20 mg/kg) in 0.9 % saline, which is referred to as KX in the text. In **paper IV**, we used isoflurane (4% for induction, 2–2.3% for maintenance) to anesthetise the mice that underwent optoacoustic imaging.

Different types of anesthetics have been shown to affect glymphatic transport in different ways (Hablitz, Vinitsky, Sun, Stæger, et al. 2019), with KX, which yields a similar electroencephalogram power spectrum to natural sleep, being the preferred option to mimic sleep-state and study glymphatic function.

LPS injections

In **paper IV**, we injected LPS (1 mg/kg, Sigma) or its vehicle (ddH_2O) intraperitoneally 3 hours before cisterna magna injections/perfusion/ physiological measurements.

Meningitis induction

In **paper II,** acute bacterial meningitis was induced by subarachnoid injection of *S. pneumoniae* (strain SP001) in rats, which is the most common pathogen causing bacterial meningitis, in a volume of 20µl at a rate of 2µl/min, or saline as control.

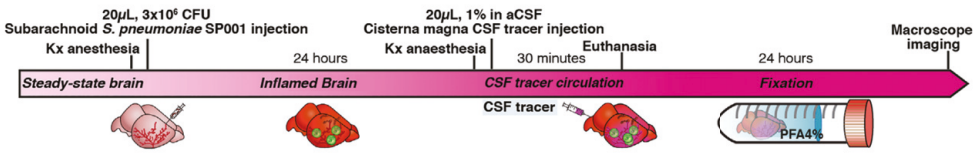

Fig 10. Meningitis experimental timeline. Twenty-four hours after S pneumoniae or saline subarachnoidal injection, a cerebrospinal fluid tracer (Alexa Fluor 647–conjugated ovalbumin) was injected into the cisterna magna of anesthetised rats. Adapted from paper II

Bacteria were introduced by opening a window on the skull and carefully puncturing the meninges with a needle, and placing a catheter inside. Previous studies have introduced bacteria by injection into the cisterna magna instead (Bally, Grandgirard, and Leib 2016; Holler et al. 2014; Liu, Zhang, and Han 2015; Simões et al. 2017). However, in our study, we injected fluorescent tracers in the cisterna magna at a later time point. Thus, we chose to inject the bacteria by means of a craniotomy to avoid leaking of tracers because of a previous puncture in the cisterna magna.

EAE induction

To induce EAE, in **paper III,** C57BL/6 mice were injected subcutaneously at the flank of the back with 200µl consisting of 50 µg of mouse MOG_{35-55} peptide emulsified in CFA with an additional 200 µg of *Mycobacterium tuberculosis* H37RA. *Bordetella pertussis* toxin was injected 2 and 24 hours later. In **paper IV** mice were injected similarly with an emulsion containing 75 µg of mouse $PLP_{139-151}$ peptide (synthesized by Innovagen, Lund, Sweden) emulsified in CFA with 200 µg *Mycobacterium tuberculosis* H37RA. *Bordetella pertussis* was also administered on day 0 and again after 24 h.

In the C57BL/6 mouse, immunization with $MOG_{35–55}$ induces monophasic or chronic EAE, which resembles secondary progressive multiple sclerosis (SPMS) and is the most commonly used model of EAE (Constantinescu et al. 2011). In SJL/J mice $PLP_{139–151}$ injection results in a relapsing–remitting disease form. However, in **paper IV,** we didn´t take advantage or seek to study patterns in the remission/relapse phases but focused only on the acute state of the first relapse. Instead, the reason for using SJLJ/J mice was that these are albino, and the MSOT imaging modality is most effective on less pigmented skin.

The mice were observed daily for symptoms, and scores were assigned as previously described (Ramos-Vega et al. 2022), following the scale:

Score	Symptoms
0,5	Partial tail weakness
1	Limp tail
1,5	Limp tail and hind leg inhibition
2	Limp tail and weakness of hind legs
2,5	Limp tail and dragging of one hind leg or both legs slightly dragging at the feet
3	Paralysis of the hind legs
3,5	All the previous and unable to right itself or hind quarters are flat like a “pancake”
4	All the previous and partial front leg paralysis (score 4 was also a humane endpoint and was reached only for very few mice)

Table 2. EAE mice scoring. EAE is usually scored on scale 0 to 4 but 4 is usually a humane endpoint. Most researchers also give mice "in-between" scores (i.e., 0.5, 1.5, 2.5, 3.5) when the clinical picture lies between two defined scores. Modified from Hooke Laboratories.

Treatments

In **paper II,** rats were treated with a bolus dose of DNase, an enzyme that breaks down the extracellular DNA backbone of NETs, 6 hours after administration of *S. pneumoniae*. We parallelly treated other rats with antibiotics (ceftriaxone, 50mg/ml), or saline (control). Following the bolus dose, rats were infused intravenously by a continuous infusion of either treatment for the next 18 hours, as previously described (Mohanty et al. 2019). Direct infusion in the blood appears to be a clinically relevant and translational method to administer DNase, and therefore it was chosen. We delivered a continuous infusion of DNase in the blood to ensure

that the DNase was constantly present since intravenously administered DNase has a half-life of 3-4 hours (Davis Jr et al. 1999).

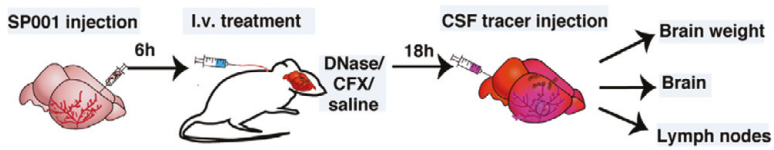

Fig 11. DNAse treatment. 6 hours after subarachnoid injection of *Streptococcus pneumoniae* or saline, rats received treatment with DNase I or antibiotic ceftriaxone or vehicle (saline). Thereafter, rats were injected in the cisterna magna with CSF tracer and euthanized 30 minutes after the injection. Adapted from paper II.

In **paper III,** mouse Interferon beta 1a (IFNb, PBL Assay Science, US), which is an anti-inflammatory cytokine and one of the most common first-line treatments for MS, was given intraperitoneally in a saline solution daily to EAE mice starting on day post-immunization (DPI) 8 and mice were scored daily until the chronic stage, DPI 28.

Reflections on animal research

All studies using animal subjects were approved by the local ethics committee. This is not always an easy process for the researcher, but it is, of course, necessary to ensure animal welfare. In the EAE model, it has been particularly important to consider the ethical endpoints since this model is recognized to have the potential to cause severe suffering in animals for a prolonged period of time.

Every scientist who uses animals for scientific purposes should implement the 3Rs in their work. After all, we all need to remember that *"the humanest possible treatment of experimental animals, far from being an obstacle, is actually a prerequisite for successful animal experiments"* (Russell and Burch 1959). Models like EAE, which exhibit a broad spectrum of neurological symptoms, are hard to *replace,* despite the increasing number of in vitro assays for investigating immune function and myelination (Wolfensohn et al. 2013). In this case, the other 2R's, *reduction* and *refinement*, become all more important.

Another critical concern in animal research is the translatability of results. Aside from the problem that our animal models are not perfect at mimicking the diseases we want to study, there are also incentives in life sciences, such as publication or funding pressure, to publish (mainly positive) results quickly and, in many cases,

underpowered studies. Using very few animals in a study because of time, cost, feasibility, or even ethics can lead to studies being underpowered, compromising the validity of the results and contributing to translational failure. Therefore, a balance must be met between the ethical reduction of animals and doing translatable research that contributes to clinical science. Otherwise, the suffering of the animals used in science can be unnecessary and futile.

Cisterna magna injections

In **papers I, II and III**, we performed cisterna magna (CM) injections of fluorescent tracers (e.g. (Alexa-647-BSA or Ovalbumin and FITC-dextran), contrast agents for MRI or fluorescent microspheres. The injection of tracers in the cisterna magna is an established method to visualize the movements of CSF flow that is less invasive than the previous methods that injected tracers into the ventricles (Ramos et al. 2019). This technique is supposed to interfere to a lesser extent with the intracranial pressure in the skull than other techniques previously used (Jessen et al. 2015). The technique has, however, been criticized regarding the volumes of tracer and infusion speeds and subsequent pressure elevations on glymphatic results. This criticism was recently solved in a study where tracer particles were injected into the CM through a dual cannulation system, with simultaneous injection and withdrawal of equal amounts of fluid. The experiment revealed no significant increase in CSF volume or intracranial pressure (Raghunandan et al. 2021). Of course, cisterna magna injection is not a perfect method, and there exist several sources of error that can affect the quality of the analysis, such as the technique of the injection operator, how far in the needle is put, etc. Therefore, the field should dedicate efforts to find newer less invasive methods to study CSF movements in a more physiological way.

Here is a brief description of the protocol that we followed for CM injections: Firstly, animals are anesthetised using a KX mix and fixed into a stereotaxic frame. It is important to fix the head to form an angle of 120° with the body to ensure consistent and appropriate delivery of tracer. To expose the cisterna magna, the skin over the occipital crest is opened, and the superficial connective tissue is pulled apart to expose the neck muscles below. The muscles at the midline are separated by carefully running the forceps along the anterior-posterior axis. The CM is then exposed, which appears as a tiny, inverted triangle, outlined by the cerebellum above and medulla below, behind the translucent dural membrane. We use a cannula consisting of a dental needle connected to a syringe filled with CSF tracer diluted in artificial CSF (aCSF). The bevelled end of the dental needle of the cannula is inserted at an angle of 45° relative to the mouse head, passing into the center of the CM. Penetration of the cerebellum or medulla or any CSF leakage should be avoided, and if this ever happens, the individual is discarded from analysis. Few drops of glue are applied onto the needle insertion site, and the tracer is injected at

a rate of 1 µl/min (mice) or 2 µl/min (rats) for 10 minutes, resulting in a total injected volume of 10 µL for mice and 20 for rats. Depending on the study, the tracer was let to circulate for a few minutes (in most cases 30 minutes, to assess the stage of influx into the brain), and then we imaged the brains/spinal cords and other organs, or we performed live imaging, or alternatively transcardially perfused the animals.

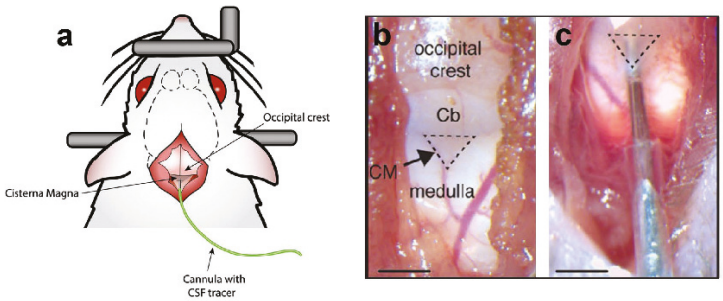

Fig 12. Cisterna Magna Injection. a) Schematic of the dorsal view of a rodent undergoing cisterna magna injection. Head is fixed in place with ear bars. b-c) Magnified view of the cisterna magna and the site of injection. Adapted from Ramos et al. 2019, Xavier et al. 2018, and Bechet (2022).

Tissue processing

Perfusion and fixation

In most cases, animals were transcardially perfused with PBS followed by 4% PFA, and then the brains, spinal cords, and other organs were immersion post-fixed overnight in PFA. In cases when we needed the meninges as a whole mount, we post-fixed the whole head of the mouse (without skin) overnight and later dissected the meninges from the skull. In **paper IV,** we first transcardially injected the vascular marker Lectin, and after a few minutes of circulation, we transcardially perfused-fixed with PFA.

One important detail was to add heparin (5 IU/ml) to the PBS that we first perfused with, to help inhibit blood clot formation and preserve the patency of the vascular system. It is always important to obtain consistently perfused samples across all animals in one experiment, but this was especially important in **paper IV**, where we aimed to compare the state of the vasculature and the vascular perfusion between groups.

Meningeal dissections

The calvarial dura mater was dissected as previously described (Louveau, Filiano, and Kipnis 2018) by carefully peeling it off from the skull. The dura mater of the spinal cord was dissected by first cutting on the sides of the vertebrae using micro scissors angled towards the outside (to prevent major damage to the spinal cord) and removing the ventral side of the vertebrae. Next, a superficial incision was made in the ventral median fissure of the spinal cord, and a piece of meninges was gently pulled to detach it from the spinal cord. Moving along the spinal canal, the spinal cord is removed to detach it from the meninges, and then the dura mater is carefully pulled from the bone, for which the nerves are pulled out of the vertebra one by one.

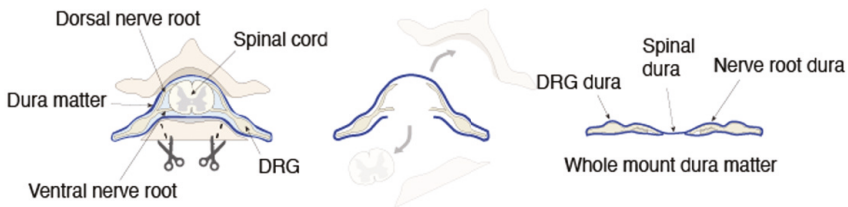

Fig. 13. Meningeal dissection. Schematic depicting dissection and whole mount preparation of spinal cord meninges. Firs the ventral vertebrae are cut on the sides, and the bone is removed from the ventral side. Next, the spinal cord is carefully removed, and the meninges are pulled from the dorsal bone with the help of forceps.

At the time of the start of this thesis, very few labs had performed a whole mount dissection of meninges and staining. In particular, the dissection of dura mater from the spinal cord was not an established protocol, and we invented our own way of doing it. It is worth noticing that with the current method, we are not yet able to separate the spinal nerves from their meningeal sheaths, and finding a way to analyse the dura covering spinal nerves on its own would be very valuable for future experiments that build on **paper III.**

Immunohistochemistry

The histological preparation for immunohistochemical analysis has been employed in different ways across the four papers in this thesis, and different types of tissues have been used for immunohistochemistry. In the great majority of cases, we used immunofluorescence. A general protocol is summarized here:

- The first step was usually blocking and permeabilizing with a solution made of donkey or goat serum, BSA, and Tween-20. These were added in

different concentrations and for different periods of time depending on what was optimal for a specific antibody and the tissue. For example, for staining meningeal samples, we usually block for longer than for brain sections because they are thicker in some regions once mounted, which adds background signal. In the case of cryosections, a previous step to blocking was washing them in PBS at room temperature a few times.

- Next, we incubated the samples overnight (sometimes longer if the antibody required so) in primary antibodies at 4°C in blocking solution or similar.
- On the second day, one would usually start with washing the primary antibodies, but I found that for some antibodies, leaving it on for an extra hour at room temperature worked much better, and at times it was the only thing that worked.
- We washed the primary antibodies with PBS and with a 1% BSA solution several times; the more washing, the better.
- The samples were then incubated with secondary antibodies in 1% BSA generally for two hours at room temperature.
- In the last step, we washed the samples with PBS and added any additional staining such as DAPI and/or Lectin. Lastly, the samples were mounted with a cover glass.

Although the general protocol is quite straightforward, some tissues, e.g., brain slices, are much easier to stain and handle than others. For example, meningeal whole-mounts are fragile and uneven, so they are the trickiest type of tissue to stain and mount used in this thesis. In addition, certain antibody protocols were especially difficult to optimize, which was the case with CD markers.

Microscopic images for analysis and representative images were captured using either a Nikon ECLIPSE Ti2 or a Nikon Confocal A1RHD. Images were analysed using Fiji software for cell counts, vessel density and fluorescence intensity measurements.

In two different studies, we analysed the AQP4 polarization. We stained the samples using an anti-AQP4 antibody and measured the fluorescence intensity both at the vessel wall and the parenchyma around the vessel using Fiji. We calculated the polarization ratio between the vessel wall and the parenchymal signal to get an idea of the amount of protein at the astrocytic endfeet versus everywhere else.

Tissue clearing

We followed the iDISCO+ (immunolabeling-enabled three-dimensional imaging of solvent-cleared organs) protocol as described by (Renier et al. 2014) to clear the spinal cords in **paper IV**. Briefly, the spinal columns were first decalcified in EDTA for 24 hours. Next, the tissue was dehydrated in increasing methanol/H_2O series (20%, 40%, 60%, 80%, 100%), delipidated with methanol/dichloromethane, and pure dichloromethane, and optically cleared with dibenzyl ether (DBE) for at least 14 days. We finally replaced DBE for ethyl cinnamate (ECi) prior to imaging because DBE is a toxic chemical that is more difficult to handle, while it is safe to handle ECi outside of the hood because it is not toxic, and both have the same refractive indexes. In our case, we also needed to incubate with EDTA to decalcify the bone. The bone decalcification adds one more layer of complexity to the clearing protocol and, of course, introduces additional sources of error, so it is generally preferred to clear the isolated spinal cord without bone if the study allows. In our case, we chose to keep the bone to maintain the spinal cord and the vasculature on the surface of the spinal cord as intact as possible.

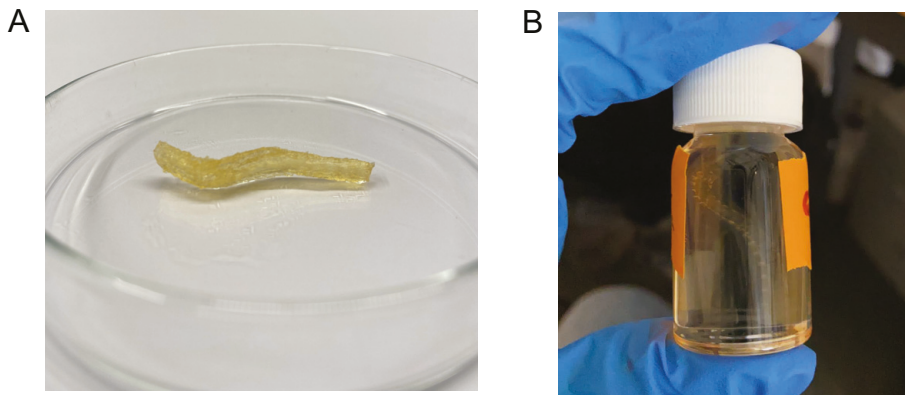

Fig 14. An optically cleared spine. (A) Mouse spine after clearing but without refractive-matching solution (in air). (B) Mouse spine after clearing and in DBE (refractive-matching solution).

Afterwards, we also performed de-clearing or rehydration. Reversing the clearing is not a well-established method yet, as there is to the best of our knowledge, only one publication currently in *bioRxiv* describing it (Bhatia et al. 2021). We followed their same protocol; rehydrated the spinal cords in decreasing methanol/H_2O series (100%, 80%, 60%, 40%, 20%) and finally washed in 1 × PBS overnight. After that, we dissected the spinal cords from the bone and sectioned them with a vibratome for immunohistochemistry/confocal microscopy. The staining was successful, and

we were able to image and quantify the number of vessels stained by Glut1. The samples were, of course, not as beautiful as freshly perfused samples, and one could argue that this adds another layer of error. However, since this was a comparison between diseased and control mice, and both underwent the same procedures, we believe that this was still worth it to reuse the same samples from light-sheet microscopy.

Imaging modalities

Fluorescent microscopy

Whole brains, spinal cords, and lymph nodes were imaged using a Nikon SMZ25 stereomicroscope with a Plan Apo 0.5x objective equipped with an Andor Zyla 4.2 Plus sCMOS camera. The same microscope or similar was used to image in vivo tracer flow through the skull, vertebrae, or skin. Vibratome slices of brain and spinal cord and meningeal whole mounts were imaged with both Nikon Ti2 Eclipse and Nikon A1RHD confocal microscopes. Cleared spinal cords were imaged using an Ultramicroscope Blaze (Miltenyi); more information about light sheet imaging is below.

Magnetic resonance imaging (MRI)

MRI has been an important method in **paper III** to elucidate the movement of CSF along the spinal cord in vivo and to assess the importance of spinal nerve swelling in EAE. The MRI procedures included in this thesis were carried out by Yuki Mori, Ph.D. A brief description of the protocols follows.

Mice were subjected to CM injections as explained previously, but in this case, the T1-enhancing contrast agent Gadobutrol was injected into the CM. MRI was performed in a 9.4T preclinical scanner housed at University of Copenhagen equipped with 240 mT/m gradient coil and using a homemade MR conditional holder. During MRI scanning, animal body temperature and respiration rate were controlled and maintained.

Dynamic contrast-enhanced MRI (DCE-MRI) is considered the most suitable technique to study CSF flow 3D mapping in the brain of living animals and humans. In **paper III,** two separate DCE-MRI scans were acquired, covering 1) cervical and thoracic levels and 2) lumbar levels of the spinal cord. Pre and post-contrast T1-weighted (T1W) imaging were collected with 3D fast low angle shot (3D-FLASH) sequence. However, in this study, we also used high-resolution tracer-free CSF

cisternography using 3D fast imaging employing balanced steady-state acquisition (3D-TrueFISP) to depict volumes of different spaces in the spinal cord.

Light sheet imaging

Optically cleared spinal columns were imaged using an Ultramicroscope Blaze (Miltenyi Biotec) with a 4x objective equipped with an organic solvent compatible immersion corrected dipping cap. The excitation wavelength used was 640 nm, and the emission filter used was 680/30 nm, since our aim was to visualize the Lectin Dylight-649 perfused. The spinal cords were imaged in the sagittal plane, from one lateral side to the other, and we chose to focus only on the lumbar region of the spinal cord, given the time-consuming nature of the method. We used both left and right-side illumination and single field of view to optimize the quality of the image. To avoid defocus, we used a 6-step sequential shifting of the focal position of the light sheet per plane and side, which considerably increased the quality of the images, facilitating the analysis. We used the vessel segmentation and analysis pipeline (VesSAP) to quantify the vascular changes in the lumbar spinal cord in an unbiased and semi-automatic way (Todorov et al. 2020).

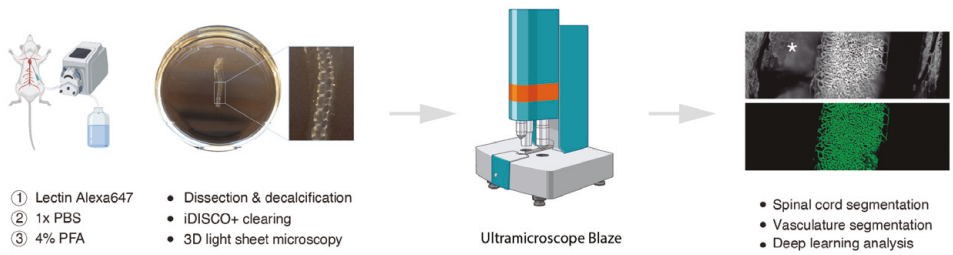

Fig 15. The process of light sheet imaging and analysis. First, we performed intracardial injection and staining with the vascular marker lectin and PFA fixation, then iDISCO+ clearing, and lastly, imaging of the lumbar cord using the Ultramicroscope Blaze. Adapted from paper IV.

Optoacoustic imaging

Multispectral optoacoustic tomography (MSOT) is an imaging modality that works by illuminating the tissue with a pulsed laser to provoke the photo absorbers that are naturally present in the tissue or contrast agents to absorb the light. The absorption of light translates in a thermoelastic expansion that runs through the tissue as an acoustic wave that we can detect with an ultrasound transducer. The optical absorption of different molecules varies depending on the wavelength, so we can

distinguish between different tissue components. In our study, we took advantage of the distinct absorption spectra of hemoglobin and deoxyhemoglobin to measure their concentrations and calculate the oxygenation in the tissue. This is one of the strengths of the technique; its capability to measure oxygen concentration in vivo and in a minimally invasive way. In fact, optoacoustic imaging has been used to study oxygenation in cancer studies and even in stroke, but never in EAE or a model of neuroinflammation (Attia et al. 2016; Ghosh et al. 2020; Ma et al. 2009; Tomaszewski et al. 2017). Some advantages of this imaging modality include avoiding ionizing radiation, good image resolution with high penetration depth, real-time imaging, needs little floor space, and is available as a small mobile unit; plus it is more economical than other imaging modalities such as MRI (Steinberg et al. 2019).

In **paper IV**, we used an inVision 512-echo (iTera Scientific GmbH, Munich, Germany) small animal imaging system. It emits excitation pulses with a duration of 9 ns at 10 different wavelengths (700, 730, 750, 760, 770, 800, 820, 840, 850, 880 nm) with an average of 10 pulses per wavelength at a repetition rate of 10 Hz. In this system, ten arms of a fiber bundle provide even illumination of a ring-shaped light strip. The system is also able to record ultrasound signals at the same time, which is used to identify anatomical structures

Mice were anesthetised with isoflurane during the whole experiment and their hair was removed to avoid interference with the light. In oxygen enhancement (OE) challenge experiments, animals were continuously imaged for 30 min (5 min breathing 21% O2; 20 min breathing 100% O2; and 5 min breathing 21% O2 again).

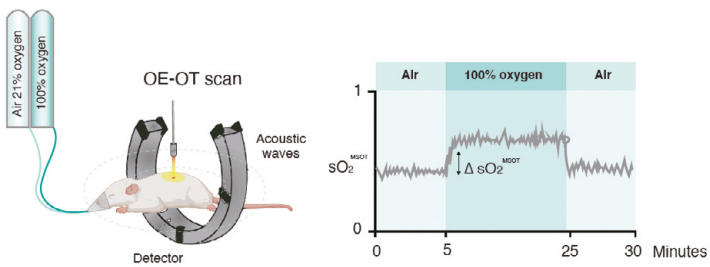

Fig 16. Oxygen enhancement challenge experiment using multispectral optoacoustic tomography (MSOT). Mice were imaged for 30 min (5 min breathing 21% O_2; 20 min breathing 100% O_2, and 5 min breathing 21% O_2 again). The difference in oxygen saturation (ΔSO_2^{MSOT}) between breathing air and 100% oxygen was calculated. Adapted from paper IV.

Statistical analysis

Statistical analyses in this thesis were performed using GraphPad prism. Data sets were tested for normality when possible. If normality was assumed, parametric tests were used: paired t-test for comparing two dependent groups, unpaired t-test to compare two independent groups, one way-ANOVA to compare more than two groups, and two-way ANOVA (or a mixed-effects model) to compare more than two groups considering more than one variable. If normality was not assumed, we used nonparametric tests such as Mann-Whitney or Wilcoxon tests. In most cases, we represented the values as mean ± SEM or as all individual values with maximum and minimum points. A p-value < 0.05 was considered statistically significant.

Reflections on statistics and preclinical research

A common concern in life sciences is the reproducibility of results. In recent years, many scientists have publicly criticized and attributed this crisis to the p-value culture (Baker 2016a). This culture has led to the practice of publishing only the findings that meet the p-value < 0.05 significance criterion, which has resulted in a division of research findings in either positive or negative. The issue has been furthered by the pressure in academia to continuously publish novel and positive results. A good approach for the future would be to pay more attention to other factors, such as the experimental design, the assumptions of the data analysis, the quality of the methods, and especially the statistical power of a study. In fact, low statistical power is known to reduce the likelihood that a significant result reflects an actual effect (Button et al. 2013). Thus, underpowered studies also contribute to the replication crisis. It is therefore essential to carefully design our studies to ensure reliable data.

Summary of results and discussion

In this section, the main results from the papers included in the thesis are summarized. However, not every detail is included here; thus, the reader is encouraged to check the complete Results and Discussion in the papers and manuscript attached in this book.

Acute systemic LPS-exposure impairs perivascular CSF distribution in mice (paper I)

The glymphatic system is believed to be involved in neurodegenerative diseases, specifically in the clearing of harmful proteins that aggregate and accumulate in the brain (Tarasoff-Conway et al. 2015; Simon and Iliff 2016). A common feature in many neurodegenerative diseases is neuroinflammation. In addition, systemic inflammation has been implicated in the pathology of AD (Erickson et al. 2012). In our first study, we aimed to address the unanswered question of how systemic inflammation (and derived neuroinflammation) impacts CSF flow dynamics in the brain. One way of modeling systemic inflammation in rodents is by injecting a bacterial endotoxin, LPS. Systemic inflammation in response to LPS has previously been shown to cause neuroinflammation and cognitive impairment, thus affecting the brain (Nava Catorce and Gevorkian 2016). In **paper I,** we injected LPS intraperitoneally in mice and studied the brain distribution of CSF fluorescent tracers injected in the CM 3 hour after LPS injection. LPS exposure decreased CSF tracer signal in the brain around the middle cerebral artery in comparison to vehicle controls (Figure 17). In coronal sections, we observed that tracer influx into the parenchyma was also lower in LPS-treated brains, indicating that LPS-derived systemic inflammation decreases perivascular CSF flow and influx into the brain.

Glymphatic function is supported by astrocytes and facilitated by AQP4 channels in astrocytic endfeet, allowing CSF transport into the brain. We assessed astrocytes and AQP4 channels in our model by measuring levels of the astrogliosis marker GFAP and of the AQP4 protein. Since we did not find any differences in GFAP or AQP4 levels in LPS-treated animals, we studied the polarization of AQP4 to the astrocytic endfeet by immunohistochemistry but also found no effects of LPS on polarization (Figure 18).

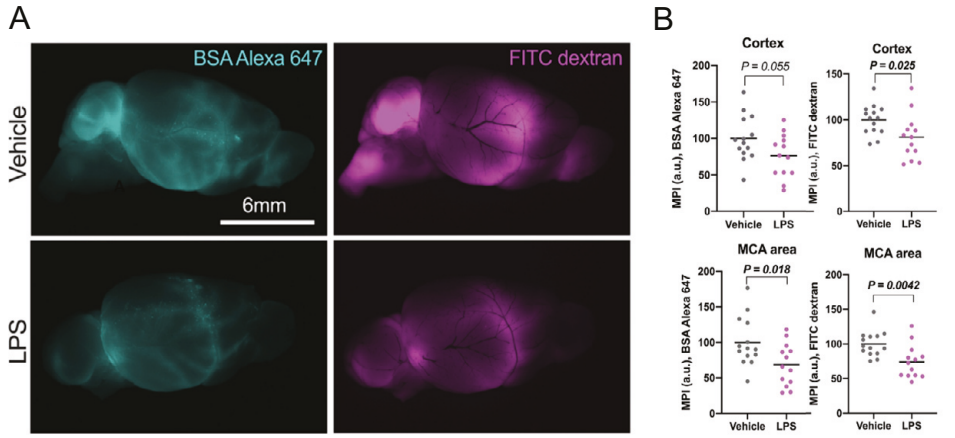

Fig 17. CSF transport of fluorescent tracers after LPS treatment. (A) Representative images of the lateral view of the brain and the MCA showing fluorescent tracer intensity and (B) Comparison of tracer intensity of the two different tracers in the cortex and the MCA area. (Adapted from Paper I)

One of the main players of neuroinflammation in the brain is microglia, the resident immune cells of the CNS, which are also some of the first responders when there is an immune response (Bilbo and Stevens 2017). In our study, we found no differences in Iba1^{+} (microglial activation marker) number of cells, but we observed an increase in the coverage of microglial (Iba1^{+}) processes in brain slices after LPS treatment, which is also an indication of activation (Figure 18). On the other hand, we did not find any effects of LPS on measured neuroinflammatory cytokines or markers of blood-brain barrier increased permeability.

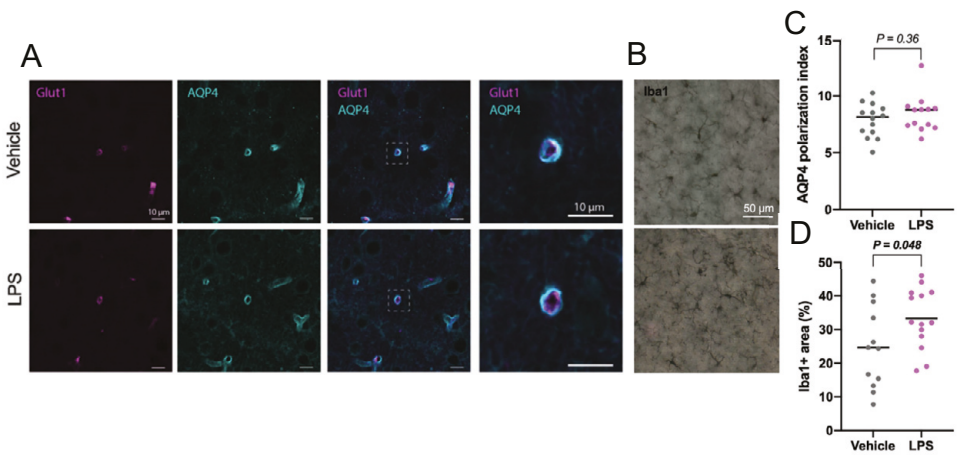

Fig 18. AQP4 polarization and Iba1 coverage after LPS exposure. (A) Representative images of AQP4 and Glut1 labeling blood vessels. (B) Iba1 DAP staining of microglial processes (C) Comparison of AQP4 polarization between LPS and vehicle controls. (D) Comparison of Iba1+ coverage. (Adapted from Paper I)

Lastly, since the movement of CSF is known to depend on physiological parameters such as the cardiac cycle, we measured cerebral blood flow, respiration rate, body temperature, and heart rate. In this experiment, we found no changes in most of these parameters, but we did find a significant increase in heart rate 3 hours after LPS exposure.

Altogether the results from **paper I** suggest that LPS-derived systemic inflammation affects the brain CSF distribution, even after one single low dose of LPS was administered. While a previous study found that higher doses of LPS affect the clearance of proteins from the brain to plasma (Erickson et al. 2012), this is the first study to assess the effect of LPS and systemic inflammation on CSF distribution in the brain. Unfortunately, we did not detect clear signs of neuroinflammation or astrogliosis in the brain at this time point, other than an increase in microglial processes. However, this might as well indicate that CSF flow responses are an early feature of the effect of LPS in the brain. It is also interesting that the heart rate of mice treated with LPS increased over time, since it has been shown that glymphatic function is higher during sleep, during which heart rate is lower, and thus suggesting that perhaps heart rate and glymphatic function might be inversely related (Xie, Kang, Xu, Chen, Liao, Thiyagarajan, O'Donnell, et al. 2013),.

Bacterial meningitis leads to CSF disturbances which rely upon neutrophil traps (paper II)

In **paper II,** we studied inflammation closer to the brain, in the meninges, and its effect on CSF transport. Acute neuroinflammation is in many cases associated with blood-brain barrier dysfunction followed by cerebral edema (Fukuda and Badaut 2012). In fact, in bacterial meningitis, a major cause of tissue damage is the accumulation of fluid in the brain, which leads to brain edema and increased intracranial pressure (Durand et al. 1993). One possible cause of this fluid accumulation is the impairment of the physiological clearance pathways of the brain. Since *Streptococcus pneumoniae* is the most common cause of bacterial meningitis, in **paper II,** we injected a clinical strain of this bacteria isolated from a meningitis patient, SP001, under the dura mater in rats.

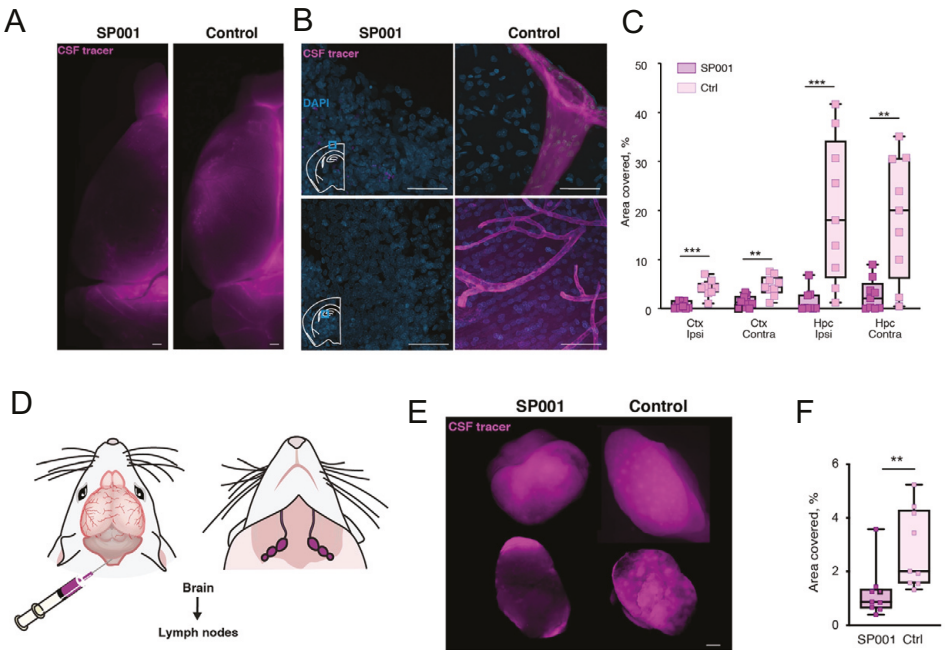

Fig 19. Meningitis impairs glymphatic influx into the brain and to the superficial cervical lymph nodes. (A) Representative images of the dorsal view of brains obtained from infected and control rats after circulation of the CSF tracer. (B) Representative images of CSF tracer distribution in the cortex (upper panel) and hippocampus (lower panel). (C) Comparison of CSF tracer distribution in cortex and hippocampus of the ipsilateral hemisphere (where bacteria/saline were injected) and contralateral hemisphere (no injection) of SP001-infected and control animals. (D) Schematic representation of CM injection of CSF tracer and distribution from the brain to the cervical lymph nodes. (E) Representative images of cervical lymph nodes 30 minutes after CSF tracer injection. (J) Comparison of total CSF tracer drainage for 2 lymph nodes per rat. (Adapted from Paper II)

Just 24 hours after SP001 inoculation, we injected a CSF fluorescent tracer into the cisterna magna, and we analysed its distribution in the brain after 30 mins circulation. The results showed that the meningitis rat brains had lower overall perivascular CSF flow and decreased influx to the cortex and hippocampus in brain slices (Figure 19). We also found that the efflux of tracer from the brain to the lymph nodes was impaired (Figure 19). This suggests that the movement of CSF is impaired in meningitis and the outflow of fluid from the brain towards lymph nodes is disrupted.

In this study, we also examined the effect of meningitis on glial cell responses. We found that microglia and astrocytes were activated (identified by changes in their cellular morphology and/or increased expression of activation markers). In addition, AQP4 polarization to the large vessel walls was decreased in meningitis, which would affect the ability of astrocytes to regulate fluid transport.

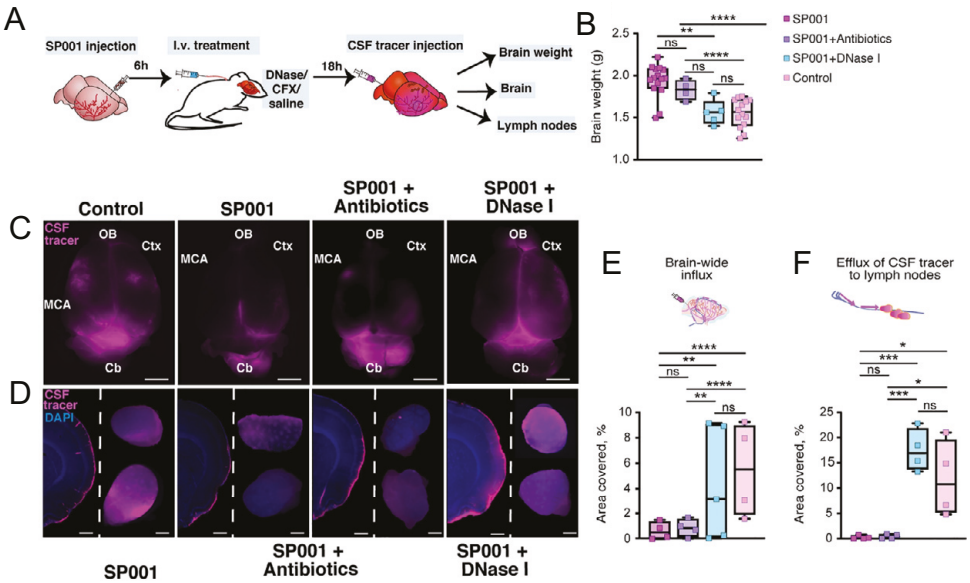

Fig 20. Targeting NETs with DNase I treatment restores CSF Flow. (A) Schema of the experimental procedure to disrupt NETs; 6 hours after subarachnoid injection of *Streptococcus pneumoniae* or saline, rats received treatment with DNase I or antibiotic ceftriaxone or vehicle (saline). Thereafter, rats were injected in the cisterna magna with CSF tracer and euthanized 30 minutes after the injection. (B) Brain weight of control, *S pneumoniae* SP001-infected saline treated, antibiotic-treated, and DNase-treated rats. (C) Representative images of dorsal brains. (D) Coronal sections and cervical lymph nodes. (E) Comparison of brain CSF tracer distribution in control, *S pneumoniae*–infected, antibiotic-treated, and DNase I–treated groups (F) Quantification of total CSF tracer drainage of 2 lymph nodes per rat.

Most importantly, we found neutrophils to play an important role. Infiltration of large numbers of neutrophils into the CSF (pleocytosis) is a known hallmark of bacterial meningitis (Mook-Kanamori et al. 2011). Neutrophils can form NETs (neutrophil extracellular traps) by expelling their DNA and enzymes to form sticky

traps with antimicrobial properties to kill pathogens (Brinkmann et al. 2004). NETs have been previously detected in the CSF during meningitis, and here we show NETosis by means of confocal microscopy in the meninges of rats.

Interestingly, DNase treatment to dissolve NETs restored the CSF circulation both inside the brain and out towards lymph nodes, in addition to decreasing brain edema formation (Figure 20). This indicates that NETs potentially hinder the flow of CSF during meningitis.

Overall, the findings described in **paper II** indicate that decreased CSF transport plays a role in edema formation during bacterial meningitis and that targeting CSF movement and dissolving neutrophil extracellular traps could be helpful to treat that aspect of the disease. Even though the purpose of neutrophils purpose is to resolve the bacterial infection, our data suggest that NETs produced by neutrophils could be harmful to the fluid homeostasis in the brain. Specifically, the accumulation of these NETs in the meninges and subarachnoid space might increase CSF viscosity, thus obstructing its circulation. The use of DNase to dissolve NETs shows potential to treat meningitis, however, the efficacy of this enzyme to treat meningitis in humans was only assessed in one anecdotal study back in 1959 and was not explored further (Johnson, Ayvazian, and Tillett 1959). Aerosolized DNase has nonetheless been approved for human use in the treatment of cystic fibrosis and has been injected safely in patients with lupus (Davis Jr et al. 1999). This shows promise that DNase could be a safe drug to try in patients with meningitis that present with edema and increased intracranial pressure.

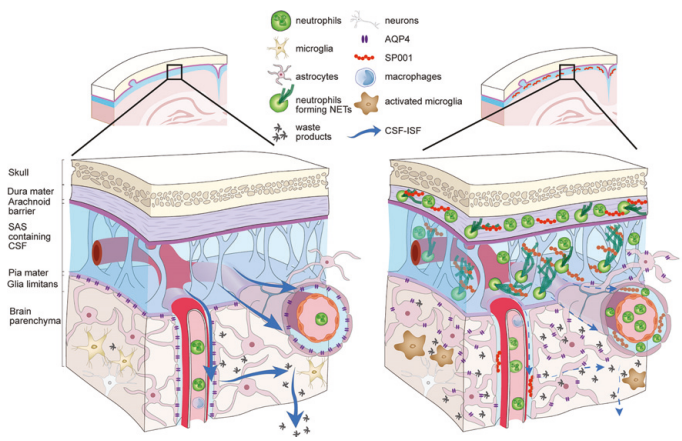

Fig 21. Proposed model of CSF impairment in bacterial meningitis. As bacteria proliferate mainly in the subarachnoid space and the perivascular compartment, circulating neutrophils are recruited in large numbers into the central nervous system, where they form neutrophil extracellular traps (NETs). Glymphatic function is impaired, possibly partly due to NETosis, followed by accumulation of immune cells and bacteria, along with astrogliosis and AQP4 depolarization. Reduced glymphatic clearance leads to cerebral edema and accumulation of waste products and metabolites in the perivascular space and the brain parenchyma. (Adapted from paper II)

Immune infiltration of the spinal nerve meninges and disrupted CSF flow in the spinal cord are early features of EAE (paper III)

In order to study neuroinflammation in more detail, in **paper III**, we investigate the experimental autoimmune encephalomyelitis (EAE) mouse model of multiple sclerosis (MS), which is usually seen as the prototypical inflammatory disease of the CNS. In fact, much of the current knowledge about neuroinflammation has been obtained from studies using the EAE model (Constantinescu et al. 2011). In patients, MS lesions are characterized by immune cell infiltration and formation of perivascular cuffs preferentially around cerebral blood vessels (Popescu, Pirko, and Lucchinetti 2013). Since these same peri-vascular pathways are CSF highways, we hypothesized that cerebrospinal fluid transport might play a role in EAE progression.

In **paper III,** mice were immunized with the myelin peptide MOG35-55 to develop EAE and were examined in the presymptomatic, acute, and chronic phases.

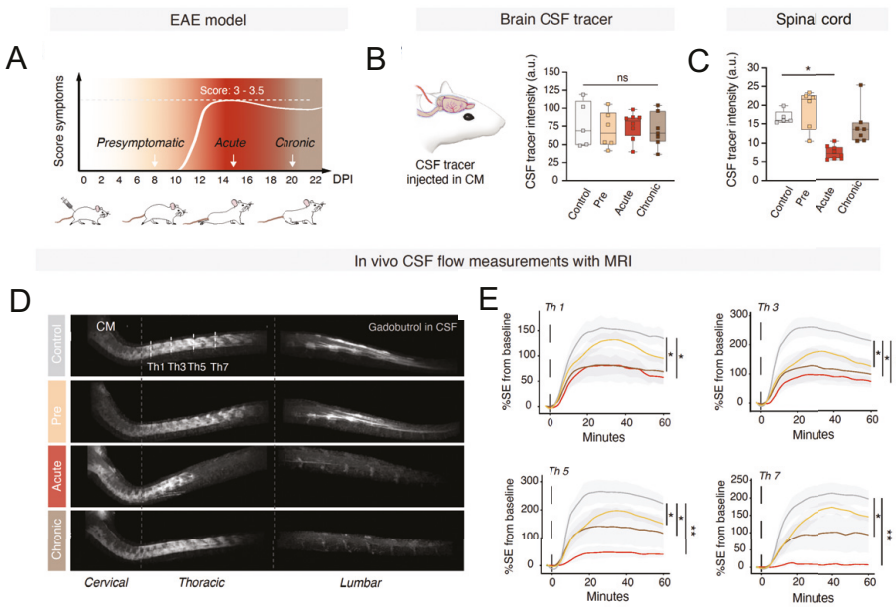

Fig 22. CSF flow patterns during EAE reveals resistance against flow in the thoracic spinal cord. (A) Schematic diagram depicting EAE disease progression in the C57BL/6 mice immunized with MOG35-55. (B-C) CSF tracer intensity in the brain (B) and spinal cord (C) of control, presymptomatic (Pre), acute and chronic EAE mice, 30 minutes after injection of OA-647 in the cisterna magna. (D) Dynamic contrast enhancement imaging (DCE-MRI) of the spinal cord 60 min after CM injection of the contrast agent Gadobutrol and indication of thoracic spinal levels Th1-Th7 analysed. (E) MRI signal enhancement ratio (%SE) from baseline during 60 min of circulation in the thoracic spinal segments Th1, Th3, Th5, and Th7. (Adapted from paper III)

We measured CSF flow both in brains using a fluorescent tracer and in vivo using a contrast agent for MRI. Perivascular CSF transport was normal in the brains of EAE mice when compared to controls. However, we found a blockage of CSF flow in the thoracic spinal cord of EAE mice, starting at the presymptomatic stage, which was most evident while imaging in vivo with MRI (Figure 22).

To elucidate possible sources of the blockage of flow, we used a comparative volumetric analysis of the spinal cord by MRI and showed that the volume of the spinal cord and subarachnoid space were respectively increased and decreased in the acute stage of EAE (Figure 23). However, the spinal cord was not swelling yet at the presymptomatic stage, which could not explain why the CSF flow had already started to decline at a presymptomatic point. Interestingly, at that earlier stage, the volume of the spinal nerves was already significantly increased (Figure 23). Since the space around nerves is known to be one of the main outflow pathways of CSF out from the CNS (Proulx 2021; Steer and Horney 1968), the swelling of spinal nerves might be a bottleneck for the flow of CSF in the spinal cord in early EAE stages.

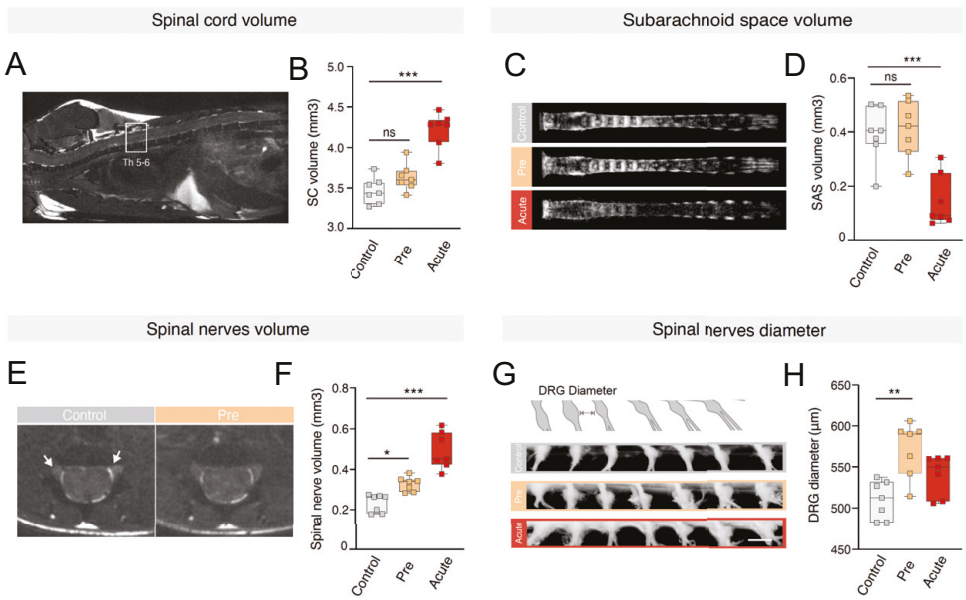

Fig 23. Enlargement of spinal nerves in presymptomatic and acute EAE. (A) Representative image of the level of the spinal cord analysed with T2W MRI. (B) Volume measurements of the spinal cord (SC) analysed by MRI cisternography in the three groups of interest. (C) Representative images of the volume of subarachnoid space (SAS) imaged with T2W MRI. (D) Volume measurements of subarachnoid space (SAS) analysed by MRI. (E) Representative images of spinal nerves in control and presymptomatic EAE mice imaged with T2W MRI. (F) Volume of the spinal nerves analysed by MRI. (G) Representative images of the post-mortem DRG attached to the spinal cord that were analysed for diameter. (Adapted from paper III)

Since previous studies have proposed that infiltration of T cells and inflammation in the meninges precede disease onset (Brown and Sawchenko 2007; Schläger et al. 2016a), we decided to study the extension of spinal dura mater that covers the spinal nerve roots and dorsal root ganglia (DRG).

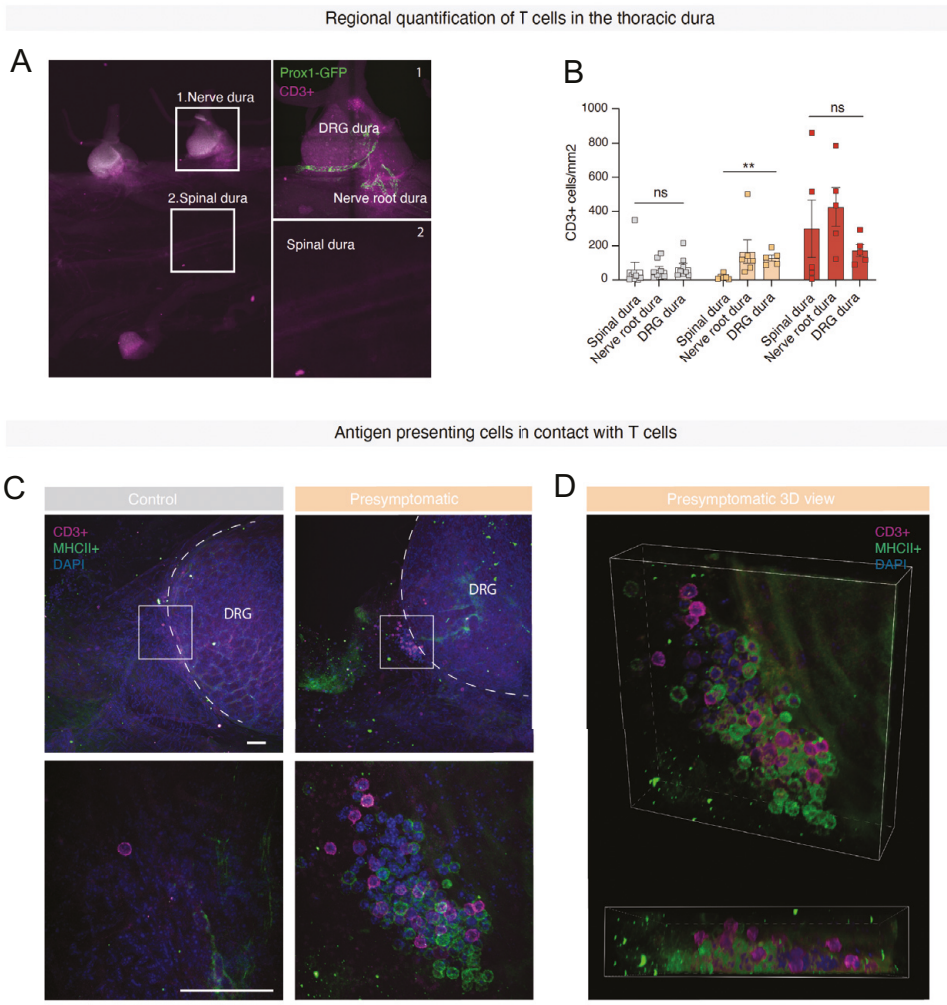

Fig 24. The dura mater around spinal nerves is a site of early T cell infiltration before onset of EAE. (A) Representation of the different regions of the thoracic spinal dura analysed. The wholemount duras were stained to mark T cells (CD3+) and lymphatic vessels are identified by the Prox1-GFP reporter. (B) Quantification of the number of T cells present on 3 regions of interest in the spinal dura: on the dura covering the spinal cord, the dura around nerve roots, and the dura attached to the DRG. (C) Confocal images of the dura around the nerve and on the DRG showing T cells (CD3+) and antigen-presenting cells (MHCII+) in direct contact with each other at the presymptomatic stage at the limit of the DRG dura. (D) 3D view of the right bottom panel in (C) showing that T cells (CD3+) and antigen-presenting cells (MHCII+) are indeed in close contact while the markers do not colocalize. (Adapted from paper III)

Immunohistochemistry of whole mount meningeal samples dissected from thoracic spinal cords showed meningeal T cell infiltration from early EAE stages, and that T cells mainly accumulate in the meninges around spinal nerves. T cells were not frequently found inside meningeal lymphatic vessels around spinal nerves in EAE (data not shown). In addition, we observed that MHCII positive cells (antigen-presenting cells) were also located in the meninges around spinal nerves, in close contact with T cells, where they may presumably present antigens to them (Figure 24). Lastly, IFNb treatment, which is thought to ameliorate EAE and MS progression by counteracting upregulation of MHCII and consequently T cell activation, reduced the number of lymphocytes present in the spinal nerve meninges and rescued CSF flow along the spinal cord, ameliorating symptoms.

Paper III identifies for the first time the spinal nerve roots and the meninges around them as some of the first sites of immune cell infiltration. The study also suggests that decreased CSF flow plays a key role in the pathogenesis of EAE and MS, potentially allowing for T cell infiltration. Our data suggest that T cell activation occurs in the paths of CSF efflux along nerves, where clearance of antigens presumably happens. This local activation might induce nerve swelling starting at the nerves, leading to obstruction of fluid outflow and fluid stagnation. Previous studies have proposed that the flow of CSF washes T cells from the pial surface (Bartholomäus et al. 2009; Schläger et al. 2016a), and we believe that this stagnation or decrease of flow might facilitate the invasion of CNS tissue by the immune cells. If this is the case, then the disturbances in CSF flow could be both an early consequence and a propagating event of the disease.

The inflamed spinal cord of EAE mice is hypoperfused and hypoxic (paper IV)

During neuroinflammation, the intense infiltration of immune cells and edema can lead to hypoxic tissue damage in the central nervous system, which has been proposed to be one way in which axons can be damaged, resulting in neurodegeneration (Pluta, Januszewski, and Czuczwar 2021; Desai and Smith 2017b). In the EAE model, we and others have shown swelling of the spinal cord, and some studies have proposed that the tissue probably suffers from hypoxia (Desai and Smith 2017a; Halder and Milner 2020; Yang and Dunn 2018). However, most studies have used postmortem samples or indirect measures of hypoxia. In **paper IV,** we aimed to image *in vivo* the oxygenation and hemodynamics of the spinal cord during EAE as well as study its link to vascular perfusion.

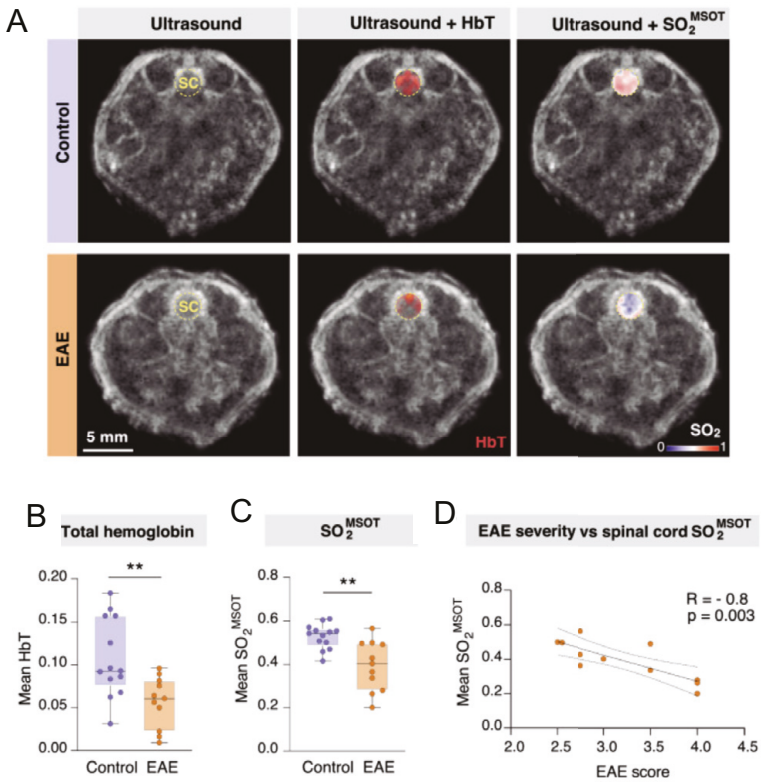

Fig 25. Low oxygen saturation in the lumbar spinal cord of EAE mice. (A) Representative images of ultrasound, total hemoglobin (HbT), and MSOT-derived oxygen saturation (SO_2^{MSOT}) in the lumbar spinal cord (SC). (B) Total hemoglobin in the spinal cord of EAE and control mice. (C) MSOT-derived oxygen saturation in the spinal cord of EAE and control mice. (D) Correlation between MSOT-derived oxygen saturation and EAE severity.

We investigated the oxygen saturation (SO_2) in the spinal cord of symptomatic acute EAE mice using multispectral optoacoustic tomography (MSOT). EAE mice exhibited lower SO_2 in the spinal cord compared to controls in addition to lower total hemoglobin concentrations. Our results suggested that the spinal cords of EAE mice are subjected to poor vascular perfusion and hypoxia that seem to correlate with the severity of the disease (Figure 25).

Oxygen administration relieved hypoxia in the spinal cord of EAE, however, we observed that EAE mice took longer to achieve their highest oxygen saturation point, suggesting the vasculature is not as functional as in control (Figure 26). This difference was especially remarkable in the ventral half of the spinal cord.

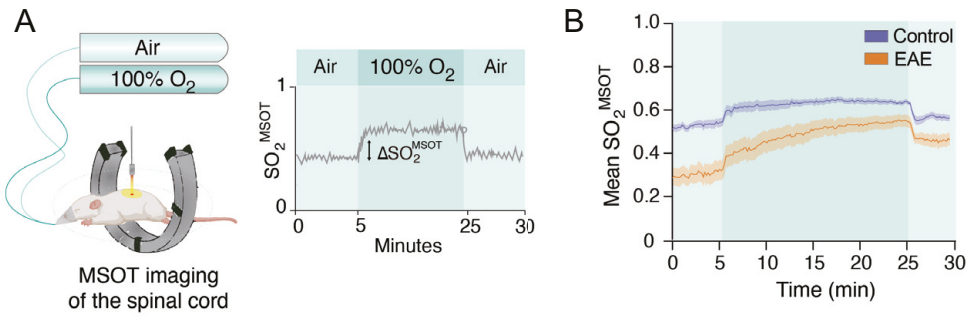

Fig 26. Oxygen administration improved oxygenation of the spinal cord in EAE mice. (A) Diagram showing the experimental procedure of the oxygen enhancement challenge (OT-OE) using MSOT. (B) Measurement over time of the oxygen saturation in the spinal cord of EAE and control mice during OE-OT. (Adapted from paper IV)

In order to assess if poor vascular perfusion of the spinal cord was perhaps to be blamed, we transcardially perfused the mice with the vascular tracer Lectin-Dylight649 and imaged the spinal cords with a light sheet microscope. We analysed the 3D data obtained from imaging using a novel vessel segmentation and analysis pipeline (VesSAP), which revealed that the vascular network perfused is reduced in EAE compared to controls (Figure 27). The data also suggests that the decrease was related to poor vascular perfusion and not related to the number of vessels per se, which in fact, was higher in EAE, indicating angiogenesis as a compensatory mechanism.

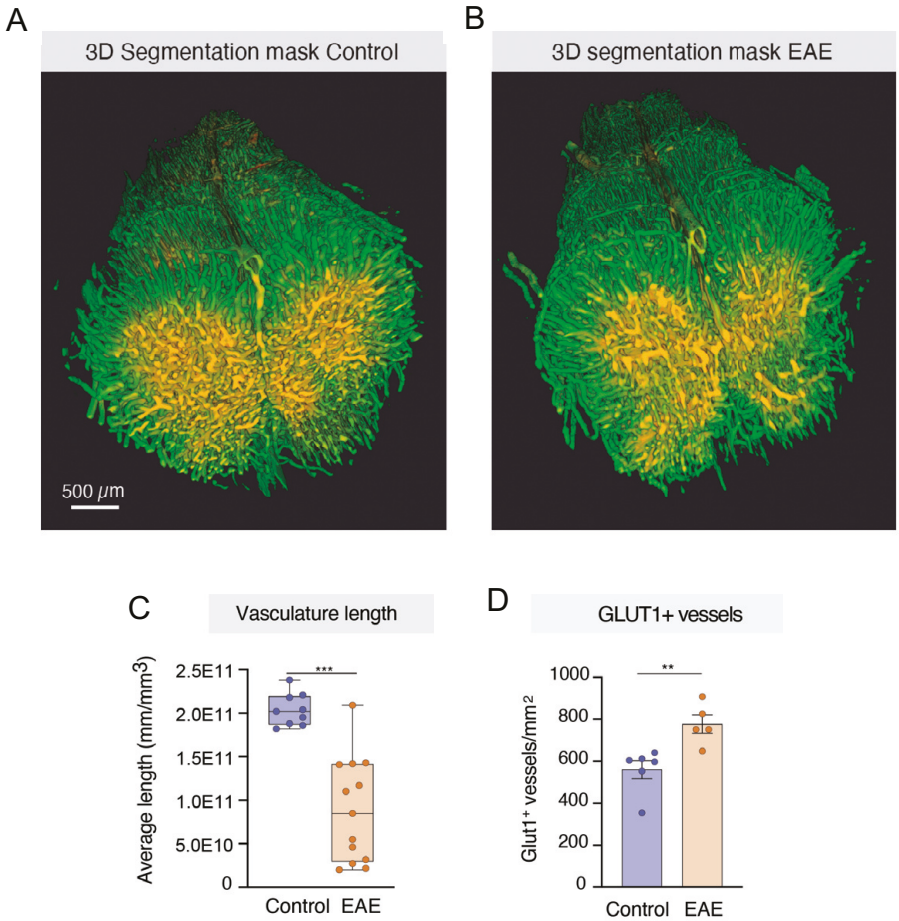

Fig 27. 3D imaging shows a reduced perfused vasculature network in the spinal cord of EAE mice. (A) 3D rendering of the lumbar spinal cord segmentation from a control mouse. (B) 3D rendering of the lumbar spinal cord segmentation from an EAE mouse. (C) Quantification of the vasculature length using VesSAP. (D) Quantification of the number of Glut1$^+$ vessels in spinal cord sections from EAE and control mice. (Adapted from paper IV)

Overall, the results of **paper IV** support the idea that inflammation and edema in the spinal cord hinder the perfusion of part of the vasculature, which could be the basis for the decrease in blood flow and hypoxic state that we describe, and the hypoxia presumably leads to activation of an angiogenic response to increase vessel density. This study also brings up optoacoustic imaging as a promising tool to further investigate vascular and hypoxic changes in the central nervous system in mice, with the real possibility of translating the findings to humans.

Conclusions and future perspectives

Paper I – Understanding how general inflammation influences the brain through CSF transport

In **Paper I**, we sought to elucidate how the transport of CSF is affected by a peripheral acute inflammatory challenge. We found that the perivascular distribution of CSF and its influx into the brain was impaired already 3 hours after a single systemic injection (1mg/kg) of LPS. However, most of the factors expected to influence glymphatic transport and/or neuroinflammation, such as respiration, brain blood flow, proinflammatory cytokines, astrocytes, and AQP4 polarization, were not affected at this early time point (Nava Catorce and Gevorkian 2016). The only measured parameters that were altered were the heart rate and the activation of microglia, in addition to the mice showing the typical sickness behavior that is expected from a reaction to LPS.

LPS is known to lead to cognitive impairment in rodents, most likely through neuroinflammation (Zhao et al. 2019; Oh et al. 2020). It has been shown that LPS can elicit neuroinflammation through the TLR4 receptor and via microglial activation. (Kloss et al. 2001). Considering that in most cases microglia are the first responders to brain injury, it is not surprising that we only found activation of microglia, of all parameters measured, accompanying the decrease in CSF flow. However, this gives rise to many questions. Is the activation of microglia leading to the reduction of CSF flow? Or is, on the contrary, the slowing of the flow of CSF making the microglia reactive? Further studies looking at the isolated relationship between CSF flow and microglial activation are needed to understand this particular circumstance.

The other parameter that was affected at this time point was the heart rate, which was increased after LPS injection, similarly to what was reported previously (Ehrentraut et al. 2007). It is worth noting that a study found that glymphatic influx in the brain correlates positively with deep sleep and negatively with heart rate (Hablitz Lauren et al.). Thus, it is possible that the increase in heart rate induced by LPS is an element contributing to slowing down CSF influx. However, as explained in our publication, we measured physiological parameters only in a limited number of animals. Therefore, it would be very valuable to study further the effect of

slowing heart rate on CSF distribution in the CNS by means of LPS or other modulators without an inflammatory component (Mestre, Tithof, et al. 2018).

Another factor that could have potentially influenced the CSF flow might have to do with the fact that TLR4-dependent inflammation causes hypersecretion of CSF from the CP in the ventricles (Karimy et al. 2017). Karimy and colleagues found that the TLR4 pathway activates the NKCC1 cotransporter in the CP epithelium, leading to increased production of CSF. Since LPS acts by binding the TLR4 receptor, it is possible that it results in overproduction of CSF. Unfortunately, CSF production was not measured in our study. Thus, future investigations that measure the production in response to LPS would be helpful to confirm this hypothesis. However, a potential increase in CSF production should lead to an increase rather than the observed decrease in CSF perivascular distribution. Some authors have hypothesized that inflammation-derived CSF oversecretion may initially lead to increased export of fluid and cytokines from the brain, but eventually, as a result of inflammation, swelling and closure of CSF exit routs may lead to fluid stagnation and reduced transport (Mogensen, Delle, and Nedergaard 2021). These are, of course, mere speculations, and further studies are needed to discern how CSF production is affected in different stages of neuroinflammation and in what ways CSF production affects CSF flow.

This first paper contributes to the knowledge of how peripheral inflammation impacts the brain and CSF transport, but additional research on the topic is needed in order to understand and develop new therapeutic strategies for the consequences of central inflammation in the CNS. The main conclusion of this paper was that glymphatic function is affected at a very early stage of peripheral inflammation.

Paper II – The role of the CSF in edema formation in meningitis

In **paper II**, we found that CSF influx to the brain and drainage into the cervical lymphatic system was suppressed in a rat model of bacterial meningitis. Glial cells were reactive in our model, and AQP4 polarization to astrocytic endfeet in large vessels was disrupted, likely contributing to the decrease in CSF influx to the brain. In addition, neutrophils were found to release NETs, and disrupting these NETs by means of DNase treatment restored CSF flow, indicating that the formation of NETs contributed to disrupting CSF transport in meningitis. Lastly, the removal of NETs also ameliorated the brain swelling seen in this model, suggesting that blockage of CSF transport contributes to edema formation in meningitis.

In the blood, NETs activate coagulation systems, which makes the fluid consistency thicker (Longstaff et al. 2013). In patients with cystic fibrosis NETs also increase

mucus viscosity because of the presence of the DNA fibers (Zawrotniak and Rapala-Kozik 2013). We hypothesize that NET accumulation in the CSF in the subarachnoid space potentially changes the properties of the CSF and modifies or slows down its circulation. In addition, NETs were observed in the pia and dura mater, thus, one possibility is that the NETs hinder the brain's CSF efflux pathways (meningeal lymphatics and/or at the skull base) to the cervical lymphatic system.

Another novel aspect of our study is the connection between the decrease in CSF efflux to peripheral lymph nodes and the presence of edema in meningitis. Acute edema is a known consequence of meningitis in patients that predicts disease outcome in some cases (Durand et al. 1993). Even though it has usually been simply attributed to inflammation, edema and increased ICP in meningitis remains an issue without proper treatment because the mechanisms behind it are poorly understood (McGill et al. 2016; Quagliarello, Long, and Scheld 1986; Tariq et al. 2017; Keep, Andjelkovic, and Xi 2017). Our study points at a failure in transport and efflux of CSF during bacterial meningitis as a possible source of edema formation and proposes DNase I or other treatments targeting neutrophil NETs as a solution. Targeting NETs is a potential way of bypassing the use of antibiotics, which is continuously challenged by new resistant strains. Of course, additional preclinical and clinical investigations are needed to prove the efficacy of DNase I in the context of bacterial meningitis. In addition, further studies analysing the water content in the brain in this model and in relation to CSF transport are needed, given that in this study, we only assessed the total brain weight, which is a less direct measure of brain swelling. It would also be very interesting to investigate in more detail the spatial-temporal distribution of NETs in the CSF in order to better understand in what way they can disrupt CSF movement or outflow paths.

Lastly, the fact that restoring CSF flow by means of DNase treatment decreased glial activation indicates that the normal flow of CSF helps to relieve or clear neuroinflammation, which increases our understanding of how CSF flow affects existing inflammation in the brain.

Paper III – Swelling of nerves, blocked outflow of CSF and lymphocytes invading the CNS-PNS meninges

In **paper III** we show that in the EAE model of neuroinflammation, T cells invade the dura mater covering thoracic spinal nerves in the presymptomatic phase, before T cells populate the spinal cord itself. Seemingly, the T cell activation also occurs in these areas along the spinal nerves, where we found close contact between $CD3^+$ lymphocytes and $MHCII^+$ APCs, and which are also paths of CSF efflux and potentially of clearance of antigens from the CNS (Proulx 2021). We demonstrate

that the spinal nerves start to swell in the presymptomatic stage before any changes are observed in the spinal cord, leading to obstruction of CSF efflux and presumably to fluid stagnation in the spinal cord.

A previous study also found impaired CSF flow in the spinal cord during EAE (Fournier et al. 2019). We hypothesize that the fluid stagnation could contribute to further T cell invasion of the spinal cord. This is also in line with previous studies that suggest that CSF flow washes out lymphocytes that invade the meninges (Bartholomäus et al. 2009; Schläger et al. 2016b). In pathological conditions, it might be that the stagnation of CSF allows T cells to further invade the tissue. Additionally, we find that T cell numbers were increased in the dura mater of spinal nerves before we detected any increase in numbers in the CSF, which shows that lymphocytes do not reach the site through the CSF. Our results are compatible with the idea that lymphocytes could access the meninges through meningeal blood vessels like it has been reported (Schläger et al. 2016a) or even through specialized channels connecting meninges to the bone marrow in the vertebrae (Mazzitelli et al. 2022). Our data suggest the meninges of the CNS-PNS (central-peripheral nervous systems) border as the main point of T cell accumulation and perhaps entry. We also found MHCII^{+} antigen-presenting cells in that region of the dura in contact with T cells, presumably making it a point of T cell activation. These results support the two-wave hypothesis of EAE in that antigen presentation to T cells in the dural compartment precedes the onset of clinical symptoms (Bartholomäus et al. 2009; Brown and Sawchenko 2007).

According to our results, the transport of CSF in the spinal cord might be both an initiating event and a propagating source of EAE. Many questions remain, and future studies should focus on understanding why T cells may accumulate in the dura around spinal nerves, which could give us clues as to how the disease is initiated. Even if we did not find that T cells were frequently inside meningeal LVs, a closer look at their role in all the above-mentioned processes and in the progression of EAE might aid in understanding why this area is important. Additionally, further investigations of how the flow of CSF affects the migration of T cells in the meninges would be useful to confirm that the stagnation of fluid is indeed contributing to the disease progression. It also seems necessary to further confirm that antigens such as myelin leave the CNS along these paths and to study the activation status of lymphocytes and APCs on the nerves in detail. Answering these questions would be of relevance for gaining insights into the pathogenicity and trajectory of T cells in multiple sclerosis.

Lastly, recent advances in the field of meningeal immunity have discovered communicative channels from the skull bone marrow to the meninges (Rustenhoven et al. 2021; Pulous et al. 2022; Mazzitelli et al. 2022; Herisson et al. 2018; Cai et al. 2019). Understanding how this recently discovered pathway relates to the immune infiltration seen in the spinal nerve meninges during EAE appears an exciting topic of future research.

Paper IV – The interplay between neuroinflammation, vascular dysfunction and hypoxia

In the last paper, we show that the spinal cord is both hypoperfused and hypoxic in the EAE model. We show that there is hypoxia in the tissue in vivo and that, even though oxygen administration ameliorates the oxygen concentration, the vasculature is compromised. We also show that the vascular perfusion of the spinal cord of EAE is disrupted in spite of the angiogenic response in the area. A novel aspect of the study is that we introduce optoacoustic imaging (MSOT) as a technique with the potential to further decipher the role of hypoxia in neuroinflammation.

Interestingly, we also observed that the ventral side of the spinal cord was most vulnerable to hypoxia, being the limiting factor for the total tissue to respond adequately to oxygen. This was in line with previous studies that proposed that hypoxia may happen in "watershed areas" of the CNS with the poorest or most vulnerable blood supply architecture (Halder and Milner 2020). We know that in the ventral spinal cord, the anterior spinal artery's conformation makes the area vulnerable to poor perfusion (Satran 1988). In our study, we did not analyse the vasculature of the ventral versus dorsal spinal cord in EAE, but future studies looking at this particularity could shed light on the vulnerable regions of the CNS during EAE.

We applied MSOT in the spinal for the first time and were the first to visualize hypoxia in vivo in the EAE model. The technique is a novel non-invasive imaging modality that can measure tissue oxygenation in real-time and is being developed as a new experimental diagnostic tool in the field of cancer but has not yet been used in the context of MS or EAE (Ma et al. 2009; Attia et al. 2016; Tomaszewski et al. 2017; Ghosh et al. 2020). We believe that the results from this study have clinical translation potential because optoacoustic imaging is a technique undergoing rapid development for clinical use. We hope to see more research looking at how hypoxia correlates with damage in the CNS and if it is predictive of inflammation and relapses.

One hypothesis that could explain our results is that hypoxia in watershed regions, which may happen due to injury or hypoperfusion in the area, activates an inflammatory and angiogenic response. At the same time, inflammation could cause dysfunction and even occlusion of the existing and new vessels, hindering the perfusion of the tissue and perpetuating the hypoxic state. This might in turn, contribute to prolonging and exacerbating the neuroinflammatory response and damage to the tissue. In line with these observations, another study observed a partial occlusion of spinal vasculature in a different EAE model by means of MRI

(Mori et al. 2014). Further studies are required to decipher how inflammation may elicit hypoxia, or the other way around since the order of events is not yet clear (Halder and Milner 2020). We also need additional research to understand how hypoxia can be alleviated to reduce the damage caused by neuroinflammation, considering that most studies manipulating oxygen in MS patients have failed (Halder and Milner 2020; Bennett and Heard 2010). Understanding the interplay between hypoxia, vascular dysfunction, and neuroinflammation better would provide an improved basis for developing new treatments and diagnostic tools for patients of MS and of other diseases with a significant neuroinflammatory component.

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It makes what is excellent in others belong to us as well.

- Voltaire

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About the author

MARTA RAMOS VEGA started her doctoral studies at Lund University in 2018, after serving as research assistant in labs all over the world; in Spain, Australia, Malta, and France. She holds a BSc in Biotechnology and MSc in Neuroscience and is interested in neuroinflammation as well as imaging & visualization techniques. During her doctoral studies, Marta has researched fluid dynamics in the inflamed brain and spinal cord. The work from this thesis contributes to the scientific understanding of how neuroinflammation interacts with cerebrospinal fluid transport.

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