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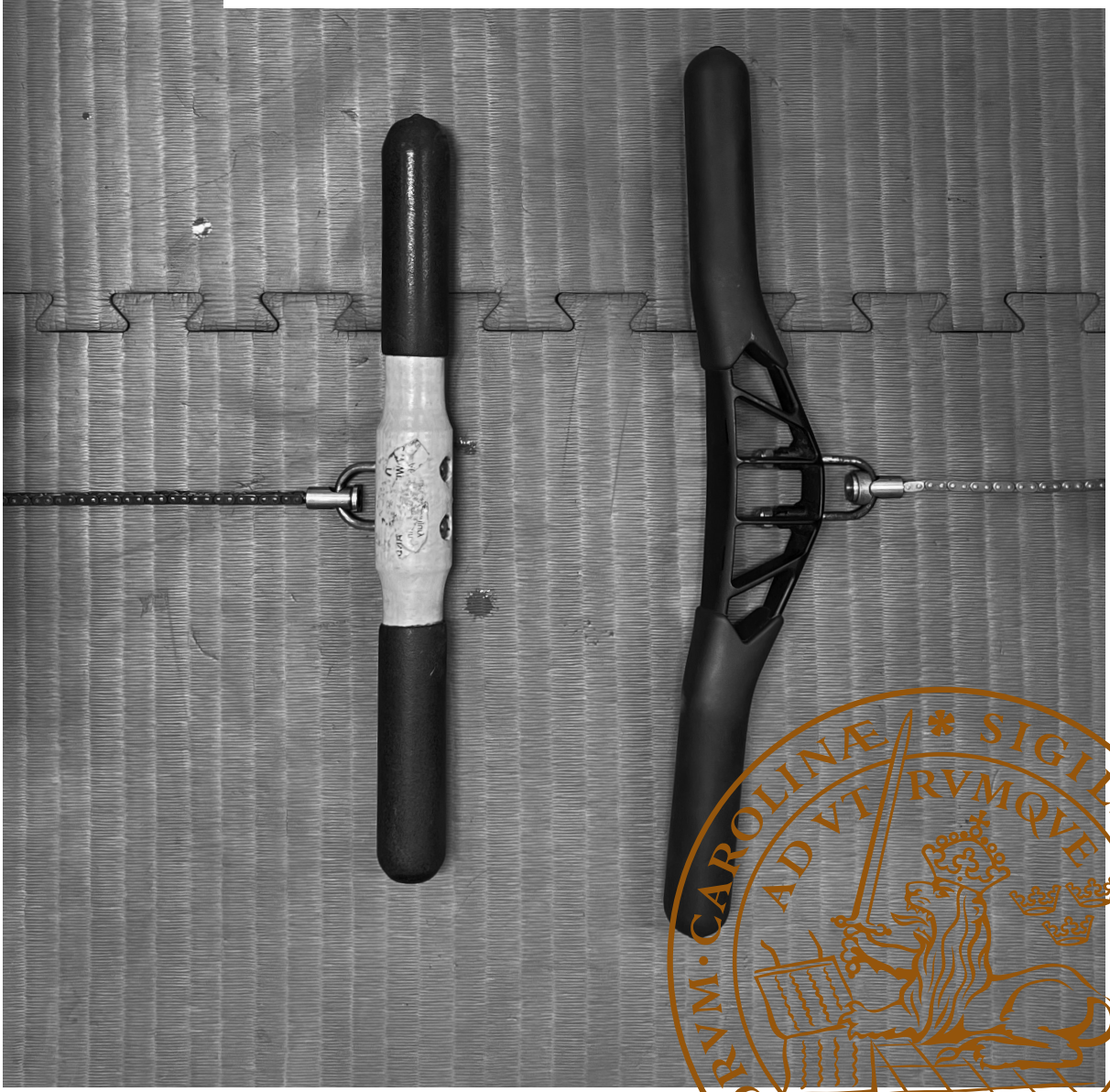

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Oscar Manouchehrian

LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract According to the World Health Organization, 50 million people are affected by dementia, and with aging populations, these numbers are expected to increase. Alzheimer's disease (AD) is the most common form of dementia, followed by vascular dementia (VaD). One is believed to be caused by abnormal accumulation of specific protein aggregates, the other one by impaired blood flow to the brain, but the two also share inflammatory components. Recommended preventative measures for both AD and VaD include cognitive stimuli, healthy diets, and regular physical activity. However, the exact mechanisms of exercise as attenuation in these common diseases remain to be elucidated.

Many experimental studies show that neuroinflammation after cerebral focal ischemia can exacerbate the insult. However, most studies have been performed in adolescent male mice when modeling these stroke-related diseases that affect elderly women the most. Galectin-3 is an inflammatory mediator that has been implicated in many diseases, including brain ischemia. In some conditions, it exerts protective and anti-apoptotic effects, and in others, it exacerbates inflammation and injury. Our group has previously shown that Galectin-3 ablation improves outcomes in experimental models of neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease. In Paper I, we subjected 24-month-old galectin-3 null female mice to pMCAO and compared behavioral disturbances and lesion size to wild-type controls.

In Paper II, we studied long-term voluntary running in a mouse model of familial AD, and in Paper III, we trained mice on treadmills for a month before subjecting them to a model of hypertension (a well-known risk factor for VaD).

Furthermore, in patients with small vessel disease (a leading cause of VaD), enlarged perivascular spaces can be seen with MRI. These spaces are believed to play an important role in cerebral waste clearance through the so-called glymphatic system, where cerebrospinal fluid (CSF) flushes through the brain during sleep. It has been speculated that the perivascular spaces are enlarged when harmful residues are accumulated, generating a dysfunctional and inflammatory environment, which in turn causes defective glymphatic function, hypoxia, and tissue damage (Brown et al. 2018). In Paper IV, we have tested a hypothesis of whether inflammation can affect the flow of CSF, a piece of the puzzle in this serious story.

The projects in this thesis aim to increase understanding of how exercise can affect murine models of neurological diseases, the effect of inflammation on CSF movement as well as age and sex in experimental models of focal brain ischemia.

Key words Inflammation, exercise, neurology, dementia, stroke, neuroinflammation

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To the mice that sacrificed everything for these studies

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NeuroReport, 2022, Mar 29; 33:6. doi: 10.1097/WNR.0000000000001778

Paper II

Voluntary running does not reduce neuroinflammation or improve non-cognitive behavior in the 5xFAD mouse model of Alzheimer's disease.
Martina Svensson, Emelie Andersson, **Oscar Manouchehrian**, Yiyi Yang, Tomas Deierborg.

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Paper III

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Oscar Manouchehrian*, Nicholas Don-Doncow*, Sara Bachiller, Anja Meissner†, Tomas Deierborg†.

Manuscript in preparation.

Paper IV

Acute systemic LPS-exposure impairs perivascular CSF distribution in mice.
Oscar Manouchehrian, Marta Ramos, Sara Bachiller, Iben Lundgaard, Tomas Deierborg.

Journal of Neuroinflammation, 2021 Jan 29;18(1):34. doi: 10.1186/s12974-021-02082-6.

*/† Equal contribution

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Who let the dogs out?: detrimental role of Galectin-3 in hypoperfusion-induced retinal degeneration.
Oscar Manouchehrian, Karin Arnér, Tomas Deierborg, Linnéa Taylor.

Journal of Neuroinflammation. 2015 May 14;12:92. doi: 10.1186/s12974-015-0312-x.

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Current Eye Research, 2017 Jun;42(6):948-961. doi: 10.1080/02713683.2016.1254247.

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Under revision in *Journal of Neuroinflammation,* 2021.

Abbreviations

AD	Alzheimer's disease
ADP	Adenosine diphosphate
AngII	Angiotensin-II
ANOVA	Analysis of variance
AQP4	Aquaporin-4
BBB	Blood-brain-barrier
BDNF	Brain-derived neurotrophic factor
CD	Cluster of differentiation
CNS	Central nervous system
CO_2	Carbon dioxide
CSF	Cerebrospinal fluid
DAB	3, 3'-diaminobenzidine
ddH_2O	Double-distilled water
ELISA	Enzyme-Linked ImmunoSorbent Assay
EPO	Erythropoietin
GFAP	Glial fibrillary acidic protein
FACS	Fluorescence-activated cell sorting
H^+	Hydrogen ions
Iba1	Ionized calcium-binding adapter molecule 1
IgG	Immunoglobulin G
IFN	Interferon
IL	Interleukin
KO	Knock-out
K^+	Potassium ions
LD	Laser doppler
LPS	Lipopolysaccharide
LTM	Long-term memory
Ly6	Lymphocyte antigen 6

MCA	Middle cerebral artery
NFTs	Neurofibrillary tangles
NLRP3	NLR family pyrin domain containing 3
N.s.	Not significant
PBS	Phosphate buffered saline
PBS-T20	PBS with Tween-20
PBS-TX	PBS with Triton X100
PFA	Paraformaldehyde
pMCAO	Permanent middle cerebral artery occlusion
qRT-PCR	Real-time quantitative reverse transcription polymerase chain reaction
RIPA	Radioimmunoassay
Run	Running
SDS page	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
Sed	Sedentary
STM	Short-term memory
TBI	Traumatic brain injury
TNF	Tumor necrosis factor
VaD	Vascular dementia
VEGF	Vascular endothelial growth factor
3R	Replacement, reduction, refinement
5xFAD	Five different human mutations of familial Alzheimer's disease

Lay summary

According to the World Health Organization, 50 million people are affected by dementia, and with aging populations, these numbers are expected to increase. Alzheimer's disease (AD) is the most common form of dementia, followed by vascular dementia (VaD). One is believed to be caused by abnormal accumulation of specific protein aggregates, the other one by impaired blood flow to the brain, but the two also share inflammatory components. Recommended preventative measures for both AD and VaD include cognitive stimuli, healthy diets, and regular physical activity. However, the exact mechanisms of exercise as attenuation in these common diseases remain to be elucidated.

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Populärvetenskaplig sammanfattning

Enligt WHO är 50 miljoner människor drabbade av demens, och med en åldrande befolkning förväntas dessa siffror öka. Alzheimers sjukdom är den vanligaste formen av demens, följt av vaskulär demens. Den ena tros vara orsakad av abnormal ackumulering av vissa proteinaggregat, den andra av försämrat blodflöde till hjärnan, men de två delar också inflammatoriska komponenter. Rekommenderade förebyggande åtgärder för både Alzheimers sjukdom och vaskulär demens inkluderar kognitivt stimuli, en hälsosam kost och regelbunden fysisk aktivitet. De exakta mekanismerna för träning som dämpning vid dessa vanliga sjukdomar återstår dock att klarlägga.

Många experimentella studier att neuroinflammation efter cerebral fokal ischemi kan förvärra skadan. De flesta studier har dock utförts på unga hanmöss, trots att dessa strokerelaterade sjukdomar hårdast drabbar äldre kvinnor. Galectin-3 är ett inflammatorisk protein som har varit inblandad i många sjukdomar, inklusive hjärnischemi. I vissa tillstånd utövar det skyddande och har anti-apoptotiska effekter, och i andra förvärrar det inflammation och nervskada. Vår grupp har tidigare visat att genetisk deletion av galektin-3 förbättrar resultaten i experimentella modeller av neurodegenerativa sjukdomar, såsom Parkinsons sjukdom och Alzheimers sjukdom. I projekt I utsatte vi 24 månader gamla galektin-3-fria honmöss för pMCAO och jämförde beteendestörningar och lesionsstorlek med vildtypkontroller.

I projekt II studerade vi långvarig frivillig löpning i en musmodell av familjär Alzheimers sjukdom. I projekt III tränade vi möss på löpband i en månad innan vi inducerade högt blodtryck (en välkänd riskfaktor för vaskulär demens) med hjälp av infusion av en vasokonstriktor, Angiotensin-II.

Hos patienter med småkärlsjukdom (viktig orsak till vaskulär demens), kan förstorade perivaskulära utrymmen ses med magnetkamera. Dessa utrymmen tros spela en viktig roll i rensandet av hjärnans avfall, genom det så kallade glymfatiska systemet, där cerebrospinalvätska (CSF) sköljer genom hjärnan på natten. Det har spekulerats i att de perivaskulära utrymmena förstoras när skadliga rester ackumuleras, vilket genererar en dysfunktionell och inflammatorisk miljö, vilket i sin tur orsakar defekt glymfatisk funktion, hypoxi och vävnadsskador (Brown et al. 2018). Om denna teori är sann återstår att se. I projekt IV har vi testat en hypotes om huruvida inflammation kan påverka flödet av CSF, en pusselbit i denna allvarliga historia.

Projekten i denna avhandling syftar till att öka förståelsen för hur träning kan påverka musmodeller av neurologiska sjukdomar, effekten av inflammation på CSF-rörelser samt ålder och kön i experimentella modeller av fokal hjärnischemi.

Resumen en español

Según la OMS, 50 millones de personas padecen demencia y, con el envejecimiento de la población, se espera que estas cifras aumenten. La enfermedad de Alzheimer es la forma más común de demencia, seguida de la demencia vascular. Se cree que la primera es causada por la acumulación anormal de ciertos agregados proteicos, mientras que la otra se debe a un deterioro en el flujo sanguíneo al cerebro; pero las dos enfermedades comparten componentes inflamatorios. Las medidas preventivas recomendadas tanto para la enfermedad de Alzheimer como para la demencia vascular incluyen estímulos cognitivos, una dieta saludable y actividad física regular. Sin embargo, todavía no están claros los mecanismos exactos por los que el ejercicio atenúa estas enfermedades tan comunes.

Muchos estudios experimentales muestran que la neuroinflamación después de una isquemia focal cerebral puede agravar la lesión. Sin embargo, la mayoría de los estudios modelando estas enfermedades relacionadas con accidentes vasculares en animales se han realizado con ratones macho jóvenes, cuando en realidad estas enfermedades afectan más gravemente a las mujeres mayores. La Galectina-3 es una proteína inflamatoria implicada en muchas enfermedades, incluida la isquemia cerebral. En algunas condiciones tiene un efecto protector y tiene efectos anti-apoptóticos, pero en otras agrava la inflamación y el daño nervioso. Nuestro grupo de investigación ha demostrado previamente que la eliminación genética de Galectina-3 mejora los resultados en modelos experimentales de enfermedades neurodegenerativas, como Parkinson y Alzheimer. En el Proyecto I, expusimos ratones hembra envejecidos (24 meses de edad) sin Galectina-3 a pMCAO (oclusión de la arteria cerebral media) y comparamos los trastornos del comportamiento y el tamaño de la lesión con ratones control.

En el proyecto II, estudiamos en ratones con Alzheimer de tipo familiar los efectos del ejercicio voluntario (correr en una rueda) a largo plazo. En el Proyecto III, entrenamos a los ratones en una cinta de correr durante un mes antes de inducirles hipertensión (un conocido factor de riesgo de demencia vascular) usando un vasoconstrictor, Angiotensina-II.

En pacientes con microangiopatía (causa importante de demencia vascular), se puede ver usando resonancia magnética que los espacios perivasculares están agrandados. Se cree que estos espacios juegan un papel importante en la limpieza de los productos de desecho en el cerebro, a través del llamado sistema glinfático, donde el líquido cefalorraquídeo (LCR) limpia el cerebro mientras dormimos. Se ha especulado que estos espacios perivasculares se agrandan cuando se acumulan residuos nocivos, lo que genera un ambiente disfuncional e inflamatorio, que a su vez provoca una función glinfática defectuosa, hipoxia y daño en el tejido (Brown et al. 2018). En el Proyecto IV, estudiamos si la inflamación puede afectar el flujo de LCR en el cerebro, una pieza del rompecabezas en esta importante historia.

Los proyectos de esta tesis tienen como objetivo ampliar el conocimiento de cómo el ejercicio puede afectar las enfermedades neurológicas en modelos de ratones, el efecto de la inflamación en los movimientos del LCR y por último, la edad y el género en modelos experimentales de isquemia cerebral focal.

پرطرفدار علمی خلاصه

،جمعیت سن افزایش با و هستند مبتلا عقل زوال به نفر میلیون بهداشت۵۰ جهانی سازمان گزارش طبق دمانس آن از پس و است عقل زوال نوع ترین شایع آلزایمر بیماری .یابد افزایش تعداد این رود می انتظار ،خاص پروتئینی تجمعات غیرطبیعی تجمع دلیل به آنها از یکی که شودمی تصور .دارد قرار عروقی التهابی اجزای دارای همچنین دو این اما ،شودمی ایجاد مغز در خون جریان در اختلال دلیل به دیگری محرک شامل عروقی عقل زوال و آلزایمر بیماری دو هر برای شده توصیه پیشگیرانه اقدامات .هستند مانند ورزش دقیق های‌مکانیسم ،حال این با .است منظم بدنی فعالیت و سالم غذایی رژیم ،شناختی های .است مشخص هنوز رایج های‌بیماری این در تضعیف

آسیب تواند می مغزی کانونی ایسکمی از پس عصبی التهاب که دهد می نشان تجربی مطالعات از بسیاری این رغم‌علی ،است شده انجام جوان نر های‌موش روی بر مطالعات بیشتر ،حال این با .کند تشدید را یک گالکتین۳ .دهندمی قرار تأثیر تحت را مسن زنان شدت به سکته با مرتبط های‌بیماری این که واقعیت شرایط برخی در .دارد نقش مغزی ایسکمی جمله از ها بیماری از بسیاری در که است التهابی پروتئین .کند می تشدید را عصبی آسیب و التهاب دیگر برخی در و دارد آپوپتوز ضد اثرات و دارد محافظتی اثر گالکتین۳ ژنتیکی حذف که بود داده نشان قبلاً ما گروه

،آلزایمر بیماری و پارکینسون بیماری مانند ،عصبی کننده‌تخریب های‌بیماری تجربی های‌مدل در را نتایج تجربی مغزی سکته معرض در را گالکتین۳ فاقد ماهه ۲۴ ماده های موش ما ،پروژه۱ در . میبخشد بهبود .کردیم مقایسه وحشی نوع های کنترل با را ضایعه اندازه و رفتاری اختلالات و دادیم قرار

خانوادگی آلزایمر بیماری به مبتلا موش مدل در را مدت طولانی داوطلبانه دویدن ما ،دوم پروژه در شده شناخته خطر عامل یک(بالا خون فشار ایجاد از قبل را ها موش ما ،۳ پروژه در .کردیم مطالعه روی آآنژیوتانسین۲ ،عروق کننده منقبض تزریق از استفاده با ماه یک در)عروقی عقل زوال برای دادیم آموزش تردمیل

بزرگ عروقی اطراف فضاهای)عروقی عقل زوال مهم علت کوچک عروق بیماری به مبتلا بیماران در پاکسازی در مهمی نقش فضاها این که شود می تصور .کرد مشاهده مغناطیسی دوربین با توان می را شده در نخاعی مغزی مایع که جایی ،کنند می ایفا گلیمفاتیک اصطلاح به سیستم طریق از مغزی زائد مواد بقایای شدن انباشته با عروقی اطراف فضاهای که است شده زده حدس .یابد می جریان مغز طریق از شب عملکرد باعث خود نوبه به که ،کند می ایجاد التهابی و ناکارآمد محیط یک که ،شوند می بزرگ مضر التهاب آیا اینکه مورد در را ای فرضیه ۴ پروژه در .شود می بافت آسیب و هیپوکسی ،گلیمفاتیک معیوب داستان این در پازل از ای قطعه ،کردیم آزمایش ،بگذارد تأثیر نخاعی مغزی مایع جریان بر تواند می .جدی

های‌بیماری موش های‌مدل بر ورزش تأثیر چگونگی درک افزایش هدف با نامه‌پایان این های‌پروژه کانونی ایسکمی تجربی های‌مدل در جنسیت و سن و نخاعی-مغزی مایع حرکات بر التهاب تأثیر ،عصبی .است مغزی

Introduction

Homeostasis

Optimal body function relies on keeping many physiological variables, such as blood pressure, oxygen, energy levels, blood pH, and temperature within specific pre-set limits, also termed homeostatic ranges (Silverthorn 2019). Arterial blood pressure is needed for desired perfusion of organs, providing energy (at correct concentrations) at proper temperatures (for optimal function of enzymes and substrate reactions). Regulatory mechanisms involved in maintaining equilibriums include, but are not limited to; baroreflex and the renin-angiotensin system that result in water retention and vasoconstriction; pancreatic islets cells sensing high glucose levels and releasing insulin; thermoreceptors stimulating thermogenesis or sweating (Silverthorn 2019).

Homeostatic functions are often controlled centrally, and in the brain itself, inhibitory neurons balance neuronal activity, and there are more mechanisms in place to avoid overstimulation. Like in many other tissues, brain cells produce an excess of metabolites that need removal. Since the brain lacks lymphatic vessels to clear its extracellular buildup – it is believed that this function is instead provided by the movement of cerebrospinal fluid (Jessen et al. 2015).

Homeostasis is often described as the dynamic balance between the autonomic branches; the parasympathetic and the sympathetic nervous system (Buijs 2013). The former is primarily active during rest and the latter more during physical activity (Silverthorn 2019).

Exercise

Physical exercise shifts the homeostatic pre-sets to meet the new needs and challenges. To meet the energy demands in skeletal muscles, the myogenic response shunts blood to active muscles due to vasodilation resulting from increasing concentrations of H^+, CO_2, K^+, lactate, and ADP. In order to keep mean arterial blood pressure high enough, blood flow is shunted away from non-vital organs, and

cardiac output[1] is increased. A lot of heat is generated in metabolic processes, and to counter hyperthermia, the skin produces sweat to cool off (Silverthorn 2019).

Physical exercise is also a physiological stressor, likely because of myofibrillar breakdown (Peake et al. 2017). In the acute stages (immediately post-exercise), there is an inflammatory response with markedly higher levels of circulating interleukin-6 and leukocytes. Recovery to baseline levels depends on exercise intensity; after moderate exercise, baseline levels of circulating lymphocytes are reached within 30 minutes of recovery. If exercise has been more intense, this lymphocytopenia goes beyond the baseline and stays low for some hours (Risoy et al. 2003). In contrast, levels of neutrophils decrease in the acute exercise setting but then increase above baseline for several hours (Risoy et al. 2003).

Neurological disease

Diseases are harmful deviations from physiological and structural functions. This, of course, also applies to neurological diseases, where the function or structure of the brain or spinal cord deteriorates. Neurological diseases are the leading causes of disability worldwide and the second leading cause of death (after ischemic heart disease) (Feigin et al. 2020). The two most significant contributors to human suffering and death among neurological diseases are cerebrovascular disorders and Alzheimer's disease (Feigin et al. 2020).

Cerebrovascular disease

Cerebrovascular diseases are disorders of cerebral blood supply. From a physiological view, the brain is particularly sensitive to reductions in blood supply, mainly because of the high metabolic requirements[2] and the presence of excitotoxic glutamate (Lee et al. 2000). Loss of oxygen and nutrients will quickly cause energy and ion imbalance, leading to the release of excess glutamate from damaged neurons (Lee et al. 2000). Extracellular increases of glutamate lead to overactivation of Ca^{2+}-channels, that in turn results in, among other things, mitochondrial dysfunction and release of reactive oxygen species. The ensuing necrosis of neurons and glial cells can propagate to neighboring cells (Lee et al. 2000).

Clinically, examples of cerebrovascular diseases are ischemic stroke, hemorrhagic stroke, and subarachnoid hemorrhage. Ischemic strokes are the most common, and

[1] Cardiac output = heart rate x stroke volume (Silverthorn 2019).

2 The brain constitutes only 2% of our body weight, but consumes 20% of its oxygen (Jain, Langham, and Wehrli 2010).

these can be stratified into different subtypes, i.e., cardioembolic strokes, thromboembolic strokes, and lacunar strokes (Ornello et al. 2018). Cardioembolic strokes are often formed due to clot formation in atrial fibrillation, then the embolus travels to and occludes a brain vessel. Thromboembolic strokes are caused by thrombus formation in an atherosclerotic artery. This thromboembolism can then occlude a brain artery (Norrving 2022). Lacunar infarcts affect the small vessels deep inside the brain and are considered to be manifestations of small vessel disease (Behrouz, Malek, and Torbey 2012).

All of these diseases are due to poor blood flow in the brain, and their clinical manifestations will depend on the brain regions that are affected. This, in turn, depends on which vascular trees or branches have disrupted perfusion. For example, an embolic stroke in the left middle cerebral artery (MCA) can result in the typical acute hallmarks common in public-awareness messages; paralysis and sensory loss of the contralateral body half (lower part of face, arm, and hand), as well as visual field deficits and impaired language ability (Nogles and Galuska 2022).

Intervention in acute stages can include dissolving blood clots with plasminogen activators (thrombolysis) or surgical removal of the blood clot (thrombectomy). Having had a stroke is one of the biggest risk factors for getting another stroke. Therefore, secondary interventions target modifiable risk factors, usually homeostatic failures such as diabetes, hyperlipidemia, atrial fibrillation, and hypertension. At the secondary intervention stage, acetylsalicylic acid (aspirin) or clopidogrel targeting platelets and clotting factors are also indicated for stroke patients (Nogles and Galuska 2022).

Strokes can be fatal, transient, or permanently impairing – and, in addition to paresis, sensory loss, etc. often include decreased cognitive abilities and neuropsychiatric symptoms such as fatigue or anxiety (Nogles and Galuska 2022). When cerebrovascular diseases significantly affect cognition, they are referred to as vascular dementia (VaD) – regarded as the second most common form of dementia (Battistin and Cagnin 2010).

Alzheimer's disease

The most common form of dementia is Alzheimer's disease (AD). Compared to stroke disease, much less is known about AD pathophysiology, but the two proteins amyloid precursor protein (APP) and Tau appear to have central roles (Roos et al. 2021). APP is cleaved into amyloid peptides by beta- and gamma-secretases, and the peptide monomers are eventually accumulated in plaques extracellularly. The rate of amyloid peptide generation can be accelerated with certain mutations in either the APP-gene or in the genes coding for presenilin 1 and 2 (PSEN 1 and PSEN 2), which are part of gamma-secretase (Shepherd, McCann, and Halliday 2009).

Later in the pathology, hyperphosphorylated tau-proteins are misfolded in neurofibrillary tangles (NFTs) inside neurons.

Clinical manifestations of AD include non-sudden impairments in learning, reasoning, language functions, as well as changes in personality and behavior (NIH 2021). The onset of clinical disease is mostly after 65 years (late-onset), but circa 5% develop the disease before 65 (early-onset).

The overwhelmingly most common form of AD is sporadic. This is in contrast to familial AD (1% of AD cases), in which genetic mutations in the APP, PSEN 1, or PSEN 2-genes drive the pathology. These mutations also predispose to the younger onset (Mendez 2017). There are no cures for AD, and current treatments mainly target the breakdown of the neurotransmitter acetylcholine (Atri 2019).

As with other pathological processes in the body, cerebrovascular disease and AD is accompanied by inflammatory responses (Degan et al. 2018).

Inflammation

Inflammation is how the body reacts to harmful stimuli. Immediately after trauma or tissue damage resulting from an infectious agent, histamine is released from mast cells which causes vasodilation of the smooth muscle cells around the vessels, and constriction of the endothelial cells leading to increased permeability (Zachary 2017). This increased gap-formation of the vessels causes exudation[3] of blood cells; mainly red blood cells, neutrophils, and monocytes, but also platelets and blood proteins, such as fibrinogen. Later, monocytes express Tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1), which among many other things activate phospholipases to cleave arachidonic acid from cellular membranes into prostaglandins and leukotrienes. These mediators will further cause increases in vascular permeability and leukocyte infiltration (Zachary 2017).

[3] Active flow of fluid with high protein content through vessel walls into interstitial fluids or unto other tissues (Zachary 2017).

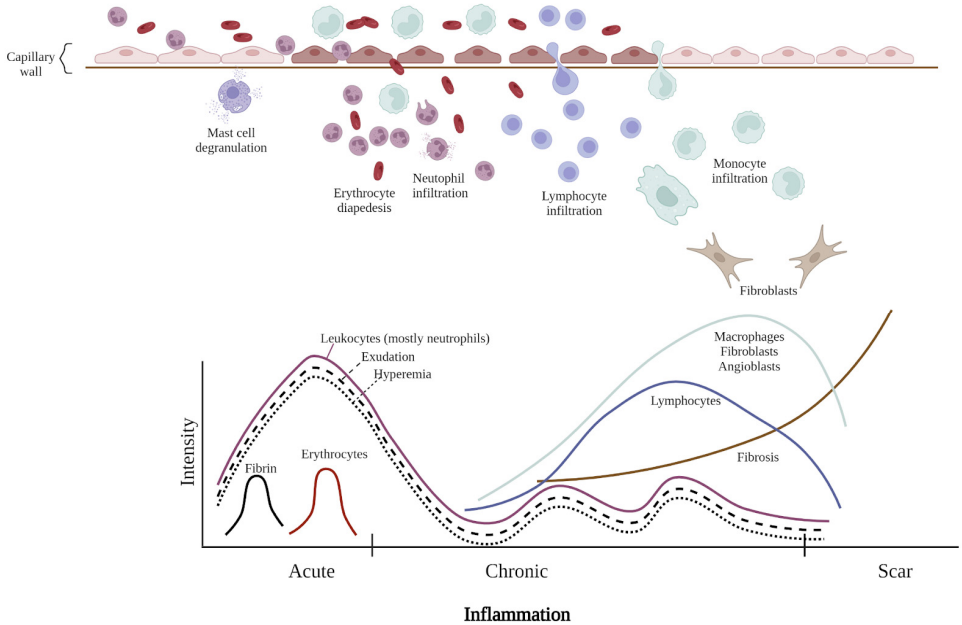


Figure 1. Inflammation. A schematic overview of a peripheral inflammatory cascade. A hurtful stimulus triggers mast cell degranulation, leading to endothelial contraction and leukocyte chemotaxis, in turn resulting in extravasation of blood proteins and cells. In the acute setting, neutrophilic granulocytes and erythrocytes penetrate the capillary wall, together with the exudated plasma proteins. At later stages of inflammation, lymphocytes, macrophages and fibroblasts are more numerous. Reparation processes indicate the chronic phase of inflammation. Modified from Zachary 2017. Made in biorender.com.

These initiating events will soon result in the display of the five cardinal signs of acute inflammation; heat (*Calor*), redness (*Rubor*) and swelling (*Tumor*) from increased blood flow and vessel leakage, pain (*Dolor*) after nociceptive stimulation from the swelling or prostaglandins and leukotrienes, as well as loss-of-function (*Functio laesa*) from pain and disrupted tissue structure (Zachary 2017).

Ideally, the noxious stimuli that initiated the inflammation are neutralized in the acute setting. Otherwise, the body needs to enter a chronic phase of inflammation. This stage is characterized by a reparatory phenotype, with collagen-producing fibroblasts (fibrosis), angiogenesis, and infiltration of a different set of immune cells, mainly macrophages and lymphocytes. Here, the noxious stimuli can be encapsulated as seen in tuberculoid granulomatosis, healed with granulation tissue, or neutralized with plasma-cell released antibodies (Zachary 2017).

These acute and chronic immune reactions occur in most body tissues, such as the skin, liver, or heart. But the initiation of inflammation in the brain is different (Galea, Bechmann, and Perry 2007).

Neuroinflammation

Since the brain is covered with a bony skull, the swelling, as we normally see in acute inflammatory reactions in the periphery, can for the brain quickly become dangerous (Stamatovic et al. 2006). Peripheral immune cell surveillance inside the brain tissue under physiological settings is neither considered wanted (Zachary 2017). This immune "privilege"[4] is upheld by multicell-constituted barriers along the blood vessels in the brain parenchyma, the blood-brain barrier (BBB) (Daneman and Prat 2015). The BBB is comprised of vascular endothelial cells; their basal membrane; and astrocytic foot processes. The BBB doesn't mean that the brain is devoid of immune surveillance or peripheral immune interactions. White blood cells in the periphery continuously move around inside the tiny capillaries of the brain and sense for disease processes, likely communicated by the numerous glial populations of the brain – astrocytes, oligodendrocytes, and microglia (Takeshita and Ransohoff 2012; Boccazzi et al. 2021).

Microglia are the resident macrophage-like cells of the brain. These small-bodied cells are derived from mesodermal stem cells in the yolk sac, which enter and populate the central nervous system during embryonal development and early postnatal life (Ginhoux et al. 2013). Microglia orchestrate the future mature brain during development by trimming (pruning) excess neuron-neuron connections (synapses) through phagocytosis (Ginhoux et al. 2013). These cells take on a much more surveilling nature in the adult brain, moving around in the brain, sensing for cell debris or foreign agents to phagocytose. Upon phagocytosis and activation, microglia acquire a more significant amoeboid morphology and start producing cytokines and other chemicals that can induce proliferation of other microglia, the breakdown of the BBB, and infiltration of peripheral immune cells (da Fonseca et al. 2014). In fact, in many neurological diseases, such as multiple sclerosis, stroke, or traumatic brain injury, there is leukocyte infiltration into the central nervous system (da Fonseca et al. 2014; Arumugam, Granger, and Mattson 2005; Beuker et al. 2021).

Inflammation in stroke and Alzheimer's disease

After the cessation of blood flow in stroke disease, neurons start dying within minutes inside the ischemic core (Lee et al. 2000; CDC 2021). Here, the cells are directly dependent on the affected blood vessels, and can't be saved (Sun et al. 2008). Dead cells will release cellular contents that will cause noxious stimuli in the penumbra, the area surrounding the infarct core. The processes occurring in the core and penumbra will lead to pro-inflammatory cytokine production following the

[4] Immune privilege sites typically tolerate foreign antigens (Forrester, McMenamin, and Dando 2018)

activation of microglia, astrocytes, neurons, and endothelial cells (Gauberti, De Lizarrondo, and Vivien 2016). Inflammatory cascades weaken the BBB (da Fonseca et al. 2014). Neutrophil infiltration will follow hours later, and they will, together with activated microglia and infiltrated monocytes, phagocytose and remove cell debris and toxic metabolites. T-lymphocytes are also present after stroke, especially in the ischemic core (Beuker et al. 2021).

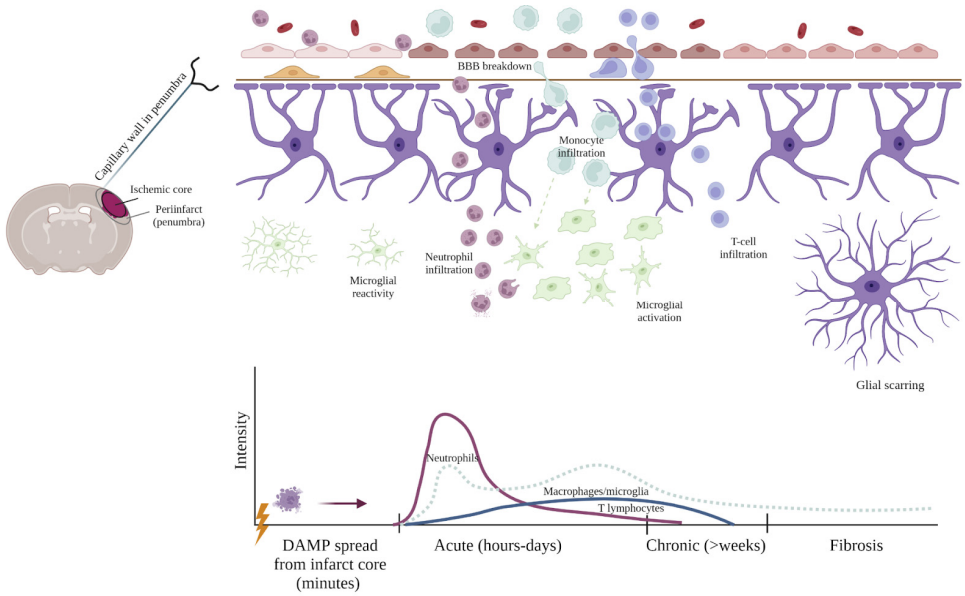


Figure 2. Inflammation in stroke. Immune cell activation and infiltration into the peri-infarct area. Neutrophils dominate the early response, and macrophages are also early responders with presence throughout the whole process. A large subset of macrophages are likely from resident microglia population. T-cells infiltrate later, albeit not as much as they do in the infarct core. Glial scars present in the penumbra. Modified from Beuker et al. 2021. Made in biorender.com.

Immune cell reactivity in the adult brain is sometimes described as a “double-edged” sword (Wyss-Coray and Mucke 2002). While performing cleanup after an injury or infection could be beneficial, microglia and infiltrating leukocytes have been shown to be detrimental to stressed but viable cells in the brain (Neher, Neniskyte, and Brown 2012). Thus, neuroinflammation has for many years been considered a target in preventing secondary injury in many neurological diseases. Our group has shown that genetic ablation of an inflammatory protein improved neuronal survival in a model of global ischemia (Burguillos et al. 2015). This protein is called galectin-3, and in his Ph.D. thesis, Boza-Serrano (Boza-Serrano 2017) showed that this protein also was contributing to the pathogenesis of Alzheimer’s mice. In contrast, Lalancette-Hebert showed that the presence of the same protein was beneficial in an experimental stroke model (Lalancette-Hebert et al. 2012).

Age is the biggest risk factor for having a first stroke (Yousufuddin and Young 2019). Although males are at a higher risk of getting strokes, women are usually more affected by stroke events (Reeves et al. 2008). Unfortunately, young male mice are overrepresented in stroke research (Sommer 2017). To balance the scale, we aimed in Paper I to explore whether an inflammatory mediator, galectin-3, would have a role in the post-stroke outcome in aged female mice.

As indicated by the reference to Boza-Serrano's thesis (Boza-Serrano 2017), inflammation also occurs in Alzheimer's disease. Although it is not uncommon for Alzheimer's disease and stroke disease to affect the same patients (Zekry, Hauw, and Gold 2002), the inflammatory responses are not identical. While the cellular and cytokine responses after a stroke much more resemble that of similar inflammatory cascades in the periphery, the response in AD is different.

As mentioned previously, AD is in part defined by the manifestation of peptide aggregates. Another histological hallmark in AD pathology are the activated microglia[5] around the above-mentioned amyloid-β plaques (Boza-Serrano 2017). Experimental studies indicate that the inflammation in AD could contribute to the pathology, which makes it an interesting target for intervention (Boza-Serrano 2017).

[5] It is not possible to differentiate activated microglia from infiltrated monocytes, but with the exception of perivascular monocytes, peripheral infiltration in AD is of unclear importance (Heppner, Ransohoff, and Becher 2015).

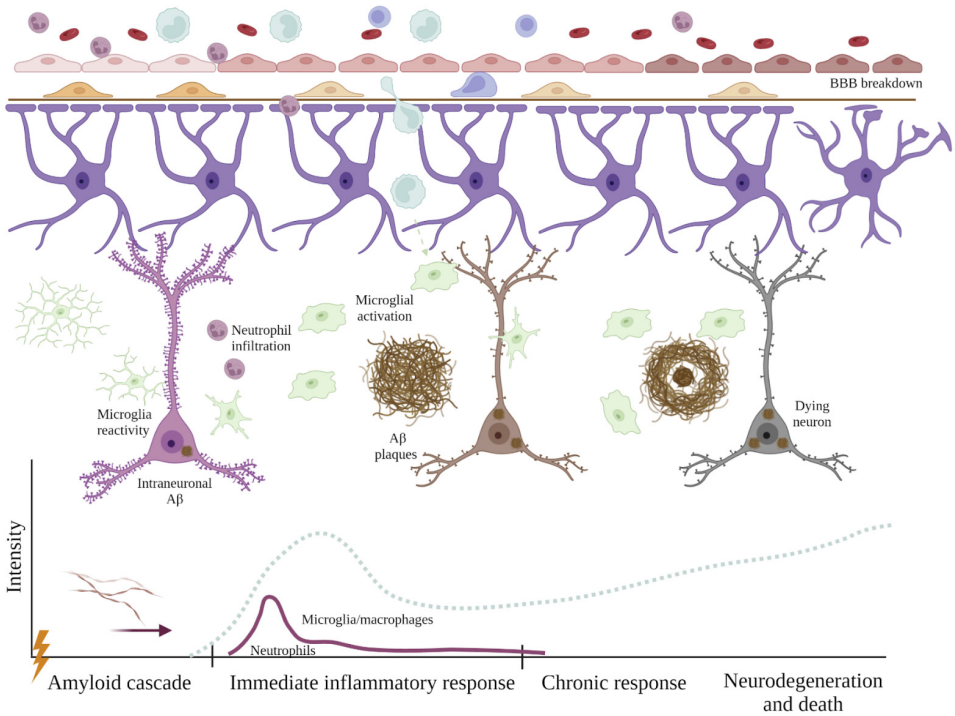


Figure 3. Inflammation in AD. Immune cell response after accumulation of amyloid-beta. The inflammatory response is throughout dominated by microglia. Neutrophils can be found in the early response. T-cells infiltrate the perivascular spaces, but in contrast to the more acute stroke scenario, they are not as involved in parenchymal effector functions. Made in biorender.com.

Exercise and Alzheimer's disease

Exercise is thought to be a protective factor in AD pathology (Meng, Lin, and Tzeng 2020). Proposed protective mechanisms of exercise include brain trophic factors and anti-inflammatory modulation (Svensson 2020). However, in a large prospective cohort study, cross-country skiers did not run a lower incidence of developing Alzheimer's disease compared to non-skiing controls (Hansson et al. 2019). This was in line with some previous findings (de Bruijn et al. 2013; Yamada et al. 2003; Ravaglia et al. 2008) and in contrast to several others (Guure et al. 2017; Andel et al. 2008; Rovio et al. 2005; Hamer and Chida 2009). Experimentally, there have been many studies subjecting different AD-mouse models to physical exercise (Ryan and Kelly 2016). Overall, most studies show a reduced inflammatory response to the AD-pathology with exercise, as well as a lower amyloid β-load (Ryan and Kelly 2016).

In Martina Svensson's Ph.D. thesis, she presented two experimental papers using voluntary running and the 5xFAD model (Svensson 2020). One of them is also part of this thesis, Paper II.

Exercise and vascular dementia

Physical exercise is widely believed to improve longitudinal health and longevity. In 2020, our group showed that the incidence of vascular dementia, but not Alzheimer's disease, was significantly decreased in a large-scale study of cross-country skiers (Hansson et al. 2019). The beneficial effects of exercise in vascular dementia are often ascribed to better cardiovascular health, such as avoiding high blood pressure (hypertension) (Cornelissen and Fagard 2005). How exercise lowers or stabilizes blood pressure is not fully understood but is believed to be due to alterations in the renin-angiotensin system and the sympathetic nervous system changes (Cornelissen and Fagard 2005). In fact, exercise counteracts blood pressure increases in models of hypertension (both spontaneous in rats) or with angiotensin-II infusions (Silva et al. 2015; Waldman et al. 2017). Furthermore, angiotensin-II models are used to mimic vascular dementia in mice (Meissner et al. 2017). Moreover, as previously mentioned, it is known that exercise affects immune cells and cytokine signaling and also that specific immune cell populations are required for developing hypertension (Saleh, Norlander, and Madhur 2016; Chan et al. 2015; Guzik et al. 2007; Marko et al. 2012; Rudemiller and Crowley 2016).

Could it then be that the immune system mediates positive exercise effects with regard to vascular dementia? In Paper III, we wanted to mechanistically explore how exercise affected hypertension-induced cognitive dysfunction and global inflammation.

Inflammation and CSF-flow

As mentioned previously, the blood-brain barrier is partly constituted by astrocytes. This is not the only function of this cell type. Astrocytes are derived from pluripotent neuroepithelial progenitor cells and are heavily involved in maintaining and regulating the neuronal microenvironment to have normal neurotransmission (Meldolesi 2020). These homeostatic functions include but are not limited to: ionic and water balance, turnover, and metabolism of potential neurotoxins; ammonia, heavy metals, or neurotransmitters such as glutamate (Meldolesi 2020). It is not then surprising that astrocytes are the most abundant cell type in the central nervous system. Another essential function has been ascribed to this very vital cell population within the last decade (Plog and Nedergaard 2018).

When we sleep, the spaces in between the endothelial basal membranes and the astrocytic end-feet expand, and cerebrospinal fluid (CSF) flushes the brain parenchyma from metabolites and toxic aggregates, including the peptide aggregates in Alzheimer's disease (amyloid β) (Liu et al. 2020; Boespflug and Iliff 2018). In 2012, Danish neuroscientist Maiken Nedergaard coined the term "*glymphatics*" to describe this phenomenon – combining *glia* (from astroglia) and *lymphatics*, hypothesizing that this system makes up for the lack of a cerebral lymph system (Iliff et al. 2013; Iliff et al. 2012).

At the start of this thesis project, the function of the CSF-mediated clearance system was believed to primarily be dependent on astrocytic expression of the water channel AQP4 (Iliff et al. 2012), but even with a knocked down expression, the function was still there, albeit decreased (Iliff et al. 2012). We hypothesized that since inflammation rapidly influences many other CNS homeostatic functions, perhaps this newly discovered glymphatic system could also be affected. This is what Paper IV aimed to elucidate.

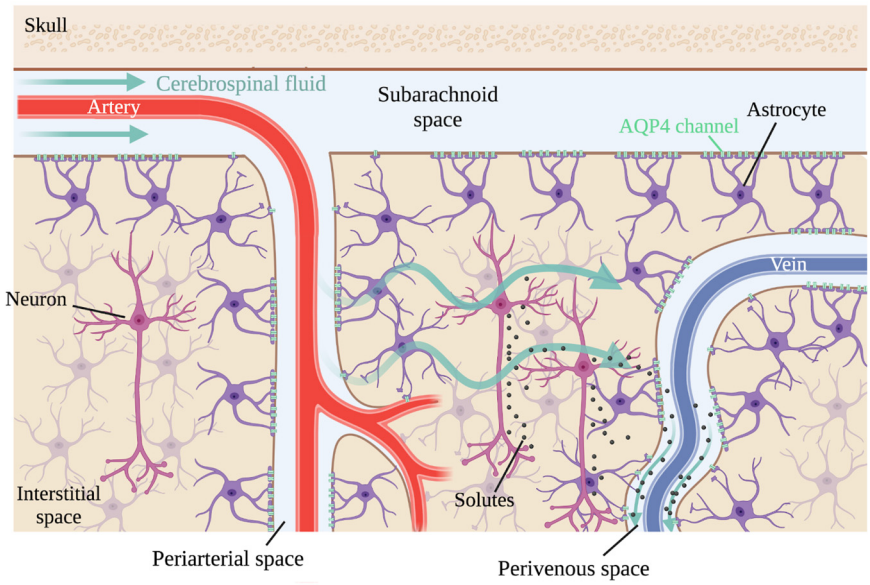


Fig 4. Glymphatic system. Proposed movement of cerebrospinal fluid (CSF) in the brain. From the subarachnoid spaces, CSF flows into the perivascular spaces, where it crosses into the brain interstitium facilitated by AQP4-channels located on the astrocytic endfeet. This movement of CSF fluid is thought to flush interstitial fluid and solutes (eg. waste products) out of the brain. Figure made by Marta Ramos, in biorender.com.

Aims

In this thesis, we aimed to explore how exercise can affect murine models of neurological diseases, how age and sex in experimental models of focal brain ischemia, as well as how inflammation influences CSF movement. The specific aims of the Papers included in the thesis are:

Paper I

Here we aimed to explore whether an inflammatory mediator, galectin-3, would have a role in the post-stroke outcome in aged female mice.

Paper II

In Paper II, we aimed to investigate whether running could alter AD pathologies, such as insoluble Aβ levels, neuroinflammation, and non-cognitive behavioral impairments.

Paper III

Here we explore how exercise affects hypertension-induced cognitive dysfunction and global inflammation.

Paper IV

Our aim was to elucidate the consequences of systemic LPS-exposure on perivascular CSF distribution as an indication of glymphatic function.

Timeline of this thesis

A reader of this thesis will most likely be surprised by the combination of different projects and publications. My focus during all these years has not only been on one specific area. Not even in one field. It might look incomprehensible, but just as historical occurrences are hard to puzzle from afar, understanding where the different turns came to be will result in everything making sense.

The main focus of my research group has been microglia, the small immune cell residing in our brains. When I was transferred to the Deierborg lab, I took over a study based on experimental focal stroke in mice lacking an important microglial protein. The mice were already analyzed for behavioral changes and sacrificed. I supervised a master's student through some analyses in this project, did some more analyses afterward, but then the project fell into a drawer for some years. But since these findings were still important to us, we thought that they should get the chance of being published. Thus, I wrote the manuscript, made the submission, and got it accepted just some months before this thesis was finalized. This was Paper I.

In my previous lab, I had worked a lot with a 5xFAD mouse cohort that was subjected to voluntary running, but I had to leave the project when I left the lab. However, these same mice (although another tissue) were the focus of a project led by my office-mate Martina, where she showed that voluntary running did not ameliorate any symptoms of the AD pathology seen in this model. I was asked to help with parts of the project and gladly accepted. The paper was later published, with me as a third author. This was Paper II.

At the time of my arrival in Tomas' group, Iben Lundgaard was starting a group in Lund, wanting to research the newly discovered glymphatic system. This sleep-dependant system was thought to be primarily controlled by another glial cell in the brain, the astrocytes. I needed a project to start with, and Tomas got us a meeting. Quickly thereafter, I was planning for a study on the role of microglia in the glymphatic system. Ethical applications were written and approved, and experimental studies were launched. During the World cup of 2018, I was in and out of the animal research facility to give my mice a special (and very expensive) drug mixed in the chow that would wipe out the whole microglial population. Then these mice would be compared to normal mice, and we also added a positive control group that was to be given LPS, activating the microglia. Here we had three groups, no microglia vs. normal microglia vs. activated microglia. After some weeks, they

were examined histologically – then we realized that the drug hadn't worked. The company blamed it on a bad batch and quickly sent us another one. But it would have been a wasted summer of working and unnecessary research animal suffering – if it wasn't for a serendipitous finding. The LPS-mice showed significantly lower glymphatic flow. We ran another cohort to confirm our results, and the results were published in the Journal of Neuroinflammation, where I was the first author. This was Paper IV.

Thus far, I then had some finished projects, but none that really was inviting for follow-up studies: Paper I with negative results. Paper II is a study, led by someone else with negative results. Paper IV with a happenstance significant finding (but not with a large enough effect that would make mechanistic studies easy).

I wanted another project. And I had grown more interested in the understudied field of vascular dementia, and I had gotten to know a new group at BMC that was using hypertension models to induce cognitive dysfunction in mice. I was already involved in another project (Paper III) with physical activity, and since *I am* interested in exercise, another project with exercise and vascular dementia was fitting for my already sprawling thesis plan. Martina Svensson had also, in another publication, showed that cross country skiers had an especially lower risk of developing vascular dementia. So, I contacted the Meissner group that had experience with the hypertension model and suggested an amendment to their ethical permit. After the amendment was accepted, we ran the cohort, did all of the behavior testing, prepared for electron microscopy, Golgi stains, immunohistochemistry, mesoscale and corticosterone ELISAs, Western blot, etc. But we really didn't find a real indication that we had replicated the disease model. Lucky enough, a postdoc, Nicholas, had measured blood pressures and done FAC-sorting and found some interesting correlations. Since Nicholas later got a job in industry and had little time to make follow-up experiments in this project, I was given the chance of continuing investigating these peripheral effects. I was looking at blood levels of different molecular pathways, remodeling of vasculature, and also writing the manuscript itself, and claiming shared first authorship. That's why Paper III is mostly focused on exercise and hypertension.

There are, of course, drawbacks to completing a Ph.D. project like this. I am not a real expert in anything because I haven't had the opportunity to dig deep into any specific subject. This is also the case for my technical skills. I have used and written about so many that I obviously cannot be an expert in any of them.

On the other hand, my acquired theoretical knowledge has become broad, and my toolbox of technological skills has grown quite heavy. I don't know everything about everything, but I probably could answer a few questions about many different things in biomedicine. Very likely more things than if I had stayed in one small area only. And there is value in that, too.

Methodological considerations

Here are brief descriptions of the methods I have used in this thesis. For more detailed protocols, see attached papers.

Animals and disease models

All experiments in this thesis were approved by the Malmö-Lund Ethical Committee for Animal Research in Sweden. In Paper I, we used aged female galectin 3-KO and wild-type mice from a C57BL/6-background. In Paper II, we solely used 5xFAD females on a C57BL/6*SJL background (Jackson). In Paper III and IV, we used male C57BL/6 mice from Taconic and Janvier, respectively.

pMCAO

In Paper I, aged (24-month-old) female mice were subjected to a left-sided permanent middle cerebral artery occlusion (pMCAO). Here, mice were anesthetized with isoflurane, and then a small head incision was made. After separating the temporal muscle, a small hole in the skull was drilled, exposing the MCA that could be cauterized. After the incision was sutured, mice were returned to their cages.

Angiotensin-II hypertension

In Paper III, we used osmotic pumps with angiotensin-II (200 ng/kg/min) and induced hypertension in the previously forced exercise mice and their controls. During the surgery, mice were anesthetized with isoflurane, and pumps were inserted subcutaneously in the neck area. After suturing, the mice were locally anesthetized and returned to their home cages.

Personal reflections

Research animals provide us with close-to-human models of health and disease. Complex organs such as the brain, or intricate cellular interactions as seen in the immune system, simply cannot be modeled in any other way at this point. However, these are sentient beings that suffer. That must be considered, always, and the 3R's (replacement, reduction, and refinement) are not just words to hide in methods-sections of our manuscripts. These thoughts must run like common threads through

our whole scientific thinking in medical research. The accumulated suffering from our studies must also be outweighed by the potential benefits it likely could result in for patients.

Furthermore, it is essential to understand the differences of mice and men when considering in vivo experiments. While pMCAO represents a similar insult to a large vessel stroke in patients, rodents have incredible abilities to recover rapidly. Wait a few weeks after an experimental stroke, and behavioral traits associated with the stroke phenotype could be gone (Rewell et al. 2017).

As mentioned in the introduction, most AD cases are sporadic. That is, patients lack any of the mutations in the APP, PSEN 1, or PSEN 2-genes. Still, current mice models of AD are all familial. The one we used in this thesis, 5xFAD, has five different mutations associated with familial AD in humans. Thus, we are modeling a disease that develops over decades with mice that develop the disease over a few months (Alzforum 2019).

Our rapid hypertension model with AngII-infusions is similar in this regard, where blood pressure increases appear after some weeks. In contrast, hypertension usually develops over many years in humans. Moreover, the cause of most hypertension in patients is not from a dysregulated renin-angiotensin system.

These differences between disease models and human conditions must be kept in mind when interpretations or translational efforts are made.

Exercise

In Papers II and III, we subjected mice to different regimens of physical exercise.

In Paper II, young adult 5xFAD mice had free access to low-profile running wheels in their home cage for 24 weeks. Running distance was recorded telemetrically, and visual observation during active hours confirmed that running mice were indeed more active than “sedentary” control mice.

In Paper III, mice were instead subjected to forced running in treadmills. During two training sessions à ten minutes, mice were accustomed to the moving bands. Later, the mice were exercised five days a week for four weeks, averaging 17cm/s for 60 minutes. In order to avoid mice getting off the treadmill or stuck in the sides or in the back, their position was sometimes manipulated with a soft-edged plastic ruler.

Personal reflections

While the readouts from the forced exercise regimen were intriguing (see manuscript for Paper III), I would probably not recommend anyone to use the treadmills for any experiment. I did not use the electrical stimulation experimental set-up described before (Svensson et al. 2016) and just instead physically blocked

mice from getting off the treadmills with soft plastic blocks. I imagined this was less stressful, but one never knows. Even though mice in this experiment didn't vary in sex, age or genetics, they behaved very differently on the treadmills. Some just ran, regardless of speed. Some needed a little bit of manipulation with the plastic ruler a few times per session. Others just didn't want to run and were the limiting factors for setting the speed. Unfortunately, one mouse broke his leg during the four weeks of running (and had to be euthanized), and several were injured (blood from paws or penis). These incidents occurred at speeds much lower than the recommended protocols, and the affected mice were not always lazy runners. Stressful for the human, dangerous for the mouse → not the best intervention.

Behavior

We assessed cognitive and non-cognitive behavior in our mice in Papers I, II, and III. In Paper I, grip strength, inclined plane test, and cylinder test were used to study sensorimotor function before and after experimental stroke.

In the grip strength test, mice were lowered onto a metallic grid with a force sensor, and front paw strength could be determined.

In the inclined plane test, mice were placed head down on an inclined plane platform, where the inclination was gradually increased – the last angles that mice were able to hold were recorded.

The cylinder test was used for the assessment of asymmetric forelimb use; mice were placed in a glass cylinder, and right/left forelimb use was recorded as one weight-bearing contact with the glass wall.

In Paper, II and III, locomotion and anxious behavior were evaluated in the Open field test, where mice were placed in an arena and allowed to explore it freely for ten and five minutes, respectively. Time spent in the center and in the periphery was assessed, with more time spent in the periphery being regarded as anxiety-like behavior.

In Paper II, other non-cognitive behavioral assessments were made using clasping scoring, elevated plus maze, rotarod test, and sucrose preference test. Throughout the experimental duration of Paper III, hindlimb clasping was assessed regularly. Mice were held at the tail base for 30 seconds, and clasping was scored. In the elevated plus-maze, exploratory and anxious behavior were studied; where a curious mouse spends more time exploring the open arms and a more anxious mouse prefers the closed arms. The rotarod test was used to evaluate motor coordination and balance by placing mice on the rotating rod and tested by increased speed of rotation in five-minute testing bouts. Anhedonic behavior was assessed with the Sucrose preference test, where temporarily single-housed mice were introduced to a 2% sucrose solution, and during a test night, given a choice to drink the sucrose solution

or normal water. The two drinking bottles were weighed before and after, and a sucrose preference index was calculated for each mouse.

In Paper III, short- and long-term memory was assessed in our mice by novel object recognition testing. For 15 minutes, mice could explore the testing arena containing two identical objects (training). One hour (short-term) after training, mice were returned for ten minutes to the arena with one object from the training session and a novel object. For long-term, the time between training and testing was 24h. Mice usually prefer exploring new objects to known ones, and active exploration of either was recorded, and a discrimination index was calculated.

Personal reflections

Reproducibility is a general problem in biomedical research (Baker 2016), and particularly one that burdens researchers using animal behavior experiments (von Kortzfleisch et al. 2020). In fact, the outcome can change depending on what time of day the behavioral experiments are conducted (Bodden et al. 2019), or whether the experimenter is male or female (Sorge et al. 2014). Efforts to standardize experimental batteries are, of course, under way (von Kortzfleisch et al. 2020). Still, until a day with no idiosyncratic rodent behavior – we should interpret all behavioral data with a lot of caution.

LPS injections

In Paper IV, LPS (Sigma) diluted in ddH_2O was injected intraperitoneally at a 1 mg/kg concentration three hours before tracer experiments, transcardial perfusions, and physiological experiments.

CSF tracing

In Paper IV, mice were anesthetized with a ketamin/xylazine mix and placed in a stereotaxic frame. Next, the cisterna magna was exposed, and ten microliters of fluorescent tracers (BSA-647 and Dextran-488) were injected into the CSF through a 30 GA needle. After 30 minutes of tracer circulation, mice were decapitated and brains fixed in PFA overnight. Brains were then imaged in a stereoscope (Nikon SMZ25), and intensity and distribution of fluorescent tracers was quantified using Fiji. Later, the brain was sectioned with a vibratome and imaged at 4x with a epifluorescence microscope (Nikon ECLIPSE Ti2). Tracer penetration into the brain sections was quantified using a macro for Fiji developed by SciLifeLab Uppsala.

Laser doppler and physiology tests

In Paper IV, mice were put under general anesthesia (ketamine/xylazine) and placed on a surgical monitor platform to record heart rate, temperature, and respiration rate. To record cerebral blood flow (actually blood *velocity* – thanks for pointing that out Johan A!), we used a Laser Doppler flowmeter (Perimed) with an optical fiber glued directly to the exposed skull. Mice were kept under general anesthesia for almost three hours, and measurements were recorded every 15 minutes.

Histology

The histological preparation for immunohistochemical and morphological analysis has differed among my papers.

In Paper I, non-fixed brains were cut in 30 µm coronal sections and stored both on glass slides and in 1.5ml Eppendorf tubes for later protein extraction.

In Paper II, PFA-fixed brains were sagitally sectioned (30µm) in the cryotome and saved free-floating.

In Paper III, fixed brains were coronally sectioned (35µm) in the cryotome and saved free-floating. Here, fixed mesenteric vessels were also OCT-embedded and cut 10µm in a cryostat and directly mounted on object-glass slides.

In Paper IV, fixed 200 µm thick coronal slices used in CSF tracer imaging were also used for immunohistochemical labeling.

For immunofluorescence, free-floating slices or mounted specimens were washed in PBS, then blocked in a blocking solution (for most experiments PBS-T20 with donkey or goat serum) and incubated overnight in primary antibodies at 4°C in blocking solution. After washing with PBS-T20, samples were incubated with secondary antibodies for two hours at room temperature, then washed in PBS-T20 and mounted with a cover glass.

In Paper IV, we performed immunostainings of microglia for brightfield imaging. Here slides were quenched in peroxidase and blocked with glycine (this step made a big difference in reducing background – thank you, Douglas Eurenius!) in PBS. After washing with PBS Triton X100 (PBS-TX), primary antibodies (against Iba1) were incubated for 48 hours in the fridge. After rinsing in PBS-TX, secondary antibodies were applied for two and a half hours. Following another rinse with PBS-TX, samples were incubated in ABC elite reagent for one hour, rinsed in PBS-TX, treated with DAB (with metal enhancer) for five minutes, and mounted.

In Paper III, we used stained pyramidal neurons with Golgi's method using the FD Rapid GolgiStain kit in full accordance with the manufacturer's protocol.

Microscopic images for analysis and representative images were captured using either a Nikon ECLIPSE Ti2 or a Nikon ECLIPSE 80i microscope, or scanned with a Hamamatsu digital slide scanner.

Images were analyzed using Fiji (Schindelin et al. 2012) for cell counts (Papers II, III, and IV), vessel thickness (Paper III), labeling intensity (Papers II, and III), area positivity (Paper IV), infarct measurements (Paper I) and sholl analysis (Paper III).

Personal reflections

Immunohistochemistry makes for beautiful visualization of the inner mysteries of our specimens. However, there are many sources of error. Such as overlapping spectra in double-stainings, or autofluorescence from tissue and vessels (especially tricky when labeling vessel markers), bleaching from the microscope laser (especially bad if you are planning to do intensity measurements), unevenly sectioned hemispheres for infarct measurements, not finding the identical anatomical region for fair analysis, unspecific binding of primaries or secondaries, and much more. Be cautious.

Brain protein extraction

In Papers I, III, and IV, brain tissue homogenates were used in Western blot analyses and for MesoScale ELISAs.

After transcardial perfusion with PBS, brain tissue was carefully removed and snap-frozen. Later the tissue was homogenized using 1ml syringes and 20-21 GA needles in RIPA buffer with proteinase and phosphatase inhibitors from Roche. Protein concentration was then determined using BCA kits.

Western blot

In Papers II, III, and IV, we performed Western blots. Here, brain tissue homogenates from brain protein extraction were mixed with LAEMMLI buffer and heated to 95°C for five minutes. Ten µg of protein were loaded per well in pre-cast gels from Bio-Rad, and proteins were separated by SDS-PAGE in TGS-buffer. Proteins were then transferred to membranes using the Bio-Rad Trans-Blot Turbo transfer system, and after blocking in PBS T20 with skim milk, membranes were incubated with primary antibodies overnight at 4°C. For some analytes (AQP4, Paper IV), control antigens were loaded together with the other protein samples.

After the primary incubation, membranes were washed in PBS-T and incubated with secondaries (peroxidase-conjugated) from Vector for two hours at room temperature. Membranes were then developed with ECL clarity buffer from Bio-Rad or Super Sigma West Femto Sensitivity Substrate from ThermoScientific and

imaged using a ChemiDoc XRS or XRS+ system. Actin or tubulin was used as housekeeping controls. Image Lab from Bio-Rad was used to measure band intensities, which were then divided by actin band intensities.

Reflections: Since the development of Western membranes rarely result in perfect images with no (or very even) background, making sure that samples from treatment groups haven't been loaded next to each other has been key.

ELISA

MesoScale V-plex (Papers I, II, and IV) and U-plex (Paper III) plates were used to quantify levels of IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, and TNF-α (proinflammatory panel; Papers I, II and IV) and BDNF, EPO, IFN-γ, IL-10, IL-17A, IL-1β, IL-21, IL-6, TNF-α, and VEGF-A (personalized panel; Paper III) in the brain and in the blood (Paper II: serum; Paper III: plasma). The procedures were carried out according to the manufacturer's protocol. For plasma measurements, samples were diluted two-fold.

In Paper III, circulating levels of renin in plasma were measured using an Invitrogen ELISA kit, according to the manufacturer's protocol. Here plasma samples were diluted five-fold.

In Papers II and III, the stress hormone corticosterone was extracted from frozen fecal pellets through drying and homogenization in methanol before measurements with a corticosterone ELISA from Enzo life sciences, according to the manufacturer's protocol.

Reflections

ELISA kits are, in my personal experience straightforward to use and analyze, and they introduce few steps of human error (good!). However, run experiments with dilution series beforehand to avoid wasting the whole (sometimes very expensive) plates with reagents, only get too weak or too strong signals. Also, remember to include samples from both experimental and control groups in dilution optimization since levels can fluctuate (especially with regards to inflammatory cytokines).

FACS and qRT-PCR

In Paper III, we assessed immune cell profiles and cytokine gene transcription with fluorescent activating cell sorting (FACS) and quantitative real-time PCR (qRT-PCR). I was not involved in gathering these data, and I will thus entirely refer to the protocol in the manuscript for Paper III.

Statistical analyses

Statistical analyses in this thesis were performed either in SPSS (Paper II) or in GraphPad Prism (Paper I, III, and IV). Datasets were tested for normality when applicable. If normality was assumed, parametric tests were performed, such as the Student's t-test for pairwise comparisons or ANOVA test if more than two groups or more than one variable was measured (2- and 3-way ANOVAs). If normality was not assumed, we used nonparametric tests such as Mann-Whitney U- or Wilcoxon tests. P values ≤ 0.05 were considered significant.

Personal reflections

In preclinical research, we rely on statistical analyses to prove/disprove our hypotheses. Still, these tools are being misused on a regular basis by many researchers, often unintentionally. Extreme but not uncommon instances in our field include having n=3 in three or more groups, running a 1-way ANOVA, making column graphs, and showing multiple comparison differences only with P-values and variations with SEM. Using parametric tests with such low n's, hiding the sample size and variation behind columns and SEM, and only relying on P-values, is wrong (Simon 2005). There are, however, many situations that don't have as clear answers. Statistical tests are often based on complicated mathematical equations and are simply too difficult for every researcher to truly understand. Unfortunately, statisticians are not numerous in our midst, and in Lund, only Ph.D. students are required to pass basic statistics courses.

More knowledge is needed.

Key results

In this section, I have summarized results from the three papers and the one manuscript included in this thesis. For more details, see the complete papers and manuscript at the end of this thesis.

Galectin-3 ablation does not affect infarct size or inflammatory cytokines after experimental stroke in 24-month-old female mice (Paper I)

Experimental focal stroke-induced weaker grip strength in aged females, but more so in galectin-3 null specimens. In the cylinder and inclined angle test after pMCAO, genotype did not affect the outcome.

Galectin-3 ablation did not affect lesion size after pMCAO in 24-month-old female mice.

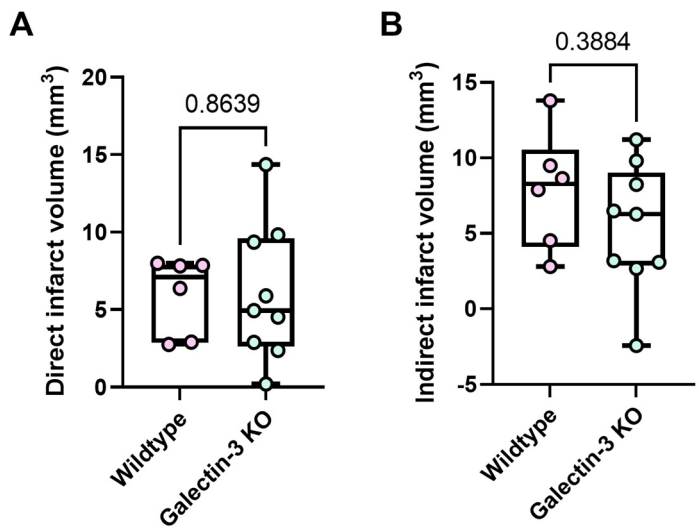


Figure 5. Infarct volumes. (A) Direct ($V_{ipsilateral}$-$V_{unaffected\ ipsilateral}$) and (B) indirect ($V_{ipsilateral}$-$V_{contralateral}$) infarct volumes in mm^3. Wildtype n = 6, Galectin-3 null n = 9. Mann-Whitney test P-values.. Error bars = min/max, and exact P-value on top of comparison brackets. (Figure adapted from Paper IV)

Inflammation is a hallmark sign of stroke disease. Therefore we wanted to measure the following cytokines in brain tissue: IFN-y, IL-1β, Il-2, Il-4, IL-5, IL-6, IL-10, and IL-12p70. Inflammatory cytokine analysis from whole-brain homogenate did not present any significant differences between the two genotypes seven days after pMCAO.

In summary, galectin-3 ablation does not affect lesions size, several motor functions, and inflammatory cytokines after experimental stroke in aged females.

Voluntary running does not reduce neuroinflammation or improve non-cognitive behavior in the 5xFAD mouse model of Alzheimer's disease (Paper II)

Running 5xFAD mice spent significantly more time exploring the open arms in the elevated plus-maze, indicating increased disinhibitory function. In contrast, in the open field test, the running mice spent less time in the center, which would indicate increased anxiety, although corticosterone stress levels were not elevated.

The development of sensorimotor dysfunction was measured with hindlimb clasping tests throughout the experimental timeline. Running 5xFAD mice developed hindlimb clasping earlier, at week 19 and 23, though the significant differences disappeared at week 26. Sensorimotor dysfunction was also evaluated with the rotarod test, where running mice did not improve after training in contrast to the sedentary mice.

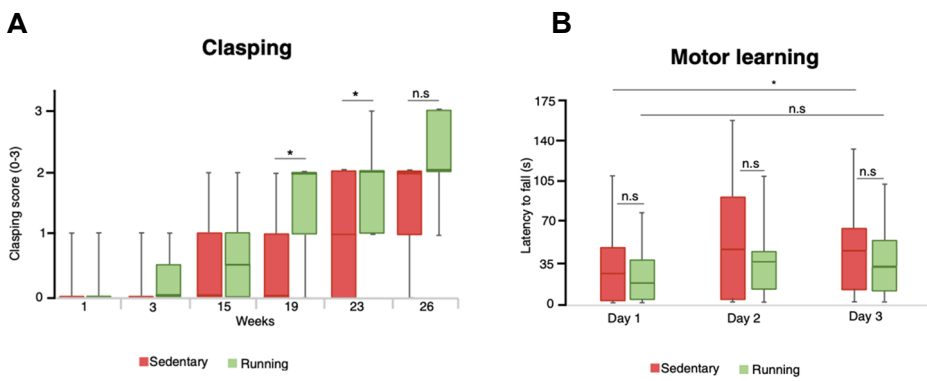


Fig 6. Hindlimb clasping and Rotarod test. (A) Clasping scores during the experimental timeline. (B) Motor learning evaluated with Rotarod test. Latency to fall of rotating rods at different training days. Box plots showing median values with interquartile ranges. Error bars = range. * = $p < 0.05$ (Mann Whitney U-test was used for each time point; for Rotarod the Wilcoxon test was used). Sedentary n = 14, running n = 14. (Figure adapted from Paper I)

No differences were detected with regards to general motor function, or sucrose preference test.

These results suggest that running does not improve many of the non-cognitive behavioral dysfunctions in the 5xFAD model, and might even make progression faster.

Using immunofluorescence imaging, we measured microglial markers, such as Iba1 and galectin-3 in the hippocampus. Running did not affect the labeling intensity of these markers. Neither did it alter levels of NLRP3 and many other inflammatory markers such as IFNy, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, CXCL1 and TNFα (in both hippocampus and blood).

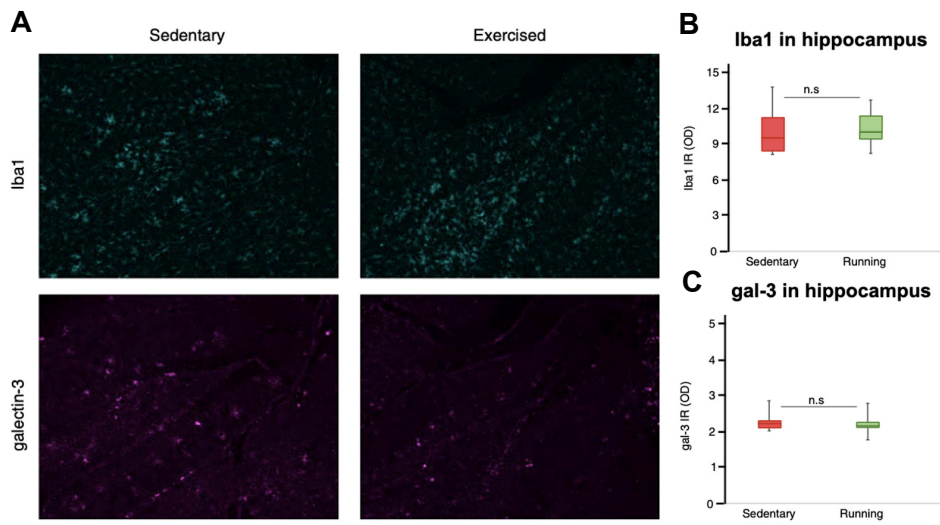


Fig 7. Hippocampal sections with Iba1⁺ and galectin-3⁺-cells. Scale bar 200 µm and box plots showing median values of Iba1 and galectin-3 labeling intensities (n = 10 + 10). (Figure adapted from Paper I)

Finally, voluntary running in 5xFAD mice did not affect levels of soluble amyloid β in CSF, insoluble amyloid β and amyloid plaques (Thioflavin-S) in hippocampus and cortex.

Our findings in this study indicate that voluntary wheel running is not a beneficial intervention for attenuating the 5xFAD pathology.

Forced treadmill running attenuates angiotensin-II induced blood pressure increase in mice (Paper III)

Four weeks of forced exercise lowered circulating levels of several immune populations and inflammatory cytokines in adolescent male mice but did not affect renin levels. Moreover, after the exercise regimen, mice improved both short and long-term memory in novel-object recognition tests in comparison with sedentary controls.

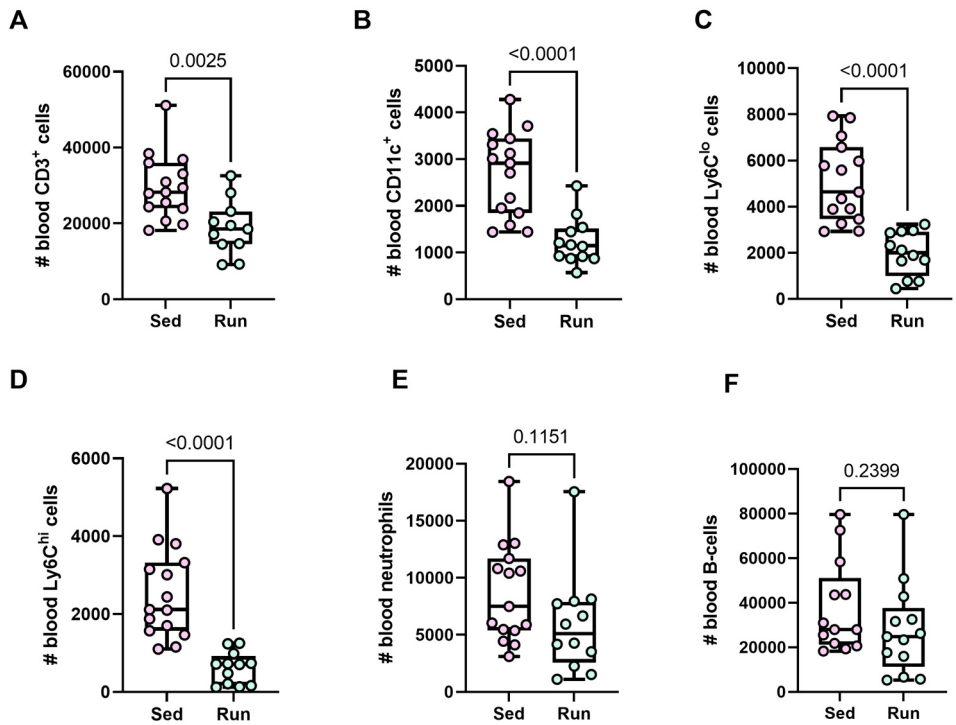
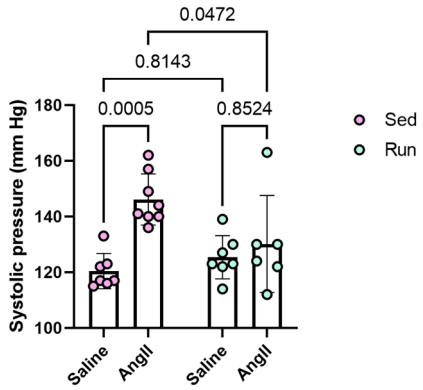


Fig 8. Running decreases several immune populations. FACS of immune cells in the blood at the end of the four-week running regimen. A) T-cells, B) CD11c+-cells, C) Ly6Clo-monocytes, D) Ly6Chi-monocytes, E) Neutrophilic granulocytes, F) B-cells. Sedentary n = 15, running n = 11. Error bars indicating min/max. Exact P-values (Student's t-test) above comparison brackets. *CD - cluster of differentiation, Ly6C - lymphocyte antigen 6, Sed - sedentary, Run - running.* (Figure adapted from Paper III)

After exercise, mice were fitted with minipumps releasing angiotensin-II (AngII) for four weeks until sacrifice. At the end of the experiment, blood pressure was significantly increased in sedentary AngII-mice, but not in running controls.



Source of Variation	% of total variation	P value
Interaction	13.75	0.0157
Treatment Factor	28.99	0.0009
Pretreatment Factor	3.758	0.1867

Figure 9. Systolic blood pressure. Systolic pressure measured with tail-cuff plethysmography, analyzed with 2-way ANOVA with Tukey's multiple comparisons. Sedentary saline n = 7, Sedentary AngII n = 8. Error bars showing SD and comparison brackets have exact P-values.

Several immune populations in bone marrow, thymus, and spleen were altered in sedentary AngII-mice but not in running counterparts. Bone marrow monocytes, thymic T and T-helper-cells, splenic B and dendritic cells were decreased in sedentary, but not (or not as much) in running, AngII-mice. Spleenic T-cells were increased after AngII-treatment, but only in sedentary mice.

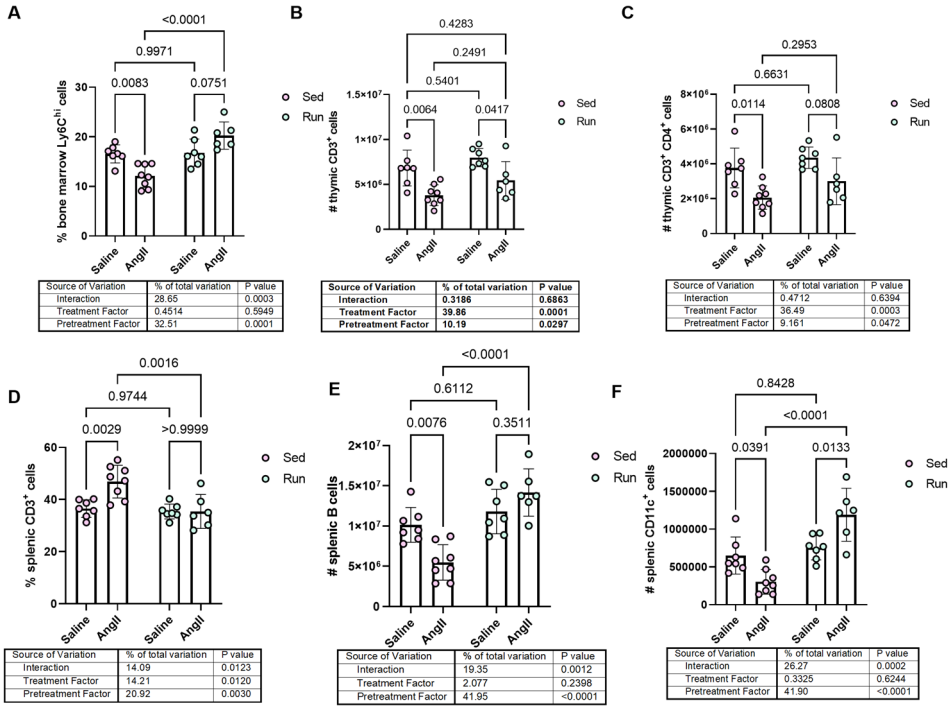


Figure 10. Running affects bone marrow, thymic and splenic immune cell responses to AngII-infusion. FACS of immune cells in bone marrow, thymus, and spleen at the end of AngII infusions. (A) Ly6C^{hi} cells in bone marrow, (B) CD3^{+}cells and (C) CD3^{+} CD4^{+} cells in thymus, and (D) CD3+ T-cells, (E) B cells and (F) CD11c^{+} cells in spleen. Sedentary saline n = 7, Sedentary AngII n=8, Run saline n = 7, Running AngII n=6. 2-way ANOVA's with Tukey's multiple comparisons. Error bars = SD. Numbers on top of comparison brackets show exact P-values. *Ly6C - lymphocyte antigen 6, CD - cluster of differentiation, Sed - sedentary, Run - running.* (Figure adapted from Paper III)

Furthermore, running appeared to counteract AngII-induced decrease of splenic IFNγ and IL-10. Interestingly, we did not detect any significant differences with regards to mesenteric artery wall thickness, inflammation, or remodeling. Not surprisingly, circulating levels of renin were lower in AngII-treated mice. Likely due to negative feedback pathways of the renin-angiotensin-system.

Since hypertension is a risk factor for vascular dementia in patients, and AngII-stimulation in mice is known to cause similar outcomes, we wanted to evaluate whether running could affect memory, neuroinflammation, and neurite complexity. Labeling intensity of microglial Iba1 and galectin-3 did not differ after four weeks of AngII. The pretreatment (running) did not appear to have an effect here, either. Sholl analysis of Golgi-stained pyramidal neurons did not reveal any differences after running or AngII. Interestingly, we did not observe memory deficits in the sedentary AngII-mice, but we did, quite surprisingly, observe an AngII-induced decrease in running mice.

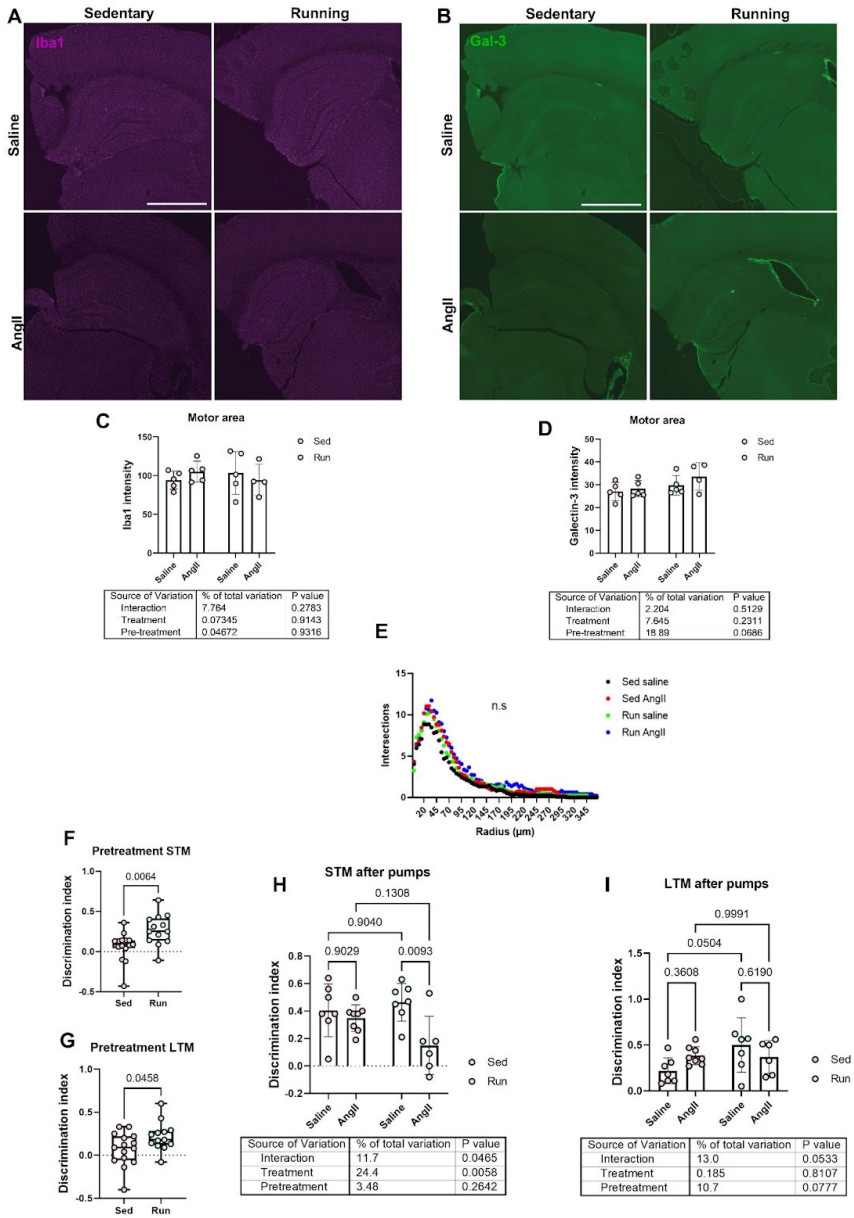


Figure 11. **Neuroinflammation, neurite complexity and novel-object recognition.** (A) Iba1 in microglia. (B) Galectin-3 in a few structures, mainly microglia. (C) 2-way ANOVA with running (pretreatment) and AngII (treatment) with regards to Iba1^{+} in motor cortex. (D) 2-way ANOVA with running (pretreatment) and AngII (treatment) with regards to Galectin-3^{+} in motor cortex. Error bars = SD. Scale bar 1mm. (E) Mixed effect analysis of sholl analysis (intersections per radius). (F-G) Novel-object recognition after four weeks of running. Short-term memory (STM) and long-term memory (LTM). Student's t-test with exact P-values. Error bars indicate range. (H-I) STM and LTM after AngII. Error bars = SD. *Iba1 - ionized calcium-binding adapter molecule 1, Gal-3 - galectin-3. STM - short-term memory, LTM - long-term memory, Sed - sedentary, Run - running.* (Figure adapted from Paper III)

In summary, running decreased several kinds of circulating immune cell populations, inflammatory cytokines, and improved memory in male mice. AngII-infusions after the running regimen increased blood pressure but not in runners, which also didn't show the same immune cell and inflammatory reactions as seen in sedentary AngII controls. However, we were not able to detect typical hypertensive traits in this AngII-model, such as memory deficits, neuroinflammation, brain structure alterations, or vascular inflammation and remodeling.

Acute systemic LPS-exposure impairs perivascular CSF distribution in mice (Paper IV)

Three hours after intraperitoneal injections LPS (1 mg/kg), CSF fluorescent tracer signal was decreased around the middle cerebral artery, in comparison to vehicle controls. Furthermore, in coronal sections tracer influx was lower in LPS-treated mice. These results indicate that systemic LPS-inflammation decreases both perivascular flow and brain tissue penetration of CSF.

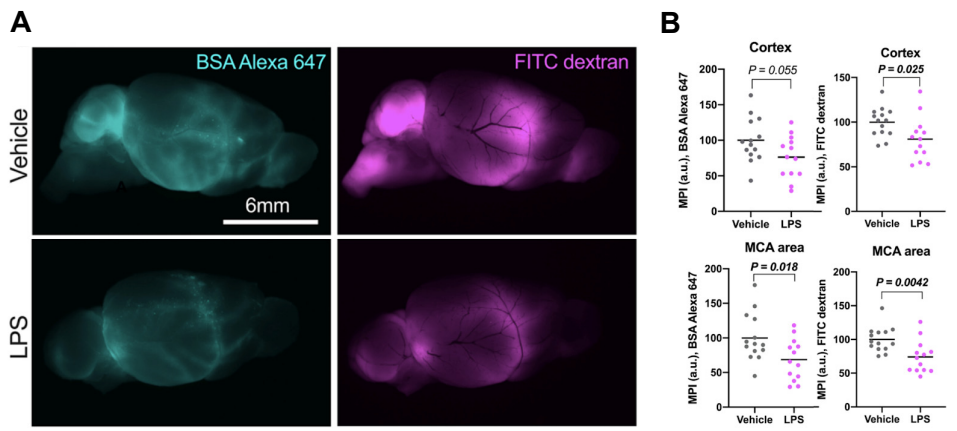


Fig 12. CSF-tracers 3h after LPS. (A) Representative images of whole hemisphere CSF-tracer (BSA Alexa 647/FITC dextran) intensity and (B) statistical analysis of tracer fluorescence (mean pixel) intensity in arbitrary units, MPI (a.u.). LPS n = 14, vehicle n = 15. Data is showing mean and individual values, with exact p-value (Student's t-test) on comparison brackets. Scale bar = 6 mm. (Figure adapted from Paper II)

Since CSF flow dynamics have been shown to be affected by physiological parameters, we sought to measure cerebral blood, respiration rate, rectal temperature, and heart rate. Within three hours, LPS did not affect cortical blood flow, temperature, or respiration, but did increase heart rate.

Because glymphatic function is believed to take place using AQP4-channels in astrocytes, we aimed to measure changes in AQP4 polarization at the astrocytic endfeet. Polarization of APQ4 did not change 3h after LPS-exposure.

Systemic injections of LPS have been shown to induce neuroinflammation, and we indeed did detect a higher Iba1$^+$-area in LPS-treated mice. We did, however, not find any differences with regards to GFAP, claudin-5, galectin-3, IgG extravasation, or IL-10.

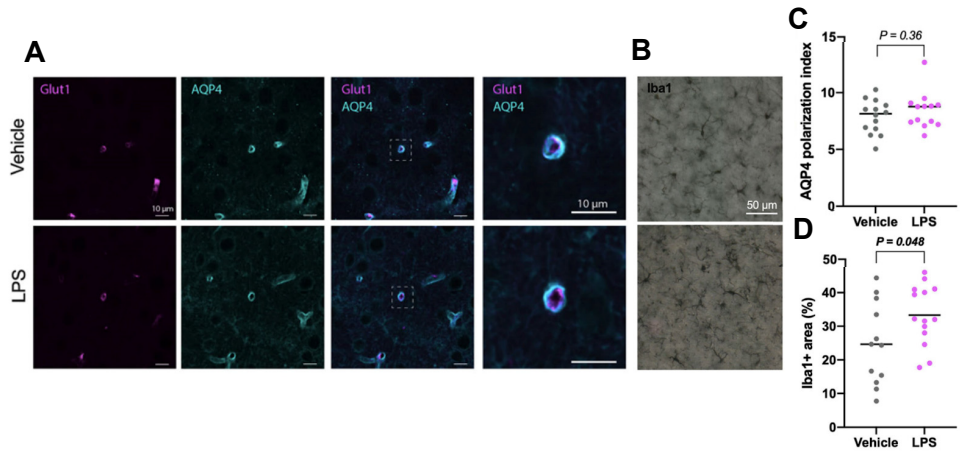


Fig 13. AQP4 and Iba1 expression 3h after systemic LPS. (A) AQP4 and Glut1 labeling cerebral vessels. (C) Analysis of AQP4 polarization index. (B) Iba1 DAB staining of microglia and (D) analysis of staining$^+$ area. LPS n = 13, vehicle n = 14. Unpaired t-tests and exact p-values above comparison brackets. Scale bars indicate 10 and 50 µm, respectively. (Figure adapted from Paper II)

In summary, acute systemic LPS exposure decreases CSF distribution in the brain and does so before many other hallmarks of brain inflammation.

Discussion and concluding remarks

Paper I

Microglia are believed to contribute to secondary injuries in stroke by killing viable cells in the penumbra and by recruiting peripheral cells that do the same (Qin et al. 2019). One molecule that is known to mediate both phagocytosis and leukocyte chemotaxis is the carbohydrate-binding molecule galectin-3 (Puigdellivol, Allendorf, and Brown 2020; Sano et al. 2000; Robinson et al. 2019). In Paper I, we compared 24-month-old female galectin-3 null and wildtype controls in a model of experimental focal stroke. Here we did not observe any significant differences in motor behavior as tested in the inclined plane and cylinder tests. However, grip strength was weaker in galectin-3 KO mice, both before and after stroke. After sacrifice, we analyzed infarct lesion size and brain inflammatory cytokine levels, with no significant differences between the two genotypes.

These results contrast previous findings in younger male animals, where galectin-3 ablation has resulted in significant reductions in inflammatory responses and concomitant changes in neuronal survival. The fact that we did not observe these differences here could be related to differences in experimental models – Lalancette-Hébert used a transient focal stroke model and sampled 72h after (Lalancette-Hebert et al. 2012)), while we performed permanent strokes for seven days. This discrepancy could, of course, also be related to sex differences in stroke vulnerability and inflammation response (Reeves et al. 2008; Manwani et al. 2013; Liu et al. 2009). Indeed, both aged female patients and mice are more sensitive to stroke pathology, likely because of decreased estrogen levels (Manwani et al. 2013; Liu et al. 2009).

Due to in-cage-fighting, we were not able to keep our male mice alive for this many months and thus lacked any aged-matched males in our comparisons. Since inflammatory changes are different between the sexes, especially in older age (Liu et al. 2009), galectin-3 could still be a target for reducing secondary stroke injury in males. With this said, galectin-3 did not appear to be an important player in the post-stroke pathology in aged female mice.

Paper II

Physical activity is widely believed to ameliorate AD pathology (Meng, Lin, and Tzeng 2020). In Paper II, we showed that voluntary running did not reduce neuroinflammatory proteins in the brain or amyloid-β in the hippocampus or CSF (Svensson et al. 2020). In contrast, running appeared to worsen several non-cognitive features of the 5xFAD model, such as anxiety, disinhibition, and hindlimb clasping. Taken together, our findings suggest that voluntary wheel running is not a beneficial intervention to halt disease progression in experimental AD. There are, however, experimental AD studies with positive data from voluntary running interventions, both with regards to amyloid load, behavior, and neuroinflammation (Tapia-Rojas et al. 2016; Francis et al. 2020; Belaya et al. 2021). It is, of course, possible that we sampled our mice too late to detect an effect of the running intervention.

The 5xFAD model is known to be aggressive (Alzforum 2019), and it is possible that the positive effects of running were simply drowned out. However, sex might also be a factor. In our study, we only used females to reduce cage violence. The mice were housed in pairs for enabling maximal access to the running wheels while not confounding results with the stress of isolation.

There are also other benefits of modeling AD in females – it is more clinically relevant. Women are way more likely to develop AD, and the disease affects women and men differently (Mielke 2018). Unfortunately, negative data and data, including female mice, are scarce in the primary literature (van Aert, Wicherts, and van Assen 2019; Sommer 2017). It is perhaps not then surprising that positive studies with running in the 5xFAD model use male mice (Belaya et al. 2020; Belaya et al. 2021).

Paper III

Physical activity is often recommended as a preventative strategy to lower the risk for vascular dementia, i.e., cognitive deficits caused by poor brain blood perfusion (Hansson et al. 2019). The mechanisms are in part explained by lower blood pressure in the physically active, in turn, believed to be due to the effect of exercise on the renin-angiotensin-system and the autonomic nervous system (Cornelissen and Fagard 2005). However, reports suggest that the immune system could also link exercise and the attenuation of hypertension development (Saleh, Norlander, and Madhur 2016; Chan et al. 2015; Guzik et al. 2007; Marko et al. 2012; Rudemiller and Crowley 2016).

In Paper III, we wanted to elucidate whether a period of forced exercise in mice would decrease angiotensin-II-induced hypertension and related hypertensive brain

phenotype (which has been described to mirror cerebral small vessel disease in humans (Meissner et al. 2017). Four weeks of running decreased circulating immune cell populations and inflammatory cytokines but did not affect renin levels. In memory tests, running mice were also higher performing. As in line with previous findings, our exercise period attenuated an angiotensin-II-induced response with regard to blood pressure (Oliveira et al. 2020). Runners also did not present the same angiotensin-II-infused changes in several immune cell populations in the thymus, bone marrow, and spleen. This might suggest protective priming by the immune system against the typical angiotensin-phenotype.

In contrast to other studies (Lanz et al. 2010; Meissner et al. 2017), we could not observe any signs of neuroinflammation or pathological brain changes in AngII-mice. Interestingly, behavioral deficits were not recorded after AngII-infusions, except in the runners, who performed worse than sedentary controls.

Since we failed to replicate earlier findings showing brain pathology after angiotensin infusions, we cannot claim any positive exercise effect with regard to vascular dementia. We believe that the lack of an effect stems from our use of a low dosage of angiotensin-II, about five times lower than what is normally used (Gomolak and Didion 2014; Iulita et al. 2018). Still, it is known that hypertension is a leading risk factor for many debilitating diseases, including vascular dementia (Emdin et al. 2016). So even if we could not induce phenotypical brain traits of hypertension in this study – we still find our results relevant.

Our findings could offer an alternative explanation to why physical activity in midlife prevents the incidence of hypertension and related diseases with aging. As with any study using rapid disease models for pathologies that typically develop over decades, caution is required when interpreting the results.

Paper IV

In Paper IV, we aimed to elucidate how acute systemic inflammation would affect CSF distribution in the brain. We observed that a single systemic LPS-injection decreased the flow of CSF-tracer after three hours. Since CSF-flow is known to be sensitive to changes in arterial pulsation, heart rate, and respiration (PMID: (Mestre et al. 2018; Villega et al. 2017; Friese et al. 2004; Hablitz et al. 2019), we measured physiological outcomes using laser doppler flowmetry and a surgical monitor. Here we failed to detect changes in cortical blood flow, temperature, and respiration, but the heart rate was found to be increased. We also could not detect changes in the expression of astroglial markers AQP4 and GFAP, indicating that the acute LPS-induced decrease of CSF-flow is not dependent on astrocytes. Neither did we detect measurable levels of proinflammatory cytokines in the brain after 3h, although microglial Iba1 showed an increase in immunostaining positivity.

Decreased CSF flow is believed to be a feature of neurological diseases, such as AD, TBI, or small vessel disease (Liu et al. 2020; Boespflug and Iliff 2018; Benveniste and Nedergaard 2022; Li et al. 2021). For instance, CSF-mediated clearing of extracellular aggregates appears to be impaired in experimental AD and TBI (Iliff et al. 2014; Iliff et al. 2012). Our results suggest that also inflammation could link neurological histopathology, behavioral deficits, and decreased CSF flow. These claims, however, would require observed effects in later time points of LPS-exposure, as well as behavioral data, neither of which we had in our study.

Conclusion

This thesis aimed to explore inflammation in the brain (Papers I, II, III, and IV), half of the projects used exercise as an experimental variable (Papers II and III). In most of these studies, our preconceptions based on previous reports (galectin-3 is important in stroke pathology, angiotensin-II causes brain dysfunction, exercise helps in AD pathology) were proven wrong. It is likely that if our experimental setups had been more similar to previous reports, we would have replicated the previous results, but one never knows. It is impossible to know how many negative studies out there have ended up in the drawers.

While we still believe that the findings in this thesis are interesting, they highlight, if nothing else, the importance of balanced reporting.

Future perspectives

I would like to use these paragraphs to address some of my main concerns with research, particularly within academia. First, the non-matching incentives of PI's and graduate students. Second, on the use of sentient animals in research. Third, on negative data.

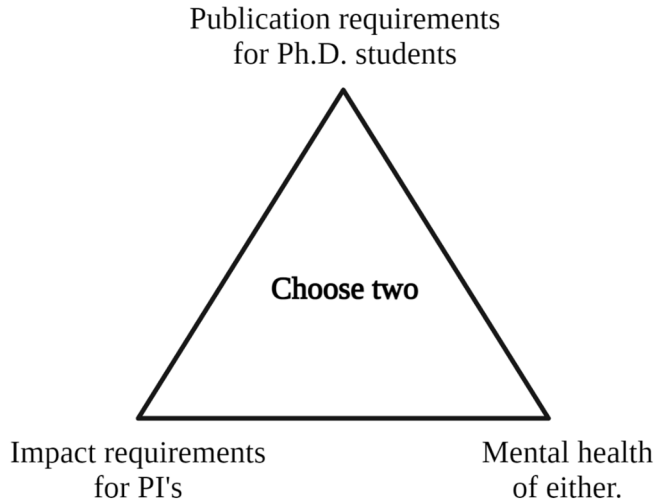
Incompatible incentives

After a completed PhD-programme, the graduate should have acquired many of the tools of a researcher. In fact, in Sweden, the Ph.D. program is referred to as *forskarutbildning* (*sv.* research education). These research tools not only consist of techniques or knowledge but also the skills to communicate science to a wider audience and an understanding of the publication process. The laws and legislations from the Swedish government (Högskolelagen 1992; Högskoleförordningen 1993), don't address any production or publication requirements, but universities have their interpretations. At Lund University, the requirements state that a Ph.D. thesis should consist of three to four original papers, of which **two** should be **published**, with at least **one** with the student as a **single first-author**, and the **second** being at least a **co-shared first-author** paper in manuscript form (PhD-requirements 2021).

On the other hand, supervisors of Ph.D. students must abide by other more or less unwritten laws – the need to publish in high-impact journals. Grant and position reviewers use high-impact publications for discriminating amongst the scientists that shall be given resources and career advances.

These requirements inevitably create different incentives for students and supervisors and often conflict and hardships. I have been spared from these pressures from supervisors, but many (if not most) of my friends and colleagues at the university have not been so lucky.

How is this issue of incentives solved?



Research animals

As mentioned in the Methods section of this thesis, animals in research are precious. They provide us with models of health and disease that are better than any alternative. Some parts of the body, like the brain or immune organs, are, at this moment, just too complex to fully recapitulate in vitro or even ex vivo. However, since the annual worldwide use of sentient animals in research possibly ranges up to 100 million (Taylor and Alvarez 2019), we need to be very careful not to cause *more* suffering in the world than we are trying to *prevent* with our studies. The 3R's (replacement, reduction, and refinement) are not to be forgotten in notebooks from the ethical course weeks and only to be reminded of when writing ethical permits. These principles must be with us always in our daily life as researchers working with animals. And we need to ask ourselves – is this experiment worth it from a utilitarian viewpoint? Is it advancing medical science or just our careers?

Negative data

This concern relates to both of the previous ones mentioned. There is a replication crisis in modern science. When scientists attempt to reproduce findings described in the primary literature, the vast majority fail (Baker 2016). This is likely very related to the publication bias, the fact that inconclusive or negative data rarely makes the print (Easterbrook et al. 1991). The fault not only lies with researchers wanting to find groundbreaking results or advocate techniques, disease models, or drug candidates. High-impact journals only publish studies reporting novel results (Nature 2022; Science 2022). Thus, studies that aim to replicate former findings or show no differences between treatment groups will end up in low-impact journals at best. I would guess most of these studies are forgotten in the deepest of drawers. The incentives for the individual researcher then clearly speak against publishing their negative data. However, the consequences are simply not acceptable.

Naturally, proving that there *isn't* any effect of a treatment is always more complicated than showing that there *is* evidence of a treatment effect (just as it is impossible to disprove the Spaghetti monster or any other deity). But here, we have to remind ourselves that we (usually) haven't tested whole populations for treatment effects but simply more or less representative samples. If we were completely transparent – our articles would be titled along the lines of "Substance X once improved survival in a handful of inbred rodents." If not stated in the title, at least this should be very clear in our interpretations. Of course, we should be as cautious when interpreting negative results.

While the experimental studies we perform neither are at the top nor at the bottom of the hierarchical evidence pyramid (Murad et al. 2016), the credibility of the systematic meta-analyses (on top) relies on the research that sits below. Thus, if the building blocks of this pyramid are as one-sided as they supposedly are now – it is no more than a house of cards.

Paraphrasing my co-supervisor Fredrik, when planning for future experiments, one has to play the guessing game whether "no one has ever tried this!" or "some have tried this, and it didn't work." The latter leads to even more waste of resources, unnecessary animal and researcher suffering.

With this said, I am proud to have co-authored two papers with mainly negative data (Papers I and II), one of which (Paper II) was featured in Scientific Reports[6] Editor's choice collection of negative results in February 2022 (Scientific-Reports 2022).

[6] One of the few journals accepting negative or replicated studies. Kudos!

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No man is an island

- John Donne

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This book is also yours.

Paper I

Galectin-3 ablation does not affect infarct size or inflammatory cytokines after experimental stroke in 24-month-old female mice

Oscar Manouchehrian[a], Emelie Andersson[b], Björn Eriksson-Hallberg[a] and Tomas Deierborg[a]

Background **The tissue damage following a focal stroke causes an inflammatory response that is thought to aggravate the disease state. Galectin-3 is a proinflammatory molecule that has been shown to play a significant role in the inflammatory responses in brain diseases and following experimental stroke. In most animal experiments, young animals are used, although attempts are often made to model diseases that affect the elderly. Therefore, in this project, we intended to investigate the role of Galectin-3 in experimental stroke in older mice.**

Methods **In this project, 24-month-old (aged) female mice were subjected to an experimental stroke (permanent middle cerebral artery occlusion) 7 days before sacrifice. We wanted to investigate whether the absence of the inflammatory protein Galectin-3 could affect motor phenotype, neuroinflammation and infarct size. Number of mice without Galectin-3 (Galectin-3 KO)=9, number of wildtype controls of the same age=6.**

Results **In our aged female mice, we could not observe any significant differences between Galectin-3 KO and wildtype regarding the inclined plane test or cylinder test. We could not observe different infarct sizes between the two genotypes. In brain homogenates, we measured levels of 10 inflammatory cytokines, but we could not see any significant differences in any of them.**

Conclusion **In summary, it can be said that the absence of the inflammatory mediator Galectin-3 does not seem to have a strong poststroke effect in aged females. Unfortunately, we could not analyze these mice with immunohistochemistry, which limited our study.** *NeuroReport* **33: 266–271 Copyright © 2022 The Author(s). Published by Wolters Kluwer Health, Inc.**

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Introduction

Age is the most substantial nonmodifiable risk factor for ischemic stroke [1], and in the elderly, women are more affected than men [1]. Neuroinflammation mediated by microglia, the main innate immune cells of the central nervous system, has emerged as a key event in the pathophysiology of stroke disease. In response to the neuronal damage initially caused by deprivation of oxygen and essential nutrients, these cells are rapidly activated and recruited to the injury site [2]. The activated microglial cells express Galectin-3 [3], a β-galactosidase binding protein that has been suggested to play a significant role in regulating the inflammatory response [4] and the outcome after brain ischemia in experimental models [5,6]. For example, Lalancette-Hébert showed that the infarct lesion was increased in Galectin-3 null mice after transient middle cerebral artery occlusion (tMCAO) [5], while our group has shown that neuronal survival was higher after global ischemia [6]. However, these studies have been performed in young male mice, and it is not known how age or age combined with sex affects similar insults. Therefore, we aimed to measure the outcome after experimental stroke in aged female mice, with and without the expression of Galectin-3.

Methods

Animals and surgery

All proceedings and animal treatment were by the guidelines and requirements of the government committee on animal experimentation at Lund University (2012, Dnr: M427-12). Animals were housed in groups of 3–6 mice per cage under a 12:12 light-dark cycle with access to water and food ad libitum. In this study, we used female wildtype ($n=6$) and Galectin-3 knockout ($n=9$) C57BL/6 mice, aged 24 months. Galectin-3 KO mice were originally obtained for Dr. K. Sävman from Gothenburg University, where they were generated as

described previously [7]. The genotype of each mouse was confirmed with PCR as described before [8]. Mice were subjected to a left-sided permanent middle cerebral artery occlusion (pMCAO) as previously described [9]. Mice were anesthetized with 5% isoflurane in oxygen, followed by 2% isoflurane in oxygen during the whole surgical procedure. A small incision was made between the left eye and ear, and the temporal muscle was separated from the skull. The middle cerebral artery was made visible by drilling a small hole in the skull. A distal permanent occlusion was made using a vessel cauterizer. The incision was sutured (nylon), and animals were returned to their cages.

Behavioral tests

Grip strength

The grip strength test was performed prior to and 6 days after pMCAO. The mouse was lowered over a metallic grid connected to a force sensor whereby it was allowed to attach to the grid with only its forelimbs. The mouse was then gently pulled back by its tail until the grip was released, and the displayed grip strength value was recorded. The grip strength of the left forelimb, right forelimb and both forelimbs was determined. For each condition, the mean value of three trials was calculated and used for analysis.

Inclined plane test

The inclined plane test was performed prior to and 6 days after pMCAO. Each mouse was placed head down on an inclined plane platform with a grooved plastic surface. The angle was gradually increased until the mouse was unable to hold its position. The last angle that the mouse was able to hold was recorded. Three rounds per mouse were performed and the mean angle was calculated and used for analysis.

Cylinder test

To assess for asymmetric use of forelimbs, the cylinder test [10] was performed 2 days after the pMCAO. Mice were put in a glass cylinder with a 90° angled mirror behind it for 3 min, and movements were recorded. Right and left forelimb use was analyzed as one weight-bearing contact with the glass wall accounting for one 'touch unit', using a VLC media player.

Cryosectioning

Animals were sacrificed on day 7 following surgery by isoflurane anesthesia and transcardial perfusion with PBS. Brains were then carefully removed and stored at −80 °C. Using a cryostat (Leica CM3050 S Cryostat, Wetzlar, Germany), a series of six 30 µm thick coronal brain sections was obtained, mounting sections in series 1–4 directly on glass. The remaining two sections (series 5 and 6) were put in an Eppendorf tube each. Sections were collected from approximately Bregma −2 to Bregma 2 (www.mbl.org). Glasses were dried and stored at −80°C. Tubes were stored at −80°C.

Morphology staining

Sections were fixed in paraformaldehyde (4%, HistoLab Products AB, Gothenburg, Sweden) for 10 min and rinsed in PBS for 2 × 3 min. Sections were incubated in Mayers-HTX (HistoLab) solution for 3 min and rinsed in a continuous flow of tap water for at least 5 min. Sections were incubated in eosin (Sigma-Aldrich, St Louis, Missouri, USA) for 1 min, and then rinsed in 70% ethanol (SOLVECO, Rosersberg, Sweden) for 1 min, followed by a rinse in 95% ethanol (SOLVECO) for 1 min and then rinsed in absolute ethanol (SOLVECO) for 2 × 1 min. Sections were incubated in HistoLab-Clear (HistoLab) for 3 × 1 min plus 1 min. Sections were mounted with Pertex (HistoLab) and air-dried on plastic, then stored at room temperature.

Infarct volume

The hematoxylin/eosin stainings were scanned, and image analysis was performed. Using ImageJ (https://imagej.nih.gov), whole coronal face areas of the contralateral hemisphere, the intact part of the ipsilateral hemisphere, and the entire ipsilateral hemisphere measured in all slides containing a distinguishable infarct (approximately responding to Bregma: 2 to −2 mm). Two infarct volumes, direct and indirect, were calculated. Direct volume was obtained by subtracting the intact ipsilateral area from the entire ipsilateral and indirect infarct area by subtracting the intact ipsilateral from the contralateral area.

Brain tissue homogenization

Every 5th and 6th slice at cryosectioning from whole brain tissue were frozen in −80 °C. Tissue mass weight was calculated for each tube. radioimmunoprecipitation assay (RIPA) buffer was prepared by adding one complete tablet (Roche, Basel, Switzerland) and one PhosStop (Roche) tablet to 10 mL of RIPA solution (Sigma-Aldrich). RIPA buffer was added in ratio 1 mL RIPA buffer/100 mg of brain tissue and stored on ice. Samples were manually homogenized using a plastic grinder. A sonicator (Branson, Ferguson, USA) was set on 10 s pulses with 60% amplitude and three pulses with 10-s intervals, and then stored on ice for a minimum of 30 min. Homogenized samples were centrifuged (Eppendorf Centrifuge 5430 R, Hamburg, Germany) on 10.000 rcf for 20 min. Supernatants were collected in Eppendorf tubes. Later, protein concentration was measured using a bicinchoninic acid (BCA) kit according to the manufacturer's protocol (BCA Protein Assay-Kit, ThermoScientific, Sweden).

Cytokine assay

MesoScale plates were used to evaluate the cytokine levels (proinflammatory panels for IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 and tumor necrosis factor-α) from every whole brain homogenate (see in Brain tissue homogenization). Analyses were carried out according to the protocol provided by the manufacturer, as described before [11,12]. Samples were prepared by a 2-fold dilution of sample protein solution (containing 120 mg protein) in diluent 41.

Statistical analysis

All statistical analyses were performed in GraphPad Prism 9 (GraphPad, San Diego, California, USA). *P* values ≤0.05 were considered significant. Since the wild-type group *N* was too low for normality testing, we used Mann–Whitney rank tests for pairwise comparisons. For some behavioral tests with more than one variable, we used 2-way analysis of variance (ANOVAs).

Results

Behavioral tests

We first set out to study sensorimotor function in established experimental stroke tests previously used by us [13–16].

Grip strength with both front paws was decreased after pMCAO (Fig. 1a; 2-way ANOVA; F (1, 22) = 16.72; P = 0.0005), and interestingly, was overall lower in Galectin-3 KO females (Fig. 1a; 2-way ANOVA; F (1, 22) = 8.889; P = 0.0069). Furthermore, it was only in mice without Galectin-3 where grip strength was significantly lower after pMCAO (Fig. 1a; Sidak multiple comparison; P = 0.023). Grip strength with the injured (right) forepaw was not significantly weaker 6 days after pMCAO (Fig. 1b). In addition, there was no genotype difference with regards to right vs. left paw grip strength, 6 days after stroke (Fig. 1b).

Use of impaired forelimb after stroke was determined with the cylinder test. There was a nonsignificant trend of higher use of the impaired forelimb in the Galectin-3 KO mice (Fig. 2a; Mann–Whitney; P = 0.1447).

To further analyse limb motor function, we subjected the mice to an inclined plane test before and after pMCAO. We did not observe any differences regarding genotype or stroke (Fig. 2b; 2-way ANOVA). Taken together, we are not able to find a clear effect of Galectin-3 ablation on sensorimotor behavior following stroke.

Infarct volume

Infarct size was measured with a series of HTX slices. The absence of Galectin-3 did not significantly affect direct or indirect infarct volume 7 days after pMCAO in 24-month-old females (Fig. 3a,b; Mann–Whitney; P = 0.8639 and 0.3884, respectively).

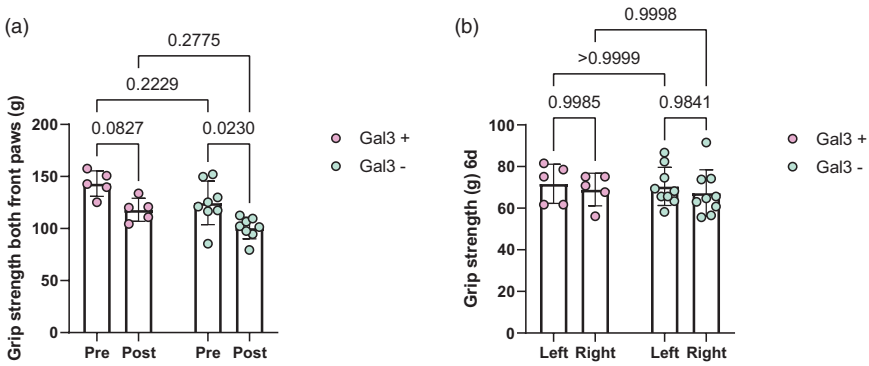
Immunohistochemistry

We tried to label these brains with microglial markers, using multiple protocols, but we were not successful.

Cytokine expression

With a mesoscale U-plex Proinflammatory panel, we measured inflammatory cytokines from brain homogenates (from slices). Except for a few analytes (IFN-y and IL12p70), we could read values within the detection range for all samples. Trends with lower expression in Galectin-3 KO could be seen for IL-1b and IL-6, but these differences were not statistically significant (Fig. 4b,f; Mann–Whitney; P = 0.18 and 0.0879, respectively).

Fig. 1

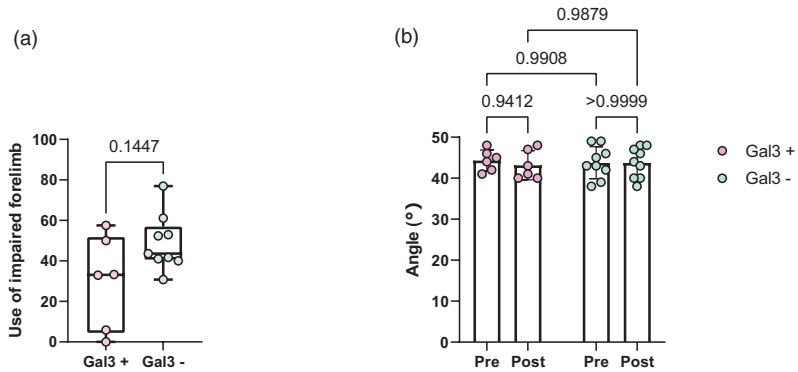


Source of Variation	% of total variation	P value
Interaction	0.01526	0.9323
Stroke	34.55	0.0005
Genotype	18.37	0.0069

Source of Variation	% of total variation	P value
Interaction	0.01179	0.9573
Hemisphere	2.374	0.4503
Genotype	0.5761	0.7087

Grip strength. Wild-type n=5 (one mouse did not grip with right forelimb), Galectin-3 KO n=9. (a) Grip strength before and after pMCAO. (b) Grip strength with left and right (injured) front limb 6 days after pMCAO. 2-way ANOVA with Sidak multiple comparisons. Error bars SD, and exact P-values on top of comparison brackets. ANOVA, analysis of variance; pMCAO, permanent middle cerebral artery occlusion.

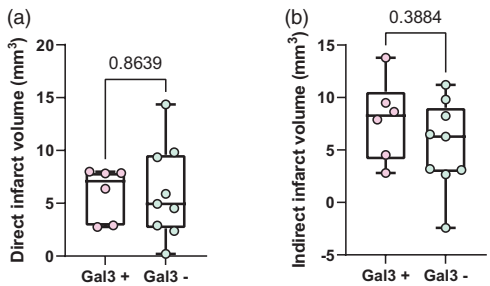
Fig. 2



Source of Variation	% of total variation	P value
Interaction	0.7305	0.6646
Stroke	0.7305	0.6646
Genotype	0.001657	0.9835

Cylinder test and inclined plane test. Wildtype $n=6$, Galectin-3 KO $n=9$. (a) Cylinder test with Mann–Whitney. Error bars range and exact *P*-values on top of comparison bracket. (b) Inclined plane test. 2-way ANOVA with Sidak multiple comparisons. Error bars SD, and exact P-values on top of comparisons brackets. ANOVA, analysis of variance.

Fig. 3



Infarct measurements. (a) Direct ($V_{ipsilateral}$—$V_{unaffected\ ipsilateral}$) and (b) indirect ($V_{ipsilateral}$—$V_{contralateral}$) infarct volumes in cubic millimeters. Wildtype $n=6$, Galectin-3 KO $n=9$. Mann–Whitney test is used for pairwise comparison. Error bars=min/max, and exact *P*-value on top of comparison bracket.

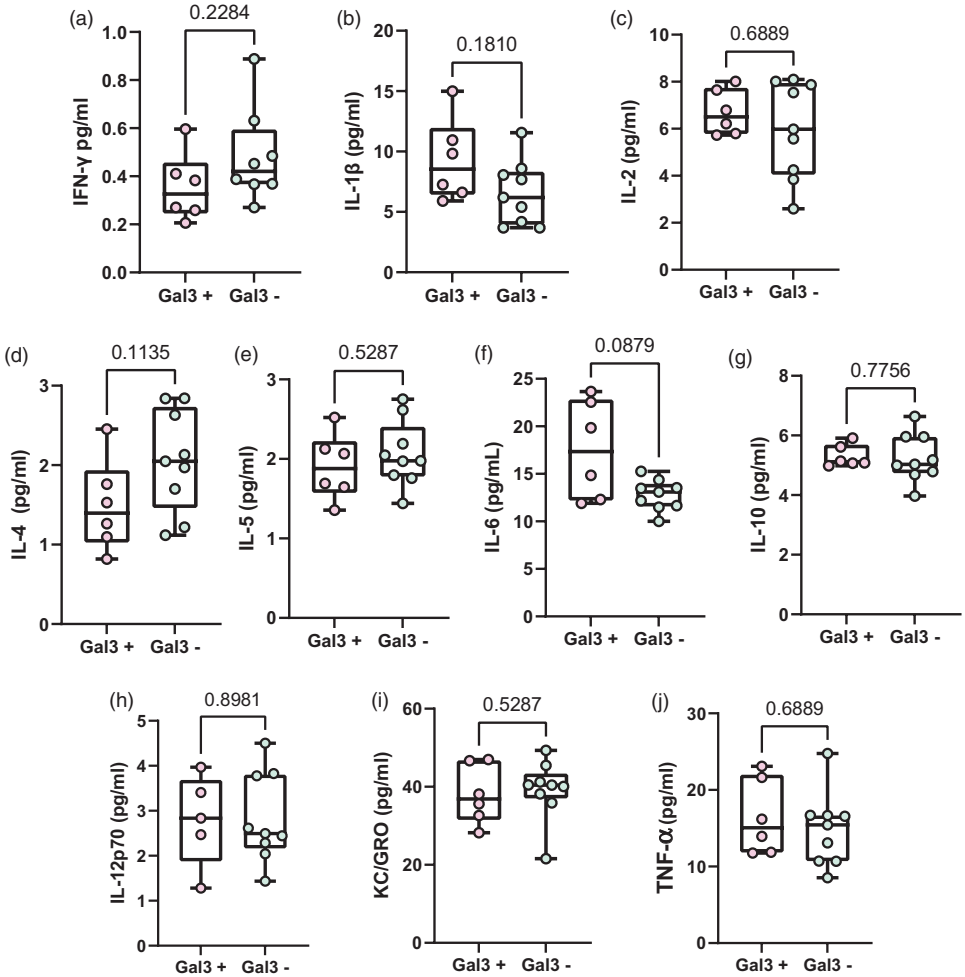
Likewise, levels of IFN-y and IL-4 appeared higher in KO females, but differences were not statistically significant (Fig. 4a,d; Mann–Whitney; $P=0.2284$ and 0.1135, respectively).

Discussion

Many experimental studies show that neuroinflammation after cerebral focal ischemia can exacerbate the insult [17]. However, most studies have been performed in adolescent mice when modeling these stroke-related diseases that usually affect the elderly [18]. Galectin-3 is an inflammatory mediator that has been implicated in many diseases, including brain ischemia. In some conditions, it exerts protective and antiapoptotic effects [5], and in others, it exacerbates inflammation and injury [6,19]. Our group has previously shown that Galectin-3 ablation improves outcomes in experimental models of neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease [6,8,20]. In this study, we subjected 24-month-old female mice to pMCAO. In contrast to a previous study by Lalancette-Hébert *et al.*, (2012), we do not report differences in lesion size between the genotypes [5]. Of course, discrepancies between our observations and Lalancette-Hébert were not only limited to the age and sex of the mice but could also be related to differences in the stroke model (permanent versus transient) and duration (7 days and 72 h) [5]. Our study design enabled us to perform several behavioral tests, and we did detect a significant genotype effect in grip strength with both front paws, where Galectin-3 ablated females were weaker. Both genotypes were weaker after stroke, but Galectin-3 KO mice were weaker before and decreased more after the pMCAO surgery. In the grip strength test with only one paw and the inclined plane test, we could not detect any effect regarding either genotype or injury/stroke.

Our study is narrow in its scope and the lowered power due to the relatively small control group may have

Fig. 4



Inflammatory cytokines in whole brain homogenates. Wildtype $n=6$, Galectin-3 KO $n=9$. (a) IFN-y. Here one Galectin-3 KO sample was below the detection range. (b) IL-1b, (c) IL-2, (d) IL-4, (e) IL-5, (f) IL-6, (g) IL-10 (h) IL-12p70. One wildtype sample was below detection range (i) KC/GRO, (j) TNF-a. Mann–Whitney test is used for pairwise comparison. Error bars=min/max, and exact *P* value on top of comparison bracket. TNF, tumor necrosis factor.

resulted in nondetection of significant differences. In addition, it is possible that our cytokine results were diluted when we homogenized both the ipsilateral and the 'healthy' contralateral hemispheres into one sample. Moreover, we do not have a young group of mice, or any males, for comparisons to our aged female results. Initially, we aimed at having both female and male mice, but due to males killing one another in the home cages, we were only able to focus this study on female mice. Because publications including female mice are underrepresented in the primary literature, we believe that this study is important to be made available. In fact, females are especially vulnerable to stroke disease, likely related to decreasing levels of estrogen [21,22]. It is difficult to say whether the results in aged male Galectin-3 null mice would have differed from male wild-type controls, but

because the inflammatory response differs between the sexes [21], the outcome is not guaranteed to be similar to our findings.

In conclusion, the ablation of Galectin-3 did not affect infarct size and levels of brain cytokines in aged female mice. However, Galectin-3 null mice appeared to be a bit weaker in grip strength, both before and after stroke. How this relates to outcomes in human patients is not easy to decipher. But we would suggest that researchers in future studies concerning diseases of primarily aged women take age and sex into consideration when designing their experiments.

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Conflicts of interest

There are no conflicts of interest.

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Paper II

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Voluntary running does not reduce neuroinflammation or improve non-cognitive behavior in the 5xFAD mouse model of Alzheimer's disease

Martina Svensson*, Emelie Andersson, Oscar Manouchehrian, Yiyi Yang & Tomas Deierborg*

Physical exercise has been suggested to reduce the risk of developing Alzheimer's disease (AD) as well as ameliorate the progression of the disease. However, we recently published results from two large epidemiological studies showing no such beneficial effects on the development of AD. In addition, long-term, voluntary running in the 5xFAD mouse model of AD did not affect levels of soluble amyloid beta (Aβ), synaptic proteins or cognitive function. In this follow-up study, we investigate whether running could impact other pathological aspects of the disease, such as insoluble Aβ levels, the neuroinflammatory response and non-cognitive behavioral impairments. We investigated the effects of 24 weeks of voluntary wheel running in female 5xFAD mice (n = 30) starting at 2–3 months of age, before substantial extracellular plaque formation. Running mice developed hindlimb clasping earlier ($p = 0.009$) compared to sedentary controls. Further, running exacerbated the exploratory behavior in Elevated plus maze ($p = 0.001$) and anxiety in Open field ($p = 0.024$) tests. Additionally, microglia, cytokines and insoluble Aβ levels were not affected. Taken together, our findings suggest that voluntary wheel running is not a beneficial intervention to halt disease progression in 5xFAD mice.

Alzheimer's disease (AD) is the most common form of dementia, affecting around 30 million people worldwide (WHO 2016). Even though cognitive dysfunction is a hallmark of AD, a majority of AD patients also suffer from other, non-cognitive symptoms such as depression and anxiety[1,2]. AD is characterized by accumulation of extracellular amyloid-beta (Aβ) plaques and progressive neurodegeneration. Further, the inflammatory response is also altered in the AD brain[3]. Postmortem studies using AD brains have revealed than pro-inflammatory cytokines, such as IL-1β and IL-6, accumulate around Aβ plaques[4,5]. In addition, microglial activation is increased[6] and correlates with the Aβ deposition[7,8]. Recently, a genome-wide association study revealed that genetics variants related to increased risk of developing AD are specifically enriched in enhancers of myeloid cells[9]. Interestingly, microglia are capable of phagocytosing Aβ aggregates and, thereby, facilitate Aβ clearance[10]. Contrastingly, neuronal Aβ production can induce cytokines in microglia and this can up-regulate the expression and enzymatic activity of β-secretase, thereby enhancing Aβ production[11]. Thus, it is likely that the microglial response in the AD brain contribute with both protective and harmful effects. Hence, future therapeutic interventions may focus on modulating different aspects of these responses.

Several studies suggest that physical exercise is beneficial by reducing the risk of AD and slowing the progression of the pathology[12–14]. Exercise intervention may improve cognition[15,16] and ameliorate Aβ levels in patients[17]. Moreover, exercise was associated with larger gray matter volumes in cortex and hippocampus and improved cortical connectivity of cognitive networks in patients with mild cognitive impairment[18,19]. However, many studies show no beneficial effects of exercise on AD[20–23]. We recently investigated how physical activity affects the risk of developing AD in two large study populations (>410 000 participants in total) over an extended period (>20 years) under different conditions[24]. Physical activity did not significantly affect the risk of developing AD in

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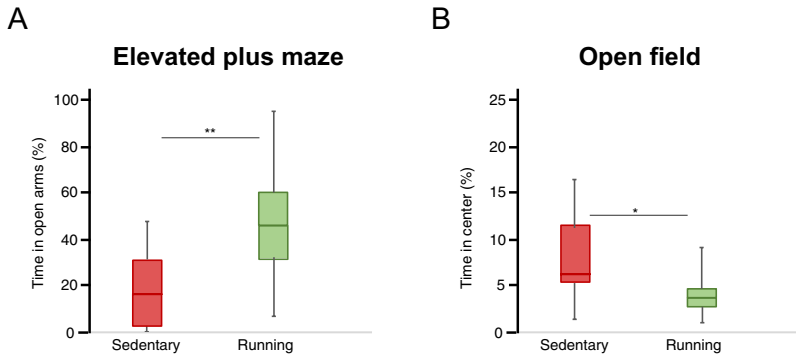

Figure 1. Exploratory behavior in Elevated Plus Maze and Open Field tests. Exploratory behavior is presented as the percentage of time spent in the open arms of the Elevated Plus Maze (**A**) or in the center zone in the Open Field (**B**) tests conducted during weeks 20–22. Box plots represent the median values for each group with interquartile ranges and error bars indicating the minimum and maximum. **Represents $p < 0.01$ and *represents $p < 0.05$ (Mann Whitney U-test). For sedentary mice $n = 14$ and for running mice $n = 14$.

any of our study populations. Hence, we questioned the effect of physical exercise on AD incidence and disease progression.

Several transgenic mouse strains have been developed to model different aspects of AD[25]. The 5xFAD strain is a mouse model with a fast development of AD pathology, showing accumulation of extracellular Aβ plaques and signs of neuroinflammation as early as 2–3 month of age[25–28]. Studies investigating the effects of exercise in other AD models have shown inconsistent results[14], for example with regard to the effects on Aβ levels[14,29,30]. We have recently shown that 6 months of voluntary running in 5xFAD mice did not result in any beneficial effects on soluble Aβ-levels, synaptic protein levels or cognitive behavior[24]. Interestingly, prior studies in other AD models suggest that exercise may reduce neuroinflammation by reducing microglial activation and levels of pro-inflammatory cytokines[31,32]. Because of its features, we view the 5xFAD model as suitable for studying the effects of exercise on neuroinflammatory and non-cognitive behavioral features of AD. We recently reported on the appearance of neuroinflammation in this model before extracellular amyloid deposition[28] and the important role of pro-inflammatory microglial galectin-3 in development of pathology and behavioral deficits[33]. In light of the pathological importance of myeloid cells in AD, the aim of this study was to further investigate the effects of 6 months of voluntary wheel running on neuroinflammation and non-cognitive behavior in the 5xFAD model.

Results

Voluntary wheel running does not induce a corticosterone stress response. Body weights did not differ between groups at the beginning or end of the study (Supplementary Table 1). Since we previously reported that forced running induces a harmful corticosterone stress response in mice[34], we controlled for stress induction by the voluntary running intervention used in this study. The fecal corticosterone levels did not significantly differ between sedentary and running mice at baseline or after 19 weeks of exercise intervention (Supplementary Table 2). Both groups displayed decreased levels of corticosterone at the end of the study compared to the baseline levels (Supplementary Table 2, median (IQR) concentrations were 2617 (1699–4455) and 1523 (1331–2205) pg/ml for the sedentary group, Wilcoxon test $p = 0.001$ and 2167 (1644–4053) and 1506 (1237–1722) pg/ml for the running group, Wilcoxon test $p = 0.02$).

Voluntary wheel running affects exploratory and anxious behavior. In the Elevated plus maze, running mice spent significantly more time exploring the open arms compared to their sedentary counterparts (Fig. 1A, median (IQR) 15.2 (3.1–30.9) % and 46.1 (29.2–60.7) % of time respectively, Mann-Whitney U-test $p = 0.001$). In the open field, running mice spent significantly less time exploring the center compared to sedentary controls (Fig. 1B, median (IQR) 6.3 (5.3–13.0) % and 3.2 (2.3–5.0) % of time, Mann-Whitney U-test $p = 0.024$). General motor function did not differ between groups as they traveled the same distance both in the Elevated plus maze and Open field (Supplementary Table 3). There was no significant difference in sucrose preference between sedentary and running mice (Supplementary Table 4).

Voluntary wheel running does not improve motor learning. The sedentary mice significantly improved their rotarod performance over time (Fig. 2, median (IQR) 27.8 (1.7–48.0) seconds and 44.3 (12.0–65.0) seconds on day 1 and 3 respectively Friedman test, $p = 0.008$). In contrast, running mice did not significantly improve over the same amount of time (Fig. 2, median (IQR) 17.2 (4.3–39.3) seconds and 31.5 (13.5–56.5) seconds on day 1 and 3 respectively Friedman test, $p = 0.47$). However, running mice did not spend significantly less time on the rotarod compared to sedentary littermates on any of the three test occasions. Taken together, these results suggest that voluntary wheel running does not improve motor learning in 5xFAD mice.

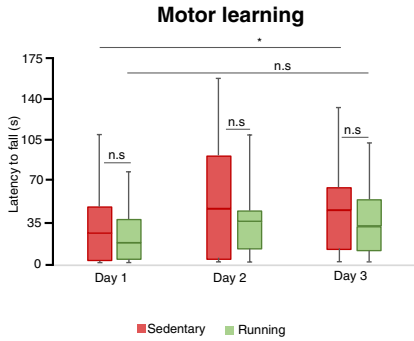

Figure 2. Motor learning in Rotarod test. The latency to fall off the rotarod at different days of training. Box plots represent the median values for each group with interquartile ranges and error bars indicating the minimum and maximum. *Rrepresents $p < 0.05$ (Wilcoxon test). For sedentary mice $n = 14$ and for running mice $n = 14$.

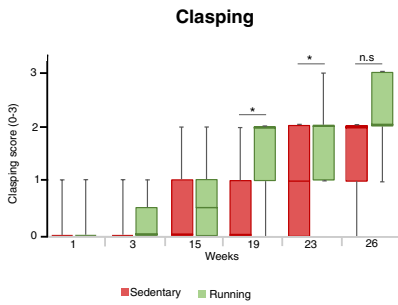

Figure 3. Hindlimb clasping at different time points. The hindlimb clasping scores at different time points. Box plots represent the median values for each group with interquartile ranges and error bars indicating the minimum and maximum. *Represents $p < 0.05$ (Mann Whitney U-test used for each given time-point). For sedentary mice $n = 14$, and for running mice $n = 14$.

Exercised mice developed hindleg clasping earlier. To measure the development of sensorimotor dysfunctions in the 5xFAD model, we performed hindlimb clasping tests during experimental weeks 1,3,15,19, 23 and 26 (Fig. 3). There were significant changes in clasping scores in both groups from the beginning to the end of the study (Friedman tests, $p<0.001$ for both sedentary and running groups). Up to week 15, there was no significant difference in hindleg clasping between sedentary and running mice (week 15, median clasping scores (IQR) were 1 (0–1) and 0.5 (0–1) respectively, Mann-Whitney U-test, $p = 0.64$). Thereafter, running mice developed hindlimb clasping earlier than sedentary controls (week 19, median clasping scores (IQR)were 1 (0–1) and 2 (1–2) for sedentary and running mice, respectively, Mann-Whitney U-test, $p = 0.009$. Week 23, median clasping scores (IQR) were 1 (0–2) and 2 (1–2) for sedentary and running mice, respectively, Mann-Whitney U-test, $p = 0.029$). Nonetheless, at the end of the study, hindlimb clasping scores did not differ significantly between the groups (week 26, median clasping scores (IQR) were 2 (1–2) and 2 (2–3) for sedentary and running mice, respectively, Mann-Whitney U-test, $p = 0.20$).

Voluntary wheel running does not ameliorate levels of insoluble Aβ. The levels of different insoluble Aβ species in hippocampus and soluble Aβ species in CSF did not differ between the running and sedentary mice groups (Supplementary Table 5). Further, the number of ThioflavinS-positive amyloid plaques in hippocampus and cortex did not differ significantly between groups (Fig. 4, median plaque numbers (IQR) in hippocampus were 35.2 (29.3–39.7) and 40 (35.7–49.7) for sedentary and running groups respectively, Mann-Whitney U-test, $p = 0.077$. Median plaque numbers (IQR) in cortex were 60.7 (52.3–65.3) and 55.8 (52.0–63.0) for sedentary and running groups respectively, Mann-Whitney U-test, $p = 0.54$).

Voluntary wheel running does not significantly reduce neuroinflammation. The total amount of microglia in hippocampus was measured by Iba1 immunohistochemistry. Intensity levels of Iba1 did not differ

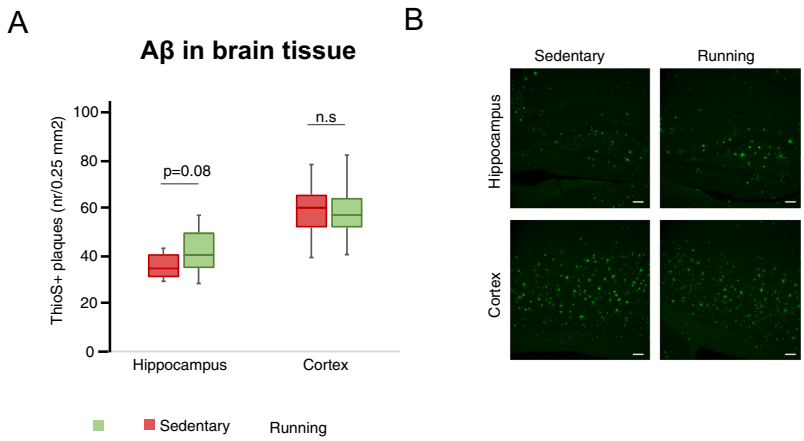

Figure 4. Aβ plaques in hippocampus and cortex. Thioflavin-S-positive Aβ plaques in hippocampus and cortex. Box plots represent the median values for each group with interquartile ranges and error bars indicating the minimum and maximum (**A**). Representative images at 10x, with scale bar representing 100 μm (**B**). p-value from Mann Whitney U-test. For sedentary mice n = 14, and for running mice n = 14.

between running and sedentary mice (Fig. 5A). Further, the levels of galectin-3 was not affected by running, as measured using Western blot (Supplementary Table 6) and immunohistochemistry (Fig. 5A). There were no differences in cytokine levels between the groups for any of the cytokines analyzed in serum or hippocampus (Supplementary Table 7). Likewise, the protein levels of NLRP3 (Supplementary Table 6) as well as the levels of iNOS (Fig. 5B, median (IQR) were 94.9 (82.3–116.1) % and 63.2 (57.2–65.4) % for sedentary and running groups, respectively, Mann-Whitney U-test, p = 0.109) in hippocampus did not significantly differ between groups.

Discussion

In the present study, we investigated the effects of voluntary wheel running on the development of neuroinflammation, insoluble Aβ load and non-cognitive behavioral deficits in the 5xFAD mouse model of AD. Our main findings show that 6 months of voluntary wheel running does not ameliorate these pathological events in 5xFAD mice. On the contrary, running may even aggravate the pathology as our running mice showed increased exploratory behavior and developed sensorimotor hindleg clasping earlier. Furthermore, the running intervention did not reduce insoluble Aβ levels, the total amount of microglia, as measured by Iba1 staining intensity, or pro-inflammatory inflammatory cytokine levels.

Running led to increased exploratory behavior in the Elevated plus maze test and increased anxiety in the Open field test. This may reflect the typical phenotypical differences that this AD model displays compared to wild-type mice in these two tests in sedentary control settings[35,36]. At 8 months of age, 5xFAD mice typically develop increased exploratory behavior in the Elevated plus maze, which correlates with the deposition of Aβ in the brain[35–37]. This increased exploratory behavior has been suggested to reflect disinhibitory tendencies, similar to what is seen in AD patients[35]. Thus, the increased exploratory behavior seen in our running mice might be interpreted as an aggravation of the behavioral dysfunction in this model. However, in this study, we had no direct comparison to wildtype mice. Hence, we cannot know if the behavior we observe in our 5xFAD really deviates from wildtype in our settings, even though existing literature strongly indicate this.

Concurrently, under sedentary conditions, 5xFAD mice have been shown to develop reduced exploratory behavior in Open field as the disease develops[36]. Hence, the increased anxious behavior seen in the Open field in our running mice can also be interpreted as an aggravation of the behavioral dysfunction. Still, we do not have any direct comparison with wildtype mice in our study to conclude this. In addition, we have previously shown that anxious behavior in Open Field is associated with increased corticosterone levels in feces collected during this test[34]. Since the corticosterone levels in feces collected during the Open Field test performed at 8 months of age in our study did not differ, it is possible that the readout of this test does not really reflect the anxiety levels during that day. Therefore, we should be careful with conclusions drawn from this test.

Hindlimb clasping and motor deficits normally develops at 9–12 months in the 5xFAD model and are suggested to reflect the Aβ accumulation and damage in spinal cord motor neurons[35,38]. In our study, running mice developed clasping earlier and motor performance and learning in the rotarod was not improved by the running intervention. Therefore, it is tempting to speculate that the increased clasping behavior in the running mice reflects a faster development of the pathology in the central nervous system. However, we do not control for development hindlimb clasping in wildtype mice since there is already robust evidence that wildtype mice do not develop this abnormal clasping behavior. Importantly, the distance traveled in the Open field and Elevated plus

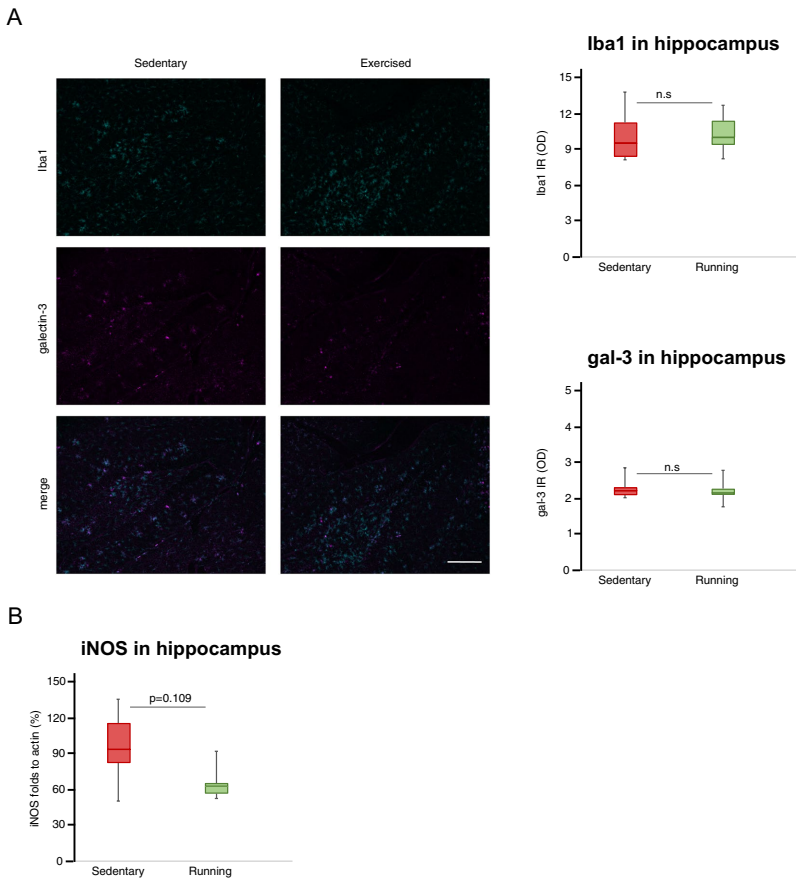

Figure 5. Neuroinflammation in hippocampus. Representative images of the Iba1 (labeling all microglia) and gal-3 (labeling activated microglia) staining in hippocampus at 10x with scale bar representing 200 μm (**A**) and box plots representing the median values of Iba1 and gal-3 intensities (n = 10 + 10). The level of iNOS (n = 6 + 6) in hippocampus normalized to actin (**B**). Box plots represent the median values for each group with interquartile ranges and error bars indicating the minimum and maximum. p-value from Mann Whitney U-test. For sedentary mice n = 6, and for running mice n = 6.

maze tests did not differ between the groups, indicating that the motor deficits in the clasping test did not bias the outcome of these anxiety tests.

Although many experimental studies demonstrate reduced Aβ levels in the brain after exercise[39], we could not detect any statistically significant changes on the levels of soluble Aβ[24] and insoluble Aβ. Interestingly, we observed a nonsignificant trend towards increased Aβ plaques in the hippocampus (p = 0.08) of running mice, in line with the effects of running found in a model of cerebral amyloid angiopathy[40]. In addition, other studies showed no effects of exercise on Aβ levels in mouse models of AD[41,42]. Further, a patient study with exercise intervention found no effects on Aβ levels in CSF[43], similar to what we observed in CSF from our 5xFAD mice. Hence, the clinical benefits regarding the effect of exercise on Aβ pathology in AD indicated in other experimental studies can be questioned.

Our group has previously showed that the 5xFAD model displays increased levels of inflammatory cytokines and neuroinflammation as early as 2–3 months of age, the same time period when the first Aβ plaques can be observed[28]. Further, manipulating cytokine and galectin-3 levels has been shown to affect Aβ pathology in the 5xFAD model[33,44]. Since exercise is known to affect the levels of several cytokines[32,42,45], these studies led us to introduce the running intervention early in our study. However, running did not affect brain or blood cytokine

levels or total microglia, as measured by Iba1 staining intensity, in our mice. Further, we could not detect any significant effects on other inflammatory markers, such as galectin-3, iNOS and NLRP3. Even though running tended to reduce iNOS levels, the effect was not statistically significant. The failure of our running intervention to affect the inflammatory reaction in the brains of our mice may be one explanation as to why the intervention did not influence Aβ accumulation or behavioral outcome, although it is interesting to note that exercise ameliorated pathology and cognitive dysfunction in other AD models without affecting cytokine levels[46].

Taken together, running exercise did not ameliorate any pathological hallmarks in our study. We do not compare with wildtype mice in our study. Still, our results indicate that a running intervention may aggravate the disease phenotype, such as increasing exploratory behavior in the Elevated plus maze, shown to be an abnormal behavior compared to wildtype in other studies. Similarly, our previous publication revealed that the intervention also aggravated cognition in the 5xFAD model[24]. Nevertheless, numerous studies have demonstrated beneficial results of exercise on AD pathology in other mouse models of the disease[14,39]. These differences may be due to several factors. First, the 5xFAD model is an aggressive model with a fast progression and a genetically driven pathology whereas most AD mouse models have a slower progression[25]. Thus, the aggressive pathology in 5xFAD mice might be more difficult to impede compared to the slower development of AD-like pathology in other models. Second, discrepancies between studies may be explained by the duration and timing of the intervention and sample collection. Many studies, compared to this study, investigate the effects of exercise over a shorter time period, making it difficult to draw conclusions about the effects of a long-term, active lifestyle initiated before pathology develops. In our study, the running intervention is started at two months of age, when AD pathology begins to develop in 5xFAD mice. In addition, our mice exercised for six months, until eight months of age, when this model has fully developed the pathology. Moreover, the mice in many exercise intervention studies are socially isolated, which some researchers suggest, may influence the results[47]. Importantly, this was not an issue in our study as our mice were housed in pairs.

Nevertheless, Choi *et al*. recently reported that running was beneficial in this model and reduced Aβ levels and improved cognition[48]. We have previously observed that forced running paradigms may induce stress in mice, which can aggravate the pathology[34]. Therefore, we compared corticosterone levels from running mice with the sedentary controls both before and after the running intervention. We did not find any signs of stress in our running mice as the corticosterone levels did not differ between groups. Interestingly, the corticosterone levels even decreased significantly in both running and sedentary groups at the end of our study. Moreover, our study followed the mice until 8 months of age, whereas the study by Choi *et al*. followed the mice until 6 months of age. Hence, it is possible that exercise may have beneficial effects in this model when measured at an earlier timepoint but cannot counteract the pathology at more advanced stages. Additionally, Choi *et al*. do not investigate effects on neuroinflammation or anxiety in their study, so it is impossible to know how these aspects were affected. Unlike their beneficial effects, we continuously monitored hindlimb clasping in our study and observed that running accelerated the development of this pathological behavior. The reasons for the discrepancies between our study and the study presented by Choi *et al*. are not likely to be explained by the genetic background as they use the same background strain as the 5xFAD mice used in our study. Discrepancies between our studies are more likely to be attributable to differences in the running protocol. Our running mice had *ad libitum* access to running wheels in their home cage, whereas the mice in Choi *et al*. study were only allowed 3 hours of running per day when they were transferred to another cage for their exercise intervention. In addition, while we house our mice in pairs, their mice also seem to be singly housed, which may induce depression and, in turn, affect behavior of mice[49]. Thus, it is possible that running counteracts some of the negative effects caused by single-housing in that study.

To the best of our knowledge, the ability of exercise to aggravate AD pathology has not been reported before. Rather, a handful of studies, using other AD models, show no effects of exercise on cognition[30]. This may be due to publication bias since it is less likely for a study reporting primarily negative data to be accepted in respected scientific journals.

In addition to the above-mentioned limitations, our study includes other obstacles regarding the translation of our results to the clinic. First, animal models do not fully recapitulate all hallmarks of AD. Second, the 5xFAD model has a genetically driven, aggressive form of the pathology, whereas the majority of human AD cases are sporadic[50,51]. Hence, we cannot exclude the possibility of exercise to be protective for development of the sporadic forms of the disease in AD mice with a slower progression, modelling most of the cases seen in the clinic.

Conclusions

Our study shows that running exercise may not only lack protective effects on the development of the AD phenotype in 5xFAD mice but may also accelerate and aggravate it.

Methods

Animals. Animal experiments were approved by the Malmö/Lund animal ethics committee (2012, Dnr: M427-12) and performed in accordance with the Directive of the European Parliament. The setup of this study has been described before[24]. As single housing can affect behavior[49], we housed our mice in pairs. Since housing male mice together may induce aggressive behavior influencing the outcome as described previously[34], we only used females.

Briefly, we used 30 female 5xFAD mice on a C57Bl/6*SJL background, obtained from Jackson laboratories, aged 9–12 weeks at the beginning of the study. Mice were housed in pairs, and each pair was randomly assigned to one of two groups: mice with access to a running wheel ("running mice") or mice without access to a running wheel ("sedentary mice"). There was no significant difference in body weights between groups at the beginning and end of the study (Supplementary Table 1). The experimental outline can be seen in Fig. 6.

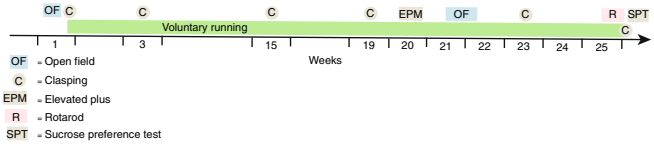

Figure 6. Experimental design. Mice had access to running wheels during experimental weeks 2–25. Before the introduction of exercise intervention, during week 1, Open Field test (OF) and Clasping test (C) were conducted. Clasping tests were then repeated in weeks 3, 15, 19, 23 and 26. During week 20 Elevated Plus maze tests (EPM) were conducted. During weeks 21–22, Open Filed tests were performed. During weeks 25–26 Rotarod tests (R) were conducted. During the last week, week 26, a sucrose Preference test (SPT) was performed before the mice were sacrificed (+) to collect brain, blood and CSF samples.

Voluntary wheel running exercise. At 9–12 weeks of age, mice were caged with (n = 16) or without (n = 14) running wheels for 24 weeks, until the end of the study. Running mice had *ad libitum* access to low-profile wireless running wheels (med-associates) in their home cage. The running distance was measured telemetrically to control that mice were running (Supplementary Fig. S1). Visual observation during the active period confirmed that running mice were significantly more active than sedentary mice in their home cages.

Behavioral tests. *Open field test.* In order to evaluate the locomotion and anxiety levels of the mice, the Open field test was conducted as described previously[34]. The test was performed one week prior to introducing the running wheels as well as after 19 weeks of voluntary wheel running. The mice were placed in an empty white box (45 × 45 cm) and allowed to freely explore it for 10 minutes. An automated behavioral system (SMART, Panlab, Barcelona, Spain) was used to measure the velocity of the movements, the distance traveled and the time spent in the center and periphery of the box. More time spent away from the center zone was regarded as a sign of anxiety. The box was cleaned with ethanol followed by water before each mouse was introduced to the Open field arena.

Clasping scoring. Throughout the study, hindlimb clasping behavior, a pathological motor reflex, was assessed regularly at six different time points (experimental weeks 1, 3, 15, 19, 23 and 26). The mice were held near the base of their tail and allowed to hang free for 30 seconds, during which the clasping behavior was recorded and scored. Clasping was scored using a scale between 0 and 3, where 0 represented no clasping (normal), 1 represented initial signs of clasping or only clasping of one hindleg for at least 50% of the time, 2 represented clasping of both hindlegs for at least 50% of the time, and 3 represented clasping of both hindlegs for nearly 100% of the time as described previously[52].

Elevated plus maze test. To examine exploratory and anxiety-like behavior, the mice were subjected to elevated plus maze test after 18 weeks of running. The elevated plus maze apparatus consisted of two open arms and two closed arms (29 × 6 cm). The entire maze was elevated about 40 cm from the floor. Each mouse was placed in the center of the maze with their head facing towards the open arm. During a 5-min test, the time spent in the open arms and the total distance traveled were recorded from above using the SMART system. A healthy mouse is curious and spend more time exploring the open arms, while a mouse with anxiety spends most of its time in the closed arms[53,54].

Rotarod test. To examine motor coordination and balance, mice were subjected to the rotarod test after 23 weeks of running. The rotarod apparatus (8200 model, Letica Scientific Instruments, LE, US) consists of a rotating spindle (3 cm diameter, 15 cm long base) with five individual, 3 cm-wide, compartments allowing for up to five mice to be tested simultaneously. Mice were placed on the rotating rod and tested by increasing the rotating speed from 4 to 40 rpm over 300 seconds. The mean time that a mouse remained on the rotarod was recorded and calculated from three trials. The mice were allowed to rest in their home cage for at least 45 min between trials. The mice were subjected to the rotarod test for three days in order to examine their motor learning abilities.

Sucrose preference test. The Sucrose preference test is described in Supplementary Methods.

Fecal corticosterone levels. Corticosterone measurements are described in Supplementary Methods.

Collection of samples. After 24 weeks of voluntary wheel running, mice were sacrificed to collect samples. The mice were anesthetized with isoflurane and CSF was collected from cisterna magna using a transparent glass capillary checking for no contamination of blood when mice were under deep anesthesia. CSF samples were snap-frozen immediately in dry ice and stored at −80 °C until analysis. Afterwards, the mice were euthanized and blood samples were collected through cardiac puncture. Blood samples were kept at room temperature for 25 min and then stored on ice for a few hours until the samples were centrifuged at 1300 g at 4 °C for 10 min. The serum supernatants were collected and stored at −80 °C until analysis. Mice were perfused with saline solution before the brains were removed. The right hemisphere was fixed in 4% paraformaldehyde in phosphate buffer for 24 hours before being stored in 30% sucrose solution at 4 °C until analysis. From the left hemisphere, the cerebellum, hippocampus and cortex were dissected, snap-frozen in dry ice and stored at −80 °C until analysis.

Immunohistochemistry. Sagittal brain sections (30 μm) were prepared from the right hemisphere as previously described[24].

Aβ plaques in cortex and hippocampus. Amyloid plaques were labeled with 0.5% Thioflavin S. Briefly, Thioflavin S was dissolved in ddH_2O and filtered through a 0.22 μm syringe filter. Sections were incubated during 5 min, rinsed for 3*10 min in PBS and mounted in aqueous mounting media. Three sections per brain (lateral 0.84–1.2 mm) were analyzed using an epifluorescence (Nikon Eclipse 80i microscope, Europe) microscope. The thioflavinS-positive plaques were counted in a 0.25 mm^2 area within regions of interest; dentate gyrus/CA4 in hippocampus and cortical layer 4 and 5 in the neocortex area above the lateral ventricle.

Microglia in hippocampus. Microglia were labeled with primary antibodies against Iba1 (rabbit, Wako, product nr 27981192, 1:750) and galectin-3 (goat, R&D, product nr AF1197, 1:1000) and secondary Alexa Fluor antibodies against rabbit (647 nm, Invitrogen, product nr A32795, 1:500) and goat (488 nm, Invitrogen, product nr A-11055, 1:500). Three sections per brain (lateral 0.84–1.2 mm) were imaged using an epifluorescence microscope (Nikon Eclipse 80i microscope, Europe). The immunofluorescence intensity was analyzed using ImageJ from 10x pictures of the dentate gyrus/CA4 in hippocampus.

Homogenization of brain tissue. The hippocampus was homogenized to extract proteins in three different fractions. The first fraction containing soluble proteins was extracted by grinding the tissue 20 times with a dounce homogenizer in 120 μl of TBS buffer (20 mM Tris-HCl, 137 mM NaCl, pH 7.6) containing protease and phosphatase inhibitors. The homogenate was incubated 30 min on ice before it was centrifuged at 14 000 g at 4 °C for 30 min after which the supernatant was collected. To obtain the second fraction containing the membrane-bound proteins, the remaining pellet was re-suspended in 120 μl of TBS with protease and phosphatase inhibitors and 1% Triton-X100. The suspension was incubated for 30 min on ice before it was centrifuged at 14 000 g at 4 °C for 30 min and the supernatant was collected. The third fraction containing insoluble protein aggregates, such as Aβ plaques, was obtained by re-suspending the remaining pellet in 120 μl of 70% formic acid. The suspension was then sonicated at an amplitude of 60% with repeating 10-second pulses followed by 10-second pause for a total of 2 minutes before it was centrifuged at 14 000 g at 4 °C for 30 min. The supernatant was neutralized 1:20 in 1 M Tris. Protein concentrations were determined (Pierce microplate BCA Protein Assay kit for the first and second fraction and the Pierce Coommassie Plus Assay kit for the third fraction). Samples were stored at −80°C until use.

Multiplex ELISA. Cytokine and Aβ ELISA are described in Supplementary Methods.

Western blotting. Protein levels of iNOS, galectin-3 and NLRP3 in the second fraction of homogenized hippocampus were measured by Western blot. Briefly, samples were loaded into 4–20% Mini-Protean TGX precast pels (Bio-Rad), then transferred to nitrocellulose membranes (Bio-Rad) using the Trans-Blot Turbo System (Bio-Rad). The membranes were then blocked with 3% casein (Sigma-Aldrich) diluted in PBS. After blocking, the membranes were incubated with primary antibodies against galectin-3 (1:3000, AF1197, R&D Systems), iNOS (1:500, SC650, Santa Cruz) and NLRP3 (1:1000, AG-20B-0014-C100, Adipogen) at 4 °C over night. The membranes were then incubated with peroxidase-conjugated secondary antibodies (1:5000, Vector Labs) and the blots were developed using Clarity Western ECL Substrate (Bio-Rad). Protein levels were normalized to beta-actin (1:10000, A3854, Sigma).

Statistical analyses. All statistical analyses were performed using SPSS version 22.0. Body weight and cytokine data was considered normally distributed and analyzed with student's T-tests. Data obtained from brain tissue stains and western blots were analyzed with Mann-Whitney U-tests. To compare the behavioral performance data between the sedentary and running groups, Mann-Whitney U-tests were used. To compare evolution of Rotarod and Clasping behavior over time within groups Friedman tests were used. For specific time-points of these tests, groups were compared with Mann-Whitney U-tests. To compare pre- and post-intervention of corticosterone levels Wilcoxon tests were used. P-values below 0.05 were considered statistically significant.

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Author contributions

M.S. was responsible for the experimental design, brain tissue collection, behavioral tests, brain homogenization, analysis of cytokines and Aβ levels with ELISA in brain, serum and CSF as well as manuscript writing. E.A. was responsible for the baseline Open field test, corticosterone ELISA, brain sectioning, staining and image analysis of Thioflavin S in hippocampus and cortex. O.M. performed immunohistochemical staining and image analysis of Iba1 and galectin-3 in hippocampus. Y.Y. performed the Western blot analysis. T.D. was responsible for the experimental design and collecting CSF and serum. All authors critically revised the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Voluntary running does not reduce neuroinflammation or improve non-cognitive behavior in the 5xFAD mouse model of Alzheimer's disease

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Supplementary Data

Supplementary Table 1- Body weights

	Sedentary (Mean±SD)	**Running** (Mean±SD)	**T-test (unpaired) Sedentary vs. Exercised**
Start Weight (g)	16.8±1.1	16.1±1.3	P=0.14
Final Weight (g)	23.4±1.3	22.6±3.0	P=0.35
Weight gain (%)	40±11	40±16	P=0.98

Supplementary Table 2- Corticosterone levels in feces (ELISA)

	Sedentary Median (IQR)	**Running** Median (IQR)	**Mann Whitney U-test Sedentary vs. Running**
Baseline levels (pg/ml)	2617 (1699-4455)	2167 (1644-4053)	P=0.66
After 19 weeks (pg/ml)	1523 (1331-2205)	1506 (1237-1722)	P=0.85
Wilcoxon test Baseline vs. After 19 weeks	P=0.001	P=0.02	

Supplementary Table 3- distance traveled in EPM and OF

	Sedentary Median (IQR)	**Running** Median (IQR)	**Mann Whitney U-test Sedentary vs. Running**
Distance moved Elevated plus maze	1048 (741-1357)	1278 (964-1616)	P=0.23
Distance moved Open field	4569 (3115-5561)	4017 (3746-5568)	P=0.93

Supplementary Table 4- Sucrose Preference

	Sedentary Median (IQR)	**Running** Median (IQR)	**Mann Whitney U-test** P-value
Sucrose Preference (%)	79.6 (76.4-86.1)	79.7 (73.2-88.4)	1.0

Supplementary Table 5- Aβ levels (ELISA)

	Different Aβ species	**Sedentary** concentration (ng Aβ /mg protein) Median (IQR)	**Running** concentration (ng Aβ /mg protein) Median (IQR)	**Mann Whitney U-test** p-values prior to Bonferroni correction
Insoluble fraction in hippocampus	Aβ-38	164.5 (131.5-322.4)	255.3 (147.8-303.4)	0.57
	Aβ-40	1048 (712.4-1286)	1194 (989.7-1526)	0.35
	Aβ-42	8007 (5909-10173)	9612 (4285-11546)	0.78
CSF (n=7+7)	Aβ-38	0.59 (0.54-0.88)	0.44 (0.19-0.54)	0.21
	Aβ-40	3.87 (2.17-4.92)	2.28 (0.76-3.23)	0.32
	Aβ-42	1.81 (1.18-2.46)	1.25 (0.35-1.68)	0.32

Supplementary Table 6- Iba1 and gal-3 in hippocampus (immunohistochemistry)

	Sedentary Median (IQR) Fold to actin %	**Running** Median (IQR) Fold to actin %	**Mann Whitney U-test** P-value
Galectin-3 (n=6+6)	116 (84-125)	114 (102-127)	0.94
NLRP3 (n=3+4)	22 (18-33)	17 (16-20)	0.63

Supplementary Table 7- Cytokine levels (ELISA)

	Cytokine	**Sedentary** Mean concentration (pg/ml) ± SD	**Running** Mean concentration (pg/ml) ± SD	**T-test** P-values prior to Bonferroni correction
Hippocampus	IL-1 β	1.29 ± 0.7	1.34 ± 0.8	0.85
	IL-2	0.022 ± 0.01	0.029± 0.02	0.26
	IL-4	0.045 ± 0.02	0.052 ± 0.04	0.58
	IL-5	0.008 ± 0.003	0.010 ± 0.005	0.26
	IL-6	0.45 ± 0.3	0.68 ± 0.7	0.27
	IL-10	0.15 ± 0.09	0.15 ± 0.07	0.99
	IL-12p70	1.57 ± 0.8	1.78 ± 0.9	0.52
	IFNγ	Below detection	Below detection	
	TNFα	0.067 ± 0.02	0.082 ± 0.06	0.40
	KC/GRO	2.75 ± 1.9	2.94 ± 2.6	0.82
Serum	IL-1 β	0.27 ± 0.2	0.20 ± 0.2	0.37
	IL-2	0.12 ± 0.1	0.13 ± 0.1	0.84
	IL-4	Below detection	Below detection	
	IL-5	1.48 ± 1.3	1.68 ± 1.0	0.64
	IL-6	3.25 ± 2.6	3.59 ± 4.2	0.80
	IL-10	7.14 ± 3.9	6.14 ± 3.6	0.48
	IL-12p70	Below detection	Below detection	
	IFNγ	0.33 ± 0.1	0.20 ± 0.1	0.06
	TNFα	3.66 ± 1.0	4.17 ± 1.8	0.38
	KC/GRO	34.14 ± 29.8	23.52 ± 10.2	0.22

Supplementary Figure S1- Distance in running wheels

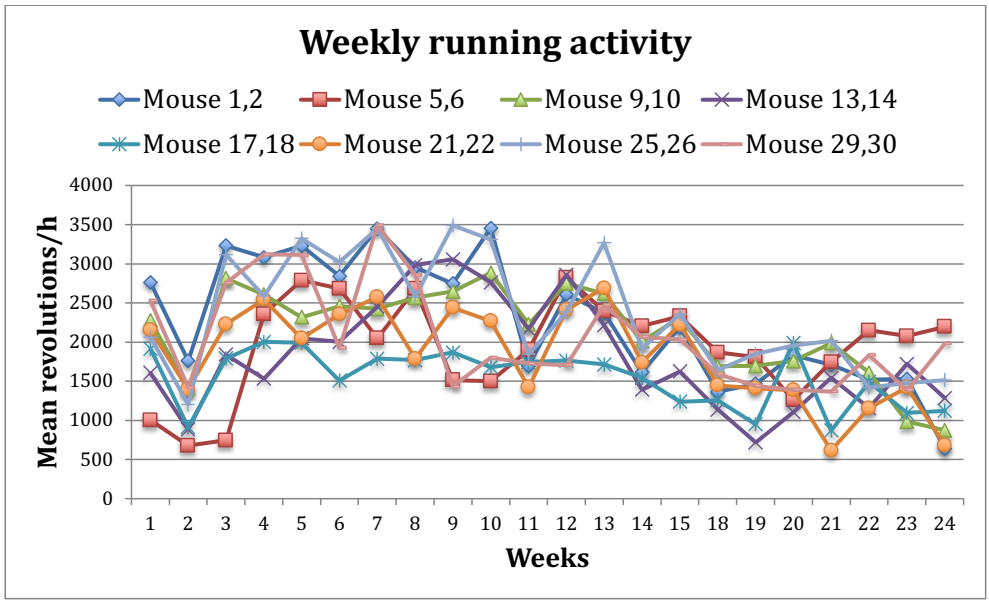

Figure S1. Running activity displayed as mean revolution per hour for each week and each couple sharing a running wheel in their home cage. Each revolution corresponds to a running distance of around 42 cm. ANOVA repeated measurements showed a change in the amount of running over time ($p<0.001$) and a paired T-test comparing the running during the first week with that of the last week of intervention revealed a trend towards decreased running over time (means±SD were 2039±549 revolutions/h during the first week compared to 1288±578 revolutions/h during the last week, paired T-test, $p=0.07$).

Supplementary Methods

Sucrose preference test

To assess anhedonic behavior, a Sucrose preference test was performed during the night before sacrifice. Mice were introduced to a sucrose solution in their home cages one night before the test. A bottle containing 2% sucrose solution was put in the place where the regular bottle with tap water used to be during the night. The regular bottle with tap water was placed in the other corner of the cage, allowing the mice to choose. The day before the test, mice were deprived from drinking five hours prior to the test. Later, mice were individually caged with access to nesting material, food pellet, as well as two bottles, one tap water and one sucrose solution as described

before [1]. Bottles were weighed before and after the test and the volume consumed was calculated. A sucrose preference index was calculated using the following formula: *Sucrose preference index=weight of consumed sucrose/total weight consumed of both solutions*

Multiplex ELISA

Cytokine ELISA

The concentrations of different cytokines in serum as well as in the pooled first and second fraction of homogenized hippocampus (25 µl/sample) were measured with the MSD Mouse Proinflammatory V-Plex Plus Kit (IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, CXCL1, TNFα; K15012C, Mesoscale) using a QuickPlex SQ120 (Mesoscale Discovery, Rockville, USA) Plate Reader according to the manufacturer's instructions. The recorded data was analyzed using MSD Discovery Workbench software. For the brain homogenate samples, the cytokine concentrations were normalized to the total protein concentrations measured in the BCA or Bradford assay.

Aβ ELISA

The concentration of different Aβ species in the insoluble fraction of homogenized hippocampus as well as in the CSF were measured with the MSD MULTI-SPOT Human (4G8) Aβ Triplex Assay (Aβ38, Aβ40 and Aβ42; K15199G-1, Mesoscale) using QuickPlex SQ120 (Mesoscale Discovery, Rockville, USA) Plate Reader according to the manufacturer's instructions. The recorded data was analyzed using MSD Discovery Workbench software. For the brain homogenate samples, Aβ concentrations were normalized to total protein concentrations measured in the BCA or Bradford assay.

Fecal corticosterone levels

Fecal samples were collected from the Open field arena after conducting the Open field test in order to measure the stress levels of the mice. The feces were stored at -80°C until use. Corticosterone was then extracted and analyzed with a corticosterone ELISA kit (Enzo Life Sciences) described by Touma et al.[2] except that feces was homogenized in 1 ml of 80% Methanol per 100 mg sample, as we have done before[3].

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Paper IV

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Acute systemic LPS-exposure impairs perivascular CSF distribution in mice

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Abstract

Background: The exchange of cerebrospinal (CSF) and interstitial fluid is believed to be vital for waste clearance in the brain. The sleep-dependent glymphatic system, which is comprised of perivascular flow of CSF and is largely dependent on arterial pulsatility and astrocytic aquaporin-4 (AQP4) expression, facilitates much of this brain clearance. During the last decade, several observations have indicated that impaired glymphatic function goes hand in hand with neurodegenerative diseases. Since pathologies of the brain carry inflammatory components, we wanted to know how acute inflammation, e.g., with lipopolysaccharide (LPS) injections, would affect the glymphatic system. In this study, we aim to measure the effect of LPS on perivascular CSF distribution as a measure of glymphatic function.

Methods: Three hours after injection of LPS (1 mg/kg i.p.), C57bl/6 mice were (1) imaged for two CSF tracers, injected into cisterna magna, (2) transcardially perfused with buffer, or (3) used for physiological readouts. Tracer flow was imaged using a low magnification microscope on fixed brains, as well as using vibratome-cut slices for measuring tracer penetration in the brain. Cytokines, glial, and BBB-permeability markers were measured with ELISAs, Western blots, and immunohistochemistry. Cerebral blood flow was approximated using laser Doppler flowmetry, respiration and heart rate with a surgical monitor, and AQP4-polarization was quantified using confocal microscopy of immunolabeled brain sections.

Results: LPS-injections significantly lowered perivascular CSF tracer flow and penetration into the parenchyma. No differences in AQP4 polarization, cytokines, astroglial and BBB markers, cerebral blood flow, or respiration were detected in LPS-injected mice, although LPS did elevate cortical Iba1^{+} area and heart rate.

Conclusions: This study reports another physiological response after acute exposure to the bacterial endotoxin LPS, namely the statistically significant decrease in perivascular distribution of CSF. These observations may benefit our understanding of the role of systemic inflammation in brain clearance.

Keywords: LPS, CSF, Microglia, Glymphatic System, AQP4, Inflammation

Introduction

Lipopolysaccharides (LPS) are molecules found in the outer membrane of Gram-negative bacteria, and are frequently used in many animal models to mimic inflammatory responses, e.g., in sepsis, depression, and neurodegeneration [1–3]. In 2012, Erickson et al. showed that repeated systemic LPS-exposure lowered bulk flow of CSF, and impaired amyloid-β clearance [4]. The authors thus hypothesized that systemic inflammation could contribute to Alzheimer's disease [4]. The bulk flow of cerebrospinal fluid (CSF) along periarterial spaces, to be exchanged with interstitial fluid, has since 2013 been known as the glymphatic system [5]. Thereafter, many publications have implicated this sleep-dependent system [6] in the clearance of neurodegenerative protein aggregates [7, 8], but none (to our knowledge) have imaged the direct effect of an

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inflammatory stimuli on the periarterial CSF-movement. Elucidating how inflammation impairs flow of CSF could give valuable insight into the pathology of common neurodegenerative diseases. Imaging CSF-tracers after Cisterna magna-injections is now a standard method of tracking CSF movement [6, 9, 10]. We used this method to assess the acute effect of a single systemic LPS injection on perivascular CSF dynamics in the mouse. In addition, important facilitators of glymphatic function such as AQP4 polarization and cortical blood flow were measured.

Materials and methods

Animals

All procedures, handling, and housing in this study were carried out in accordance with the international guidelines on experimental animal research and were approved by the Malmö-Lund Ethical Committee for Animal Research in Sweden (M250-11, M30-16, Dnr 5.8.18-01107/2018). Male C57BL/6/N mice (age 12 weeks and weight between 23 and 32 g) were acquired from Janvier Labs (France). Mice were housed according to the regulations of Lund University and compliant with the international guidelines on experimental animal research. Mice were group-housed in 12 h dark/light cycle at a stable temperature with access to water and chow, ad libitum.

Drugs

LPS (1 mg/kg, Sigma, catalog no. L4516, lot 014M4107V or 01M4049V) or its vehicle (ddH_2O, volume corresponding to that of LPS-injected mice, i.e., 4 μL/g body weight) were injected intraperitoneally, 3 h before (1) the injection of the CSF-tracers into the cisterna magna (LPS n = 18, vehicle n = 15), (2) transcardial perfusion (LPS n = 5, vehicle n = 5), or (3) experiments with physiological readouts (LPS n = 4, vehicle n = 5).

Anesthesia was administered before cisterna magna injections as a mixture of racemic ketamine (Ketaminol®, catalog no. 511519, 100 mg/kg) and xylazine (Rompun®, catalog no. 022545, 10 mg/kg) in 0.9 % saline, referred to as KX, intraperitoneally (i.p.).

Animals for physiological readouts were anesthetized with KX and were redosed with ketamine (50 mg/kg) 1 h after initial dose. Two hours after initial dose, mice were given a third of the initial KX dose, and another ketamine dose (20 mg/kg) after 3 h. Ketamine redosing, as opposed to using more conventional anesthetics, was chosen as not to mix anesthetics.

Cisterna magna CSF tracer injection

The fluorescent CSF tracers (Alexa-647 conjugated 66 kDa-BSA and FITC-conjugated lysine-fixable 3 kDa-dextran, ThermoFisher) were dissolved together in 0.9% saline at a concentration of 0.5% (w/v).

LPS-treated and vehicle mice were put under general anesthesia and placed in a stereotaxic frame. The cisterna magna was surgically exposed and a 30G needle connected to a 50 μL Hamilton syringe through a polyethylene tube (I.D. 0.28 mm) was inserted in the cisterna magna. Ten microliters of CSF tracer were injected at a rate of 1 μL/min for 10 min with a Harvard apparatus pump. After 30 min circulation time, mice were decapitated and brains were quickly removed and immersed in 4% paraformaldehyde (Histolab), PFA, overnight.

Seven animals in total were removed from glymphatic measurements, due to death during anesthesia, or cerebellar injections, and have not been included in this communication, giving us n = 13 for the LPS group and n = 14 for the vehicle animals (however, mice with cerebellar injections were still used for later immunohistochemistry).

Brains were imaged using a × 0.5 objective on a SMZ25 Stereo Microscope (Nikon) and tracer distribution was quantified using ImageJ software. Briefly, areas corresponding to cerebellum, cortex, olfactory bulb, and ROI confined to the perivascular space of the middle cerebral artery (MCA area) were outlined with the polygon tool in ImageJ, and fluorescent intensities were measured. Brain coronal slices (200 μm) were sectioned with a vibratome and imaged with a Nikon ECLIPSE Ti2 microscope at × 4 magnification. Tracer penetration was measured in slices using a Fiji [11] macro developed by SciLifeLab Uppsala (anna.klemm@it.uu.se, Supplementary materials) which uses the PerObjectEllipsefit plugin [12], to automatically detect the slice and calculate the threshold and then measures the mean intensity of the slice.

Laser Doppler and physiological readout

Mice were anesthetized with KX, as described above, and placed on a surgical monitor platform (Harvard apparatus, catalog no. 75-1500, Holliston, USA) fitted with a heating pad and sensors for temperature (kept stable at 37 °C), heart, and respiration rate. Cortical Laser Doppler (Perimed, PeriFlux System 5000, Stockholm, Sweden) flowmetry was measured by surgically exposing the right hemisphere skull and gluing (Loctite, EAN 5010266241173) an 0.5 mm optical filament (Perimed, product no MT B500-0L120), connected to the probe (Ellab, product no PRA22002A275M), on the skull close to the MCA. Once a stable Laser Doppler value could be recorded, LPS (n = 4) or vehicle (n = 5) mice were injected intraperitoneally, and measurements were recorded every 15 min for 165 min, and then animals were sacrificed. One out of four LPS-treated mice, and two out of the five controls died during anesthesia, and their measurements were thus disregarded from our analyses, giving us n = 3 for both groups in the LD/physiological experiments.

Immunohistochemistry and analysis

Cortical slices (200 μm) used in CSF tracer imaging were also used for immunohistochemical analysis. For IgG extravasation measurements, we used 35 μm cryotome sections from transcardially perfused brain hemispheres. For the immunolabeling with AQP4/Glut1, slices were first rinsed in phosphate-buffered saline (PBS), followed by 3 h block in Normal donkey serum 10% (v/v) in PBS with Tween-20 (Sigma, 0.25% v/v), PBS-T20. Primary antibodies were incubated for 72 h at 4 °C in blocking solution. For immunolabeling, sections were immunoreacted with primary antibodies for AQP4 (rabbit, Merck Millipore, catalog no. AB3594, 1:300) and GLUT1 (mouse, Merck Millipore, catalog no. MABS132, 1:350). After the primary incubation, slices were rinsed 1 h in PBS-T20, followed by blocking solution for 15 min, then 3-h incubation of secondary Alexa Fluor antibodies against rabbit (488 nm, Invitrogen, catalog no. A-11055, 1:500) and mouse (568 nm, Invitrogen, catalog no. A-10037, 1:500) in blocking solution. After rinsing in PBS 3 × 5 min, the samples were air-dried and mounted with Diamond Antifade Mountant (ThermoScientific, Sweden). In the 200 μm slices with successful staining, a total of 13 LPS-treated mice and 14 controls were represented and analyzed. The polarization of AQP4 along vessels in tissue was imaged and quantified blindly in the cortex, as described before [10, 13, 14]. Six different blood vessels per animal from comparable regions of the cortex were selected at random and imaged with a Nikon Confocal A1RHD microscope at ×40 magnification. For AQP4 polarization quantification, a cross-sectional line was drawn using the line plot tool in ImageJ and centered on the blood vessel in order to include both AQP4 signal from the vascular endfeet and signal from the parenchyma. The AQP4 polarization was calculated by averaging the peak intensity of the AQP4 signal in the vascular endfeet divided by the average of the parenchymal fluorescence signal, and differences between groups were assessed with an unpaired *t* test.

For the staining against Iba-1, the slices (successfully stained controls $n = 12$, LPS $n = 14$) were first rinsed in PBS, followed by 1 h quench in 1% peroxide dissolved in PBS. After washing with PBS, slices were blocked with glycine (Sigma, 0.5 M) in PBS for 1 h, and then washed with PBS and later PBS Triton X100 (Sigma, 0.5% v/v), PBS-TX, for 30 min. For immunolabeling, the primary antibodies against Iba1 (rabbit, Wako, catalog no. 27981192, 1:20000) was applied for 48 h at 4 °C in PBS-TX. After the primary incubation, slices were rinsed 2 × 30 min in PBS-TX, followed by 2.5-h incubation of secondary antibodies (biotinylated anti-rabbit IgG (Vector), 1 μg/L) in PBS-TX. After rinsing in PBS-TX 2 × 30 min, the samples were treated with ABC elite reagent (Vectastain, catalog no. PK-7100) 1 h, then rinsed in PBS-TX, and thereafter treated with DAB with metal enhancer (Sigma, catalog no. D0426). The reaction was stopped with PBS after 5 min, and slices were mounted on microscope slides, rinsed shortly with Xylene (Sigma) and subsequently mounted with DPX (Sigma). Slices were scanned (Hamamatsu digital slide scanner) and Iba1-positive cells were manually counted in 3 random fields of cortical images in 2 sections (at bregma 0 and bregma − 2 mm) from each animal. Image analysis (Fiji) using the cell-counter plugin were made blinded to assess changes in microglial cell density. In addition, in order to estimate the $Iba1^+$ area, we blindly and manually thresholded images to omit the background and measured the $Iba1^+$ area fraction in Fiji. For IgG extravasation measurements, the perfused and fixed 35 μm slices were first rinsed in PBS, followed by 2 h block in bovine serum albumin 10% (v/v) in PBS-T20, incubated overnight in 4° with secondary Alexa Fluor antibodies against mouse (488 nm, Invitrogen, catalog no. A-21202, 1:500) in blocking solution. After rinsing in PBS 3 × 5 min, the samples were air-dried and mounted with Diamond Antifade Mountant (ThermoScientific, Sweden), and then imaged with a Nikon ECLIPSE Ti2 microscope at ×20 magnification. IgG intensity was measured in cortical areas corresponding to around bregma 0 mm using Fiji.

Western blot and cytokine ELISA

LPS-treated ($n = 5$) and control mice ($n = 5$) were transcardially perfused under deep anesthesia with PBS. The brains were removed, snap frozen, and homogenized in RIPA buffer (Sigma-Aldrich, Germany) with proteinase and phosphatase inhibitors (Roche, Switzerland). Later, protein concentration was measured using a BCA kit according to the manufacturer's protocol (BCA Protein Assay-Kit, ThermoScientific, Sweden), and mixed with 2× LAEMMLI buffer (Bio-Rad, Sweden) and boiled at 95 °C for 5 min. Each well was loaded with 10 μg protein and was separated by SDS-PAGE using pre-cast gels (4–20%, Bio-Rad) in TGS buffer (Bio-Rad, Sweden). For AQP4 blotting, 2 ng of control antigen was diluted in RIPA, mixed with LAEMMLI as the other samples, and loaded together with the protein samples. The proteins were transferred to nitrocellulose membranes (Bio-Rad, Sweden) using the TransBlot Turbo system from Bio-Rad. The membranes were washed 1× with PBS, and subsequently blocked for 1 h with skim milk at 3% (w/v) in PBS 0.1% (w/v) Tween 20 (PBS-T), then washed 3 × 10 min in PBS-T. Then, blots were incubated with primary antibodies in PBS-T overnight in 4 °C, for AQP4 (rabbit, Merck Millipore, AB3594) 1:1000, GFAP (goat, Santa Cruz, catalog no. SC-6170) 1:5000, and Galectin-3 (goat, R&D, catalog no. AF1197) 1:1000.

After washing as abovementioned, the membranes were incubated with peroxidase-conjugated secondaries (Vector, 1:5000) for 2 h RT. After washing 3 × 10 min with PBS-T, the blots were developed using Super Signal West Femto Sensitivity Substrate (ThermoScientific, Sweden) or ECL Clarity (Bio-Rad) according to the manufacturer's protocol and imaged using the ChemiDoc XRS system from Bio-Rad. For AQP4, blots were imaged using the ChemiDoc XRS+ system (Bio-Rad). After developing the primary blot, actin conjugated with secondary (Sigma, catalog no. A3854), 1: 10000 in PBS-T for 20 min, then washed and developed as above. Band intensities were measured in Image Lab software (Bio-Rad) and normalized to actin levels.

MesoScale (MSD) plates were used to evaluate the cytokine levels (proinflammatory panels for IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, and TNF-α) from cortical together with hippocampal homogenates, dissected from both perfused and tracer-injected frozen hemispheres. Analyses were carried out according to protocol, as described before [15].

Statistical analyses

Tracer experiments were made in two cohorts, 6 months apart, where measurements from the second cohort of controls animals (n = 7) were normalized to the first batch of controls (n = 6). The fluorescence intensity adjustment index from the controls was then applied to the second batch of LPS mice as well (n = 7).

For the arbitrary measurement values of Laser Doppler, values were decimalized (baseline set to 100). For analysis of heart rate, respiration, and temperature, values were instead normalized to the average baselines.

Datasets were assessed for normality, where possible (N too low in Western and IgG assays), with D'Agostino and Pearson tests. All sets passed normality testing. Thus, student's t tests were used for comparisons of whole-hemisphere tracer, tracer penetration per animal, AQP4, Iba1, GFAP, galectin-3, IgG, and IL-10 comparisons. For comparison of tracer penetration per animal, we averaged tracer intensity from all slices per animal, decimalized all values, and set the vehicle means to zero—this way treatment differences could be understood as percentages of controls. Differences in tracer area penetration across different brain levels were evaluated with a two-way ANOVA, using multiple comparisons with a Šidák correction. Differences in cortical blood flow, respiration, and temperature were determined with two-way ANOVA. For analysis of heart rate, we used a mixed-effects model (because of missing values for two timepoints in one animal) to assess differences after LPS-exposure compared to baseline.

Statistical analyses were all performed in GraphPad Prism version 8.4.2. P values ≤ 0.05 were considered significant; however, differences were also described with 95% confidence intervals to illustrate treatment effects.

Results

Cisternae Magna injections

LPS injection decreases distribution of CSF tracer in the brain

We assessed the effect of LPS to CSF flow in the brain by injecting two tracers (Alexa-647 conjugated BSA (66kDa) and FITC-conjugated lysine-fixable dextran (3 kDa)) into the cisterna magna, from where they could distribute for 30 min before collection of brains.

Stereoscopic examination revealed tracer distribution in cerebellum, general cortex, and olfactory bulb (measurement areas in Fig. 1a). Cortical distribution corresponded to area around the MCA. In LPS-treated mice, cortical tracer signal, measured as mean pixel intensity (MPI), was lower (although not quite significantly so in BSA-Alexa 647), compared to control animals (unpaired t test, 95% CI of difference = − 48 to 0.57 and − 35 to − 2.6, respectively; Fig. 1e, f). When we only measured an area corresponding to the perivascular space around the MCA, thereby omitting proximity to the injection site, tracer signal differed even more between treatment groups (31% and 26% lower in the two tracers, unpaired t test, 95% CI of difference = − 57 to − 5.8 and − 43 to − 8.9, respectively; Fig. 1g, h). Tracer intensity in olfactory bulb and cerebellum were not significantly different between groups (Fig. 1c, d, i, j).

We then collected six 200-μm-thick coronal sections of the brains at bregma 2, 1, 0, − 1, − 2, − 2.7 mm, which exhibited tracer influx along cortical arterioles. LPS-treated mice showed a lower BSA Alexa 647 and FITC dextran tracer distribution in the brain, compared to control animals (two-way ANOVA, P = 0.0002 and P = 0.018, respectively; Fig. 2a–c). At bregma, the decrease was most pronounced with 25% lower BSA Alexa 647 tracer penetration in the LPS-treated mice (95% CI = − 48 to − 2.2; Fig. 2b). On average, tracer penetration was 16% (BSA Alexa 647) and 18% (FITC dextran) lower in LPS animals (albeit not significant for the FITC-measurement; unpaired t tests, 95% CI of difference = − 30 to − 2.8, and − 51 to 16 respectively; Fig. 2d, e).

Taken together, observations from CSF tracer experiments suggests that LPS decreases perivascular flow of CSF, as well as penetration of CSF into the brain parenchyma.

Surgical monitoring

LPS-exposure changes heart rate, but not cerebral blood flow, respiration, or core temperature

Because physiological parameters such as cerebral blood flow, heart rate, and respiration have been linked to CSF movement in the brain [16–20], we wanted to measure these variables using laser Doppler flowmetry and

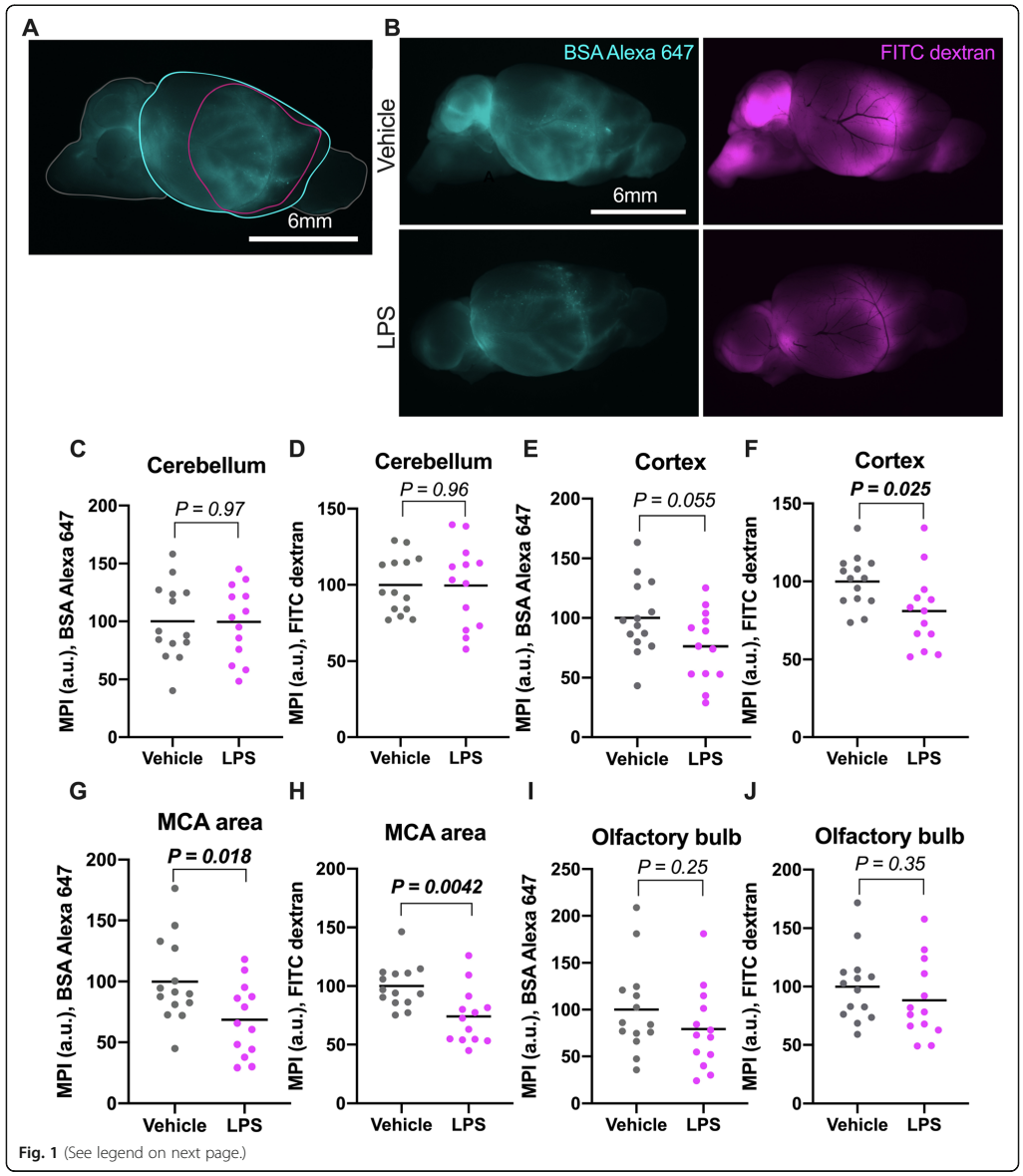

Fig. 1 (See legend on next page.)

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Fig. 1 CSF-tracer intensity 3h after systemic LPS-injection (n = 14), compared with vehicle (n = 13). **a** Color coded areas used for mean fluorescence intensity measurements in ImageJ. Left (grey) cerebellum. Middle (magenta), MCA area (ROI confined to the perivascular space of the MCA). Middle (cyan), cortex. Right (grey), olfactory bulb. Scale bar 6 mm. **b** Representative tracer (BSA Alexa 647 and FITC dextran) images for Vehicle and LPS-groups. **c**–**j** Statistical analyses of mean pixel intensity in arbitrary units, MPI (a.u.). **c** MPI (a.u.) in area corresponding to cerebellum, BSA Alexa 647. Unpaired t test, 95% CI of difference = − 26 to 25. **d** MPI (a.u.) in area corresponding to cerebellum, FITC dextran. Unpaired t test, 95% CI of difference = − 19 to 18. **e** MPI (a.u.) in whole cortical brain area, BSA Alexa 647. 24% lower in LPS-mice, albeit not significantly so. Unpaired t test, 95% CI of difference = − 48 to 0.57. **f** MPI (a.u.) in cortical brain area, FITC dextran is 19% lower in LPS-treated animals. Unpaired t test, 95% CI of difference = − 35 to − 2.6. **G:** MPI (a.u.) in MCA area, BSA Alexa 647 was 31% lower on average in LPS-mice. Unpaired t test, 95% CI of difference = − 57 to − 5.8. **h** MPI (a.u.) in MCA area, FITC dextran. LPS 26% lower on average. Unpaired t-test, 95% CI of difference = − 43 to − 8.9. **i** MPI (a.u.) in area corresponding to olfactory bulb, BSA Alexa 647. Unpaired t test, 95% CI of difference = − 57 to 16. **j** MPI (a.u.) in area corresponding to olfactory bulb, FITC dextran. Unpaired t test, 95% CI of difference = − 37 to 14. Mean pixel intensity in arbitrary units, MPI (a.u.). Data are shown as mean and plotted individual samples, with P values above comparative brackets. Scale bars 6 mm

surgical monitoring after LPS-injections. Cortical blood flow measurements with laser Doppler showed no significant differences after acute LPS treatment, tested with two-way ANOVA (Fig. 3a). Simultaneously, we recorded respiration, temperature, and heart rhythm using a surgical monitor. During the several-hour long anesthesia, curves from LPS and control mice were stable and changed similarly, most likely because of additional ketamine injections (Fig. 3b, c). Heart rate, however, was significantly increased within 3 h of systemic LPS-administration (two-way ANOVA, 95% CI of difference = 0.09 to 28; Fig. 3d).

These results indicate that within 3 h of systemic exposure, LPS does not affect cerebral blood flow or respiration, but might affect heart rate.

Immunohistochemistry, Western blot, and ELISA

LPS does not affect astrocytic AQP4 and GFAP within 3 h

Since glymphatic function is tightly linked to AQP4 expression and astrocytes, we wanted to measure if LPS changed AQP4 expression in astroglial endfeet and expression of general astrocyte marker GFAP. Immunofluorescence in cortical and hippocampal areas revealed AQP4 to be expressed in astrocytic endfeet localized around blood vessels (Fig. 4a). AQP4 polarization, defined as vessel intensity to parenchyma ratio, did not significantly differ between LPS and control animals (unpaired t test, 95% CI of difference = − 0.67 to 1.8; Fig. 4b).

Immunoblotting of AQP4 and GFAP in cortical and hippocampal homogenates showed the presence of a band at 38 kDa and GFAP at 52 kDa, respectively, with no detectable difference between LPS and control mice (unpaired t test, 95% CI of difference = − 0.032 to 0.044, Fig. 4c, d; − 0.26 to 0.91, Fig. 4e, f).

LPS changes microglia morphology, but not microglial numbers or expression of galectin-3 after 3 h

In cortical and hippocampal areas, Iba1 antibody labeled microglial cells and their ramified processes. No significant differences were found in cell numbers between LPS and control mice quantified from cortex (unpaired t test, 95% CI of difference = − 36 to 150; Fig. 5a, b). However, the Iba1$^+$ area was larger in the LPS mice compared to controls (unpaired t test, 95% CI of difference = 0.078 to 17; Fig. 5c).

Immunoblotting of galectin-3 (a marker for activated microglia) in cortical and hippocampal homogenates showed the presence of a band at 30 kDa with no difference between LPS and vehicle-treated mice (unpaired t test, 95% CI of difference = − 1.5 to 0.56, Fig. 5d, e).

LPS does not change BBB permeability markers and cytokines within 3 h

Measuring the tight junction protein claudin-5 with immunoblotting in cortical and hippocampal homogenates did not reveal any significant differences between treatment groups (unpaired t test, 95% CI of difference = − 0.32 to 0.12; Fig. 5f, g). Analysis of blood brain barrier permeability with IgG in cortical areas showed labeling in cerebral vessels in both vehicle and LPS injected animals with no statistical differences in intensity (unpaired t test, 95% CI of difference = − 12 to 7.1, Fig. 5h, i).

We measured neuroinflammatory cytokine expression 3 h after systemic LPS injection. Almost no signals were given for the 10 proinflammatory analytes (IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, and TNF-α). Eight samples were above detection threshold, and only for IL-10. These eight samples, five vehicle controls and three LPS-treated brain homogenates, showed no group differences (unpaired t test, 95% CI of difference = − 0.31 to 0.26, Fig. 5j).

Altogether, our experiments of acute systemic LPS exposure did not generate detectable changes with regards to astrocytes, BBB permeability markers, and neuroinflammatory cytokines. We did, however, observe an increase in Iba1$^+$ area, indicating microglial reactivity [21].

Discussion

This study shows significantly decreased perivascular CSF tracer flow as early as 3 h after systemic exposure to LPS in male mice. The reduction was also visible in analysis of CSF tracer penetration into brain parenchyma. This rapid response upon LPS challenge on perivascular flow seems to occur earlier than we can detect

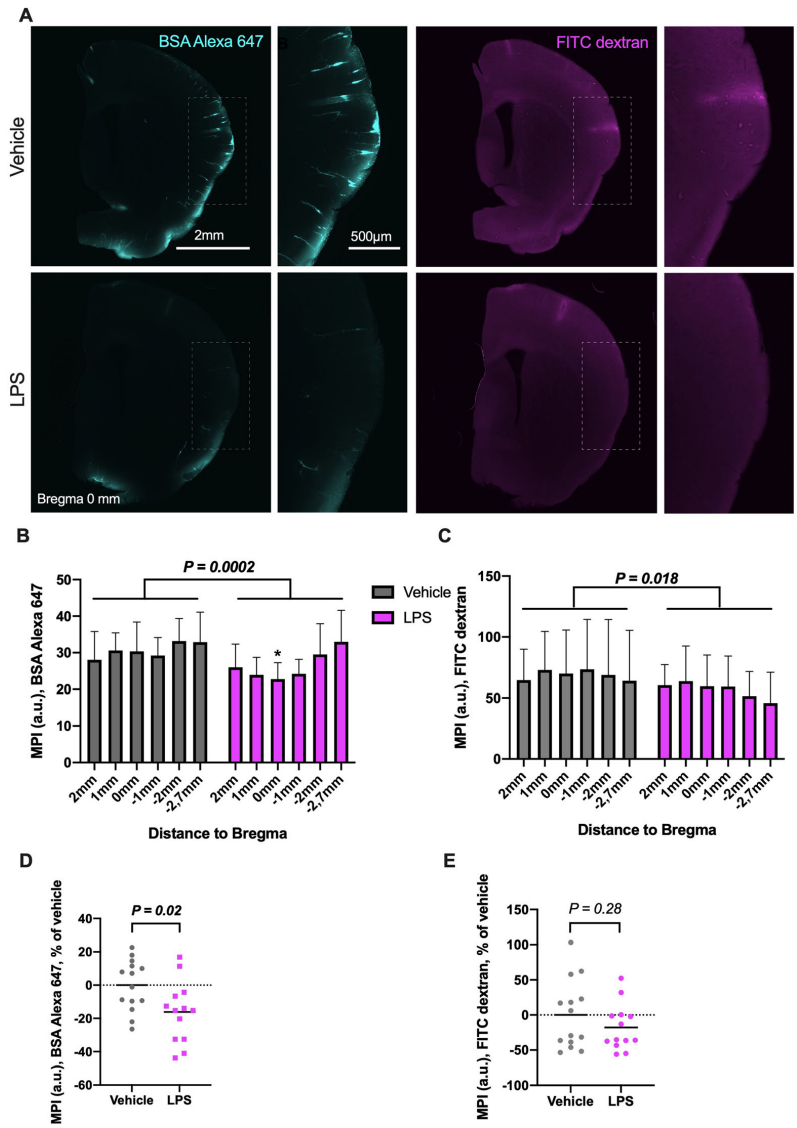

Fig. 2 (See legend on next page.)

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Fig. 2 CSF-tracer penetration in coronal slices 3 h after systemic LPS-injection (n = 14), compared with vehicle controls (n = 13). **a** Tracer intensity in coronal slices, at bregma 0 mm. FITC dextran, being a much smaller and not as easily fixed molecule compared to the previous tracer, appears blurrier in slice images. **b** Two-way ANOVA, BSA Alexa 647 mean pixel intensity in arbitrary units, MPI (a.u.), treatment difference P = 0.0002. At bregma 0 mm, tracer signal was significantly lower in LPS-mice, multiple comparison with Šidák correction. **c** Two-way ANOVA, area of FITC dextran MPI (a.u.), treatment difference P = 0.018. **d** Average BSA Alexa 647 tracer MPI (a.u.) per animal, normalized to vehicle mean. LPS 16% lower on average. Unpaired t test, 95% CI of difference = − 30 to − 2.8. **e** Average FITC dextran tracer MPI (a.u.) per animal, normalized to vehicle mean. LPS 18% lower on average, albeit not significantly so. Unpaired t test, 95% of difference = − 51 to 16. Mean pixel intensity in arbitrary units, MPI (a.u.). X-axis indicates distance to bregma. Means are shown as staples with error bars = SD (**c**, **d**), as well as mean with plotted values (**e, f**). Scale bars 2 mm and 500 μm. P values above comparative brackets. * indicate multiple comparison difference P < 0.05

an effect on many inflammatory readouts such as astrocytic response and a rise in cytokine levels.

These results may be relevant for several reasons. A decreased flow of CSF has been implicated in many diseases of the brain, such as Alzheimer's disease, traumatic brain injury, and cerebral small vessel disease [4, 22–24]. Non-cleared aggregates are often highlighted in this correlation, but impaired CSF flow and neurodegeneration might also be linked by inflammation. In fact, a link between the CSF and the peripheral immune system has previously been described by Louveau et al. (2018), showing that the drainage of CSF and immune cells into cervical lymph nodes through lymphatic vessels is key for the development of experimental autoimmune encephalomyelitis (EAE), a neuroinflammatory condition and animal model of multiple sclerosis [25].

Systemic exposure to the bacterial endotoxin, LPS, has been shown to elicit cognitive dysfunction in rodents [26] — which is believed to have neuroinflammatory causes [27]. In fact, our results could thus indicate another possible mechanism to LPS-induced cognitive impairment—namely a decrease in CSF movement. This assumption, however, essentially rests on observed effects in later time points of LPS-exposure, as well as behavioral data, neither of which we have.

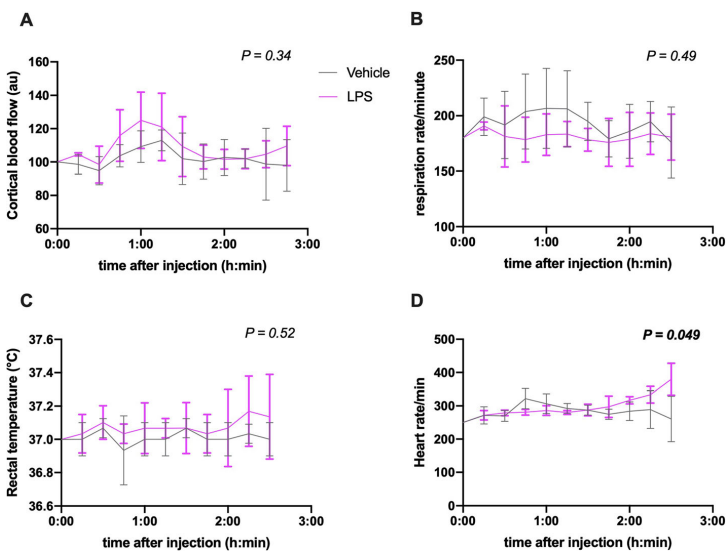

Fig. 3 Cortical blood flow, respiration rate and rectal temperature within 3 h of systemic LPS injection. Vehicle n = 3, LPS n = 3. **a** Cortical blood flow in arbitrary units (au), measured with laser Doppler flowmetry. Two-way ANOVA, treatment factor 95% CI of difference = − 9.4 to 22. **b** Respiration rate measured with surgical monitor. Two-way ANOVA, treatment factor 95% CI of difference = − 52 to 30. **c** Basal temperature measured rectally. Two-way ANOVA, treatment factor 95% CI of difference = − 0.30 to 0.18. **d** Heart rate measured with surgical monitor ECG. Mixed-effects analysis (Two-way ANOVA), treatment factor 95% CI of difference = 0.09 to 28. Data are shown as mean and SD (error bars). P values for treatment factor differences

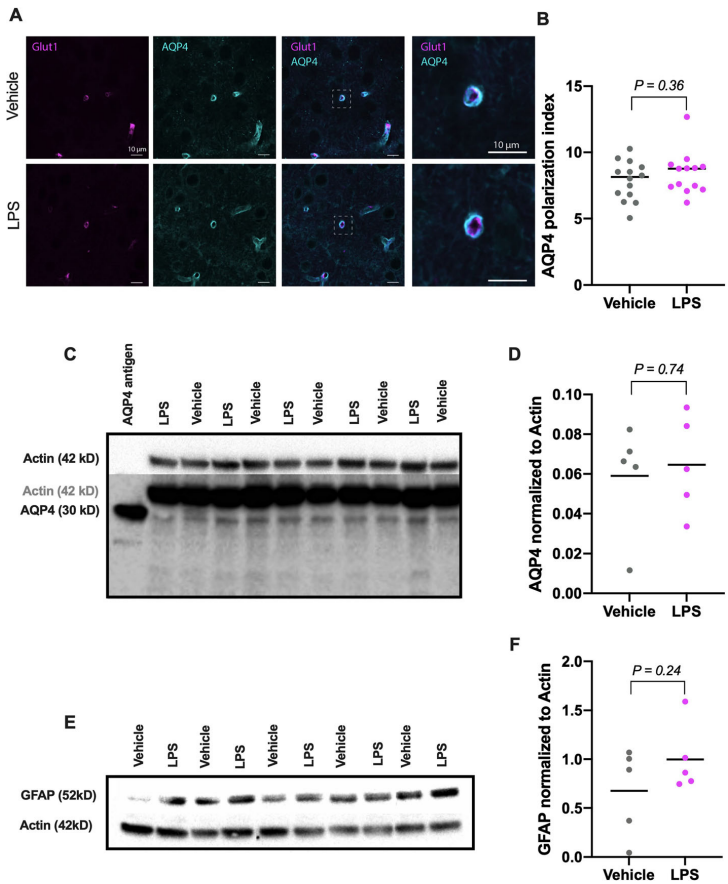

Fig. 4 AQP4 expression (vehicle $n = 5$, LPS $n = 5$) and polarization (vehicle $n = 14$, LPS $n = 13$), as well as GFAP expression (Ctrl $n = 5$, LPS $n = 5$) after 3 h of systemic LPS injections. **a** AQP4 and Glut1 show cerebral vessels. **b** AQP4 polarization index differences were not observed 3 h after LPS. Unpaired *t* test, 95% CI of difference = − 0.67 to 1.8. **c** AQP4 detected bands at 30 kD. **d** Changes in AQP4, measured with Western blot, were not observed 3 h after LPS. Unpaired *t* test, 95% CI of difference = − 0.032 to 0.044. **e** GFAP showed bands at 52 kD. **f** GFAP was not significantly upregulated in LPS-treated mice. Unpaired *t* test, 95% CI of difference = − 0.26 to 0.91. Scale bars 10 µm

As briefly mentioned in the introduction, Erickson et al. observed that repeated systemic injections of LPS (3 mg/kg) lowered intracerebroventricularly injected CSF tracers in serum [4]. In our study, mice only received a single LPS injection, and at a lower concentration (1 mg/kg). While Erickson studied more parameters with very interesting results, including decreased amyloid beta efflux after LPS exposure [4], our study used a less invasive method of CSF tracing [9]. Since intracerebroventricular injections may cause inflammation, they could influence CSF flow readouts [28]. Observations even suggest that inserting a cannula through the cortex decreases glymphatic flow [29]. In contrast, tracers injected into the cisterna magna does not penetrate any brain tissue and thus does not cause parenchymal gliosis [9]. Furthermore, as opposed to sampling CSF efflux in serum [4], we used tracer imaging and show the decreased flow of CSF in the brain. However, our results are in line with the previous findings of Erickson et al. [4], which we find assuring.

Interestingly, a 2017 study by Karimy et al. showed that an intraventricular hemorrhage-induced and TLR-4 dependent inflammation caused CSF hypersecretion from the choroid plexus epithelium [30]. Since TLR4 is the main receptor for an LPS-response in mice [31], it could be possible that our effect was caused by an

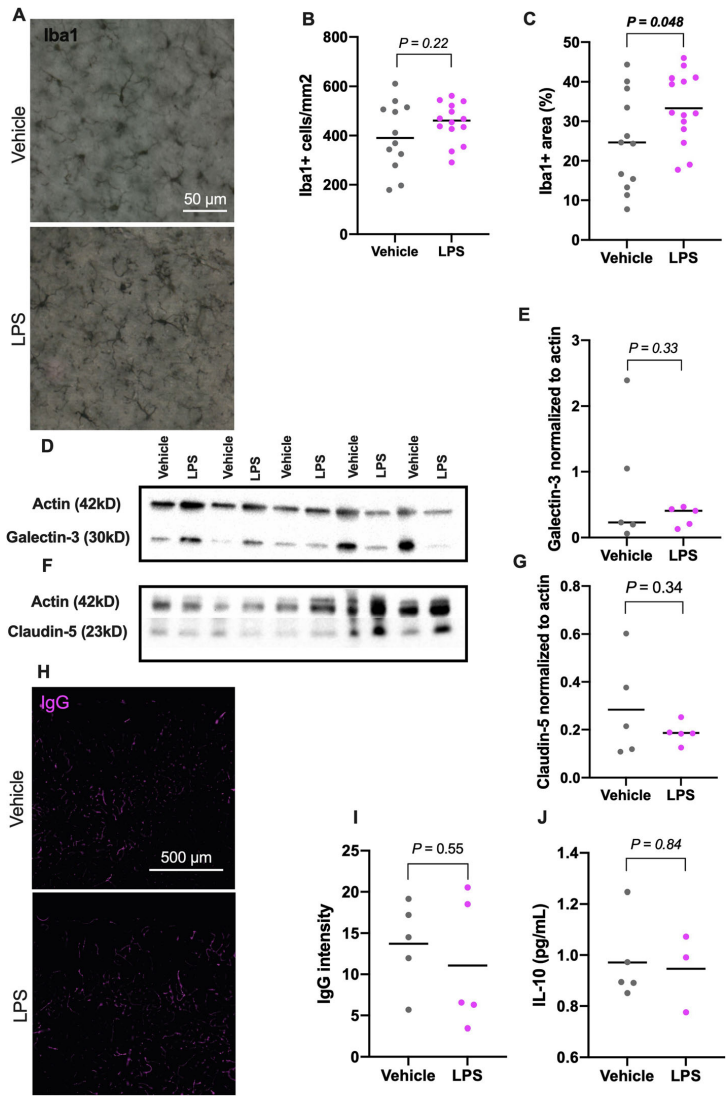

Fig. 5 Markers of microglia, BBB permeability and cytokines. **a** Iba1^{+} cells in coronal cortical sections. Scale bar = 50 µm. **b** Statistical analysis of Iba1^{+} cell numbers 3 h after LPS injection. Vehicle group $n = 13$, LPS $n = 14$. Unpaired t test, 95% CI of difference = − 36 to 150. **c** Statistical analysis of Iba^{+} area in LPS injected animals. Unpaired t test, 95% CI of difference = 0.078 to 17. **d** Galectin-3 detects bands at 30 kD in both groups (vehicle $n = 5$, LPS $n = 5$). **e** Statistical analysis of galectin-3 WB, 3 h after systemic LPS exposure. Unpaired t test, 95% CI of difference = − 1.5 to 0.56. **f** Claudin-5 label bands at 23 kD. Vehicle $n = 5$, LPS $n = 5$. **g** Statistical analysis of Claudin-5 WB. Unpaired t test, 95% CI of difference = − 0.32 to 0.12. **h** IgG labeling reveal cortical vessels. Scale bar = 500 µm. **i** Statistical analysis of IgG intensity. Vehicle group $n = 5$, LPS $n = 5$. Unpaired t test, 95% CI of difference = − 12 to 7.1. **j** IL-10 mesoscale finds eight samples within detection range (vehicle $n = 5$, LPS $n = 3$). Treatment differences with unpaired t test, 95% CI = − 0.31 to 0.26. Data are shown as mean and plotted individual samples, P values for group comparisons are shown above brackets

TLR4-mediated *oversecretion* of CSF, impairing glymphatic flow. However, such a mechanistic explanation warrants further studies, both with regards to how CSF oversecretion impacts glymphatic function, and how systemic LPS affects TLR4 in the choroid plexus.

In our mice, we tried to elucidate other possible mechanisms behind our observed effect on CSF movement. AQP4-expression and polarization in astrocytic endfeet have been shown to be key factors for CSF tracer dynamics [29], as well as to be affected after a neuroinflammatory stimuli [32], such as LPS [33]. Thus, we measured both AQP4 expression and polarization, but could not find any indication that our acute effects of LPS were caused by changes to this water channel. We have previously shown that galectin-3 can contribute to full-blown inflammatory microglial response 6 h following LPS challenge in vitro [34], and be a detrimental component in Alzheimer's disease pathogenesis [35]. For this cohort with readouts 3 h post LPS exposure, we did not detect any differences in microglial activation marker galectin-3. Nor did we find any differences in astrocytic GFAP, BBB permeability markers, or an elevation of proinflammatory cytokines. Further studies are needed to evaluate the differences in these glial and cytokine responses at a later time point. We chose to set measurements at 3 h post LPS injections, since according to most published observations, that is approximately when the brain starts to be measurably affected — with neuroinflammatory markers such as IL-6, IL-1b, and TNF-alpha showing elevation [3]. However, not all reports show significant differences in markers of inflammation at our time point, not even at higher LPS doses. The LPS treatment was likely not without neuroinflammatory effects, since our LPS-treated mice displayed typical sickness behavior, which is closely tied to brain cytokine levels [36, 37]. Moreover, LPS-injected mice did show an increase in $Iba1^+$ area, which indicates microglial reactivity to inflammatory stimuli [21].

Little is known about the interaction of microglial activation and CSF dynamics. Our recorded correlation might suggest either a role of microglial reactivity in abnormal CSF flow, or of the latter as a stimulus for microglial activation. Naturally, this interrelationship may not be more than two separate downstream effects of LPS exposure.

Glymphatic CSF flow has been shown to correlate with the cardiac cycle [16], and to follow the pulse-rhythms of cerebral arteries [16]. We therefore investigated the effect of LPS on cerebral blood flow, but did not detect any changes, in contrast to a previous report with intravenous administration of LPS [17]. When measuring cerebral blood flow, we simultaneously recorded respiration and heart rate, both of which are believed to affect CSF flow [18, 19]. Of these two parameters, we observed a statistically significant elevation in heart rate after LPS-injections, similar to what was measured by Ehrentraut et al. [20]. Since heart rate is believed to be one of the factors that makes glymphatic influx sleep dependent (heart rate low, and influx high, during deep sleep [19]), this LPS-induced effect on heart rate could then, at least partly, explain our detected LPS-effect on CSF influx. However, these results must be interpreted with caution due to the low number of subjects.

The strengths of this study are the steady differences in CSF-tracer intensity, using two tracers and analyzing with an established method as described in the literature [9, 10]. Limitations, other than what has been mentioned previously, are the use of only young male mice, since both age and sex are known factors for LPS response in mice [3, 38]— which may then have influenced our findings. Analyses of plasma markers may have aided us in finding interesting correlations. The same is true for blood pressure measurements, since arterial blood pressure is known to affect CSF flow [16, 39], and to be affected by LPS exposure [40]. Moreover, some experiments in our study, such as the physiological readouts (primarily because animals died under the long anesthesia), but also our Western blots and IgG measurements, were underpowered, increasing the risk of getting both false positive, and negative, results.

Conclusion

In conclusion, our study reports another physiological response after acute exposure to the bacterial endotoxin, LPS, namely the significant decrease in perivascular distribution of CSF. We welcome future studies that endeavors to replicate, and thus confirm these findings—as we believe that they may help us grasp how inflammation affects the brain, in both health and disease.

Abbreviations

LPS: Lipopolysaccharides; KX: Ketamine and Xylazine; AQP4: Aquaporin-4; CSF: Cerebrospinal fluid; GFAP: Glial fibrillary acidic protein; Iba1: Ionized calcium-binding adapter molecule 1; PBS: Phosphate-buffered saline; T20: Tween-20; TX: Triton X100; MPI (a.u.): Mean pixel intensity in arbitrary units

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Authors' contributions

OM, IL, and TD designed the research studies. OM performed the experiments. OM, MR, and SB analyzed the data. OM, MR, SB, IL, and TD wrote the manuscript. IL and TD contributed with reagents. All authors read and approved the final manuscript.

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Availability of data and materials
Raw data can be accessed upon request by contacting the corresponding author.

Ethics approval and consent to participate
Animal procedures, handling, and housing in this study were carried out in accordance with the international guidelines on experimental animal research and were approved by the Malmö-Lund Ethical Committee for Animal Research in Sweden (M250-11, M30-16, Dnr 5.8.18-01107/2018).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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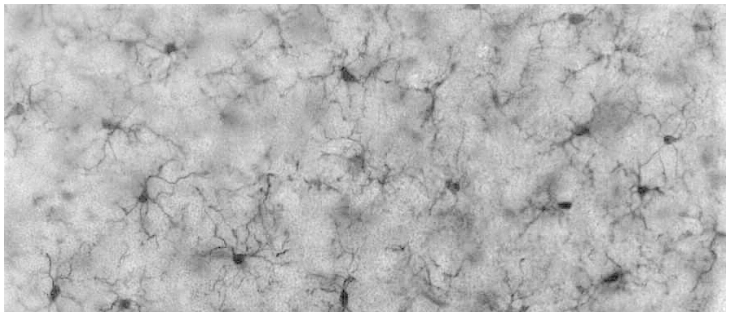

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