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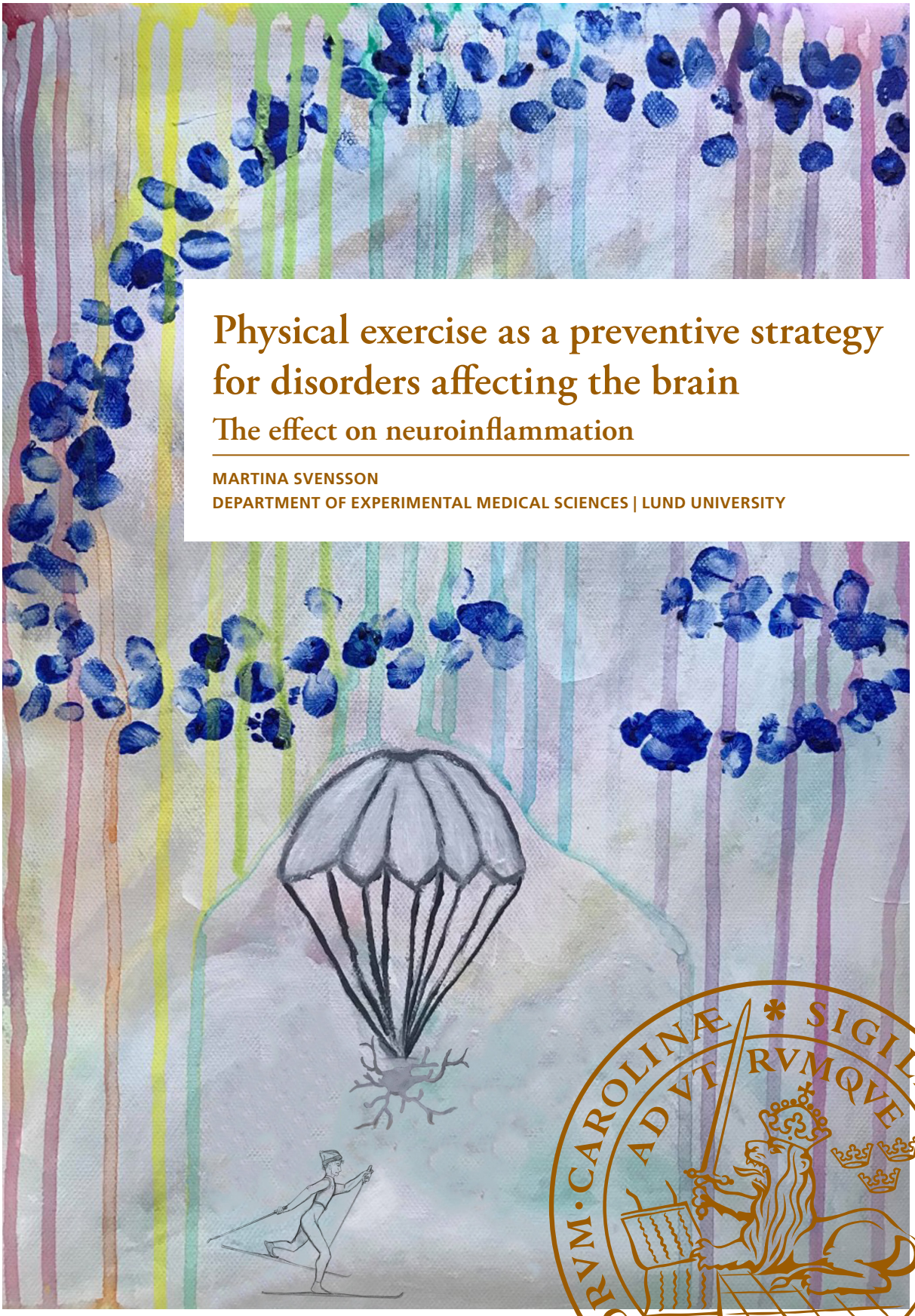

Physical exercise as a preventive strategy for disorders affecting the brain

The effect on neuroinflammation

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UNIVERSITY

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Abstract

Physical activity is associated with lower risks of developing dementia, Parkinson's disease (PD) and depression. Furthermore, being physically active prior to brain ischemia appears to help the recovery process. However, previous studies do not differentiate between the two most common forms of dementia, Alzheimer's disease (AD) and vascular dementia (VaD), when it comes to the association between physical activity and dementia. Also, these studies do not take into consideration the potential bias due to reverse causation when assessing these associations. Moreover, little is known about how exercise affects neuroinflammation and other pathological hallmarks of brain ischemia and AD.

In this thesis, I investigate the potential of physical activity to act as a preventive strategy against common disorders affecting the brain. With a focus on translational medicine, I combined an epidemiological approach with experimental approaches. By using the Vasaloppet Registry together with the Swedish population and patient registries, we investigated if physically active skiers (n= 197,685) had lower risks of AD, VaD, PD and depression compared to an age and sex-matched general population (n=197,684) during an up to 21-year follow-up. Using experimental interventions, I investigated how running affected neuroinflammation and other aspects of the molecular pathology in mouse models of brain ischemia and AD.

I discovered that forced running in mice prior to global brain ischemia may induce a stress response, leading to increased neuronal death in the hippocampus.

We observed a lower risk of depression among both sex in our skiers (hazard ratio (HR)=0.50 (0.46-0.53), p<0.001). Among men, faster skiing was associated with an even lower risk compared to slower skiers (HR=0.65 (0.49-0.87), p=0.004), whereas faster female skiers did not have any difference in risk compared to slower counterparts (HR=1.14 (0.77-1.70), p=0.51). The reasons behind these differences require further investigation.

Skiers also had a lower risk of developing PD compared to controls during the early follow-up period (HR=0.71 (0.55-0.90), p=0.004), but this association became non-significant in sensitivity-analysis excluding persons diagnosed with PD within the first 5 years after inclusion (HR=0.80 (0.62-1.03), p=0.09). With longer follow-up times, the skiers' cumulative PD incidence converged with that of the general population. These findings are in line with the motor reserve hypothesis, wherein physically active individuals may develop a motor reserve, allowing them to sustain PD pathology longer before symptoms appear.

Moreover, skiers had a lower risk of VaD (HR=0.54 (0.37-0.80), p=0.002), but not AD (HR=0.88 (0.66-1.18), p=0.40). Experimentally, in the 5xFAD mouse model, 6 months of voluntary wheel running did not ameliorate their AD-like pathology. These findings question the general view of exercise as being protective against AD.

Taken together, even though it is not equally effective against all disorders, a physically active lifestyle seems to be associated with a lower risk of developing disorders affecting the brain. Furthermore, the exact mechanisms behind these associations remain to be elucidated, and the optimal exercise settings need to be defined.

Key words

Physical activity, Exercise, Neuroinflammation, Microglia, Cytokines, Behavior, Dementia, Depression, Brain ischemia, Alzheimer's disease, Vascular dementia, Parkinson's disease

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Coverphoto by;

Decibelle & Candela Svensson (the dark blue dots are made by my daughter's fingerprints and represents DAPI-stained neurons in the rodent hippocampus)

Jimmy Persson (the Vasaloppet skier)

Anja Svensson (putting everything together)

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”Allt stort som skedde i världen skedde först i någon människas fantasi”
(Astrid Lindgren)

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List of publications

This thesis is based on the following papers, referred to by roman numerals:

I. **Microglia-secreted galectin.3 acts as a toll-like receptor 4 ligand and contributes to microglial activation.** Burguillos MA, Svensson M, Schulte T, Boza-Serrano A, Garcia-Quintanilla A, Kavanagh E, Santiago M, Viceconte N, Oliva-Martin MJ, Osman AM, Salomonsson E, Amar L, Persson A, Blomgren K, Achour A, Englund E, Leffler H, Venero JL, Joseph B, Deierborg T. *Cell Reports*. pii: S2211-1247(15)00140-0. Mar 4, 2015.

II. **Forced treadmill exercise can induce stress and increase neuronal damage in a mouse model of global cerebral ischemia.** Svensson M, Rosvall P, Boza-Serrano A, Andersson E, Lexell J, Deierborg T. *Neurobiol Stress*. 9;5:8-18, Sep 2016.

III. **Long distance ski racing is associated with lower long-term incidence of depression in a population based, large-scale study.** Svensson M*, Brundin L*, Erhardt S, Madaj Z, Hållmarker U, James S, Deierborg T. *Psychiatry Res.* 281, 112546. Nov 2019.

IV. **Delayed clinical manifestation of Parkinson's Disease among physically active: do participants in a long-distance ski race have a motor reserve?** Olsson T*, Svensson M*, Hållmarker U, James S Deierborg T. *J Parkinsons Dis*. 10 (1), 267-247, Jan 2020.

V. **Midlife physical activity is associated with lower incidence of vascular dementia but not Alzheimer's disease.** Hansson O*, Svensson M*, Gustavsson AM*, Andersson E, Yang Y, Nägga K, Hållmarker U, James S, Deierborg T. *Alzheimers Res Ther.* 11 (1) 87, Oct 2019.

VI. **Voluntary running does not reduce neuroinflammation or improve non-cognitive behavior in the 5xFAD mouse model of Alzheimer's disease.** Svensson M, Andersson E, Oscar Manouchehrian, Yang Y, Deierborg T. *Sci Rep*. 10 (1) 1346, Jan 2020

*Equal contribution

Additional publications, not included in the thesis

I. **Autophagy is increased in laminin α2 chain-deficient muscle and its inhibition improves muscle morphology in a mouse model of MDC1A.** Carmignac V, Svensson M, Körner Z, Elowsson L, Matsumura C, Gawlik KI, Allamand V, Durbeej M. *Hum Mol Genet.* 20(24):4891-902, Dec 15, 2011

II. **Effects of Physical Exercise on Neuroinflammation, Neuroplasticity, Neurodegeneration, and Behavior: What We Can Learn From Animal Models in Clinical Settings.** Svensson M, Lexell J, Deierborg T *Neurorehabil Neural Repair* 6, 577-89 July 29, 2015

III. **Genetic ablation of soluble tumor necrosis factor with preservation of membrane tumor necrosis factor is associated with neuroprotection after focal cerebral ischemia.** Madsen PM, Clausen BH, Degn M, Thyssen S, Kristensen LK, Svensson M, Ditzel N, Finsen B, Deierborg T, Brambilla R, Lambertsen KL. *J Cereb Blood Flow Metab.* Oct 19, 2015

IV. **Endogenous IFN-β signaling exerts anti-inflammatory actions in experimentally induced focal cerebral ischemia.** Inácio AR, Liu Y, Clausen BH, Svensson M, Kucharz K, Yang Y, Stankovich T, Khorooshi R, Lambertsen KL, Issazadeh-Navikas S, Deierborg T. J *Neuroinflammation* 12:211, Nov 18, 2015

V. **BID Mediates Oxygen-Glucose Deprivation-Induced Neuronal Injury in Organotypic Hippocampal Slice Cultures and Modulates Tissue Inflammation in a Transient Focal Cerebral Ischemia Model without Changing Lesion Volume.** Martin NA, Bonner H, Elkjær ML, D'Orsi B, Chen G, König HG, Svensson M, Deierborg T, Pfeiffer S, Prehn JH, Lambertsen KL. *Front Cell Neurosci*.10:14, Feb 3, 2016

VI. **Fumarate improves functional outcome of experimental ischemic stroke.** Clausen BH, Lundberg L, Yli-Karjanmaa M, Martin NA, Svensson M, Alfsen MZ, Flæng SB, Lyngsø K, Boza-Serrano A, Nielsen HH, Hansen PB, Finsen B, Deierborg T, Illes Z, Lambertsen KL. *Exp Neurol.* 2017 Sep;295:144-154.

VII. **Potent pro-inflammatory and pro-fibrotic molecules, osteopontin and galectin-3, are not major disease modulators of laminin α2 chain-deficient muscular dystrophy.** Gawlik KI, Holmberg J, Svensson M, Einerborg M, Oliveira BM, Deierborg T, Durbeej M. *Sci Rep*. 2017 Mar 10;7:44059

VIII. **Focal, but not global, cerebral ischemia causes loss of myenteric neurons and upregulation of vasoactive intestinal peptide in mouse ileum.** Cheng X, Svensson M, Yang Y, Deierborg T, Ekblad E, Voss U. *Int J Exp Pathol*. 2018 Feb;99(1):38-45.

Abbreviations

APP	Amyloid precursor protein
Aβ	Amyloid beta
ApoE	Apolipoprotein E
α-syn	Alpha-synuclein
AD	Alzheimer's disease
BACE1	Beta-secretase 1
BDNF	Brain-derived neurotrophic factor
β2-AR	Beta-2 adrenergic receptor
CAM	Cellular adhesion molecule
CD	Cluster of differentiation
CNS	Central nervous system
COX	Cyclooxygenase
CRP	C-reactive protein
CSF	Cerebrospinal fluid
DAMP	Damage-associated molecular patterns
GWAS	Genome-wide association study
HPA	Hypothalamic–pituitary–adrenal (axis)
IDE-1	Insulin-degrading enzyme 1
IFNγ	Interferon gamma
IL	Interleukin
iNOS	Inducible nitric oxide synthase
MCH	Major histocompatibility complex
MyD88	Myeloid differentiation primary response 88
NFκB	Nuclear factor kappa B
NMDA	N Methyl d aspartate
PAMP	Pathogen-associated molecular pattern
PET	Positron emission tomography
PGE2	Prostaglandin E2
PRR	Pattern recognition receptor
PD	Parkinson's disease
PS-1, 2	Presenilin 1 and 2
PSD-95	Postsynaptic density protein 95)
SSRI	Selective serotonin reuptake inhibitor
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
VaD	Vascular dementia

Popular science summary

Every winter, skiers all around the world challenge themselves by participating in Vasaloppet, the world's largest long-distance ski race held in Sweden. Not only do they display their athleticism, they also benefit in other ways: depression is only half as common, the manifestation of Parkinson's is delayed and they have lower risk of vascular dementia, but not Alzheimer's. We made these discoveries when comparing almost 200 000 long-distance skiers with a matched general population during an up to 21-year follow-up.

Globally, almost 300 million individuals are affected by depression (WHO 2019). Among both sexes, fewer skiers developed depression. Faster racing was associated with a further lowered risk of depression in skiing men.

Parkinson´s disease (PD) is a movement disorder affecting over 6 million people worldwide (EBC). Skiers were diagnosed with PD less frequently compared to controls during the first 15 years of the follow-up. Thereafter, the PD-levels among the skiers approached that of the general population. Based on this, we believe that the physically active skiers develop a motor reserve, allowing them to sustain brain pathology longer before motor symptoms develop.

Alzheimer's disease (AD) is the most common form of dementia, followed by vascular dementia. Globally, dementia affects 50 million people (WHO 2019). Vascular dementia was less common among our skiers. On the contrary, the risk of AD was not affected even though other studies show the opposite. Next, we took a closer look at how exercise affects molecular pathological hallmarks within the brain using mouse models.

Neuroinflammation seems to contribute to many disorders affecting the brain. Microglia are the main inflammatory cells in the brain. This cell type may become activated and display different characteristics, either protecting or damaging neurons. Galectin-3 is a carbohydrate-binding protein used by microglia for inflammatory communication. We have demonstrated that galectin-3 contributes to neuronal damage in our mouse model of global brain ischemia. We wondered whether exercise might affect inflammation. Therefore, we subjected half of our mice to treadmill running before inducing brain ischemia. Unfortunately, the forced running lead to stress, and running mice displayed increased neuronal damage. Furthermore, treadmill running did not affect the levels of microglia or galectin-3. On the contrary, the

running intervention seemed to increase the levels of inflammatory cytokines. We were obliged to look for another running paradigm for our mice.

Neuroinflammation also influences the development of AD in our AD mouse model. Since other studies have shown that physical activity reduces the risk of AD, we wanted to investigate if running could affect the molecular hallmarks of AD. Instead of forcing mice to run, we allowed them to run voluntarily in running wheels. Despite this, running did not ameliorate the AD pathology as measured by the levels of amyloid-beta and their memory capabilities. Cytokine levels also remained unchanged. We concluded that physical activity does not affect the development of AD, at least not the type of AD we investigated.

Taken together, physical inactivity is a major risk factor for disorders affecting the brain, such as dementia, stroke, and depression (WHO 2018). Despite this, 25 % of all adults around the globe do not fulfill the recommendations given by WHO on physical activity levels. In addition, the impact of the above-mentioned diseases increases as the aging population grows. Increasing physical activity might be the key in dealing with this challenge, even though it does not seem to help against all disorders affecting the brain. Nevertheless, train your brain!

Résumé

L’inactivité physique est un facteur de risque majeur pour les maladies cérébrales, telles que la dépression et la démence. Malgré cela, un tiers des adultes européens n’atteint pas les recommendations d’activité physique de l’OMS (OMS 2015). Dans le même temps, le nombre de personnes touchées par ces maladies augmente (EBC). Une activité physique plus importante pourrait être la clé pour relever ces défis. Nous avons donc besoin de connaissances approfondies de la manière dont l’activité physique affecte ces maladies. L’activité physique, prévient-elle vraiment toutes ces maladies du cerveau?

Aujourd’hui, la maladie de Parkinson affecte plus d’1 million d’Européens. La maladie commence par la dégradation de la région cérébrale Substantia Nigra, entraînant un manque de dopamine suivi de troubles du mouvement. Dans le cas de la maladie d'Alzheimer des régions cérébrales sont également dégradées, mais cette fois-ci il s’agit de l’hippocampe, entraînant des difficultés de mémoire. Parmi les pathologies démentielles, la maladie d’Alzheimer est la plus courante, suivie par la démence vasculaire. En Europe, 10 millions de personnes souffrent de démence (OMS 2019). La dépression est encore plus courante. Environ 40 millions d’européens souffrent de dépression (OMS 2019).

Nous avons examiné comment l'activité physique peut jouer un rôle dans le risque de développer ces maladies cérébrales. En suivant près de 200 000 skieurs ayant participé au Vasaloppet (une course de longue distance) en parallèle avec le même nombre de non-skieurs parmi la population pendant 20 ans, nous avons pu étudier comment les niveaux d'activité affectent le risque de ces maladies.

Indépendamment du sexe, les skieurs développent moins souvent de dépression comparé aux non-skieurs. Parmi les hommes, la vitesse influait aussi, plus elle est élevée, plus le risque de dépression est faible. Pendant les 15 premières années après la course, les skieurs ont également eu un risque réduit de développer la maladie de Parkinson. Au-delà, ils ont rejoint les niveaux de la population générale. Nous pensons que les skieurs, physiquement actifs, développent une réserve motrice, qui leur permet de supporter davantage de pathologie cérébrale avant que les symptômes moteurs apparaissent. Concernant les pathologies démentielles, la démence vasculaire était également moins fréquente chez les skieurs. Cependant, le risque de maladie d'Alzheimer ne semblait pas être affecté, bien que d'autres études aient montré le contraire. Nous avons ensuite examiné de plus près l'impact de l'exercice sur des processus pathologiques moléculaires dans le cerveau.

Les recherches montrent que l'inflammation cérébrale peut contribuer à la progression de nombreuses maladies cérébrales. La microglie est la principale population de cellules inflammatoires du cerveau. En étant activée de différentes manières, la microglie peut protéger ou endommager le cerveau en augmentant ou en réduisant l'inflammation. La

galectine-3 est une molécule sécrétée par les cellules de la microglie pour qu'elles puissent communiquer entre elles. Nous avons constaté que la galectine-3 joue un rôle crucial dans les lésions cérébrales dans notre modèle murin d'ischémie cérébrale globale (une situation qui affecte le cerveau pendant d'arrêt cardiaque). Nous avons fait courir un groupe de souris comparé à un groupe témoin avant de couper temporairement le flux sanguin vers le cerveau. Malheureusement, l'entraînement forcé sur les tapis roulants a stressé les souris à tel point qu'elles ont développé des lésions cérébrales majeures. En outre, l'entraînement n'a pas affecté la quantité de microglie ni la galectine-3. En revanche, l'entraînement sur les tapis roulants semblait augmenter les niveaux de cytokines, d'autres molécules de la signalisation inflammatoire. Néanmoins, nous avons pu trouver un autre moyen d'entraîner des souris sans les stresser.

L'inflammation cérébrale affecte également la progression de la maladie chez nos souris Alzheimer. Comme d'autres études ont montré que l'activité physique réduisait le risque de maladie d'Alzheimer, nous voulions tester si l'exercice chez nos souris pouvait affecter l'évolution de la pathologie au niveau moléculaire. Nous avons laissé les souris s'entraîner librement dans des roues au lieu de les forcer. Malheureusement, l'entraînement n'a pas amélioré leur mémoire. La quantité d'amyloïde bêta, un marqueur d'Alzheimer, n'a pas été affectée. L'exercice n'a pas non plus modifié les niveaux de cytokines. Notre conclusion est donc que l'activité physique n'affecte pas la progression de la maladie d'Alzheimer, tout du moins pas dans le type d'Alzheimer que nous avons étudié.

Populärvetenskaplig sammanfattning

Halverad risk att drabbas av depression. Parkinsons sjukdom fördröjs. Risken minskar för vaskulär demens, men inte för Alzheimers sjukdom. Dessa fynd gjorde vi hos nästan 200 000 Vasalopps-åkare när de jämfördes med övriga befolkningen och följdes mellan 1989-2010.

I Sverige diagnosticeras var femte person någon gång under sitt liv med depression (Folkhälsomyndigheten 2017). I vår Vasalopps-studie drabbades både manliga och kvinnliga skidåkare mer sällan av depression jämfört med övriga befolkningen. Bland de manliga skidåkarna spelade även hastigheten roll, ju snabbare skidåkning desto lägre risk för framtida depression.

Parkinsons sjukdom är en rörelsesjukdom och drabbar idag 1 % av befolkningen över 60 år (1177.se). Våra skidåkare drabbades i mindre utsträckning av Parkinsons de första 15 åren. Därefter närmade de sig befolkningens nivåer. Vi tror därför att de fysiskt aktiva skidåkarna utvecklar en motorisk reserv som gör att det tar längre tid innan sjukdomen ger symptom trots att nedbrytningen i hjärnan inte förhindras.

Alzheimers är vår vanligaste demenssjukdom, följt av vaskulär demens. I Sverige insjuknar runt 10% av befolkningen i demens i slutet av livet (Alzheimersfonden 2019). Vasalopps-åkarna utvecklade vaskulär demens mer sällan jämfört med de som inte åkte. Risken för Alzheimers verkade däremot inte påverkas, trots att andra studier visat motsatsen. Vi tittade närmare på hur träning påverkar molekylära sjukdomsprocesser i hjärnan.

Inflammation i hjärnan, neuroinflammation, kan vara en bidragande faktor vid många hjärnsjukdomar. Mikroglia är hjärnans främsta inflammatoriska cell. Genom att aktiveras på olika sätt kan mikroglia antingen skydda eller skada hjärnan. För att kommunicera utsöndrar mikroglia bland annat molekylen galektin-3. Vi har konstaterat att galektin-3 bidrar till hjärnskadorna som uppstår i vår musmodell för global ischemi i hjärnan (ett tillstånd som drabbar hjärnan vid hjärtstopp). Vi tänkte att träning skulle kunna påverka inflammationen i dessa möss. Därför lät vi hälften av mössen springa på löpband innan blodflödet till hjärnan ströps tillfälligt. Dessvärre stressade den påtvingade träningen mössen, de utvecklade större hjärnskador. Dessutom påverkade löpningen inte mängden mikrogliaceller eller dess inflammatoriska signalmolekyl, galektin-3. Tvärtom verkade löpträningen öka nivåerna av andra inflammatoriska signalmolekyler; cytokinerna. Som tur var kände vi till ett sätt att löpträna möss utan att de blev stressade.

Neuroinflammation påverkar även sjukdomsutvecklingen i våra Alzheimers-möss. Eftersom andra studier har visat att fysisk aktivitet minskar risken för Alzheimers ville vi undersöka om löpträning i våra möss kunde påverka det molekylära sjukdomsförloppet. Vi lät mössen träna fritt i löphjul istället för att tvinga dem. Trots

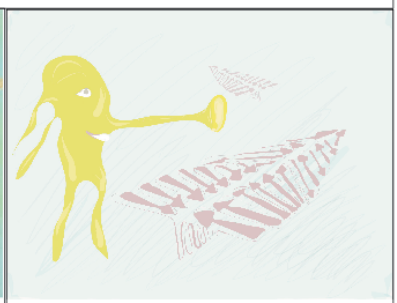
detta förbättrade träningen inte mössens minne. Nivåerna av Alzheimers-markören amyloid-beta påverkades inte heller. Cytokin-nivåerna var också oförändrade. Vår slutsats är att fysisk aktivitet inte påverkar sjukdomsutvecklingen vid Alzheimers sjukdom, åtminstone inte i den typen av Alzheimer vi undersökte.

Fysisk inaktivitet är en stor riskfaktor för hjärnsjukdomar såsom demens, stroke och depression (WHO 2018). Trots detta uppfyller var tredje vuxen svensk inte WHOs rekommendationer och antalet som lider av demens och depression ökar. Fysisk aktivitet kan vara nyckeln till en minskning, även om det inte verkar ha effekt på alla hjärnsjukdomar. Träna Hjärna!

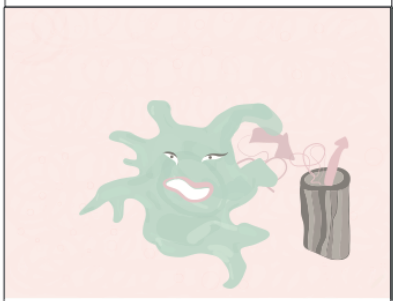
Till alla barn

MIKROGLIA MIA BLIR ARG

Djupt inne i hjärnans slingriga skogar bor våra vänner Nervcellen Ellen och Mikroglia Mia. När Nervcellen Ellen skräpar ner brukar Mikroglia Mia städa.



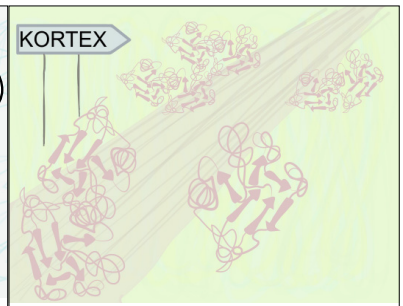
Nervcellen Ellen brukar vika pappers-flygplan att flyga med ute i trädgården. Flygplanen kallas för A-beta. En dag började Nervcellen Ellen vika flygplanen fel och de gick inte att flyga med längre.



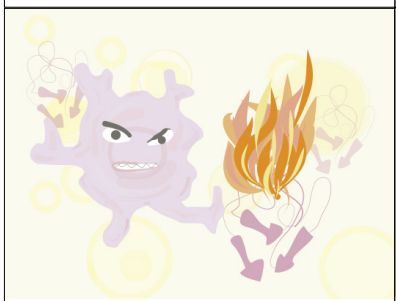
Nervcellen Ellen kastade ut de trasiga flygplanen i trädgården. Mikroglia Mia tyckte att flygplanen skräpade ner. Hon kastade dem i papperskorgen.

Nervcellen Ellen fortsatte slänga ut trasiga flygplan. Till slut hann Mikroglia Mia inte städa undan alla A-beta blåste ut från trädgården och spreds i hjärnans skogar.

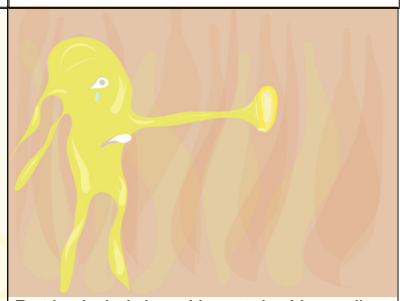
Då tappade Mikroglia Mia tålamodet. Hon blev arg på Nervcellen Ellen. Nervcellen Ellen lyssnade inte, utan tog fram klister. A-beta klibbade fast i varandra



Klumpar med A-beta skräpade ner i hjärnans skogar. Alla dessa klumpar med A-beta gör det svårt att tänka när man blir gammal.



Mikroglia Mia blev ännu argare och hällde ut bensin för att elda upp A-beta. Men det kom nya hela tiden. Då blev mikroglia Mia så arg att hon hällde ut bensin överallt.



Det började brinna i huset där Nervcellen Ellen bodde. Nervcellen blev skadad.

Brandkåren ryckte ut, men elden hade redan spridit sig i hjärnans slingriga skogar. Nu försöker de släcka bränderna i gamla människors huvuden. De väntar på att forskarna ska ta reda på hur man släcker bränderna.

I min forskning försöker vi ta reda på om träning kan hjälpa Mikroglia Mia att släcka branden i hjärnan, det är det den här boken handlar om.

Context of this thesis

I started my Ph.D. project in 2013 when my supervisor, Tomas Deierborg, received funding for investigating the effect of exercise on disorders affecting the brain within a translational setting in collaboration with Jan Lexell. By writing a critical review (not included in this thesis), I wanted to elucidate how animal models of specific disorders affecting the brain could be used to model exercise interventions using clinically relevant settings. I summarized what was already known in this research field, focusing on the effects of exercise on neuroinflammation, neurodegeneration, and behavior in different disorders affecting the brain.

After identifying research questions that needed to be answered, the first experimental step of my thesis work was initialized; I investigated the effect of treadmill running on mice before subjecting them to global brain ischemia. Before studying the effect of running in this model, I investigated how neuroinflammation contributes to the damage and behavioral deficits in this mouse model (Paper I). Unfortunately, we discovered that forcing mice to run induced a stress response that had detrimental effects on brain recovery following ischemia (Paper II). Therefore, we decided to use a voluntary wheel running setup for our next experimental study.

In parallel with my experimental work, we were looking for possibilities to make my project more translational and did so, by including data from human studies. This opportunity arose when my supervisor contacted Ulf Hållmarker and Stefan James, researchers at Uppsala University responsible for the build-up of the Vasaloppet database. This collaboration offered a great possibility to study how a physically active lifestyle (in this case, being a Vasaloppet skier) could affect the risk of developing different disorders affecting the brain later in life. Thus, during the latter half of my Ph.D. time, I was responsible for compiling, interpreting and reporting our results from this data in three different manuscripts; one investigating depression, one investigating Parkinson's Disease (PD) and one investigating dementia, distinguishing between Alzheimer's disease (AD) and vascular dementia (VaD). This was done in collaboration with clinicians to assure that the results were reported in relevant ways and that reasonable conclusions were drawn.

When investigating the association between Vasaloppet participation and incidence of depression (Paper III), I obtained a clinician's perspective and received consultation on my work by collaborating with Lena Brundin, a psychiatrist and psychiatric researcher.

In the study investigating Vasaloppet skiers and a later risk of PD (Paper IV), the working hypothesis and the manuscript were prepared in collaboration with Tomas Roos (né Olsson).

In my thesis, I had the great opportunity of combining epidemiological human data from physically active persons with experimental, molecular insights from mouse models on how AD pathology is affected by exercise. In the study looking at midlife physical activity and dementia (Paper V), I shared the first authorship with Anna-Märta Gustavsson and Oskar Hansson. They are both clinicians with extensive knowledge about dementia diagnoses. Anna-Märta and Oskar were responsible for analyzing the Malmö diet and cancer cohort included in this paper while my responsibility (together with Tomas Deierborg) was analyzing the Vasaloppet cohort and conducting and analyzing the results from the animal model reported in this paper.

In conjunction with the Vasaloppet and dementia study, I conducted the last experimental study included in my thesis (Paper VI). By allowing transgenic AD mice (5xFAD) to voluntary run in wheels, I investigated how exercise affects molecular hallmarks of AD. The effects on soluble amyloid-beta, synaptic proteins, and cognitive behavior were reported in the Vasaloppet dementia paper. Finally, I reported the effects of exercise on neuroinflammation and non-cognitive behavior in 5xFAD mice in the last experimental paper included in this thesis (Paper VI).

Taken together, my thesis should be viewed as an attempt to investigate the impact of physical activity on disorders affecting the brain from two different perspectives; one epidemiological and one experimental. The overall goal of this work is to encourage a more translational view of this research area.

Introduction

In this introduction, I will first define some of the most common disorders affecting the brain which were also investigated in my research. Next, I will give a more detailed description of the neuroinflammatory component of these disorders. Lastly, I will describe the main focus of this thesis; the therapeutic potential of physical exercise with main focuses on its neuroinflammatory effects and its potential as a preventive strategy for the diseases affecting the brain.

Disorders affecting the brain

This section will briefly introduce the disorders affecting the brain that are investigated in my research, namely global cerebral ischemia, vascular dementia (VaD), Alzheimer's disease (AD), Parkinson's disease (PD) and depression. Before introducing each disorder it is important to mention the complexity of comorbidity. Many patients do not suffer from only one of these disorders. Hence, the overall picture is complex; the determination of the exact pathological mechanisms behind each diagnosis is difficult as these mechanisms are rarely isolated and suspected to affect each other. For instance, almost 30 % of patients with AD also suffer from cerebrovascular lesions [1], and several reports suggest that cerebral ischemia impacts the development of AD [2]. Furthermore, in some studies, depression is associated with an increased risk of developing AD and PD and of having a stroke [3]. Not only are there patients affected by several of these disorders, but also many of these disorders give rise to overlapping symptoms. For example, patients with dementia are prone to getting depressive symptoms [4,5]. Depressive symptoms have even been suggested to be one of the signs of prodromal AD, the stage before cognitive symptoms are noticed [6]. Reversely, patients with psychiatric disorders tend to also have cognitive dysfunctions [7]. Also, many patients with PD develop dementia with time [8]. Depression is a common secondary complication following a stroke, as post-stroke depression has been shown to manifest in up to 70 % of all stroke survivors [4]. Thus, the below-described disorders affecting the brain are indeed distinct but overlap in certain pathological processes.

Cerebral ischemia

Globally, cerebral ischemia is estimated to affect almost 14 million people each year [9]. Even though ischemic stroke dropped in rank as now the fifth leading cause of death in men, it remains the third leading cause of death in women [10]. Despite extensive research, we are still lacking knowledge about the etiology of stroke and the pathological processes involved. The main molecular events contributing to the pathology of cerebral ischemia are described below and summarized in Figure 1.

Cerebral ischemia usually starts with an occlusion in one of the cerebral vessels, leading to the deprivation of oxygen and nutrients as well as the accumulation of waste products within the neurological tissue supplied by the affected vessel. This ultimately leads to the death of the affected neurons within a few minutes unless the occlusion is resolved and blood flow is re-established quickly. There are several molecular causes behind this neuronal cell death, but the impact of each cause is not yet clear. The lack of energy and oxygen supply immediately gives rise to a lack of adenosine triphosphate, the ultimate mediator of energy for cells. Given the lack of oxygen, lactic acid will result from anaerobic metabolism, causing a drop in pH. A lack of energy implies that cells will be unable to maintain their ion balances as they are dependent on several energy-consuming ion pumps for active transportation of ions across the cellular membranes. One such example of an energy-consuming ion pump is the sodium-potassium pump (Na^+ K^+ ATPase pump), which is required to maintain the membrane resting potential of a neuron. Non-functional ion pumps lead to dysregulated depolarization with an influx of Ca^{2+} ions. In presynaptic neurons, this will lead to the secretion of neurotransmitters, such as excitatory glutamate, into the synaptic cleft. Once this state is accomplished, it all turns into a self-propagating disaster on the molecular and cellular levels. Notably, the released glutamate binds to excitatory receptors, such as NMDA, leading to membrane depolarization and, subsequently, an even greater influx of Ca^{2+} into the cells, a phenomenon referred to as excitotoxicity.

Increased intracellular Ca^{2+}levels induce a multitude of downstream intracellular signaling pathways, affecting the activity of catabolic enzymes such as proteases and lipases. Thus, cellular structures are broken down from the inside of the cell. Moreover, mitochondria become dysfunctional as free radicals are produced and caspases involved in programmed cell death are activated. All these factors likely contribute to the apoptosis and necrosis occurring in the neuronal tissue during the hours and days following the ischemic event. In addition, the immune response elicited by the ischemic insult affects the molecular pathology following cerebral ischemia. The inflammatory contribution to the pathology will be described in more detail at the end of the next section (see *The role of neuroinflammation in brain ischemia*).

The affected brain area surrounding the site of occlusion can be divided into the ischemic core and the penumbra, where the area affected evolve with time. The ischemic core is the area in which neurons are irreversibly damaged and dying, whereas

the penumbra is the area with dysfunctional but still living neurons. The latter is an area where the fates of cells are thought to not yet be determined but rather to be rescuable through therapeutic interventions. With time, some cells within the primary penumbra will be found to be irreversibly damaged. Hence, these cells will die and become a part of the expanding ischemic core, the definitive lesion. The remaining cells within the primary penumbra will eventually recover, and this area will regain its normal functions during the weeks following the ischemic event. This process is thought to be highly influenced by the immune response.

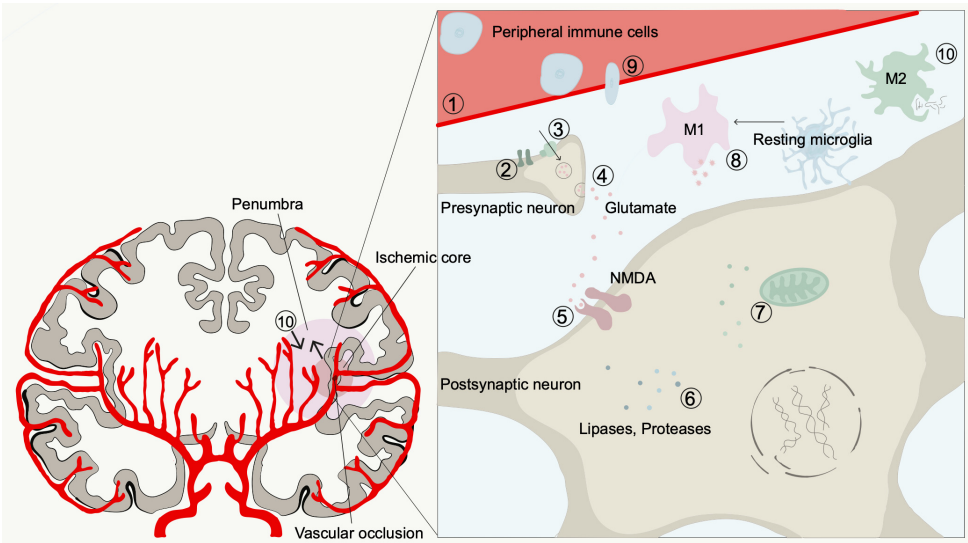


Figure 1. Molecular pathology in the brain following brain ischemia
Following vascular occlusion, the lack of vascularly supplied oxygen and glucose lead to a lack of energy within the brain parenchyma (1). The resting membrane potential is lost since the Na+K+ATPase pump maintaining this potential no longer works (2). This leads to an uncontrolled influx of Ca2+ into neurons through voltage-gated channels (3). Vesicles with neurotransmitters, such as glutamate, are released into synaptic clefts (4) and bind to NMDA receptors on postsynaptic neurons in an uncontrolled way, causing excitotoxicity (5) through excessive influx of Ca2+. This leads to abnormal activation of enzymes degrading macromolecular structures inside the cell (6). Simultaneously, mitochondrial dysfunction leads to the release of free radicals and the activation of caspases causing apoptosis (7). Dysfunctional neurons fail to maintain microglia in their resting state, and molecules from damaged tissue leads to pro-inflammatory activation of the microglia (M1) and secretion of harmful molecules (8). Peripheral immune cells infiltrate the brain (9). In the later phase, alternatively activated microglia (M2) contribute to tissue healing and inflammatory resolution (10).

The initial symptoms and long-term outcome of recovery differs widely between patients. Not only are the symptoms determined by the site of brain ischemia but also by the size of the injury. Initial symptoms range from paralysis and impairments of speech and vision to unconsciousness and, in the worst case, death. Long-term outcome following recovery is determined by the site of the lesion and the recovery rate of the neurons within the penumbra.

To improve the chances of a better outcome, it is essential to rapidly get the patient under medical surveillance and to administer treatment in the very early phase. Today, few established treatments exist. With thrombolytic treatment, the occlusion is resolved through intravenous administration of tissue plasminogen activator, improving functional outcome even for the milder cases [11]. This treatment has to be induced within a few hours of symptom onset [12,13] and even if received within this time, less than 50% achieve desirable effects [14]. Another treatment is thrombectomy, where the occlusion is mechanically removed intravenously [15]. However, since the vast majority of patients (>70%)) do not seek medical care within the first hours after onset, these treatments are limited [14,16] and new treatments are needed. Many different treatment strategies are promising in animal models but rather disappointing when translated into human settings. The reasons for these failures remain largely unknown but might, in part, be explained by differences between species and the fact that the animals used in experimental settings are rather young [17,18]. The current lack of effective treatments increases the importance of developing preventive strategies.

Recent studies have indicated the possibility to lower the mortality rate in stroke patients by managing different lifestyle risk factors [19]. Well-known risk factors related to lifestyle are smoking, poor diet and physical inactivity [20]. Hypertension, diabetes and age are other risk factors. Certainly, the stroke mortality rate has decreased over the last few years, due to risk factor control [19,20]. Nevertheless, the research presented in this thesis investigates the possibility of physical exercise as a preventive strategy. The potential effects will be further introduced in section 3 of this introduction (see *Exercise as a preventive strategy for disorders affecting the brain*).

Global cerebral ischemia

This thesis focuses on the effects of physical exercise on a specific type of ischemia: global cerebral ischemia. This refers to an ischemic insult that affects large parts of the brain, including, among others, the hippocampus and striatum. It can be caused by either drowning or cardiac arrest, but our research focuses on the latter. Cardiac arrest affects around 10000 people each year in Sweden. Out of the cases that occur outside of a hospital setting, the mortality rate is as high as 90 %. Many of the molecular events in global cerebral ischemia are similar to those of ischemic stroke, but in experimental settings of global cerebral ischemia no delayed selective neuronal death or penumbra is seen [21].

Vascular dementia

Disturbances in the cerebrovasculature is thought to account for 10-20% of all dementia cases, often referred to as vascular dementia (VaD) [22,23]. Men are typically at higher risk for this type of dementia compared to women [23,24]. VaD is divided into several subtypes depending on the cause. For example, hypoperfusion, multiple cortical infarcts, white matter lesions, or recurrent microbleeds may cause vascular dementia [25].

Cognitive deficits are varied and depend on the brain region affected. For instance, attention and executive function may be affected.

Factors that increase the risk of VaD are age, smoking, diabetes, hypertension, and atherosclerosis. Interestingly, vascular risk factors also increase the risk of developing AD [26]. Importantly, many dementia cases display a mixture of AD and vascular dementia pathology [1]. Knowledge about the pathology of VaD, alone or in combination with AD, is scarce. As there are still no effective treatments for vascular dementia, preventive strategies are highly valued. Physical activity might be one such strategy, and this will be further described and investigated in this thesis.

Neurodegenerative diseases

Due to increasing life expectancies but lack of effective treatments or preventive strategies, the number of people affected by neurodegenerative diseases, such as AD and PD, is suggested to increase. In 2015, dementia was estimated to cost societies around the globe over 800 billion dollars (WHO, 2019).

Alzheimer's disease

Today, 44 million people are estimated to suffer from dementia [27], with the prevalence of AD nearly two times higher among women compared to men [28]. Constituting 50-80% of all dementia cases, AD represents a major cause of dementia [22,27], followed by vascular dementia. AD is a neurodegenerative disorder, characterized by a sequential increase and spread of intracellular hyperphosphorylated tau proteins and extracellular aggregates of amyloid beta (Aβ) protein within the brain (see Figure 2). At the same time, there is a progressive degeneration of neuronal tissue, specifically decreasing hippocampal and cortical grey matter size and leading to enlargement of the ventricles. The primary symptom, leading to diagnosis is cognitive impairment, such as executive dysfunction, loss of memory and disorientation. Many patients also suffer from non-cognitive symptoms, like mood dysregulation and sleep dysfunction [22].

The late-onset sporadic form of AD constitutes the majority of AD cases [22]. AD is a multifactorial disorder, and the sporadic form arises from an interaction between environmental and genetic risk factors [27]. Aging is a major risk factor [22,27]. Other risk factors are lifestyle-related, such as smoking, heavy alcohol consumption, poor diet and a sedentary lifestyle [24,29]. On the contrary, having a high education level and high socioeconomic status seem to be protective [30]. Furthermore, having type 2 diabetes, atherosclerosis, a cardiovascular disease or hypertension also increases the risk of AD [24]. This increased risk could, in part, be countered by the use of antihypertensive drugs and statins [31,32]. Even for the sporadic form, there are certain known genetic factors, such as the E4 variant of the apolipoprotein E (ApoE), that increase the likelihood of developing AD [27]. In addition, genetic variants that affect inflammation and

endosomal-vesicle recycling pathways have been linked to the development of AD in large genome-wide association studies [33].

The remaining AD cases, around 1 %, are caused by familial variants, which generally causes an earlier onset of the disease, sometimes as early as 30 years of age [22]. These cases are primarily due to an inherited autosomal dominant pathogenic variant in the gene for either amyloid precursor protein (APP), presenilin-1 (PS1) or presenilin-2 (PS2). These genes encode the protein and enzymes involved in the generation of amyloid beta (Aβ). Nevertheless, the accumulation of Aβ plaques represents a hallmark of both sporadic and familial forms of AD. The normal, physiological function of Aβ is still unknown. Aβ is generated from the cleavage of APP by different enzymes, such as β-secretase and γ-secretase. Once generated, Aβ can be present in different states, such as monomeric, oligomeric, or fibrillar. Aβ aggregates cause synaptic dysfunction and neuronal cell death by harming the neuron, for example by forming ion channels, thinning the cell membrane or overexciting NMDA receptors [34]. The most abundant Aβ fragments are of the following amino acid lengths: Aβ38, Aβ40, and Aβ42, whereof Aβ42 is the most prone to aggregate [34].

Another hallmark of AD is the intraneuronal accumulation of hyperphosphorylated tau, referred to as neurofibrillary tangles. In short, the normal physiological function of microtubule-associated protein tau is to support the cytoskeleton within neurons [34]. Hyperphosphorylation of tau (p-tau) reduces its ability to bind and function as a microtubule-associated protein. Instead, it aggregates and forms neurofibrillary tangles, contributing to neuronal dysfunction and cell death from inside of the cell. Aβ and p-tau aggregation begin in specific brain areas, and the process spreads across the brain as the disease progresses according to the model proposed by Braak et al [35]. Aβ accumulation starts in the frontal/temporal lobes, hippocampus and limbic areas, thereafter, spreading into other cortical areas and lower brain structures, whereas tau accumulation starts in the locus coeruleus in the lower brain region, thereafter, spreading towards hippocampus and neocortex [34-36]. Consequently, the spread of Aβ across the brain can be measured with amyloid-PET, and the levels of secreted Aβ42 and p-tau can be measured from the cerebrospinal fluid (CSF) [37]. Today, these biomarkers are the basis for clinical diagnostic tools.

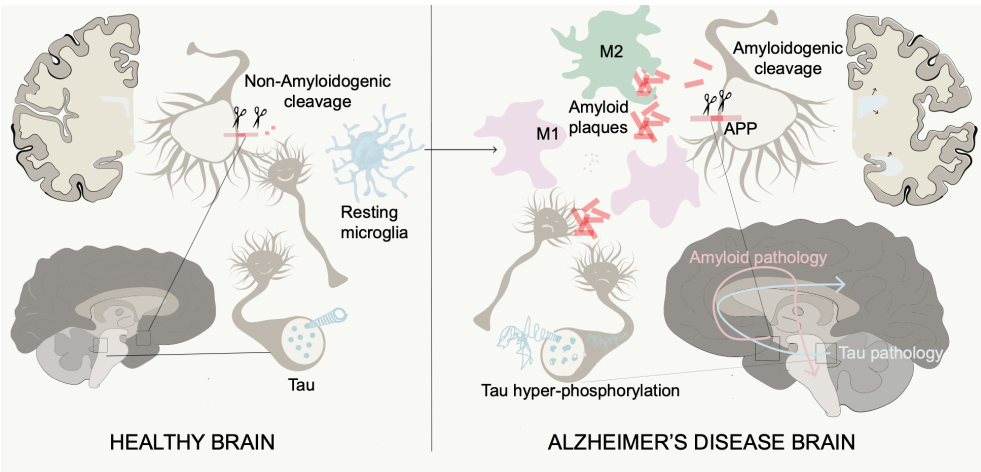


Figure 2. Alzheimer's disease pathology
In the healthy brain, APP is mainly cleaved in the non-amyloidogenic pathway, tau stabilizes microtubuli and microglia are mainly resting. In Alzheimer's disease, APP is cleaved in an amyloidogenic way, generating Aβ, which aggregates to form neurotoxic fibrils and plaques. This activates microglia into different profiles (M1, M2), resulting in phagocytosis of Aβ but also in inflammation and release of neurotoxic molecules. Amyloid pathology spreads from the temporal lobe and limbic areas towards the other cortical structures and lower brain regions (pink arrow). Additionally, tau becomes hyperphosphorylated and aggregates. Tau pathology starts in the lower brain structures and spreads towards the hippocampus and cortex (blue arrow).

Even though much knowledge has been gained since AD was first discovered, there is still no effective treatment. Additionally, it remains unknown what is inducing the abnormal accumulation and aggregation of Aβ and tau. The main clinical trials test treatments focused on reducing the amounts of aggregated proteins within the brain, so far without any obvious success. The fact that the majority of AD cases are sporadic also points out a need to investigate other contributors to the pathology in order to find new therapeutic targets. Interventions aimed at preventing the onset of AD are also highly important. The contribution of neuroinflammation to AD pathology represents one such pathological aspect and promising target. This will be further introduced in the following section. The potential of physical exercise as a preventive strategy for AD represents a major focus of my research and will be further introduced in the last section of this introduction.

Parkinson's disease

Today, 1 % of people older than 60 years of age live with PD [38], a disease affecting men more often than women. The cardinal symptoms of PD are resting tremor, bradykinesia, rigidity and postural instability. Symptoms arise due to the progressive degeneration of dopaminergic neurons within the midbrain, particularly those with cell bodies in the substantia nigra that project their axons into the striatum. These dopaminergic neurons have been suggested to be especially vulnerable due to their pacemaker-like properties causing recurrent calcium transients intracellularly [39]. Even

though the degeneration of dopaminergic neurons within the substantia nigra is the major characteristic of PD, the dysfunction also affects other neurons, such as serotonergic and noradrenergic neurons [40]. Likewise, even though motor symptoms represent the cardinal symptoms, many PD patients also suffer from additional non-motor symptoms, like dysregulated mood, cognitive deficits and sleep disturbances.

To confirm a PD case post-mortem, the affected brain regions also have to display accumulated protein aggregates of misfolded α-synuclein (α-syn). Normally, α-syn is involved in synaptic vesicle release within the axon terminals. In PD, α-syn misfolds, starts to aggregate and, eventually ends up near the cell soma as larger inclusions, called Lewy bodies. Not only is aggregated α-syn dysfunctional, but it also obtains neurotoxic properties, first leading to dysfunctional neurons and ultimately to neuronal death. Once α-syn starts to misfold, the pathology spreads across the brain as described by Braak et al [41].

Despite extensive research, the knowledge is limited about what triggers α-syn to misfold and to aggregate in the first place. It has been proposed that α-syn pathology might have different causes in different patients. PD is classified as either sporadic (around 90-95% of cases) or genetic (remaining 5-10 % of cases) [42]. In the pure genetic forms, the disease results from the familial inheritance of a known disease-causing gene variant, such as a mutation in SNCA or LRRK2 [43]. In the sporadic forms, PD is suggested to be caused by the interaction of genetic and environmental risk factors. Mutations in the gene encoding β-glucocerebrosidase is the most common genetic risk factor [43]. Head injury and pesticide exposure are environmental risk factors, whereas smoking, anti-inflammatory medication and physical activity may be protective.

As dopaminergic neurons degenerate in the substantia nigra, dopamine secretion in the striatum decreases, which explains the symptoms of resting tremor and bradykinesia. Fortunately, L-dopa treatment can supply the dopamine-starved brain with exogenously delivered dopamine, reducing the motor symptoms. However, L-dopa does not prevent the underlying α-syn-driven pathology from progressing. Eventually, neurodegeneration will reach a degree at which L-dopa treatment is no longer helpful. Additional treatment strategies have been tried, ranging from stem cell therapies to deep-brain stimulation, all with substantial limitations. Therefore, finding strategies to prevent or postpone the onset of the disease as well as to reduce the symptoms is of great importance. The research behind this thesis investigates the potential of physical activity as one such preventive strategy, which is further introduced in the third section of this introduction.

Depressive disorders

Current reports estimate that depression affects 350 million people worldwide [44]. Around 10 % of the population are estimated to suffer from at least one depressive episode during their lifetime. Importantly, being diagnosed with depression is around 2 times more common among women compared to men [45]. Given that this also affects people of younger ages, the total number of years lived with a disability caused by depression is substantial [46]. Depression is even reported to be the leading cause of disability [44,47]. Not only does this disorder cause individual suffering, but it also poses a significant economic burden to society.

Depression is characterized by depressed mood, sleep dysregulation, decreased interest in normally enjoyable activities and reduced energy, concentration, and self-esteem. The pathophysiological reasons for depression remains largely unknown. The most well-established explicatory model of depression is the monoamine hypothesis, due to the discovery that depressed patients have alterations in neurotransmitter signaling of monoamines, such as serotonin, dopamine, and noradrenaline [44]. The most prevalent anti-depressant drugs on the market today, selective serotonin reuptake inhibitors (SSRI), target these pathways. Furthermore, depressed patients often have dysregulated stress systems, for example a dysfunctional hypothalamic-pituitary-adrenal (HPA) axis leading to elevated cortisol levels [44]. This finding led to the stress hypothesis of depression. Likewise, psychological stress and adverse life events are associated with an increased risk of depression [44]. Moreover, increased neurogenesis has been showed to reduce depressive symptoms and this discovery led to the neurogenesis hypothesis of depression [44]. Similarly, signs of increased neuroinflammation in depressed patients [48] led to the inflammatory hypothesis of depression, which will be further described in the next section (see *Neuroinflammation in disorders affecting the brain*). Furthermore, genome-wide association studies (GWAS) have linked a risk for developing depression to specific gene variants [49], leading to the genetic hypothesis of depression. However, none of the above-mentioned hypotheses manage to fully explain the disorder and account for all cases. Most researchers nowadays regard depression as a complex disorder, possibly involving multiple alterations in the above-mentioned systems [44]. Many studies also indicate interactions between the abovementioned systems. For instance, inflammatory cytokines affect the monoamines and the HPA axis signaling systems [44]. Further, molecules involved in the HPA axis also influence the rate of neurogenesis [44]. Specific risk gene variants may increase the sensitivity of an individual to environmental events that would push these systems into dysregulated states [44]. Depression might even be regarded as a syndrome rather than a simple disorder that could be explained by a single pathophysiological model.

The lack of one treatment for all patients with depression supports the view of depression as being a rather complex disorder. Treatment options for depression have been revolutionized during the last century with the development of anti-depressive

drugs such as SSRIs, and the use of electroconvulsive therapy to treat drug-resistant depression. However, the frequency of relapse among patients is still high [50], and the number of patients not achieving desirable effects from these treatments is unacceptable [51,52]. Many patients also suffer from severe side-effects from the current treatments [53]. This dilemma emphasizes the importance of finding alternative therapeutic strategies. Physical activity represents a promising preventive strategy and is further described in the last section of this introduction and investigated in this thesis (see *Exercise as a preventive strategy for brain disorders*).

Neuroinflammation in disorders affecting the brain

The immune system

The immune system is an evolutionarily old part of multicellular organisms, protecting them against potentially harmful invading species and substances. It is also involved in development, remodeling, cleaning, tissue homeostasis and synapse pruning. Different cell types and a large subset of sensing and effector molecules make this system complex. It can be divided into two different parts, the innate and adaptive systems, which are continuously complementing and interacting with each other. The innate immune system is the evolutionarily oldest part and represents the first line of defense with its quick response. However, the innate immune system is rather unspecific compared to the evolutionarily younger but slower-acting adaptive immune system, which recognizes the intruding pathogen in a more specific way. The adaptive part of the immune system has an immune memory function, allowing the system to recognize a pathogen faster if it returns. Interestingly, the innate immune system is required for the adaptive immune system to function. The adaptive immune system is, indeed, too slow to solely defend against intruders. Thus, highly evolved species cannot survive without either of these two complementary systems.

The focus of this thesis is the role of the innate immune system in disorders affecting the brain, such as in cerebral ischemia and AD, where microglial cells are the main players of the neuroinflammatory response.

Microglia

As the main immune cell in the brain, microglia are the first line of innate defense. They constitute around 5-12 % of all glial cells within the human and rodent brain, depending on the brain region [54,55]. Importantly, the density of microglia is much higher in the hippocampus and substantia nigra compared to the cerebellum and brainstem [55,56]. Microglial density also differs between grey and white matter [54,55].

Furthermore, the morphology of microglial cells differs substantially between different regions across the brain [55]. Microglia are tissue-resident myeloid cells derived from mesodermal/mesenchymal tissue [57]. Originating from the yolk sac, they migrate into the CNS during embryonic development before the establishment of the blood-brain-barrier [58,59]. Microglial cells are sustained by continuous slow self-renewal, with a median rate of almost 30% per year [60]. During the embryonic development of the central nervous system, microglia contributes to neurogenesis and synaptic maturation [58]. Even in the adult brain, microglia play an important role in maintaining homeostasis and supporting synaptic pruning [58]. In the healthy brain, microglial cells are mainly in a resting state characterized by a complex morphology including branched processes, with which they scan the environment for potential threats [58]. There are two types of microglial motility: movement of their processes during the resting state to sense the environment and migration of the whole cell within the brain parenchyma after activation to translocate towards a pathological site [61,62].

Under normal brain homeostasis, microglia are maintained in their resting state by a regulatory ligand-receptor system, that involves the binding of CD200R and CX3CR1 on microglia to CD200 and CX3CL1 on neurons, respectively [63]. This direct cell-to-cell communication is suggested to prevent microglia from becoming activated. The microglial cell membrane also contains pathogen recognition receptors (PRR) that recognize different extracellular soluble pathological signaling molecules [58]. These signaling molecules can be either endogenously derived damage-associated molecular patterns (DAMPs) released following, for example, tissue damage, or exogenously derived pathogen-associated molecular patterns (PAMPs) from pathological organisms invading the brain [58].

Microglial activation requires changes in two different regulatory systems [58], referred to as "on" and "off". The "off" system has to be down-regulated or dysregulated to reduce the repressive effect on microglial activation. This system includes the neuronal suppression of microglial activation via CD200-CD200R signaling as previously described [63]. Additionally, the "on" system has to be stimulated to induce activation, for example by the recognition of any soluble DAMP or PAMP by PRR on microglia [58]. One specific example is the recognition of lipopolysaccharide (LPS, a well-known bacteria-derived PAMP) by toll-like-receptor 4 (TLR4, a well-known subtype of PRR), which leads to the activation of microglia [64]. Probably, improper microglial activation is the result of an imbalance between these two opposing regulatory systems. If the "on" signaling is strong enough or the "off" signaling is weak enough, it results in microglial activation [65].

Once activated, microglia change their morphology by retracting their processes to become shorter and thicker, and by enlarging their cell body [58]. In their activated form, microglia can secrete a higher quantity of signaling and effector molecules. These microglia-derived molecules have diverse functions. Some are prostaglandins and

cytokines, such as prostaglandin E2 (PGE2) and interleukin-1β (IL-1β), immune signaling molecules regulating the inflammatory status of the surrounding cells [66]. Other molecules include enzymes, like insulin-degrading enzyme (IDE-1), involved in the degradation of extracellular debris [67]. Further, activated microglia are capable of secreting free radicals, such as reactive oxygen species [68].

To complicate the matter further, the population of microglial cells is not homogenous. Rather, with increasing knowledge, researchers have come to view activated microglia as a highly heterogeneous cell population [69]. There is, however, a growing debate on how to classify and divide the different activated phenotypes [70-72]. The most common classification is M1-M2 profiling [72]. This concept is derived from in vitro macrophage research and has, to some extent, also been proven to be applicable to microglial cells due to their close relationship with macrophages [72,73]. The M1 and M2 activated phenotypes are induced by distinct factors, exert diverse functions and involve different secreted factors, visualized in Figure 3 below.

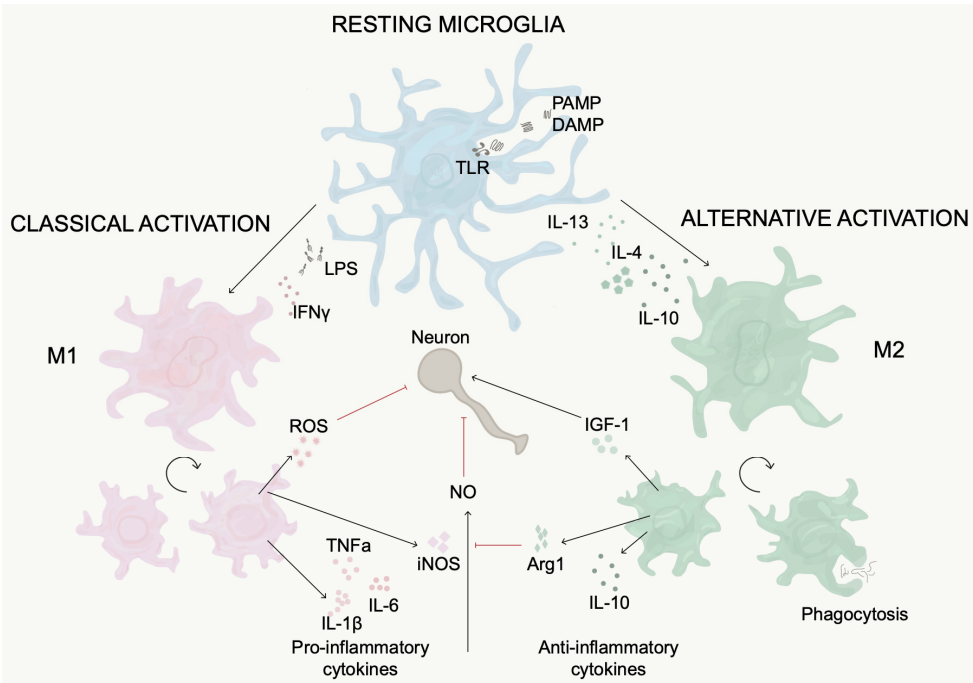


Figure 3. Microglial profiling
In the healthy brain, the majority of microglial cells are in a resting state, with a ramified morphology with long and thin processes. When microglia are activated they start to proliferate. Stimulation with lipopolysaccharides (LPS) or interferon-gamma (IFNγ) leads to the classical activation of pro-inflammatory microglia (M1), which secrete pro-inflammatory cytokines, iNOS and reactive oxygen species (ROS) that are neurotoxic. Opposingly, IL-4, IL-13, and IL-10 may alternatively activate microglia into an anti-inflammatory phenotype (M2) that secretes neurotrophic factors, arginase 1 (Arg1) and anti-inflammatory cytokines.

The M1 profile represents the classical activation of microglia, induced by LPS and IFNγ [73]. The M1 profile has neurotoxic properties, including the expression of iNOS and the secretion of reactive oxygen species and pro-inflammatory cytokines, such as TNFα, IL-1β, and IL-6 [72,73]. Opposingly, the M2 profile represents the alternatively activated microglia, which are suggested to be anti-inflammatory and neuroprotective [72,73] because of their secretion of neurotrophic factors, such as IGF-1, and anti-inflammatory cytokines, such as IL-10 [68,72,73]. The M1 and M2 phenotypes also have different metabolic profiles [74]. While M1 produce most of their energy from anaerobic glycolysis, M2 depend on oxidative phosphorylation to produce energy. The M2 category has been further subdivided into M2a, M2b, and M2c [59,72,73,75]. M2 microglia are suggested to be induced by anti-inflammatory cytokines such as IL-4, IL-10, and IL-13 [73]. M2 are characterized by their expression of arginase 1, an enzyme that opposes the effect of iNOS. They also have a higher capacity for phagocyting cellular debris [74]. Lately, it has been suggested that profiles of microglia are rather dynamic, allowing the cells to respond to changes in the environment and to acquire new phenotypes at a later timepoint [76-81]. Because of this, most experts see the M1-M2 model as a simplified description with limited, if any, applicability to reality [70]. Many scientists believe that microglia phenotypes are not classifiable into isolated, distinct categories, such as M1 and M2, but rather are better described by a continuum where M1 and M2 are the two extremes [82]. Others suggest that microglial profiling is multidimensional, including characteristics that we are not yet aware of [83]. This situation is for the future researchers to clarify.

Taken together, microglia provide a promising area for future scientific discoveries that answer questions regarding pathological processes and uncover potential targets for new therapies against disorders affecting the brain.

Other immunological cellular players within the brain

Apart from microglia, neuroinflammation is also affected by other brain resident cells. Besides supporting neuronal functions and homeostasis, astrocytes have inflammatory properties [84]. For example, they are capable of secreting cytokines and promoting immunosuppression [84]. Intriguingly, like microglial cells, astrocytes have also been suggested to have different functional phenotypes in disease conditions [85]. Indeed, in a study in *Nature,* Liddelow *et al.* showed that activated microglia could be involved in the induction of a specific neurotoxic phenotype of astrocytes [85]. Neurons also play a role in controlling the immune response [86]. Further, perivascular cells, such as pericytes have inflammatory properties [87]. Evidently, perivascular cells are plastic and possess great migratory abilities, characteristics that are demonstrated when they leave the vessel wall and acquire a microglial phenotype following a stroke [88]. Moreover, even oligodendrocytes have been shown to produce and respond to cytokines and cross-talk with microglia [89]. In disease conditions when the blood-brain barrier is compromised,

such as during brain ischemia, peripheral immune cells, such as monocytes, neutrophils, T-cells and B-cells, demonstrate their neuroinflammatory impact as they can infiltrate the brain [90]. However, my focus on neuroinflammation in this thesis is on the role of microglia.

Cytokines

Cytokines and other soluble signaling molecules are crucial for the communication between microglial cells. To date, over 300 different cytokines have been discovered [91]. Cytokines are defined as small immunomodulatory signaling proteins with the ability to work in an autocrine, paracrine and endocrine manner. When interacting with an immune cell, cytokines bind to specific receptors, inducing different downstream intracellular signaling cascades and molecular reactions within the target cell. Thus, the effect on the target cell is not only determined by the type of cytokine but also by the receptor type expressed on the target's cell membrane. Due to the pleiotropic nature of many cytokines, it has been challenging to classify cytokines based on their effects. Adding to that, different brain regions may respond very differently to the same cytokine [91]. To complicate the matter further, the effect of a specific cytokine on a specific target cell also depends on simultaneous signals from other contextual signaling molecules. It is also not certain whether a cytokine with certain properties in the periphery will display the same characteristics in the CNS. For example, IL-6 constitutes an excellent example of a cytokine previously described to have potent pro-inflammatory effects which later turned out to also display anti-inflammatory properties when studied in another context (See the section about Exercise as a preventive strategy). Moreover, much of what is known about cytokines is derived from animal studies, and the immunological functions sometimes differ between animals and humans [69,92].

Still, many researchers classify cytokines as either pro-inflammatory or anti-inflammatory. For instance, interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ) are often regarded as pro-inflammatory, whereas IL-4, IL-10, and IL-13 are regarded as anti-inflammatory. Table 1 offers a simplified overview of the classification of some of the most studied cytokines which are also investigated in my scientific work. The work in this thesis includes cytokine analyses from several different studies investigating different disorders affecting the brain. These cytokine analyses can be seen as fingerprints that are unique to the specific models and circumstances applied in my studies.

Table 1. Cytokine characteristics
Characteristics of cytokines investigated in this thesis. Source refers to the phenotype of the microglia producing the cytokine indicated; Unknown type of microglia (M), classically activated microglia (M1) or alternatively activated microglia (M2). Classification as anti-inflammatory (-) or pro-inflammatory (+) is given if available. Note that cytokines are produced not only by microglia but also by other cells in the brain.

Cytokine	Source	Classification	Effect	Reference
TNFα	M1	+	Activation of NFκB Induction of cell death pathways	73
IFNγ	M1	+	Induce M1 and MCH II	73
CXCL1	M1	+	Chemoattraction of neurophils Spinal cord development	93,94
IL-1β	M1	+	Induce COX-2 and metalloproteases induce pro-inflammatory cytokine expression	95
IL-1ra	M2	-	Antagonizing IL-1β	73
IL-2	M1	+	Brain homeostasis	73,96
IL-4	M2	-	Induce M2 Antagnoinzing IL-1β	73,97
IL-5	M	-	Microglial activation	98-100
IL-6	M1	±	Depend on context and induction source Energy homeostasis Neuroinflammation	101-104
IL-10	M2	-	Induce M2 Reduce synthesis of pro-inflammatory cytokines	73,97,105
IL-12	M1	±	Depends on stage of pathology, Early: pro-inflammatory Late; resolution, limiting pathology	97,106

Galectins

Another category of soluble inflammatory mediators is galectins. These molecules are beta-galactoside-binding lectins, characterized by their affinity for poly-N-acetyllactosamine-enriched glycoconjugates. Galectins have inflammatory properties [107-110] and are involved in homeostatic functions, such as cell adhesion, myelination, vesicle transport and proliferation [109,110]. In the brain, the major galectins expressed are galectin 1, 3, 4, 8 and 9 [110]. Galectin-3 is the only galectin investigated in my thesis work.

Galectin-3 is expressed, either constitutively or by induction, in almost all inflammatory cell types, including microglia [107]. In fact, microglia are the major source of galectin-3 in the brain. However, the expression levels are rather low in a healthy brain and induced in disease conditions. This molecule can either work extracellularly, by binding to and activating receptors on the cell surface, or intracellularly, by interacting with signaling molecules in the cytoplasm [107]. Its effects demonstrates further diversity because of its ability to oligomerize [107], thus creating additional structural sites for interactions, for example, with extracellular matrix molecules. Existing research demonstrates inconsistent properties of galectin-3 in neurological

diseases with a neuroinflammatory component [108,110]. Interestingly, depending on which disease model is used, galectin-3 has been shown to both increase inflammation and decrease neuronal death as well as to affect the remodeling capacity. These discrepancies might be attributable to the effect of timing, since galectin-3 might affect microglial cells and the surroundings differently, in different contexts and at different time points of disease progression [111]. Notably, galectin-3 is capable of inducing the production of IL-1β and TNF-α, cytokines classified as pro-inflammatory [112]. Moreover, it promotes microglial proliferation [110] and migration [113] and affects phagocytic capacity [114].

Given the above-mentioned properties of galectin-3 and its possible effects on cytokine production in microglia, it is a molecule of interest when looking at the effects of the interventions studied in this thesis.

Intracellular inflammatory signaling pathways

Once bound to receptors on the microglial cell membrane, signaling molecules, such as cytokines and galectins, activate intracellular inflammatory signaling pathways. This leads to a change in the characteristics and the function of the cell. The exact details of these downstream intracellular signaling pathways are beyond the main focus of this thesis and will, therefore, only be briefly described. Nuclear factor kappa beta (NFκB) is an important master regulator when it comes to the transcription of different inflammatory proteins. Downstream effects of cell membrane receptor signaling often involve this transcription factor complex. Once activated, NFκB translocates from the cytosol into the nucleus, inducing expression of inflammatory genes. For example, the expression levels of several cytokines and other inflammatory effector molecules are regulated by NFκB. Another intracellular protein complex involved in the inflammatory response is the nucleotide-binding leucine-rich repeat-containing family pyrin domain containing 3 (NLRP3). NLRP3 is an inflammasome, a multi-protein cytoplasmic complex which, upon stimulation, promotes the cleavage of caspases. This ultimately leads to the processing and secretion of pro-inflammatory cytokines, such as Il-1β. Taken together, NFκB and NLRP3 are important intracellular inflammatory effector molecules with potential roles as therapeutic targets in diseases with a neuroinflammatory component.

Neuroinflammation as a contributor to disorders affecting the brain

Many studies have pointed out neuroinflammation as an important contributor to different disorders affecting the brain. Below, I will describe the neuroinflammatory component of each disorder included in this thesis.

The role of neuroinflammation in brain ischemia

Within minutes to hours after the onset of ischemia, an inflammatory response is induced though the activation of resident microglia and the recruitment of peripheral immune cells. The ischemia-induced neuronal injury affects the neuronal ability to maintain microglia in their resting state as previously described. Neuronal damage in brain regions controlling the immune and sympathetic nervous systems may affect the regulation of immune functions [115]. Moreover, damaged neuronal tissue secretes inflammatory mediators, such as cytokines, that recruit and activate a variety of immune cells. For example, IL-1β has been shown to aggravate ischemic damage [116]. Other cytokines, such as TNF-α seem to have both protective and harmful effects following ischemia [115,117], most likely dependent on the target cell and environmental context. Recruited immune cells also contribute to the neuroinflammation following brain ischemia, but this thesis focuses on the microglial contribution.

Even though inflammatory cells contribute to the expansion of neurological damage following ischemia, it is important to emphasize their role in the recovery and healing phase. Experimental studies clearly show that inhibition of inflammatory cells and mediators following ischemia might also worsen the outcome [118]. For instance, microglia can also achieve the so-called M2 phenotype and promote the healing process through phagocytosis of necrotic neurons and secretion of neurotrophic factors. Thus, if we wish to interfere with the neuroinflammatory response following ischemia, we need to increase our knowledge concerning the therapeutic time window and environmental context for different inflammatory mediators. This thesis work is one step towards this crucial characterization.

The role of neuroinflammation in neurodegenerative diseases

Numerous infectious agents have been suggested to increase the risk of neurodegenerative diseases, such as AD [119]. Due to its antimicrobial properties in culture, Aβ may be produced in response to infectious agents as an evolutionary immune response [119]. Still, the causal link behind the association between infections and the development of AD has to be clarified. Nevertheless, increased activation of microglia has been confirmed in human AD brains in several post-mortem studies [120], indicating a neuroinflammatory role in the disease. Yet, rather than be cause by infectious agents, increased microglial activation may be a consequence of the amyloid pathology in AD [121,122]. Nevertheless, recent GWAS point towards microglial involvement because of certain genetic risk factors [123,124]. For instance, mutation in the microglial gene TREM-2 increase the risk of developing AD and a variation of the galectin-3 gene has also been linked to an increased risk [125,126]. Thus, neuroinflammation seems to be an early contributor to AD pathology.

Microglial contribution to AD appears diverse: initially, microglia appear to protect the neurons from Aβ [127]. In contrast, in the later stages of the disease, the protective

phagocytic capacity of microglia seems to be lost [128]. Instead, microglia may be stuck in a chronically activated state and contribute to neuronal damage through the production of pro-inflammatory molecules [128]. Interestingly, Aβ is capable of activating microglia through TLR4 [129], an ability that might remain from its suggested evolutionary role as a response to infections [119]. Further, inflammatory galectin-3 is upregulated in post-mortem human AD brains and specifically expressed in microglia closest to Aβ plaques [126]. The factors that determine the balance between different microglial phenotypes and, thus, the overall effect within the AD brain remains to be elucidated. Increased knowledge about these processes might reveal novel therapeutic targets.

Similarly to brains from AD patients, post-mortem brains from PD patients show elevated levels of reactive microglia [130]. Additionally, increased microglial activation has been shown by positron emission tomography (PET) imaging of PD patients [131]. Like Aβ, α-synuclein is also capable of inducing a microglial response [132], and, remarkably, galectin-3 is involved in this response as well [132]. Future studies that increase the knowledge about the inflammatory component of PD may uncover future therapeutic targets.

The role of neuroinflammation in depression

Depressed patients also have increased microglial activation, as measured with PET [133]. Interestingly, long-lasting and untreated depression were strong predictors of microglial activation. Further, post-mortem brains from depressed patients reveal increased cytokine levels [134]. Modern biotechnology makes it easier to investigate the causality in this association. For instance, polymorphisms in cytokine genes have been suggested to affect the risk of depression [135]. Further, Dahl *et al.* showed that serum cytokine levels were increased in depressed patients, but normalized to control levels in those recovering [136]. Moreover, interferon treatment in patients with other medical conditions increased their risk of developing depression [137]. Thus, whether inflammation is the cause or consequence of depression, it is suggested to contribute to the pathology. To reduce inflammation might be a successful strategy for the prevention and treatment of depression.

Exercise as a preventive strategy for brain disorders

Exercise is one part of an active and healthy lifestyle. Its effects on the body, including the brain, have been subject to increasing interest in modern society and healthcare. Physical activity is defined by Caspersen et al. as “any bodily movement produced by skeletal muscles that results in energy expenditure”. Further, they define exercise as “a subset of physical activity that is planned, structured, and repetitive and has as a final or an intermediate objective the improvement or maintenance of physical fitness” [138].

Adding to that, exercise also should result in a substantial increase in cardiac output. Exercise affects a multitude of physiological systems within the body: neurotrophic factors, neurotransmitters, the HPA axis, and the immune system are examples of systems affected by physical activity. Questions that are of further interest include how exercise affects specific pathological hallmarks involved in particular disorders affecting the brain, such as brain ischemia, a neurodegenerative disorder, or depression.

My thesis focuses on the effect of exercise on the risk of developing disorders affecting the brain, with a special focus on its effects on neuroinflammation and symptoms in the context of specific pathological hallmarks for these disorders.

Exercise effects outside the brain

The effect of exercise outside the brain is beyond the scope of this thesis but needs to be briefly described to illustrate the complexity of exercise-mediated effects on the body as a whole.

Muscular effects of exercise

Since exercise implies muscle movement, it seems logical that it gives rise to molecular changes within muscular tissue. Indeed, contracting muscles secrete molecules, referred to as myokines [139]. Some of these myokines are also known as cytokines, and these were originally discovered to have immunological properties within the blood, as previously described (see *Neuroinflammation in disorders affecting the brain*). After being secreted from muscle cells in response to contraction, myokines act both autocrinally, within the muscle, and endocrinally, on other tissues [140]. Myokines secreted following exercise seem to promote a more anti-inflammatory status within the body [139]. Thus, exercise-induced myokines are considered to have a beneficial, immuno-dampening effect on inflammatory diseases [139]. Myokines induced by exercise include, amongst others, IL-6, IL-15, irisin and BDNF [140]. Among these, muscle-derived IL-6 has caught great interest. Previously seen as a pro-inflammatory cytokine, IL-6 has been observed to possess anti-inflammatory properties when of muscular origin under exercise conditions [141,142]. Hence, IL-6 is a double-edged sword in the context of inflammation. Its properties are determined not only by its concentrations and source but also by simultaneous trans-signaling in the targeted tissue [143]. Remarkably, exercise-mediated muscle-derived IL-6 may also affect the inflammatory status inside the brain [144]. Moreover, through muscular expression of kynurenine-aminotransferases, exercise seems to tilt the peripheral kynurenine metabolism, trapping metabolites of these pathways in the periphery and, thereby, preventing potentially harmful metabolites from arising in the brain [145-147]. However, these exercise-mediated effects on plasma kynurenine levels in patients are not long-lasting[148], and its long-term impact on the brain has to be further investigated.

Metabolic effects of exercise

Amongst the most well-known effects of exercise are the metabolic effects. Interestingly, IL-6 has a direct effect on lipid and glucose metabolism [144,149] Exercise-mediated increase of IL-6 is suggested to play a metabolic role and not a pro-inflammatory role as previously thought [144]. Similarly to muscles, adipose tissue secretes a collection of molecules, in this context termed adipokines [140]. A growing number of researchers claim that adipocyte-derived inflammatory cytokines are responsible for the chronic low-grade inflammation seen in inactive individuals with metabolic syndromes [150]. Subsequently, the metabolic effects of exercise might ameliorate this inflammatory status by improving the composition of adipose tissue to include more brown adipose tissue [150].

Peripheral immunological effects of exercise

Exercise is well-known to affect the inflammatory status [139,151] although the exact mechanisms must still be elucidated. Figure 4 shows how the effects of exercise on various inflammatory mediators may interact at a systemic level. As mentioned previously, exercise affects the secretion of immunomodulatory molecules from muscle and adipose tissue, and these effects likely affect the inflammatory status at a systemic level. Notably, an acute bout of exercise leads to different effects on immune cells compared to effects observed during regular exercise. The effect on immune cells also differs based on whether the cells are of the innate or the adaptive immune system. Generally, within hours, exercise induces increased levels of circulating innate immune cells, like neutrophils, NK cells, monocytes and macrophages [151,152]. This might be driven by the exercise-mediated increase in adrenaline and cortisol (see *Exercise effects on the HPA axis and adrenergic stress systems*). Moreover, the expression of cellular adhesion molecules (CAMs) is reduced [153], indicating diminished migration of immune cells from the blood to surrounding tissues. In contrast, acute exercise reduces the number of circulating adaptive immune cells, such as T lymphocytes [151]. Remarkably, long-term effects of regular exercise seem to be a normalization of innate immune cells in circulation and a reduction of their inflammatory state [151].

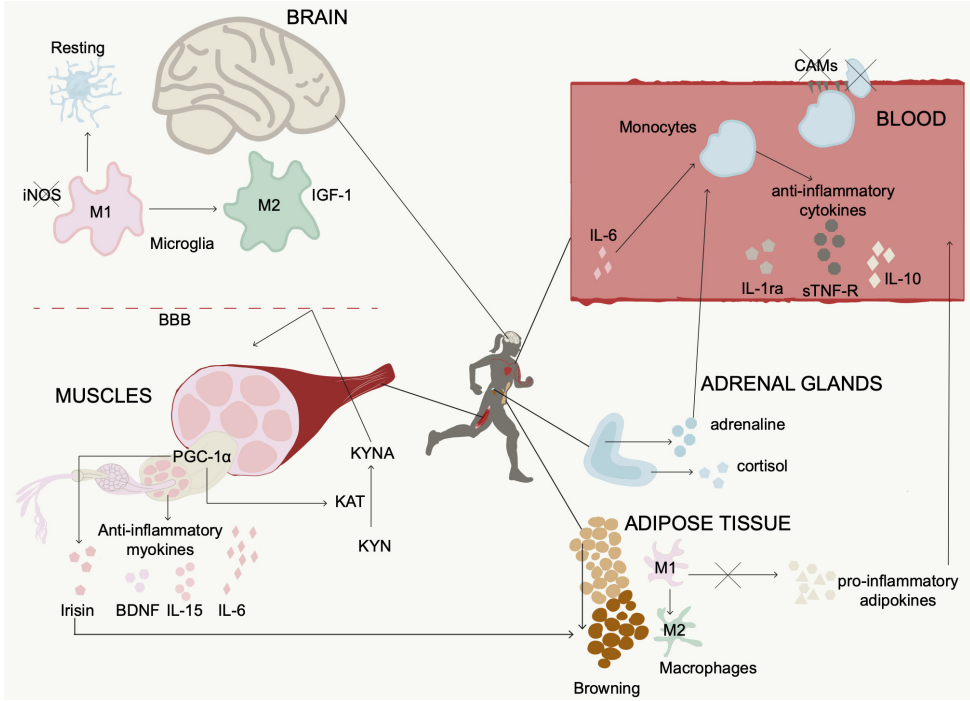


Figure 4 Anti-inflammatory effects of exercise
Exercise-induced expression of myokines, like IL-6, have systemic anti-inflammatory effects. IL-6 increases 100-fold in the blood and induces the expression of anti-inflammatory cytokines. Additionally, PGC-1α is produced in contracting muscle cells and leads to the expression of KAT, an enzyme catalyzing the conversion of KYN to KYNA, which traps these potentially neurotoxic metabolites in the periphery by making them unable to cross the blood-brain barrier (BBB). Further, cellular adhesion molecule (CAM) levels on the vessel wall are reduced, diminishing the extravasation of immune cells. Exercise induces browning of adipose tissue, which is associated with less pro-inflammatory adipokines and a phenotypic switch of adipose tissue-resident macrophages into more anti-inflammatory macrophages (M2). Adrenaline is secreted from adrenal glands and may dampen the pro-inflammatory response of macrophages and microglial cells. In the brain, exercise favors the anti-inflammatory microglial phenotype (M2), reduces microglial activation and suppresses pro-inflammatory microglial (M1) iNOS expression.

On a molecular level, exercise-mediated changes have been observed with several different immunological proteins, such as down-regulation of TLR-2 and TLR-4 [154], receptors involved in pro-inflammatory intracellular signaling. Furthermore, the levels of pro-inflammatory C-reactive protein (CRP) are elevated immediately after exercise [152], but baseline levels are reduced with regular exercise [155-157]. Acute exercise also gives rise to increased levels of cytokines, such as IL-15, IL-6, IL-1ra, sTNFα-R, and IL-10 [140,158-160]. The most profound increase is seen in IL-6 levels, peaking within an hour after an exercise session with up to a 100-fold increase of concentrations and, thereafter, returning to baseline within 24-72 hours [161-165]. In contrast, regular exercise decreases baseline levels of IL-6 [155,156,166]. Importantly, an exercise-mediated increase of IL-6 is not accompanied by increases in levels of typical pro-inflammatory cytokines such as TNFα and IL-1β, which would happen in the case of infectious or inflammatory conditions [139,167]. On the contrary, an exercise-mediated increase in

circulating IL-6 seems to inhibit TNFα production [139] and stimulate production of IL-10 and IL-1ra, cytokines with anti-inflammatory properties [141]. IL-6 affects the levels of downstream inflammatory mediators, possibly via epigenetic modifications on inflammatory target genes [168]. The exercise-mediated induction of IL-6 is followed by changes in the methylation of genes implicated in immune functions [168]. However, exercise-mediated changes in systemic IL-6 levels are highly dynamic, and caution needs to be taken regarding the timing of sample collection. Indeed, cytokines are rapidly excreted into urine [160]. Furthermore, other factors, like ethnicity, also affect the degree of change in cytokine concentrations following exercise [169].

In summary, most studies point towards the potential of exercise to reduce systemic inflammation.

Exercise effects inside the brain

Compared to the effect of exercise on other parts of the body, its effects on brain function are far less investigated. This lack of knowledge serves as a starting point for a new field of research, in which scientists have only taken the very first steps. With this mentioned, we will go through what is known and hypothesized about the effects of exercise on the brain as of today.

Exercise effects on neurotrophic factors and neurotransmitters

It is well-known that exercise affects neurotransmitters and neurotrophic factors in the brain [170]. Brain-derived neurotrophic factor (BDNF) has emerged as a key player, and tremendous efforts have been made to investigate its role in mediating the beneficial effects of exercise on the brain. Indeed, BDNF levels have been shown by many studies to increase in response to exercise [171-175]. Contrarily, other studies show that the beneficial effects of exercise are independent of BDNF [176-179]. However, the lack of an effect on BDNF following exercise might be explained by factors such as not reaching the exercise duration threshold or not accounting gender, since the effect seems to be less pronounced in women [171,172].

The noradrenergic, serotonergic, dopaminergic and endocannabinoid systems are affected by exercise [170,180,181]. Interestingly, the beneficial effects of exercise in several animal models seem to depend on the serotonergic 5-HT3 receptor [182] and the noradrenergic system [183]. Additionally, exercise may enhance the affinity between dopamine and its receptor [170] as well as increase the levels of serotonin [170] and noradrenaline [184]. The understanding of these effects is further complicated by the impact of different exercise parameters such as intensity and duration. On top of that, the effect of exercise also differs between different brain regions.

Exercise effects on the stress systems

Exercise also affects central systems implicated in the stress response [185]. For example, acute exercise increases the plasma levels of both cortisol and adrenaline [185,186], key players within two different systems related to stress. Adrenaline and cortisol are produced in the adrenal gland medulla and cortex, respectively. Adrenaline increases immediately after exercise onset and acts on adrenergic receptors composing the autonomous nervous system to regulate heartbeat and respiration in order to cope with external demands during exercise. Also, in the brain, adrenergic signaling is affected and regulates cerebral blood flow distribution [187,188]. The effect varies between different brain regions [186]. Interestingly, adrenaline appears to affect microglial expression of cytokines and response to LPS via beta-2-adrenergic receptors (β2-AR) [189,190]. Indeed, cell culture experiments show that β2-AR stimulation leads to the production of IL-1ra, which can antagonize neurotoxic IL-1β [191]. Thus, an exercise-mediated increase in adrenergic signaling via β2-AR on microglia may be anti-inflammatory. Moreover, exercise is suggested to affect macrophage polarization states in favor of the more anti-inflammatory M2 phenotype via β2-AR [192]. Interestingly, beta-2-adrenergic stimulation of microglia increases their capacity to take up Aβ [193,194].

It usually takes a few minutes from exercise onset to increase the levels of cortisol, as it requires a step-wise activation of the hypothalamic-pituitary-adrenal (HPA) axis within the brain. By inducing the release of corticotropin-releasing hormone from the hypothalamus, which subsequently induces a release of adrenocorticotropic hormone from the pituitary gland, exercise makes the adrenal glands within the kidneys produce cortisol [152]. Again, exercise parameters, like intensity and duration, affect the degree of the HPA axis response [195-197], but more exercise is not necessarily more beneficial. For instance, potential beneficial effects on the HPA axis might be lost in overtrained individuals [197]. Moderate quantities of exercise are suggested to train this system to better cope with stress as revealed by a shorter-duration HPA axis response to a stressor [198,199] and adaptation through reduced tissue sensitivity to glucocorticoids [195]. When studying these effects in animal models, it is important to consider the difference between forced and voluntary exercise, since forced exercise may also induce psychological stress [200-202]. Importantly, the effect of HPA responses on brain cells seems to differ between stress and exercise conditions [203] (see *Stress as a confounding factor in exercise research*).

The effect of exercise on neuroinflammation

Neuroinflammation is suggested to contribute to our most prevalent disorders affecting the brain. The knowledge of exercise-mediated effects on neuroinflammation is scarce, mainly due to limitations in monitoring these mediators in the brain of living patients. Measurement of cytokines in CSF offers an approximation giving insight into the cytokine levels within the brain. However, this method does not reveal differences

between brain regions. Therefore, the use of experimental animal models is of great importance.

Previously described as the main immune cell in the brain (see *Microglia*), microglia are interesting targets for exercise-mediated neuroinflammatory effects. Numerous studies reveal that exercise may affect microglia in terms of activation, phenotypic profiling, and distribution [204-206]. For instance, exercise may decrease the total number [207] of microglia as well as dampen its activation [208-211] and proliferation [212]. Exercise increases CX3CL1/CX3CR1 [205] and IGF-1 levels [206], suggesting a phenotypic shift in favor of the more anti-inflammatory and neuroprotective M2 phenotype [205,213]. Further, the pro-inflammatory neurotoxic M1 phenotype is disfavored because of iNOS suppression [210] and a reduction of CD86 and major histocompatibility complex II (MCH II) [204] in exercised mice. Phagocytic capacity is also affected [214] as exercise can stimulate microglial phagocytosis of harmful debris [214]. Still, investigating these aspects separately in different animal models does not give insight into the overall picture of how exercise affects microglia and the results are inconsistent between studies. Contrary to the above-mentioned effects, other studies show that exercise might increase microglial activation [215,216], proliferation [212] and cell numbers [217]. The different effects of exercise on microglial cells are summarized in Table 2.

Inflammatory intracellular pathways within microglial cells could be of particular interest to increase our knowledge about the mechanisms behind the neuroinflammatory effects of exercise. For instance, exercise may reduce the levels of myeloid differentiation 88 (MyD88) [101,209,218], a downstream target-protein of pro-inflammatory TLR-4-activation. Moreover, exercise reduces the phosphorylation of NFκB [209,218], a transcriptional master regulator of inflammatory signaling molecules (see *Intracellular inflammatory signaling pathways*). Depending on the site of exercise-mediated reduced phosphorylation on NFκB, it will affect the expression of different inflammatory target genes, such as cytokines [219].

Table 2. Effects of exercise on microglia.
Note that the table only list studies that reported effects. Several studies report no effects.

Microglial function	Effect of exercise	Exercise setting	Reference
Activation	↓ activation	Treadmill	205,208-211
	↑ activation	Treadmill, Wheel	215,216
Proliferation	↓ number of microglia	Treadmill, Wheel	206,207
	↑ microglial proliferation	Wheel	212,217
Phagocytosis	↑ phagocytosis of debris	Wheel	214
Profiling	↓ iNOS (M1)	Treadmill	205,210,213
	↓ CD86 (M1)	Treadmill	204,205,213
	↓ MHC II (M1)	Wheel	204
	↑ MHC II (M1)	Wheel	204
	↑ IGF-1 (M2)	Treadmill, Wheel	205,206
	↑ CD206, Arg1 (M2)	Treadmill	205,213

Cytokines are responsible for the extracellular inflammatory signaling between microglial cells. Remarkably, exercise decreases the protein levels of pro-inflammatory TNFα while increasing the levels of anti-inflammatory IL-10 in murine hypothalamus [220]. Likewise, following exercise, TNFα and IFNγ decrease in the prefrontal cortex [221], and IL-1β and TNFα decrease in the hippocampus [222]. Like in the periphery after exercise, IL-6 increases in the brain [223,224] and may even be crucial for the beneficial effects [101]. In human CSF, no changes have been detected in the levels of TNFα and IL-6, following exercise [225,226] despite increased IL-6 in plasma. This might be due to the timing of CSF sampling. Moreover, cytokine levels seem to depend on exercise duration, intensity, and type as well as on the brain region, making cytokine levels difficult to compare across studies. Altogether, most experimental studies indicate a tendency for exercise to reduce central levels of pro-inflammatory cytokines and increase levels of anti-inflammatory cytokines [220,227-229]. The effects of exercise on cytokines are summarized in Table 3.

Exercise also affects other extracellular inflammatory molecules. By increasing the expression of cyclooxygenase-2 (COX-2), exercise may lead to increased production of inflammatory lipid signaling molecules, like PGE2, a well-known prostaglandin [230,231]. This effect appears to be dependent on NFkB and to become more pronounced with increasing exercise intensity [230]. Interestingly, increased COX-2 expression in the brain is correlated with increased IL-6 in plasma [232]. Moreover, the effects of exercise on COX-2 and PGE2 are time-dependent: the levels increase immediately after exercise but return to baseline within a few days, and the long-term effect of exercise is decreased levels of these molecules [233,234].

Table 3. Effects of exercise on cytokines
The effect of exercise on cytokine production and secretion in the brain. Note that the table only displays studies that report effects. Several studies report no effects. hip=hippocampus, cxt=cortex, hyp=hypothalamus, pvn=paraventricular nucleus, str=striatum.

Cytokine	Effect of exercise	Exercise setting	Reference
TNFα	↓ TNFα in whole brain	Treadmill	229
	↓ TNFα in prefrontal ctx	Swimming	221
	↓ TNFα in hyp	Treadmill	220
	↓ TNFα in hip	Wheel, Treadmill	101,222,223
	↓ TNFα in pvn	Treadmill	228
	↑ TNFα in ctx and str	Treadmill	235
IFNγ	↓ IFNγ in prefrontal ctx	Swimming	221
IL-1β	↓ IL-1β in hip	Wheel	222
	↓ IL-1β in whole brain	Treadmill, Wheel	229,236
	↑ IL-1β in whole brain	Wheel	236
	↓ IL-1β in pvn	Treadmill	228
IL-6	↑ IL-6 in whole brain	Wheel	236
	↑ IL-6 in hip	Treadmill, Wheel	101,223
	↑ IL-6 in CSF	Swimming	224
IL-10	↑ IL-10 in whole brain	Treadmill, Wheel	229,236
	↑ IL-10 in hyp	Treadmill	220
	↑ IL-10 in pvn	Treadmill	228

In conclusion, different modeling systems and exercise parameters and a lack of gender considerations in previous studies make it difficult to draw general conclusions. Even though studies show conflicting results, the majority of published research points towards a beneficial, anti-inflammatory effect of exercise on the brain.

The effect of exercise on other cell types within the brain

Exercise-mediated effects on other cell types is beyond the scope of this thesis. Briefly, exercise affects astrocyte morphology [237-239], proliferation [240], numbers [241,242], and activation [243]. Additionally, the capacity of astrocytes to provide neurons with fuel, such as lactate, increases with exercise [244]. The glymphatic function of astrocytes improves with exercise due to increased expression and polarization of the aquaporin 4 channel (AQP4), which is suggested to improve glymphatic clearance of Aβ from the brain tissue [241]. Further, exercise increases the proliferation and development of oligodendrocyte progenitor cells [245] and improves the myelination status in animal models of ischemia, Alzheimer's disease and depression [246-248].

Exercise effects on the diseased brain

Using exercise as a preventive strategy to diminish the burden of different brain disorders is of growing interest. Research databases offer a multitude of experimental studies investigating the molecular exercise-mediated effects on specific pathological hallmarks of the disorders included in this thesis.

The majority of studies investigating the preventive potential of exercise suffers from one general limitation: the impact of a phenomenon called reverse causation is not taken into account. Reverse causation implies that early disease symptoms may reduce the ability to participate in physical activities even before diagnosis. For dementia disorders like AD and VaD, cognitive impairments may prevent a person from planning and executing structural exercise. Reduced motor function in the early, undiagnosed phase of PD is another potential source of reverse causation. For depression, reduced mood may prevent individuals from participating in physical activities even before diagnosis. Both neurodegenerative diseases and depression often have an early, sometimes quite long, phase with symptoms before getting the diagnosis. This increases the risk of introducing bias due to reverse causation in studies analyzing the effect of exercise on the development of these disorders. Study designs should consider this phenomenon.

The effect of exercise on brain ischemia

Many studies reveal protective effects of exercise on the development of brain ischemic attacks [249-251]. Both men and women are protected by exercise [252,253], although women might require higher intensity exercise compared to men to achieve these protective effects [253] and one study showed no significant protection for physically active women [254]. Interestingly, exercise may be more beneficial in women in lower age groups [255]. The type of stroke might also matter; moderate exercise intensity may be optimal since activities of very high intensity might even be detrimental for hemorrhagic stroke [256]. Still, another study demonstrates that even highly active individuals are protected from hemorrhagic stroke [249]. The type of physical fitness also matters as a large study, following over 1.5 million men for up to 42 years, revealed that the correlation between higher fitness and lower risk of stroke was only significant for aerobic fitness and not for muscle strength [250].

Individuals suffering from a stroke who had higher physical fitness before the insult have better outcomes: a lower mortality, fewer post-stroke complications and decreased stroke severity [257]. These patients are also more likely to have a better recovery [257]. The American Heart Association recommends that post-stroke patients exercise for at least 20 minutes each day, three days a week [258]. Notably, exercise as a treatment improved outcome and recovery when administrated following brain ischemia [259-261]. For example, cognition was improved [259].

To recover from a stroke may require functional neurogenesis in specific brain regions. Therefore, the ability of exercise to enhance levels of neurotrophic factors, such as BDNF, might be one explanation behind its beneficial effects [246,262]. In stroke patients, exercise-related cognitive improvements correlated with exercise-mediated increases in BDNF serum levels [259]. In ischemic rats, exercise augments proliferation, migration, and differentiation of neuronal progenitors in the penumbral area [263].

The evolution of the damage following brain ischemia is also affected by the inflammatory response. Exercise reduces microgliosis [208,264] and protects against ischemia-induced neuroinflammation according to a systemic review including almost 50 studies [265]. Furthermore, exercise reduced the accumulation of leukocytes during reperfusion of the brain [235], probably, by reducing the levels of molecules such as ICAM-1 and V-CAM1 [208,235], which are involved in the extravasation of peripheral immune cells from the blood into the brain. Moreover, exercise dampens the overexpression of TLR4 [218,266] as well as its downstream inflammatory signaling molecules MyD88 and NFκB [218]. Pro-inflammatory cytokines, like TNFα and IL-6, are also reduced [208]. Interestingly, exercise before the ischemic induction increased the TNFα [235], but following ischemia, the levels were not further elevated. Thus, exercise may chronically elevate TNFα levels and prime the brain, preventing the same pathway from responding to a later ischemic event.

In summary, exercise reduces the risk of ischemic stroke and improves the outcome if affected. The optimal exercise parameters remain to be determined, and the most important mechanisms involved in these effects need to be elucidated under clinical settings.

The effect of exercise on dementia

Since there is no cure for treating dementia disorders like AD and VaD, the interest in using physical activity as a preventive strategy is growing. Previous studies investigating the effect of physical activity on the development of dementia display inconsistent results [267-271]. Among those showing beneficial effects, the majority investigate the effect of activity later in life and suffer from short-term follow-ups [271,272]. Short-term follow-ups increase the risk for bias due to reverse causation, and the potential for undiagnosed dementia increases in participants studied later in life [267,273]. Thus, studies with longer follow-ups are warranted. Importantly, the pathological mechanisms behind different subtypes of dementia differ, as previously described (see *Vascular dementia* and *Alzheimer's disease*). Taking the distinct pathological mechanisms into consideration, it is unlikely that physical activity will be equally beneficial for these dementia subtypes. So far, only a limited amount of studies have differentiated between the effect of physical activity on AD and VaD [274]. For instance, a physically active lifestyle or exercise intervention did not affect the levels of Aβ as measured by PET and CSF analysis [275,276].

Experimental studies may further elucidate the pathological mechanisms affected by exercise and may explain how exercise may be protective against different dementia subtypes. Like patient studies [277], rodent studies show promising results of exercise on cognition [241,278-284]. However, only a few studies have investigated the effects on VaD pathology, probably due to the lack of robust animal models [285]. Those few studies show beneficial effects of exercise on VaD [286,287].

In contrast, the effects of exercise on AD have been investigated in numerous studies [278], whereof several reveal beneficial effects on pathological hallmarks, such as synaptic protein levels and Aβ [241,288]. Interestingly, exercise may affect Aβ in several ways. First, it may reduce soluble Aβ levels [222,279,281,283], potentially by diminishing the activity of beta-site amyloid precursor protein cleaving enzyme (BACE1) and PS1 [243,289], enzymes involved in the generation of Aβ. Second, exercise may decrease Aβ plaque deposition [243], possibly by affecting the production of Aβ42 [280], the subtype most prone to aggregation. Third, the levels and activities of enzymes involved in the degradation and clearance of Aβ from nervous tissue, such as IDE-1 and neprilysin, may be increased [243,290].

Interestingly, exercise seems to promote microglial removal of Aβ species through phagocytosis [214]. Extensive microgliosis [241,281] and pro-inflammatory cytokines [222,282] are also reduced in AD models. Furthermore, it normalizes the levels of postsynaptic density protein 95 (PSD-95) and synaptophysin [291], synaptic proteins that are usually disturbed and are key features of neuronal dysfunction. Figure 5 shows an overview of how exercise affects different molecular mechanisms in AD and Table 4 summarizes the effects of exercise on different AD hallmarks. However, there are also experimental studies that do not detect any exercise-related effects on microglia [243], Aβ [243] or cognitive function [292]. As described before, the lack of effects might be explained by the diversity of study setups.

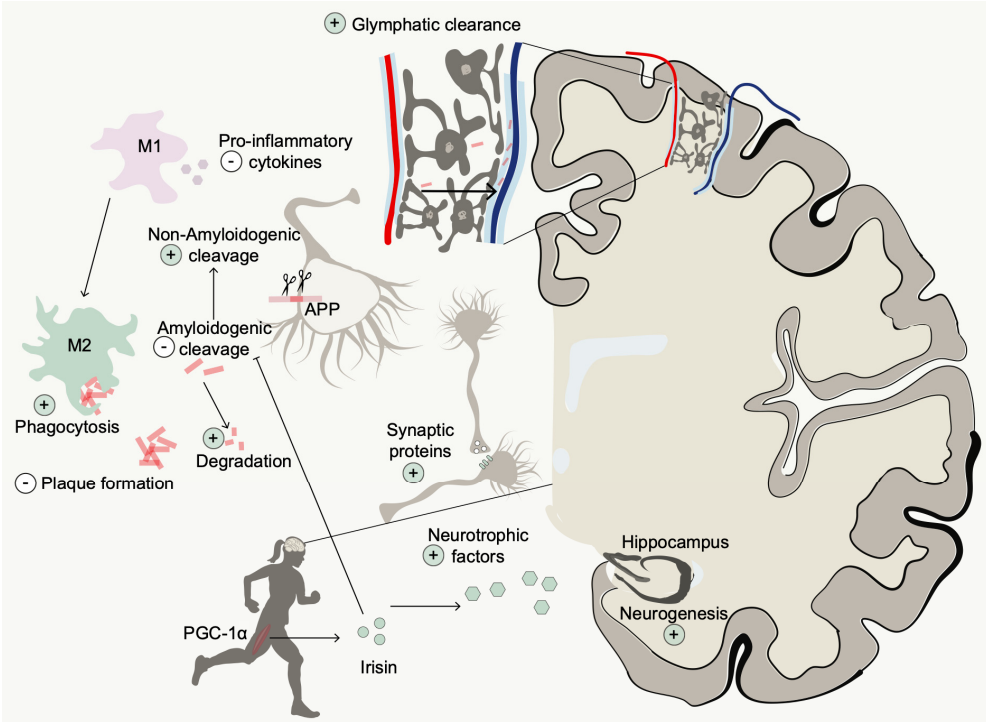


Figure 5. Potential beneficial effects of exercise on AD pathology
The majority of mechanisms displayed above have, so far, only been investigated in animal models. Several studies reveal ameliorated amyloid pathology at different levels following exercise: reduced amyloidogenic cleavage of APP in favor of increased non-amyloidogenic cleavage through regulation of APP-cleaving enzymes; reduced plaque formation and increased Aβ degradation via up-regulation of degrading enzymes and improved clearance through enhanced function of the glymphatic system. Furthermore, exercise may induce a phenotypic switch in microglial cells, reducing the levels of pro-inflammatory M1 in favor of the more beneficial M2 phenotype with increased phagocytosis of Aβ. Moreover, exercise-induced expression of PGC-1α leads to increased levels of irisin, which may inhibit Aβ-producing enzymes and increase the levels of neurotrophic factors. Additionally, increased neurogenesis has been seen in the hippocampus and synaptic function seems to improve as indicated by increased levels of synaptic proteins.

Table 4. Effects of exercise on AD hallmarks

Note that the table only displays studies reporting effects. Several studies report no effects. For clinical studies, both observational and intervention studies are included. ↑ does not necessarily mean an absolute increase but primarily indicates higher levels compared to the control group. ctx= cortex, hip= hippocampus

AD hallmark	Readout	Effect of exercise	AD model	Reference
In animal models				
Tau	Tau phosphorylation	↓ p-tau in hip	APP/PS1, hTau	289,293
Amyloid β	Production	↑ α-secretase clevage of APP	APP/PS1	288
		↓ BACE-1 and PS1	APP/PS1	243,289
	Soluble levels	↓ Aβ40 in ctx and hip	Tg2576,	222,283,290
		↓ Aβ42	APP/PS1	280,283
	Plaque levels	↓ plaque levels	APP/PS1, Tg2576	243,294
	Degradation	↓ IDE	APP/PS1	243
		↑ degrading enzymes; neprilysin, IDE, MMP9, LRP1 and HSP70	Tg2576	290
	Clearance	↑ AQP4	Thy1	241
Synaptic function	Synaptic proteins	↑ PDS-95 and synaptophysin	3xTgAD, Thy1	241,291
Neuroinflammation	Microglial actyivation	↓ microglial activation	APP/PS1, Thy1	241,281
	Microglial profiling	↑ phagocytic microglia, taking up Aβ	APP23, 5xFAD	214
		↑ M1→M2 microglia	STZ rat	213
	Cytokines	↑ IL-10 in hip and prefrontal ctx	intracer inj Ab-40	282
		↓ IL-1β and TNFα	Tg2576	222
Neurodegeneration		↓ neuonal loss in hip	Tg4-42, APP/PS1	295,296
		↑ hip volume	Tg2576, APP/PS1	294,296
Cognition	Working memory	↑ spontaneous alternation	5XFAD	175
	Object recognition	↑ object recognition	intracer inj Ab-40	282
	Spatial memory	↑ spatial cognition	APP/PS1	281,295,297
In patients				
Tau	In CSF	↓ p-Tau	Genetic AD	298
Amyloid β	In brain tissue	↓ Amyloid (PiB) PET	APOE ε4	299
	In CSF	↑ Aβ42	Genetic AD	298
Inflammation	Cytokines	↓ TNFα and IL-15	MCI	174
Neurodegenration	Grey matter	↑ Grey matter volume	Risk group	300
Cognition	Global	↑ Neurocognition	MCI, AD, Genetic AD	174,298,301,302
	Executive	↑ Executive function	Risk group	303,304

Taken together, physical activity seems to have beneficial effects on cognition and to reduce the risk of dementia, although the exact effect on different dementia subtypes needs to be assessed in larger population-based studies with longer follow-up times.

The effect of exercise on Parkinson's disease

Much effort has been made to study the effect of physical activity on PD [305-308] and most observational studies indicate that being physically active has a protective effect and lowers the risk of developing PD [306-308]. Further, exercise interventions improve walking capacity, muscle strength, balance, and other parkinsonian motor symptoms in patients with PD [305,309-313]. However, the mechanisms behind these protective effects of exercise have not yet been elucidated. Exercise-studies in rodents offer a multitude of explicatory mechanisms, ranging from an exercise-mediated reduction in α-synuclein [314-317] to the increased survival of dopaminergic neurons [314,317,318] and increased neurogenesis [315,319]. Likewise, synaptic plasticity, dopaminergic transport, and α-synuclein clearance through autophagy seem to be improved [314,317,319-323]. Interestingly, exercise reduces microgliosis [318,322] and pro-inflammatory protein levels, including TNFα and IL-1β. The expression of TLR2 and its downstream signaling mediators MyD88 and NFκB is also reduced [316,318].

However, few of the above-mentioned exercise-mediated effects have been seen in PD patients, apart from increased cytokines in plasma [324,325] and increased dopamine release in habitual exercisers compared to sedentary patients [326]. Remarkably, one study revealed that physically active patients have diminished motor symptoms compared to controls despite similar levels of dopamine [327]. Hence, it is possible that exercise does not protect the brain from the pathology but that it, rather, creates some kind of motor reserve making it possible for the physically active to cope with brain pathology to a certain degree. This would be equivalent to the cognitive reserve seen in well-educated AD patients [328].

In conclusion, exercise seems to be beneficial against PD, but the mechanisms behind these beneficial effects remain to be determined and may be due to the build-up of a motor reserve in the physically active. However, although the idea of a motor reserve in PD patients has been mentioned before [327], it needs to be investigated in a larger population-based study.

The effect of exercise on depression

Exercise may be used not only to prevent the development of depression in healthy individuals [329-331], but also to treat patients who have already been diagnosed [332]. Indeed, a recent meta-analysis including over 250 000 participants around the world revealed that exercise protected against the development of depression in people from different geographical regions and across all ages [329]. As mentioned before, reverse causation could be an issue in epidemiological studies since individuals already suffering from reduced mood but lacking a diagnosis tend to be less engaged in physical activities [333,334]. This is unfortunate since physical fitness is associated with reduced depressive symptoms in the general population [335]. Likewise, exercise may dampen already present symptoms of depression [336-339]. Conversely, other studies report no additional effect on

these depressive symptoms when compared to antidepressant medication alone [340,341]. In addition, the severity of the depression must be taken into account, since exercise has only been proposed as a treatment strategy for those with mild to moderate depression [333,342]. In more severe cases, the depressive symptoms increase the risk for failure to adhere to an exercise program [333,342].

Potential mechanisms behind the above-mentioned exercise-mediated beneficial effects on depression are beyond the scope of this thesis. Briefly, exercise may reduce inflammation [343-345] and restore the normal function of the HPA axis [346,347], two systems suggested to be altered in depression [134,136,343,348]. Neurogenesis is also suggested to be dysregulated in depression [349], and exercise may improve this [350,351]. Additionally, exercise affects tryptophan metabolism, including kynurenine metabolites [146,147], molecules known to be implicated in depression [145]. However, these effects are not long-lasting and have to be further evaluated in patients [148].

Other factors that need to be considered when studying the effects of exercise on depression are exercise parameters (see *Exercise parameters matters*) and the differences between men and women. Importantly, depression is more common among women [45], but many large-scale studies are only investigating the impact of parameters like exercise intensity and dose without taking different sex into account [352-355]. This is deplorable since the effect of physical activity on depression may differ between men and women [356]. Nevertheless, exercise appears to be protective against depression even though and the impact of reverse causation and differences between men and women need to be accounted for.

Exercise parameters matters

The heterogeneity of study setups within the exercise research field makes analysis and comparisons challenging. A multitude of parameters, such as frequency and duration of training sessions as well as exercise timing and intensity, influence the effect of exercise and must be taken into account. On top of that, endurance exercise and resistance training are two fundamentally different forms of exercise. Still, having different setups increases the possibility of finding interventions optimized for different diseases and patient subgroups.

Type of exercise

My research focuses on the effects of aerobic endurance training, in line with the majority of published studies in the field. Both aerobic endurance and resistance training induce a substantial elevation of metabolism and heart rates, but other effects seem to differ. For example, following resistance training, the effect on serum cytokines occurs at a later time point and achieves lower magnitudes compared to endurance exercise [159]. Moreover, endurance, but not resistance training, leads to increased levels

of kynurenic acid in the blood [147]. Further, when studying the effects of exercise experimentally using animal models, it is important to consider the use of voluntary versus forced exercise interventions (see *Stress as a confounding factor in exercise research).*

Exercise frequency

How often exercise is performed also matters. For instance, only intermittent running facilitated the differentiation of newborn cells into granule cells in mice [357]. The American Heart Association recommends 20-60 minutes of exercise as often as 3-7 days per week for stroke survivors to reduce the risk of additional events [258], but the recommendations may differ for different disorders.

Exercise duration

The duration of each training session and the duration of the whole intervention program should be considered. For example, longer durations lead to a higher increase in BDNF [172,358]. A longer duration is also required to reduce the resting levels of CRP [155]. The increase in the levels of IL-6 also depends on exercise duration [141].

Exercise intensity

The exercise intensity level has a great impact, especially for the immunological effects [230,359] but also on the effects on the HPA axis and BDNF [195,358,360]. Both the DNA-binding activity of NFκB and COX-2 expression increased with increasing exercise intensity [230]. Some exercise-mediated effects, like that on the β2-AR, even seem to require a certain intensity [361]. For IL-6, different impacts of exercise intensity have been seen: in some studies, the response seems to be intensity-dependent [141,224] while others do not detect any differences [163]. Following brain ischemia, low- to moderate-intensity exercise has been suggested to be the most neuroprotective [362] even though an experimental study revealed that higher-intensity reduced the amount of activated microglia and pro-inflammatory cytokines the most [363]. For stroke prevention, moderate-intensity has been advocated for since very intense training might adversely affect the development of hemorrhagic stroke [256]. For psychiatric disorders, results are inconsistent [352-355,364]; some suggest that higher intensities might be required or more beneficial [353,354] whereas others reveal no impact [355,364]. Regarding dementia, more studies discriminating between activities of different intensities are needed, but a dose-response relationship with exercise intensity has been indicated [302].

Exercise timing

The timing of an intervention with respect to disease-specific pathological events is worth considering. For instance, administering an intervention too early may be harmful in experimental brain ischemia [362], although other studies reveal beneficial effects on neuroinflammation and cognition with early initiated exercise [208,262]. Experimentally, regiments including stressors may be non-beneficial in the early phase

after a brain injury [365]. Thus, implementing voluntary instead of forced exercise may be preferred following brain ischemia as it is less stressful [362]. For more progressive diseases, such as neurodegenerative diseases, the discussion has revolved more around whether it will be too late to achieve beneficial effects of exercise if it is introduced too late in the disease process. Furthermore, the timing of sample collection with respect to the exercise execution matters. Many molecular effects of exercise have narrow windows of increase or decrease following exercise.

Taken together, little is known about how the above-described parameters affect the effect of exercise in different disorders affecting the brain. Careful consideration of different exercise parameters when designing future studies offers a greater possibility of discovering novel aspects of physical activity as an intervention.

Stress as a confounding factor in exercise research

Confounders may obscure the readout in exercise research. Stress could be a potent confounder, both in experimental settings using animal models and in epidemiological studies investigating high-performing subgroups.

While cortisol is the primary glucocorticoid involved in the stress response in humans, corticosterone is the primary glucocorticoid implicated in the stress response in rodents. In stressful contexts, elevated corticosterone may have undesired effects in the rodent brain. Still, the effects of augmented glucocorticoid levels appear to differ between exercise and stress conditions [203]. Previous stress may enhance the HPA axis response to novel stress stimuli, whereas exercise buffers the responses to novel stress. The exact mechanistic difference behind this remains unknown. Importantly, an exercise-mediated increase in glucocorticoid signaling in the prefrontal cortex leads to increased local levels of dopamine, which is essential for active coping. Opposingly, chronic stress decreases dopamine levels in the medial prefrontal cortex despite augmented levels of glucocorticoids [203]. Moreover, glucocorticoid receptors are downregulated following chronic stress [366] along with glucocorticoid resistance [367], a process suggested to ultimately lead to dysregulation of the whole HPA system. Further, stress increases the levels of adrenergic receptors on circulating immune cells [368], which might affect immune function (see *Exercise effects inside the brain*). In contrast, exercise does not have the same effects [203,368]. This might be one explanation behind the different effects of stress and exercise on neuroinflammation and other pathological hallmarks in the brain.

The effect of stress on pathological hallmarks in the brain

Stress can affect the pathological hallmarks implicated in disorders affecting the brain. For instance, chronic stress and corticosterone injections aggravate Aβ production and accumulation in AD models [369,370]. Further, in a rat model of cerebral ischemia, stress

preconditioning increased neuronal death in the hippocampus following ischemia [371]. Moreover, dysregulation of the HPA system is suggested to contribute to depression [44].

The effect of stress on neuroinflammation

As microglia express glucocorticoid receptors, they are capable of responding to differences in glucocorticoid levels [372] and this appears to lower the threshold for microglia to proliferate and release pro-inflammatory cytokines [373]. Increased microglial activity and neuroinflammation may contribute to neuronal death following stress [371], and high corticosterone levels lower the threshold for microglia to release pro-inflammatory cytokines [374]. Interestingly, stress increases the expression of NLRP3, leading to increased cleavage and secretion of pro-inflammatory IL-1β [374].

In conclusion, stress should be considered as a potent confounder, both in epidemiological and experimental exercise studies. Caution has to be made when designing studies.

Rationale

When the research projects behind this thesis were initiated in 2013, there was limited knowledge about how exercise affects pathological development, especially concerning neuroinflammation, following global brain ischemia.

As described in the introduction, it was known that a physically active lifestyle decreases the risk of dementia, Parkinson's disease (PD) and depression. However, little was known about how physical activity affects the development of different dementia subtypes as the majority of previous studies did not differentiate between the two most common, but different types of dementia; Alzheimer's disease (AD) and vascular dementia (VaD).

Moreover, there was limited knowledge about how different levels of physical performance may affect the risk of depression in regard to sex.

Furthermore, early manifestations of symptoms may reduce physical activity levels in patients already several years before the diagnosis of dementia or PD, a phenomenon called reverse causation. The majority of previous studies did not take into consideration potential bias due to reverse causation when assessing the association between physical activity and the risk of developing disorders that affects the brain.

By taking into account the points in the above-described areas, exercise-based interventions could be improved in preventive healthcare to optimize its beneficial effects.

Aims

General aims

To investigate how physical activity affects:

- neuroinflammation and behavioral deficits in mouse models of certain disorders affecting the brain.
- the risk of developing certain disorders affecting the brain in humans

Specific aims

To investigate how:

- microglial neuroinflammation contributes to the pathogenesis and behavioral deficits following global brain ischemia in mice.
- forced running affects brain pathology, including neuroinflammation and behavioral deficits, following global brain ischemia in mice.
- a physically active lifestyle affects the risk of developing depression.
- a physically active lifestyle affects the risk of developing Parkinson's disease.
- a physically active lifestyle affects the risk of developing Alzheimer's disease and vascular dementia.
- voluntary running affects Alzheimer's disease pathology, particularly with respect to neuroinflammation and behavioral deficits, in a transgenic mouse model of Alzheimer's disease.

Study overview

Figure 6 shows an overview of the scientific work presented in this thesis.

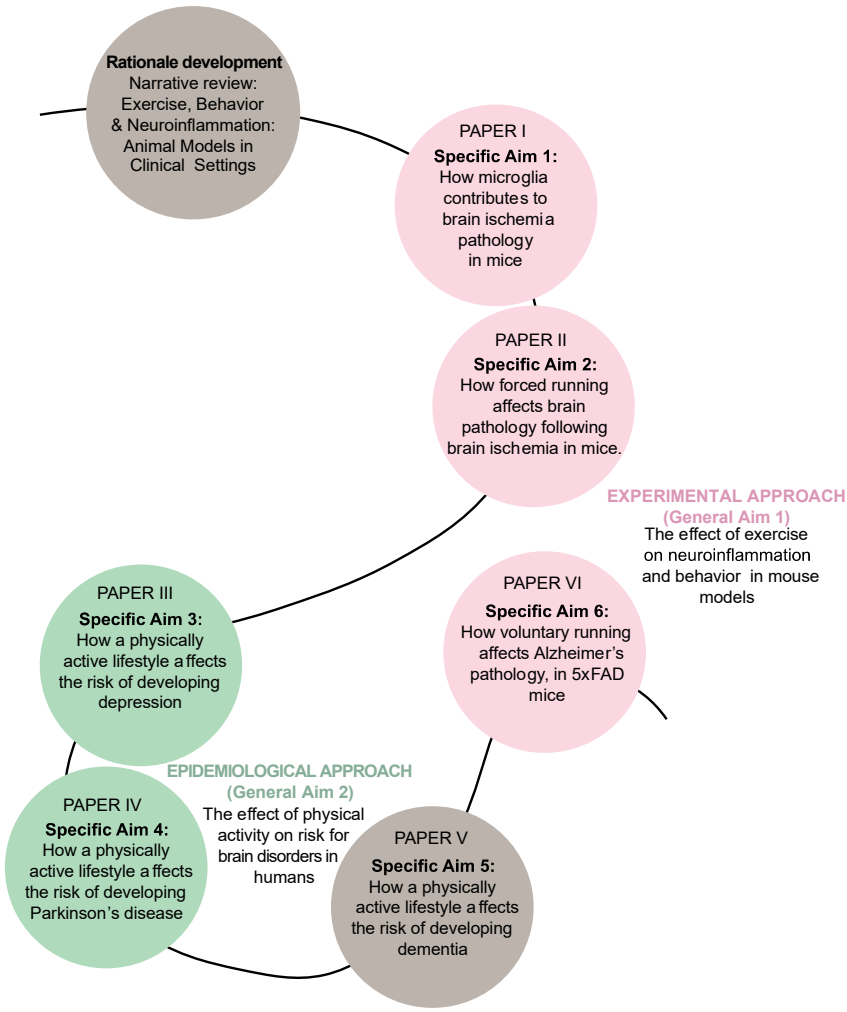


Figure 6. Study overview of this thesis
Based on the aims, the papers presented in this thesis utilize either an experimental approach (general aim 1, indicated in pink) or an epidemiological approach (general aim 2, indicated in green) or a combination of both (indicated in grey).

Methods

Detailed descriptions of the methods used in my thesis work are found in the methods sections of each paper. The purpose of this section is to point out methodological shortcomings and considerations and to explain the methodological decisions taken during my research process.

Ethical considerations

The experimental studies included in this thesis were conducted in line with the ethical permits (D.nr. M303-09, M425-12, and M427-12) approved by the animal ethics committee in Lund and in adherence to the Directive of the European Parliament. We simplified our experimental design as to contain as few treatment groups as possible and only one sex per study in order to reduce the total number of animals used without losing statistical power. We also used the Y-maze instead of Morris water maze to test cognitive behavior, as the Y-maze test is less stressful for mice [375].

The epidemiological studies were approved by the ethics board in Uppsala (D.nr. and 2010/305).

Experimental studies

Of mice and men - modeling disorders affecting the brain in mice

All molecular effects of exercise presented in this thesis are investigated in mouse models. Animal models offer unique possibilities to investigate molecular effects, but they always suffer from certain limitations and never fully mimic disorders effecting the human brain. For instance, mice have a short life span, about 2 years, making it problematic when studying aspects of the human aging processes that contribute to brain pathology. In addition, laboratory mice are inbred, so the natural genetic variation seen in a patient population is not reflected. Further, experimental animal facilities are rather sterile environments, lacking the interaction of naturally occurring

pathogens with inflammatory processes studied in my research. Nevertheless, mouse models are still needed to elucidate molecular questions.

Experimental global cerebral ischemia

We used a mouse model of global cerebral ischemia in Paper I and II. Wild-type mice of the C57bl/6 strain were bred in our facilities. By transiently occluding the two common carotid arteries for 13 minutes, we tried to mimic cardiac arrest-induced global ischemia in the brain. Since hypothermia has been suggested to reduce neuronal damage in this context, we kept our mice on heating pads to keep their body temperature at somewhere between 36.5-37.5°C (rectal measurements) during the whole procedure. This model typically develops neuronal damage in the striatum, hippocampus, and thalamus [376] and a microglial response to the insult [377].

Galectin-3 knockout

In Paper I, half of the mice used were deficient in galectin-3 [378]. This galectin-3 knockout strain originally came from Dr. K. Sävman (Gothenburg University) and is maintained in our facilities on a C57BL/6 background. Under healthy conditions, this model display no overt abnormalities [378].

Familial Alzheimer's disease

We used a mouse model of Alzheimer's disease in Papers V and VI. 5xFAD mice bred on the C57Bl/6*SJL genetic background were obtained from Jackson laboratories. The 5xFAD strain expresses 5 transgenes with mutations that cause familial AD in humans. These mutations are the following; the Swedish (K670N/M671L), Florida (I716V), and London (V717I) mutations in APP, and the M146L and L286V mutations in PSEN1. At 1.5 months, soluble Aβ42 levels are detectable, and plaques appear at around 2 months of age [379]. Synaptic function is also compromised, presynaptic synaptophysin starts to decrease from 4 months of age and post-synaptic PSD95 starts to decrease at 9 months of age [379].

We chose this model since it recapitulates the neuroinflammatory aspects of the disease early. Microgliosis begins around 2 months of age [379]. Cognitive deficits can be measured at 5 months of age [379] and abnormal exploratory behavior is seen in the elevated plus maze test at around 6 months of age [380]. Importantly, motor deficits do not develop until 9 months of age [381], allowing several months to detect deficits in cognition and anxiety before motor impairments potentially bias the readouts of these tests.

Experimental exercise

My thesis only investigates endurance exercise. We made conscious decisions regarding the exercise parameters discussed in the introduction, such as frequency, duration, and intensity. Furthermore, we aimed for proper control conditions to reduce bias in our setup. The perfect setup will never exist; there will always be methodological issues, but the most severe pitfalls can be avoided.

Forced treadmill running

We used forced treadmill running for the first exercise study (Paper II) to control the exercise intensity. With fixed speeds and a fixed duration, our aim was to expose the running group to a homogenous exercise dose to keep the variance low. We put the sedentary controls in the same environment, except that the treadmill was not moving. Our running mice were introduced slowly to the running paradigm. Despite this, we had severe problems with stress caused by forced running. The timing of the intervention during the daylight hours may have influenced the outcomes, since mice are nocturnal animals. These issues are discussed in Paper II.

Voluntary wheel running

Due to the problems with stress in the forced running protocol, we decided to use voluntary wheel running in our next study (Papers V and VI). With running wheels, mice could run at their preferred time of the day and for as much as they wanted.

The disadvantage with voluntary wheel running is that the amount and timing of the exercise cannot be controlled to the same extent as with treadmills. It is possible to reduce the variation in exercise dose by locking the wheel during certain hours or after a pre-set distance. However, we offered unlimited access to smart wheels with continuous wireless tracking of running, providing data regarding speed and distance ran. Hence, we could assure that all mice were running at least a certain distance per day. It is also possible to investigate dose-response relationships with this setup, but to track how much each mouse ran, the mice would need to be caged individually. Individual caging may lead to depression in mice which might interfere with the effects of exercise [382]. Therefore, we caged our mice in pairs. We recorded their running behavior with a video camera mounted above their cage during some nights. Observation of these recordings confirmed that both mice in a cage ran approximately equal amounts and that dominance behavior was not a problem.

Sex might matter

Both men and women were included in the Vasaloppet studies. As the risks of developing some of the most common disorders affecting the brain differ between men and women, sex is an important factor to consider. In the Vasaloppet studies, we

performed stratified analyses based on sex. In my experimental studies, we decided to focus on one sex at a time to keep the number of animals in each group high enough to get statistical power to detect differences, and at the same time, keep the total number of mice low for ethical considerations.

Male mice to model global brain ischemia

In Paper II, we wanted to investigate the effect of exercise on the outcome after global brain ischemia. In humans, global brain ischemia is usually due to cardiac arrest. We used male mice since men are more often affected by cardiac arrest [383]. Male mice are also better characterized. However, due to fighting and aggressive behavior, we had to exclude some of the mice and the majority of the remaining mice displayed signs of stress in behavioral tests.

Female mice to model Alzheimer's disease

In Papers V and VI, we wanted to investigate the effect of exercise on the development of AD pathology. Since AD is more common among women [28], we used female mice even though they are less well characterized. Another reason was problems with aggressive behavior among male mice as experienced in my previous study.

Quantification of immunohistochemical stainings

Staining intensity measurements in Image J

Ideally, the number of microglial cells should be counted and their morphology observed. For some of the brains, I had issues with low staining quality that made quantification difficult. For some conditions and brain regions (e.g. the hippocampus following brain ischemia), microglia are also too numerous to be quantified by counting if the staining quality does not allow for higher magnification images. Therefore, the total microglia population was measured as staining intensity in Image J (NIH). This is a blunt measure, but the best choice under these circumstances.

Statistical analysis

Data were analyzed in Microsoft Excel and SPSS (v. 22) as described in the original papers. In short, the data from papers I and II followed a normal distribution, and parametric tests were used (Student's t-test, ANOVA and Pearson's correlation coefficient). In contrast, most of the data from paper V and VI did not follow a normal distribution. Therefore, non-parametric tests were applied to this data (Mann-Whitney U-test, Wilcoxon tests, and Friedman test).

Sensitivity analyses

Association between physical activity and lower risk of disorders effecting the brain might be due to reverse causation, *i.e.* early symptoms prevent individuals from being physically active already before diagnosis. We perform sensitivity analysis to control for this. By excluding all cases diagnosed with the disorder of interest during the first 5 years after inclusion in our studies, we try to reduce this bias. For dementia and PD, we justify the use of 5 years for this exclusion since previous studies indicate that this is a relevant timeframe for these diagnoses [267,268,384]. Conversely, for depression, we lack similar justifications but chose to adhere to a consistent setup for our analyses. We could have presented different levels of sensitivity analyses, using for example 3, 5 and 10 years as cut-offs for exclusion. The main reason for not doing this was word count restrictions. In addition, excluding all cases during the first 10 years would imply that follow-up only last up to 11 years and lead to a significant reduction in the number of events giving less robust data.

Stratification for effect modifiers

For all diagnoses investigated in this thesis, there is an unequal distribution in the general population between men and women, indicating differences in the etiology of these disorders. Men and women may make different lifestyle choices when it comes to physical activity. Thus, sex might be an effect modifier on the association between physical activity and disorders affecting the brain. In addition to analyzing both sexes together, we stratified our analysis to analyze men and women separately.

Adjustment for confounders

The major limitation of our studies is that we could not adjust for factors indicated as potential confounders in the directed acyclic graph (Figure 7) as we lacked data on these variables. Previous self-reported questionnaires from these skiers and non-skiers revealed that skiers tend to have a better diet and smoke less [385,386]. Nevertheless, as we lacked data on diet and smoking for the majority of participants, we could not adjust for this. We began with a crude unadjusted, cox model, since adjusting for confounders is complex and may increase the risk of getting type I errors [387]. As education level differs between the populations and affects socioeconomic status, which is a potential confounder (see Figure 7), we decided to adjust for education level. Thus, we present a model adjusted for age, sex and education.

Another limitation of our studies is that we could not adjust for the level of physical activity in the control population (non-skiers). This likely led to an under-estimation of the association between physical activity and the risks for the disorders investigated. Still, previous self-reported questionnaires among these skiers and non-skiers revealed that skiers spent more time exercising [385,386].

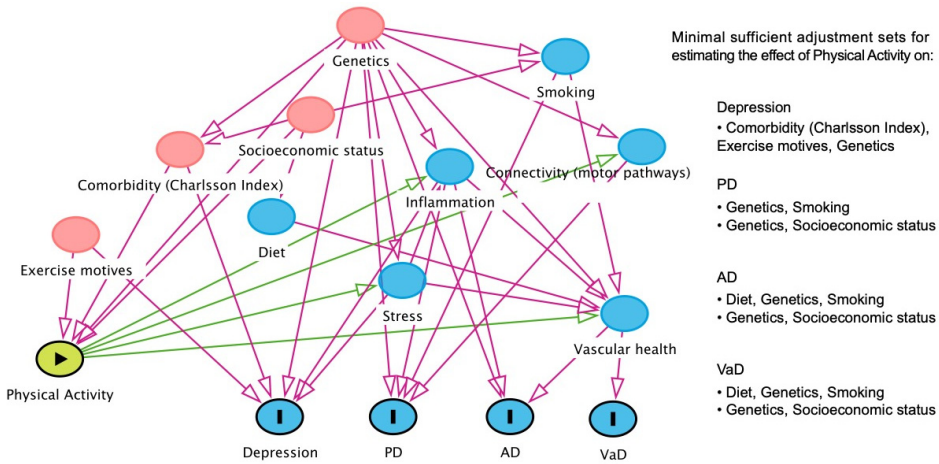


Figure 7. Pathways between physical activity and disorders affecting the brain.
Directed acyclic graph with suggestions for statistical adjustments for each outcome diagnosis investigated generated using dagitty.net. The green node indicates the exposure, and outcomes are indicated with blue nodes (I). The remaining blue nodes indicate ancestors of outcomes, and pink nodes are ancestors of both exposure and outcome. Causal paths and biasing paths are indicated with green and pink arrows, respectively.

Results

General aims

The effects of exercise on behavioral deficits in certain brain disorders

Main findings

Running intervention;

- does not improve cognition in mouse models of brain ischemia or AD.
- induces anxious behavior in mice that were forced to run.
- aggravates abnormal exploratory behavior in a mouse model of AD.

Running impaired spatial memory in AD mice

Running 5xFAD mice exhibited reduced curiosity for the new arm in the Y-maze test, indicating impaired spatial memory compared to sedentary controls (Mann-Whitney, p=0.03). In contrast, forced running did not affect cognitive behavior in wild type mice following global brain ischemia. Moreover, voluntary running did not affect working memory when assessed with the Y-maze spontaneous alternation test or object memory when assessed with the novel object recognition test.

Running aggravated abnormal exploratory behavior in AD mice

Previous studies reveal that the 5xFAD model is characterized by abnormal exploratory behavior in the elevated plus maze [380,388,389]. Our running 5xFAD mice exhibited increased exploratory behavior compared to sedentary controls.

Conclusion

Our results reveal no beneficial effects of running on behavior linked to brain pathology in these mouse models of brain ischemia and AD.

Impact

Future studies involving running intervention should consider the drawbacks of forced versus voluntary running in the experimental design.

Running does not ameliorate behavioral deficits in mouse models of common brain disorders

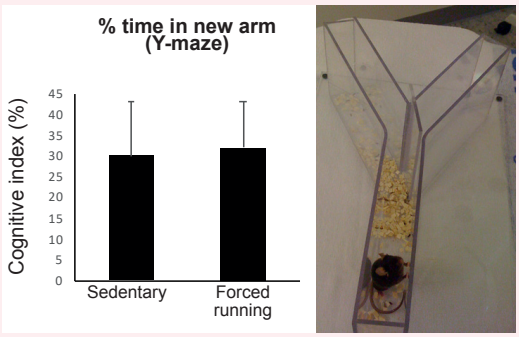
Global brain ischemia

- **Male WT mice (n=63)**
- **Common carortid artery occlusion**
- **Forced treadmill running (4 weeks)**

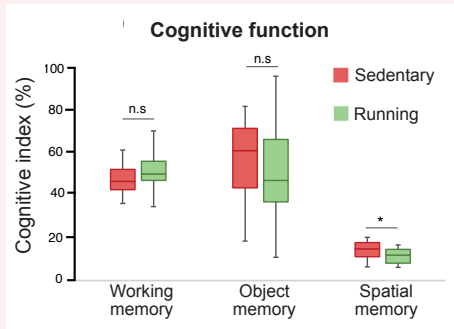
Alzheimer's disease

- **Female mice (n=30)**
- **5xFAD (transgenic AD model)**
- **Voluntary wheel running (6 months)**

Running does not improve cognition

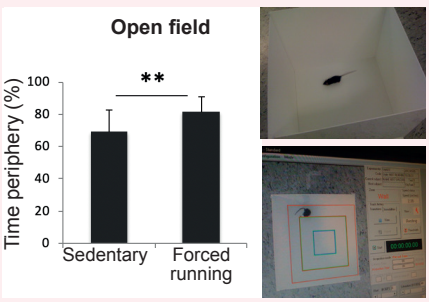


Forced running does not improve congnition in mice following brain ischemia (left picture). Spatial memory was evaluated in Y-maze test (right picture).

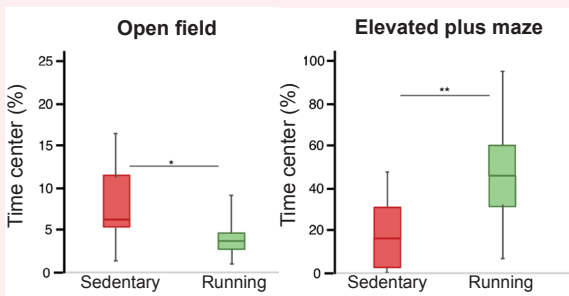


Voluntary running does not improve working or object memory in 5xFAD mice. The running intervention even aggravated spatial memeory deficits.

Running does not improve anxiety or abnormal exploratory behavior



Forced running led to increased anxiety (left). Anxious behavior was measured as time spent close to the walls in the Open Field test box (upper right). SMART software was used to objectively measure behavior (lower right).



Voluntary running aggravated abnormal exploratory behaviour, earlier reported in the 5xFAD model, as measured in the Open field and Eleveated plus maze tests.

The effects of exercise on neuroinflammation in certain brain disorders

Main findings

Running intervention;

- does not affect the number of microglia in the hippocampus of AD mice.
- tends to reduce the number of microglia in the hippocampus of mice after brain ischemia.
- has no significant effect on pro-inflammatory galectin-3 levels in the brain.
- up-regulates IFNγ in the brain and IL-10 in blood after brain ischemia if mice were forced to run.
- tends to increase pro-inflammatory iNOS in the brain after ischemia in mice given the forced running intervention.
- tends to decrease the hippocampal levels of iNOS in AD mice given the voluntary running intervention.

Forced and voluntary running may affect pro-inflammatory iNOS differently

Running did not have a statistically significant effect on iNOS levels in the brain, but tendencies were revealed. Forced running tended to increase the levels of iNOS in the brain (T-test, p=0.15) in wildtype males. In contrast, voluntary running tended to decrease the levels of iNOS in the hippocampus of 5xFAD females (Mann-Whitney, p=0.11).

Voluntary running did not affect cytokine levels in AD mice

We could not detect any effects on cytokine levels (INF-γ, IL-1β, IL-12p70, IL-2, IL-4, IL-5, IL-6 IL-10, CXCL1, TNF-α) in the hippocampus or blood of 5xFAD mice after 6 months of voluntary wheel running.

Conclusion

Our results do not show any significant effects of running on neuroinflammation but revealed opposing tendencies depending on the type of running (forced or voluntary).

Impact

Our findings reveal the importance of considering the type of running intervention in the design of an experiment, particularly for future studies.

Running does not improve neuroinflammation in mouse models of common brain disorders

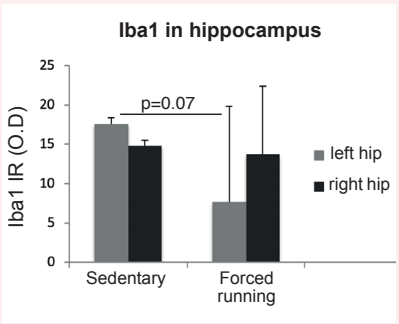
Global brain ischemia

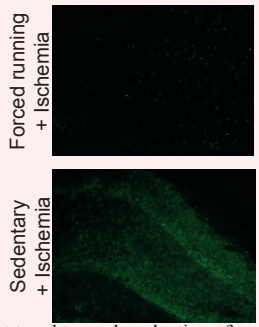
- Male WT mice (n=63)
- Common carortid artery occlusion
- Forced treadmill running (4 weeks)

Alzheimer's disease

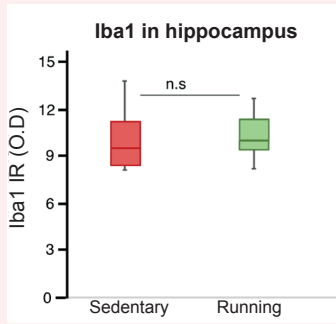
- Female mice (n=30)
- 5xFAD (transgenic AD model)
- Voluntary wheel running (6 months)

Running does not significantly affect microglia in hippocampus



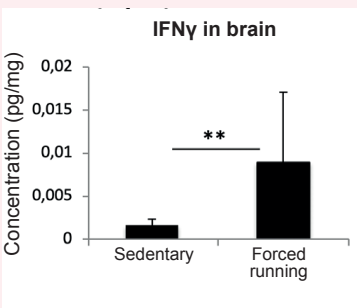


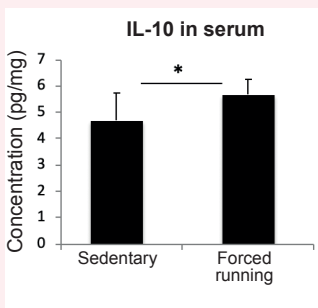
Forced running mice displayed a non-significant trend towards reduction of Iba1 in the left hippocampus following ischemia. (T-test, p=0.07)

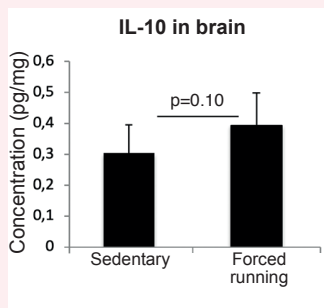


Voluntary running does not affect Iba1 in 5xFAD mice.

Forced running up-regulates the protein levels of some cytokines

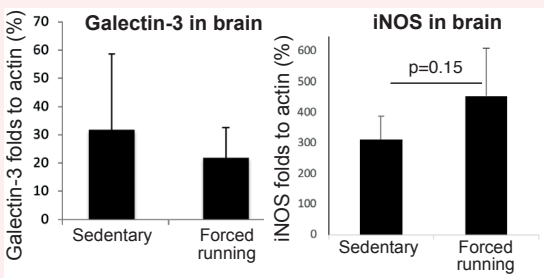




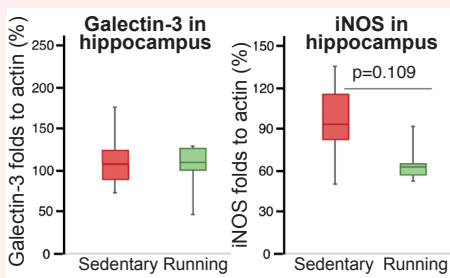


Forced running resulted in increased levels of certain cytokines in reponse to ischemia compared to sedentary conditions. (T-test) For voluntary running in 5xFAD mice, no effects were seen on cytokine levels in hippocampus or serum.

Running does not significantly affect hippocamal levels of galectin-3 and iNOS



Forced running led to a non-significant trend towards higher iNOS levels in the brain following ischemia.



Voluntary running led to a non-significant trend towards lower iNOS levels in hippocampus in 5xFAD mice.

Physical activity and the risk of certain disorders affecting the brain

Main findings

Vasaloppet skiers have;

- a 50 % lower risk for future depression diagnosis.
- a ≈ 30 % lower risk of Parkinson's disease (PD) during early follow-up.
- a ≈ 50 % lower risk for future vascular dementia (VaD).
- no significant difference in the risk of Alzheimer´s disease (AD) compared to matched general population.

The lower risk of brain disorders for skiers is unlikely due to reverse causation

Early symptoms might prevent people from being physically active even before diagnosis. This phenomenon is known as reverse causation. We tried to reduce this potential bias in our sensitivity analysis by excluding all cases diagnosed with the disorder of interest during the first five years after study inclusion. Even with this exclusion, the skiers still had a significantly lower risk of developing depression and VaD compared to controls. This indicates that the associations between a physically active lifestyle and a lower risk of these disorders are likely due to the positive lifestyle effects rather than due to reverse causation.

The lower risk of brain disorders for skiers is not explained by higher education

The skiers in our study had a significantly higher level of education compared to the non-skiers in our matched control group. Even when adjusting for age, sex and education, skiers still had a significantly lower risk of developing PD (HR=0.73 (0.57–0.93)), VaD (HR=0.49 (0.33–0.73)) and depression (HR=0.53 (0.49, 0.58)).

Not all brain disorders can be associated with a physically inactive lifestyle

Our study indicated that having a physically active lifestyle (being a skier) was not associated with a lower risk of developing AD. These results were in line with those observed in the Malmö diet and cancer study, wherein individuals that self-reported high physical activity scores had a lower risk of VaD but not AD.

Conclusion

Our results show that a physically active lifestyle is associated with a lower risk of developing depression, PD and VaD, but not AD.

Impact

Our findings support the view of physical activity as a promising preventive strategy to reduce the burden of some of the most common disorders affecting the brain.

Long distance ski racing is associated with lower risk of several brain disorders

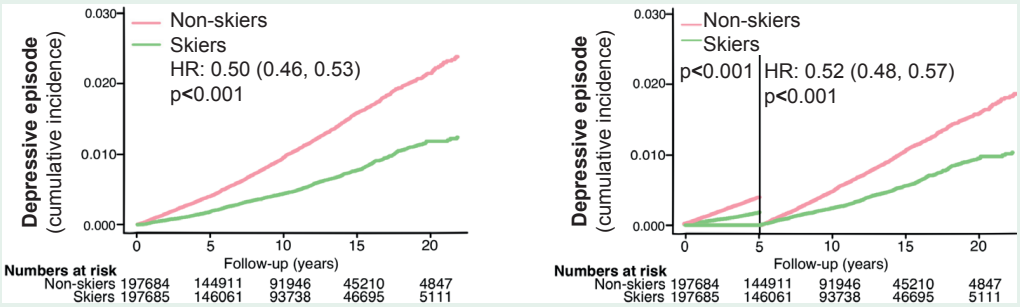
Study design

- Incidence (Patient registry)
- Vasaloppet skiers (n=197,685)
- General population (n=197,684)
- 38 % Women
- Up to 21 y of follow-up (1989-2010)
- Matched on sex & age

Participants characteristics

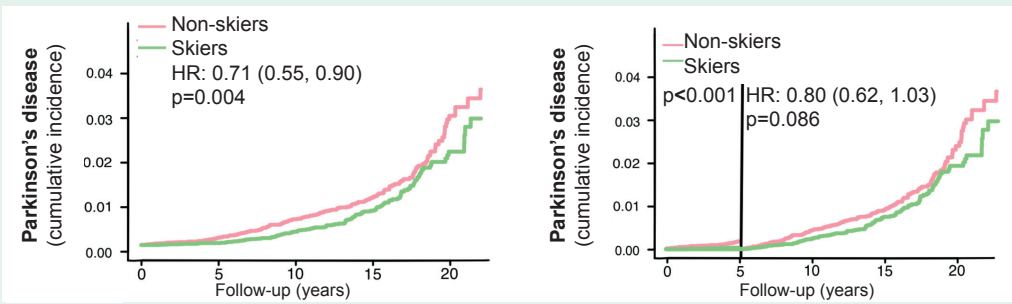
Characteristics 1989-2010	Skiers n=197,685 Median (IQR) or n (%)	Non-skiers n=197,684 Median (IQR) or n (%)
Age at baseline, y	36.0 (29.0-46.0)	36.0 (29.0-46.0)
Education:		
Primary school (8y)	14,538 (7)	34,806 (18)***
Secondary school (9-12y)	76,635 (39)	99,936 (51)
Higher education (13y)	106,147 (54)	59,986 (31)

Skiers have lower risk of depression



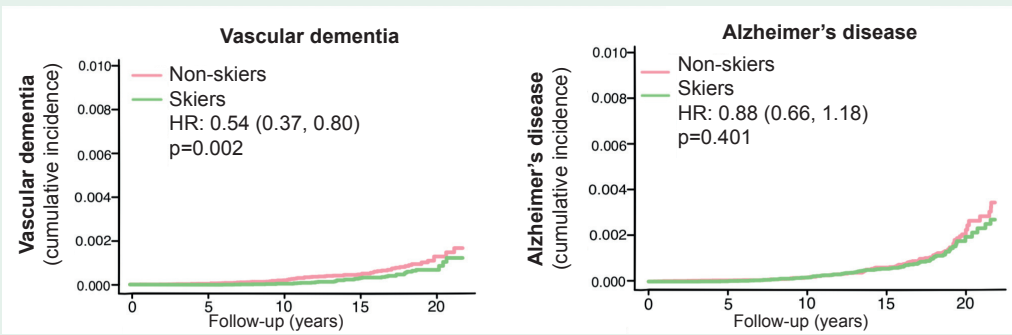
Skiers have 50% lower risk of depression compared to matched general population.

Skiers have lower risk of Parkinson's disease (PD) during early follow-up



Skiers' cumulative incidence of PD seem to converge with that of the general population with longer follow-up time.

Skiers have lower risk of vascular dementia (VaD) but not Alzheimer's disease (AD)



Skiers have almost 50 % lower risk of developping VaD, but not AD compared to matched general population.

Specific aims

Microglial contribution to pathology after brain ischemia in mice

Main findings

- Galectin-3 acts as a microglial pro-inflammatory factor following brain ischemia.

Galectin-3 deficiency leads to;

- a reduced microglial response in the hippocampus.
- improved neuronal survival in the hippocampus.
- protection from significant memory deficits.
- reduced weight loss.

Galectin-3 induces a TLR-4 dependent inflammatory response in microglia

We tested whether adding galectin-3 to primary microglial cultures could elicit an inflammatory response. Both soluble and immobilized forms of galectin-3 induced release of several inflammatory cytokines (IL-1β, IL-5, TNFα, IL-10, IL-12, and IL-4, measured with ELISA) in primary microglia. Primary microglia deficient in TLR-4 released significantly lower levels of cytokines compared to wildtype microglia when galectin-3 was added to the cell cultures.

Microglial galectin-3 has detrimental effects following brain ischemia

Mice lacking galectin-3 had reduced microgliosis and improved neuronal survival in the hippocampus after global brain ischemia. Galectin-3 deficiency also protected the mice from significant memory deficits following ischemia compared to control mice.

Conclusion

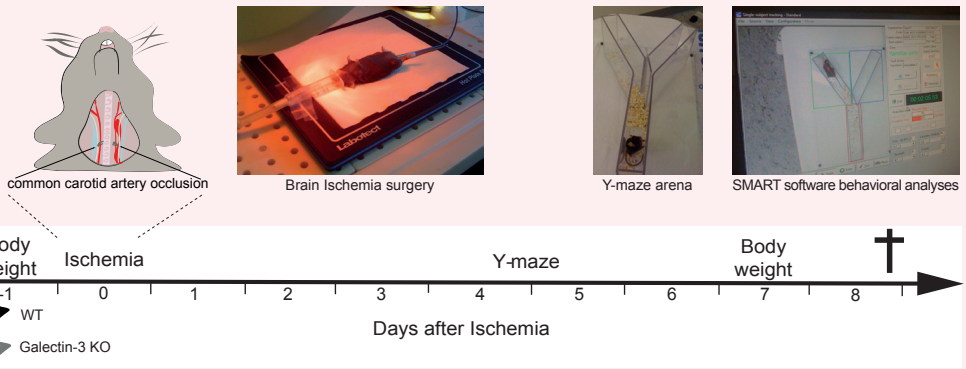
Our results show that galectin-3 is involved in microglial activation and contributes to neuroinflammation and neuronal damage following brain ischemia.

Impact

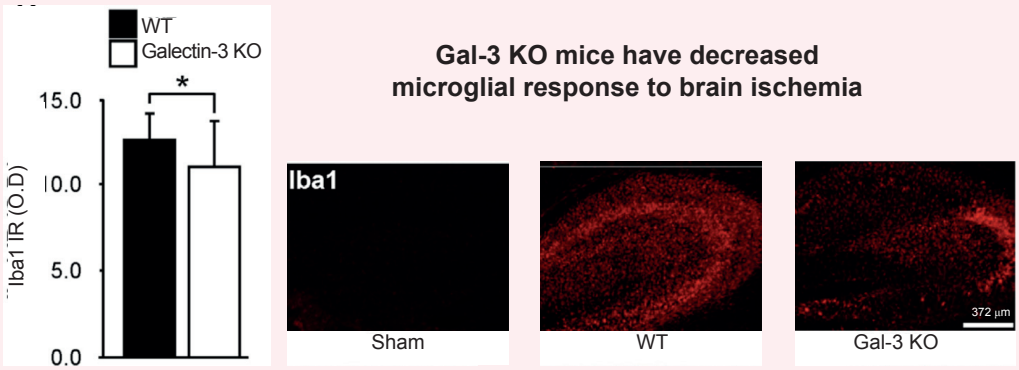
Our study identifies microglial galectin-3 as a promising target for therapeutic interventions for disorders affecting the brain.

Microglia-secreted galectin-3 acts as a toll-like receptor-4 ligand and contributes to microglial activation

Experimental design

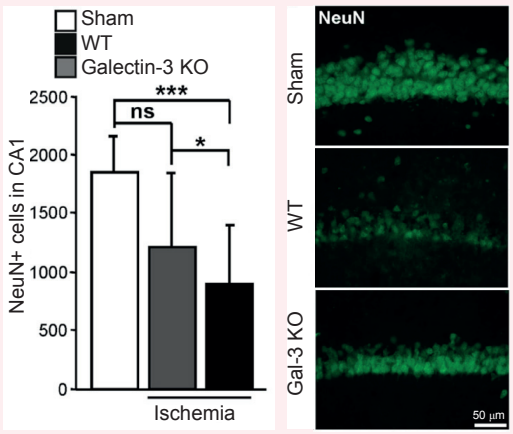


Gal-3 KO mice have decreased microglial response to brain ischemia



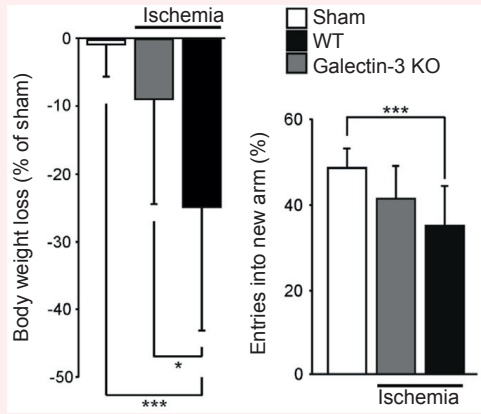
Galectin-3 KO mice show less Iba1 microglial response in hippocampus following global brain ischemia compared to WT.

Gal-3 deficiency reduces neuronal death in hippocampus after brain ischemia



Galectin-3 KO mice have higher number of surviving NeuN+ cells in hippocampus after ischemia.

Gal-3 deficiency ameliorates cognitive function and weight loss following brain ischemia



Galectin-3 KO mice lose less weight (left) and do not develop significant memory deficits in Y-maze (right) after ischemia.

The effect of forced running on pathology after brain ischemia in mice

Main findings

An intervention with forced running in mice results in;

- anxious behavior.
- increased neuronal damage that is not due to increased microgliosis in the hippocampus following brain ischemia.
- elevated corticosterone levels that correlate with neuronal damage in the hippocampus.
- Increased corticosterone and anxiety are not seen in mice given a voluntary running intervention.

Unwilful running may induce stress

We observed stressful behavior from mice in the forced running group in their home cages. The open field test revealed increased anxiety, and corticosterone levels were elevated in feces from these mice. Increased anxiety and corticosterone levels were not observed in an additional control group that had voluntary wheel running.

Stress-induced corticosterone may worsen neuroinflammation

Increased corticosterone levels in mice subjected to forced running correlated with increased levels of NLRP3 inflammasome (Pearson R=0.48, p=0.046) and its downstream proinflammatory IL-1β (Pearson R=0.61, p=0.006) in the brain. Likewise, the brain levels of IFNγ were five times higher in the forced running group compared to sedentary mice after ischemia (Fischer's post hoc test, p=0.008).

Systemic IL-10 levels associated with running may reduce the microglial response

Running mice showed increased IL-10 levels in the blood and a tendency towards increases levels in the brain (Fischer's post hoc test, p=0.10). Higher systemic IL-10 levels correlated with lower levels of microglial Iba1 in the hippocampus (Pearson R=-0.54, p=0.02). Running mice also displayed a non-significant trend (T-test, p=0.07) towards reduced hippocampal Iba1 compared to sedentary mice following ischemia. This indicates that the increased IL-10 levels induced by running may reduce the microglial response.

Conclusion

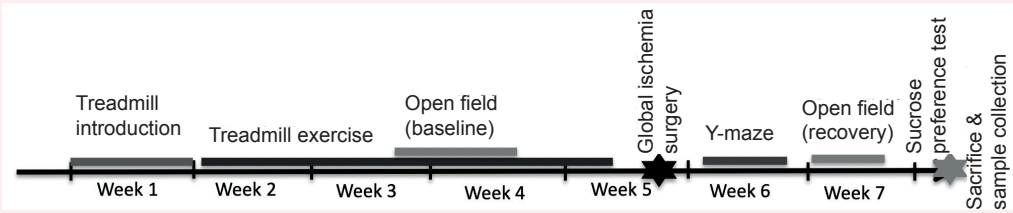
Exercise pre-conditioning may not be beneficial if the mice are forced to run as it can induce a detrimental stress response blunting the beneficial effects of exercise.

Impact

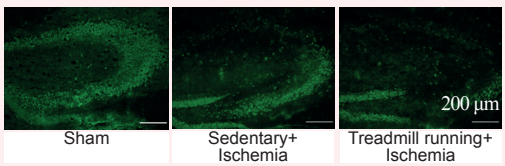
These findings are important for researchers when considering exercise paradigms for mice in experimental designs.

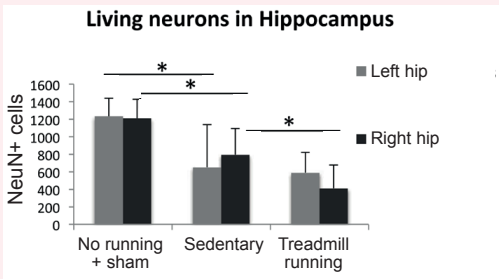
Forced treadmill exercise can induce stress and increase neuronal damage in a mouse model of global cerebral ischemia

Experimental design



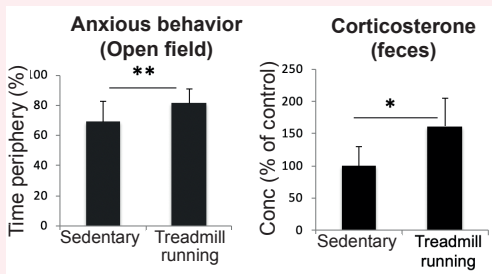
Treadmill exercise prior to ischemia aggravates hippocampal neuronal damage





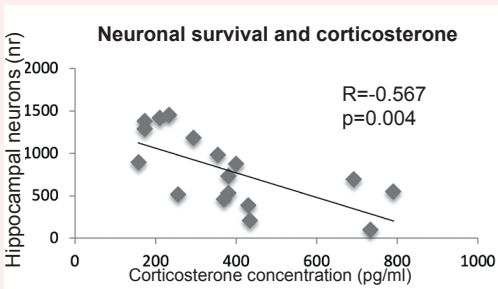
Forced running mice displayed less surviving NeuN cells in the right hippocampus following ischemia.

Forced exercise induces a stress response



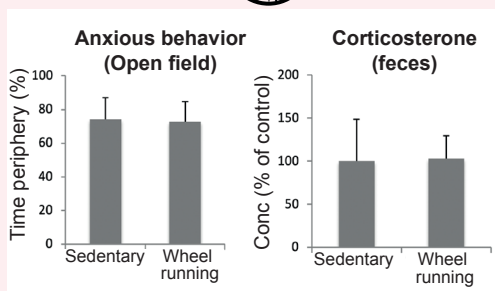
Forced running mice spend more time in the periphery of the open field and have higher corticosterone levels in feces.

The stress response correlates with hippocampal neuronal damage



The number of surviving NeuN+ neurons in hippocampus correlated negatively with the levels of corticosterone.

Voluntary wheel running does not induce a stress response



Voluntary running does not affect the time spent time in the periphery of the open field or the levels of corticosterone.

Physical activity and the risk of depression

Main findings

- Both male and female skiers had a 50 % lower risk of developing depression.
- For the male skiers, higher performance (faster finishing time) in the ski race was associated with an even lower risk of developing depression.
- For female skiers, performance in the race did not significantly relate to the risk of developing depression.

Physical performance affects the risk of depression differently for men and women

We used finishing times from the ski race as a proxy for physical performance and fitness level. When analyzing both sex together, skiers that finished the race faster had an even lower risk of depression compared to slower skiers (after adjusting for age, sex and education, HR=0.78 (0.62, 0.99)). The same pattern was seen in fast skiing men compared to slow skiing men (adjusted HR=0.65 (0.49, 0.87)). For skiing women, we saw the opposite pattern (adjusted HR=1.11 (0.74, 1.66)), although far from being statistically significant (p=0.6). Nevertheless, the fastest skiing women had a lower risk for developing depression compared to non-skiing women, but they did not receive any additional benefits.

Conclusion

Our results indicate that a less physically active lifestyle predicts depression later in life, but there may be differences between men and women regarding the association between physical performance and risk of developing depression.

Impact

Our findings support the view of physical activity as a promising preventive strategy to reduce the risk of developing depression.

Long distance ski racing is associated with lower long-term incidence of depression in a population based, large-scale study

Study design

- **Incident Depression (Patient registry)**
- **3075 cases of depression**
- **Vasaloppet skiers (n=197,685)**
- **General population (n=197,684)**
- **38 % Women**
- **Up to 21 y of follow-up**
- **Matched on sex & age**

Participants characteristics

Characteristics 1989-2010	Skiers n=197,685 Median (IQR) or n (%)	Non-skiers n=197,684 Median (IQR) or n (%)
Age at baseline, y	36.0 (29.0-46.0)	36.0 (29.0-46.0)
Education:		
Primary school (8y)	14,538 (7)	34,806 (18)***
Secondary school (9-12y)	76,635 (39)	99,936 (51)
Higher education (13y)	106,147 (54)	59,986 (31)

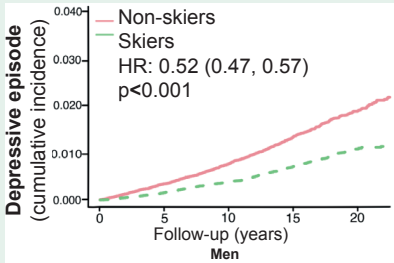
1989-2010
Swedish Vasaloppet Cohort
Skiers n=203,810
Non-skiers n=504,812

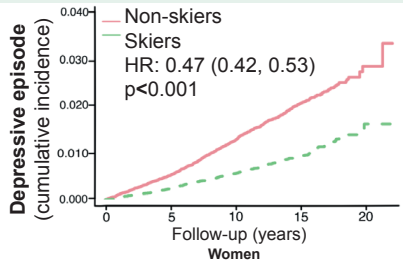
Excluded; participants already diagnosed with severe diseases
Skiers n=5,744
Non-skiers n=48,376
Excluded in rematch;
Skiers n=381
Non-skiers n=258,752

Study population
Non-depressed skiers and controls free of other severe diseases
Skiers n=197,685
Non-skiers n=197,684

Depressive episode
Skiers n=1030
Non-skiers n=2045

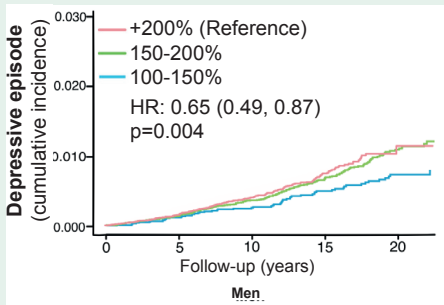
Both male and female skiers have lower incidence of depression

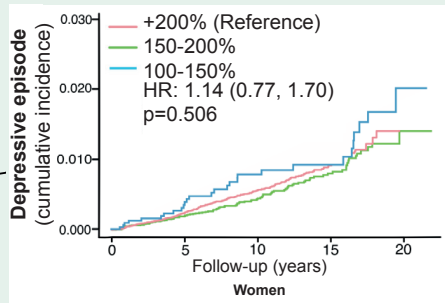




Both male and female skiers had around 50 % lower risk for depression compared to matched general population.

The impact of performance level on incident depression differs between men and women





Skiing men finishing the race faster had a lower risk of depression compared to slower skiers.

For skiing women, finishing time of the race did not significantly impact the risk of future depression.

Physical activity and the risk of Parkinson's disease (PD)

Main findings

- In the early follow-up, skiers had a 30 % lower risk of developing PD.
- In the later follow-up, the incidence of PD in skiers converged with the incidence level in the general population.

The lower risk of PD seen in skiers may partly be due to reverse causation

The significantly reduced risk of PD among the skiers became non-significant (p=0.08) in sensitivity analysis excluding all PD cases diagnosed during the first five years after study inclusion. This indicates that this association might be driven by reverse causation. Yet, after the exclusion, this association is close to statistical significance, thus reverse causation is not likely to be the only explanation.

Skiers may have a motor reserve delaying their onset of symptoms

Visual inspection of the cumulative incidence curves reveals a tendency towards convergence of the skier and non-skier curves over time. Thus, skiers are more likely to get a PD diagnosis at a later time during our follow-up compared to the non-skiers. This is especially pronounced in older age groups and among those that we followed for the longest time (included in the study 1991-2000). These observations support the hypothesis that those who are physically active may have a motor reserve that delays the onset of symptoms leading to diagnosis.

Female skiers' cumulative incidence of PD shows a greater degree of convergence

Since PD incidence generally plateaus earlier in women, the motor reserve hypothesis predicts a greater convergence of the cumulative incidence with the general population among women. In our study, we observed this pattern, further supporting this hypothesis.

Physical performance does not predict PD

We could not detect any association between race finishing time and risk of developing PD, adjusting for age, sex and education (HR= 0.98 (0.7, 1.5)).

Conclusion

Our results support the hypothesis that physically active individuals may develop a motor reserve that postpone the onset of PD symptoms.

Impact

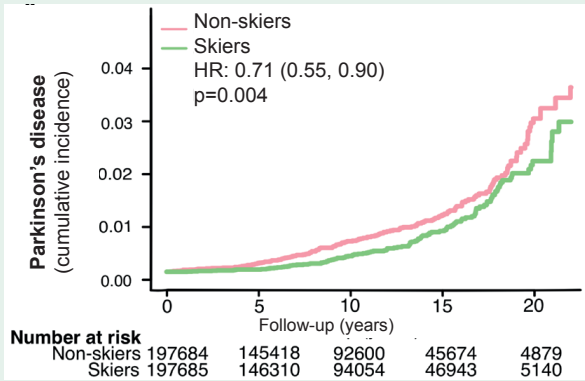
If physically active individuals develop a motor reserve, it may be possible for those individuals to have a higher quality of life for a longer time despite the current lack of treatments for PD.

Delayed clinical manifestation of Parkinson's disease among physically active

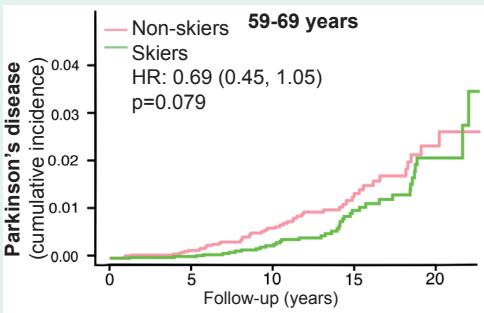
Study design

- **Incident Parkinson's disease (PD)**
- **283 PD cases**
- **Vasaloppet skiers (n=197,685)**
- **General population (n=197,684)**
- **38 % Women**
- **Up to 21 y of follow-up**
- **Matched on sex & age**

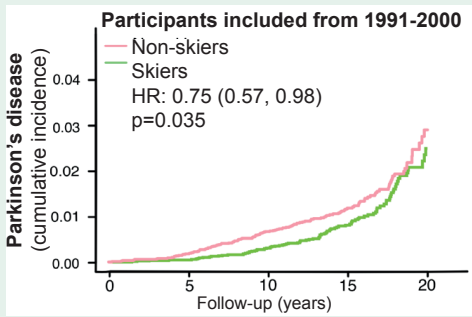
Skiers have lower incidence of PD



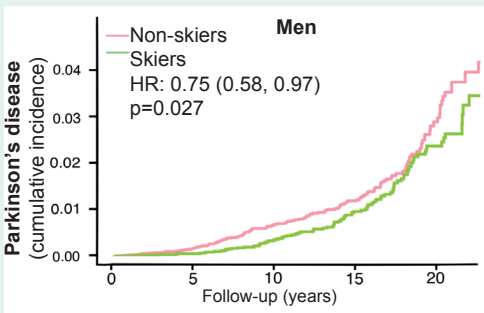
The differential of cumulative PD-incidence between skiers and non-skiers decrease with time



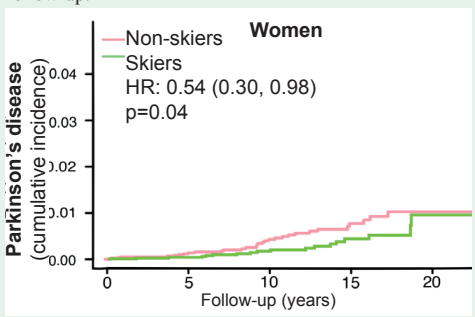
Skier's incidence of PD converges towards the level of non-skiers in late follow-up.



90 % of all PD cases comes from the group followed since 1991-2000. In this group, PD incidence among skiers converges towards the level of non-skiers in late follow-up.



The cumulative incidence of PD is higher in men compared to women and does not plateau in skiers or non-skiers during follow-up.



PD incidence plateaus earlier in women and the cumulative PD incidence of skiers and non-skiera show greater convergence over time in women compared to men.

Physical activity and the risk of dementia

Main findings

- Skiers had an almost 50% lower risk of developing vascular dementia (VaD) compared to the matched general population.
- The risk of Alzheimer's disease (AD) did not differ between groups.
- The risk of developing VaD was even lower in high-performing skiers compared to low performing skiers.
- Running did not improve synaptic proteins or soluble Aβ levels in transgenic AD mice.

A physically active lifestyle affects the risks of developing VaD and AD differently

Skiers had a lower risk of developing all-cause dementia (adjusted HR= 0.63 (0.52–0.75)) compared to non-skiers. Our results indicate that physical activity may have different effects depending on the cause of dementia. Furthermore, our results from the Vasaloppet cohort were in line with those observed in the Malmö diet and cancer study, wherein individuals with higher self-reported physical activity scores had a lower risk of VaD (adjusted HR=0.49 (0.33–0.73)) but not AD.

Low physical performance level is associated with the risk of developing VaD

Faster skiers had a further reduced risk of developing VaD. Skiers completing the race with a finishing time faster than median had a 60 % lower risk of developing vascular dementia compared to their slower counterparts (HR=0.38 (0.2-0.9) in the model adjusted for age, sex and education).

Running does not ameliorate the molecular hallmarks of AD in mice

Running did not reduce soluble Aβ levels in the cortex nor the hippocampus, when measured by 6E10 immunoreactivity and Aβ40/Aβ42 ELISA in 5xFAD mice. Moreover, levels of the synaptic proteins PSD95 and synaptophysin were not increased by exercise. On the contrary, the levels of PSD-95 seemed to decrease due to running, but the effect was not statistically significant (Mann-Whitney U-test, p=0.09).

Conclusion

Physical activity may not be equally effective against all disorders causing dementia.

Impact

Our finding that physical activity might not be associated with a lower risk of developing AD is important as it goes against the general view. Our findings might impact the setup of exercise and the investment in future studies in this field.

Midlife physical activity is associated with lower incidence of vascular dementia but not Alzheimer's disease

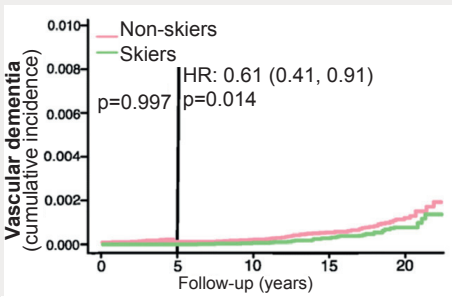
Study design

- Incidence in Patient Registry
- 181 cases of Alzheimer's disease (AD)
- 112 cases of Vascular dementia (VaD)
- Vasaloppet skiers (n=197,685)
- General population (n=197,684)
- 38 % Women
- Up to 21 y of follow-up

Experimental design

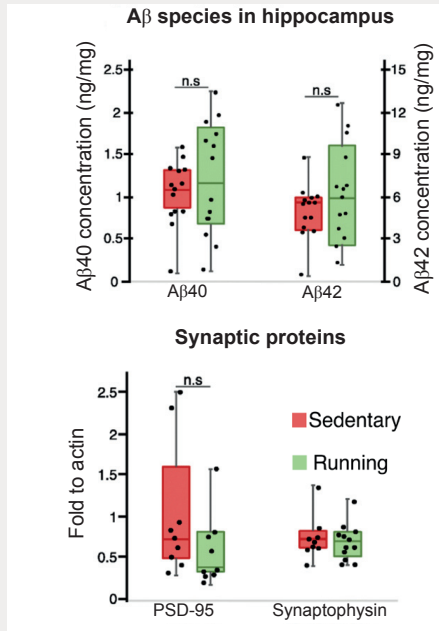
- Amyloid-β & synaptic proteins
- female 5xFAD mice (n=30)
- Voluntary wheel running (6 m)

Vasaloppet skiers have lower incidence of VaD, but not AD



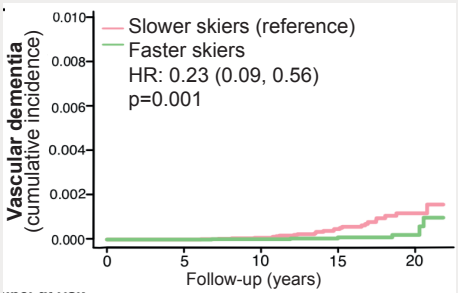
Skiers have around 40 % lower risk for VaD comppared to non-skiers even when excluding dementia cases diagnosed during the first 5 years after study inclusion. No association was seen for AD.

Running does not improve synaptic or Aβ pathology in AD mice

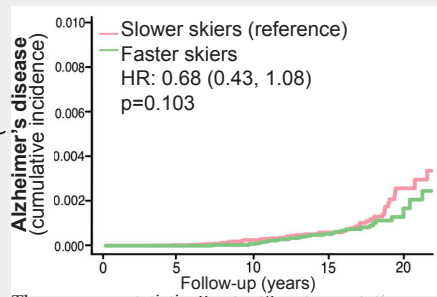


Running did not improve the levels of synaptic proteins (Western Blot) or Aβ (ELISA) in hippocampus.

Ski performance level affect incidence of VaD, but not AD



Faster skiers (below median finishing time) had almost 80 % lower risk of VaD compared to slower skiers.



There was no statistically significant association between the skier's performance level in Vasaloppet and risk for future AD.

The effects of voluntary running on Alzheimer's pathology in mice

Main findings

Voluntary running does NOT;

- affect the total amount of microglia.
- affect cytokine levels.
- reduce the levels of insoluble Aβ.
- improve motor learning in the rotarod performance test.
- Running mice developed hindlimb clasping earlier.

Running tended to augment the levels of insoluble Aβ in the hippocampus

In the cortex, running did not affect the levels of insoluble Aβ when measured using Thioflavin S staining. Conversely, running led to a non-significant trend towards increased insoluble Aβ levels in the hippocampus (Mann-Whitney U-test, $p=0.08$).

Running led to faster progression of sensorimotor dysfunction

Hindleg clasping is a sign of sensorimotor dysfunction and is typically seen in the 5xFAD model. Our running mice developed this dysfunction earlier than their sedentary counterparts.

Conclusion

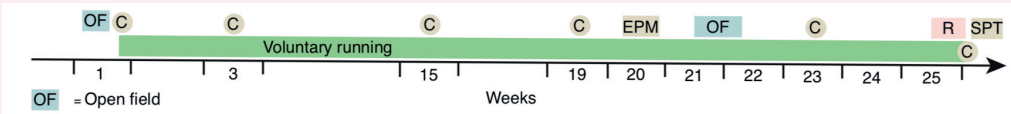
Our results indicate that exercise might not confer long-term protection against the genetic form of Alzheimer's disease.

Impact

Our findings question previous findings in the field and add to existing knowledge of the long-term effects of exercise interventions in these models.

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

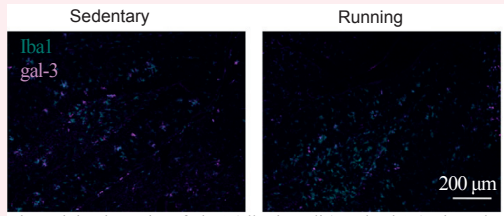
Experimental design



OF = Open field
C = Clasping
EPM = Elevated plus
R = Rotarod
SPT = Sucrose preference test

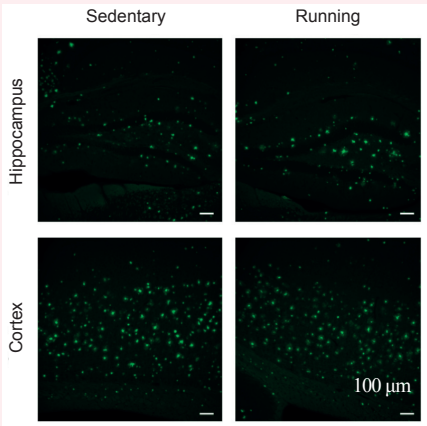
female 5xFAD mice (n=30)

Running did not significantly affect neuroinflammation



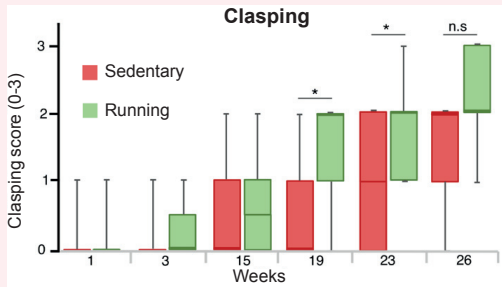
The staining intensity of Iba1 (all microglia) and gal-3 (activated microglia) in hippocampus did not significantly differ.

Running did not ameliorate the levels of insoluble Aβ



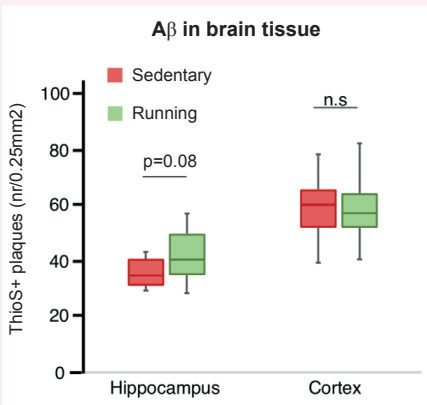
10x pictures of insoluble Aβ stained with ThioS.

Running mice developed hindlimb clasping earlier



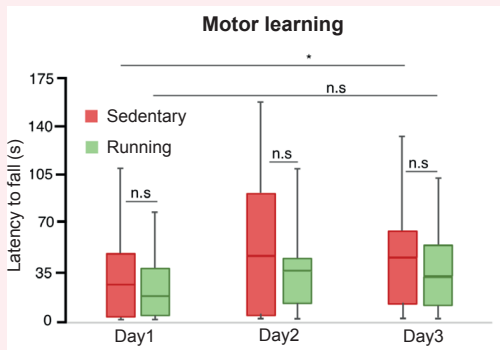
Running mice displayed significantly more clasping at 19 and 23 weeks compared to sedentary mice. (Mann-Whitney U-test)

Aβ in brain tissue



Running did not affect the levels of ThioS in cortex, but there was a non-significant trend towards increased levels in the hippocampus. (Mann-Whitney U-test)

Running did not improve motor learning



Sedentary mice significanlty improved their performance in rotarod, whereas the running mice did not. (Wilcoxon test).

Discussion

Experimental exercise

Neuroinflammation as a shared therapeutic target for brain disorders

As described in the introduction, increased neuroinflammation is associated with several disorders affecting the brain, such as depression, PD, AD and brain ischemia [116,120,130,133]. Whether this inflammation is the cause or result of each disorder can be debated. Our study on the role of microglial galectin-3 in brain ischemia demonstrates that the inflammatory response contributes to brain injury under pathological conditions. Regardless of its origin, chronic and uncontrolled inflammation in the brain is considered to negatively affect brain health [390,391]. Consequently, a treatment to target and control the inflammatory response in the brain could be useful for many disorders affecting the brain. Exercise could be such a treatment strategy. Although my experimental studies did not reveal any significant neuroinflammatory effects, many other studies have [208,235,262,265,278,392-395]. It is difficult to detect the inflammatory effects of exercise since many of these effects are transient, requiring sampling at specific time-points. Hence, it is possible that our running interventions did affect neuroinflammation and that this effect would have been detectable if samples had been collected at other time points. Nonetheless, it is important to clarify whether the inflammatory effects reported after exercise are clinically relevant from a long-term perspective. In experimental settings, my studies indicate that exercise may have different inflammatory effects depending on whether the running intervention is voluntary or enforced. Thus, in future experimental studies, interventions should be designed to prevent stress as this may interfere with the anti-inflammatory effects of exercise. In addition, sampling should be done at different time-points in order to observe the temporal dynamics of these effects.

Animal behavior as a predictor of clinical symptoms in brain disorders

Behavioral tests used in experimental research are rather poor analogies to the manifestation of pathological symptoms in patients. As such, the clinical relevance of effects seen through animal behavior can be questioned. Still, animal models are widely

used, as no better alternatives exist today. In my studies, running interventions did not ameliorate behavioral deficits. On the contrary, the mice displayed aggravated behavioral deficits in some tests. Animal behavioral testing is very complex, and potential confounders are numerous. In our animal facility, I have identified several factors that could affect animal behavior during these tests. I believe that these are common problems that can be found in most animal facilities. Large behavioral variation within groups adds to the difficulty of finding statistically significant effects. For these reasons, I think that animal behavioral readouts have a limited clinical relevance, especially from studies wherein no differences between groups are seen. In my treadmill intervention study, the forced running induced stress in the mice, and this likely affected not only the outcome in anxiety tests but also in cognitive tests. The lack of beneficial effects on our running AD mice might be because exercise does not affect AD pathology or because we used an AD model with a highly aggressive pathology. Thus, the results obtained from my experimental exercise studies do not exclude the possibility of exercise to beneficially affect behavior in other models of disorders affecting the brain, which has been observed by others [175,208,241,278,284,295,297,393,396,397].

Stress as a confounding factor in experimental exercise settings

As described previously, our forced running intervention induced a detrimental stress response in our mice, likely interfering with the potentially beneficial effects of the exercise. However, in this study, it is also possible that fighting in home cages contributed to the stress. Still, stress is a powerful confounding factor as it affects similar pathways as exercise does but with different long-term effects [203]. Therefore, it is important to consider this factor when designing experimental exercise interventions. I suggest that all experimental exercise studies address this concern by monitoring stress during and after exercise interventions. Nevertheless, it is indeed difficult to distinguish the effects of psychological stress from physical responses to exercise. A combination of behavioral tests, observation of home cage behavior and measurement of molecular markers is needed. I advocate for the development of a standardized test battery, including defined behavioral and molecular tests to control for stress induction.

Exercise epidemiology

Physical activity as a preventive strategy for brain disorders

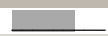
I could not detect any beneficial effects from running interventions on brain pathology in our mouse models of brain ischemia nor AD. This does not mean that running

interventions cannot exert beneficial molecular effects as many other studies demonstrated promising results [235,246,265,289,398,399]. Further, our epidemiological studies revealed that physical activity is a promising strategy to alleviate the burden of the most common disorders affecting the brain. Due to the diverse pathologies of the disorders investigated in my thesis, it is unlikely that physical activity affects all of these disorders in the same way. It is important to keep in mind that physical activity may be associated with a lower risk of a disorder without necessarily reducing the pathology. As suggested in paper IV, the association between physical activity and a lower incidence of PD might be due to the build-up of a motor reserve, allowing physically active individuals to sustain more PD pathology before the onset of symptoms. For multifactorial disorders like depression, it is possible that physical activity prevents this condition in individuals with a light tendency for depression, not necessarily through molecular effects, but rather through psychological mechanisms, such as by distracting from negative thoughts [346,400]. Additionally, a 2-sample Mendelian randomization study suggests that physical activity prevents depression rather than the inverse [401].

Importantly, we saw no association between physical activity and AD in our study. Thus, physical activity should not be regarded as a universal treatment that is equally effective against all disorders affecting the brain. Table 5 shows a qualitative evaluation of the causality of physical activity on each disorder investigated in my thesis. Further, even if physical activity causes a reduction in these disorders, there is no scientific evidence that supports physical activity as the best or only treatment option. Patients are not homogenous even though they have the same diagnosis. Many patients suffer from not only one disorder, and the recommendation to add physical activity as an additional preventive strategy needs to be evaluated for each patient. Exercise is not a miracle solution, but the adverse effects of light physical exercise are few and rare.

Table 5. Evaluation of causality

Qualitative evaluation of causality of physical activity on each disorder affecting the brain as investigated in the Vasaloppet Cohort in my thesis work according to Bradford Hill's criteria of causality. The degree of fulfilment of each criterion is indicated with a scale. The results included in my thesis serve as the basis of the evaluation and are complemented by additional studies available through PubMed.

Bradford Hill's criteria	Depression	PD	AD	VaD
Strength				
Consistency				
Specificity				
Temporality				
Biological gradient				
Plausibility				
Coherence				
Experimental evidence				
Analogy				

Physical activity vs. physical fitness as predictive factors

Few studies distinguish between the amount of physical activity and the level of physical fitness as predictive factors for disorders and functional outcomes [402-404]. Among those that do, some studies indicate that it is the fitness level that matters for brain function and mortality [403,404]. Physical activity and fitness are of course linked to some extent as being physically active improves fitness level. Nonetheless, the amount of activity required to achieve and maintain a certain fitness level differs between individuals [405-407] (see *Future perspectives*).

In our epidemiological studies, we use participation in Vasaloppet as a proxy for a high amount of everyday physical activity, which is supported by previous studies indicating that this skiing population has higher physical activity levels compared to matched non-skiers [385,386]. Additionally, we used the finishing time of the race as a proxy for fitness level, as previous studies have shown that work output (W/kg) correlates with performance in this ski race [408]. Physical activity but not physical performance predicted later development of PD. However, both physical activity and performance had significant associations to the risk of depression and VaD and could be used as predictive factors. For VaD, the predictive values of physical activity (being a skier, HR=0.49 (0.3–0.7)) and physical performance (skiing fast, HR= 0.38 (0.2, 0.9)) were in the same range, although physical activity gave a more precise predictive value (narrower confidence interval). For depression, participation in the ski race (HR=0.53 (0.49, 0.58)) had a higher predictive value than the performance in the race (HR=0.78 (0.62, 0.99)). However, Åberg *et al.* showed that the predictive values of objectively measured fitness on depression were in the same range as we see for physical activity [330]. Importantly, lower physical performance in Vasaloppet skiers predicted depression only in men and not women in our study, but Åberg *et al.* only investigated men. Taken together, additional studies are warranted to determine whether physical activity or fitness level offers the best predictive value for each disorder affecting the brain.

Ski performance affects the risk of depression differently

Among our skiers in the Vasaloppet cohort, skiing faster was associated with an even lower risk for depression if the skier was a man (HR=0.65 (0.49, 0.87)). Even if the association between fast skiing and depression in women was far from being significant (p=0.6), it is important to note that they showed an opposite pattern (HR=1.11 (0.74, 1.66)). Thus, among skiers, the impact of physical performance on depression likely differs between men and women. This is in line with other studies showing that men may benefit from higher exercise intensities [354]. On the other hand, studies suggest either that exercise intensity might not matter for women [364] or that women might benefit more from lower intensity exercise [352].

Our study cannot explain the reasons behind these differences, but I believe that the motives for exercising may influence this association since the impact of exercise reasons on life quality seems to differ between men and women [409]. Remarkably, women who reported weight loss and body toning as reasons for exercising also reported lower life quality, independent from the level of exercise [409]. The same pattern was not seen in men, wherein the only predictor of perceived life quality was the level of exercise. However, it is important to consider that the tendency for a man to seek medical care for depression has maybe increased recently compared to the period 1989-2010 when our study was conducted. Since our study relies on clinical diagnoses reported in the patient registry, we likely underestimate the true incidence of depression, especially among men, who have a lower tendency to seek care when depressed [410]. It has been speculated that the lower incidence of depression in men may be attributed to a lower tendency for men to seek medical care, which in turn, may explain their higher tendency to commit suicide compared to women. Hence, the lower incidence of depression in our high performing male skiers might be due to increased suicide rates and not seeking care to get the proper diagnosis. However, previous data demonstrates the opposite pattern: Swedish men with an objectively measured low fitness level have a higher risk of suicide [411,412]. Moreover, the awareness of mental unhealth has increased over the last decade, and, if our Vasaloppet cohort was assembled today, the incidence of depression among high-performing male skiers may have differed from what we found in our study.

General conclusions

Taken together, the results presented in my thesis suggest the following:

- A physically active lifestyle is associated with a lower risk of common disorders affecting the brain: depression, Parkinson's disease, and vascular dementia.
- Higher physical performance is associated with an even lower risk for vascular dementia.
- Physical performance level may impact the risk of later depression differently between men and women.
- Physically active individuals may develop a motor reserve allowing them to sustain more pathology before PD symptoms appear, thus leading to a later diagnosis.
- The incidence of Alzheimer's disease is not associated with a physically inactive lifestyle.
- Running does not reduce molecular or behavioral hallmarks of AD in 5xFAD mice.
- Running does not significantly affect the total amount of microglia in mice following brain ischemia or AD.
- Forced running might induce a detrimental stress response in mice, which might interfere with the beneficial effects of exercise.

Future perspectives

Our research emphasizes the potential of physical activity to be used as a preventive strategy to reduce the overall burden of some of the most common disorders affecting the brain. For an exercise intervention to reach its full preventive potential, more knowledge is needed regarding optimal dose, duration, and intensity for a given medical condition. Novel techniques offer great opportunities to uncover yet unknown effects of physical activity on the brain. It is tempting to speculate about the next generation of this research field.

New dimensions of microglial neuroinflammation

In my thesis, I have investigated the effects of running on microglial activation. The existing one-dimensional view of microglial activation states, proinflammatory M1 and anti-inflammatory M2, is already widely questioned [70,71,75,83]. Technological advancements will allow us to follow individual microglial cells in their natural environment using proteomics and transcriptomics with a spatial and temporal resolution never seen before. Novel markers linked to different functions will be discovered, adding new dimensions to our interpretation of microglial phenotypes. In fact, the previous one-dimensional phenotypic view has already started to expand into additional dimensions [83], and more are yet to come. To understand the true nature of microglia and their neuroinflammatory role in disorders affecting the brain, collaboration with experts in bioinformatics and statistics is needed. Nevertheless, a multi-dimensional view of microglial phenotypes will only be a statistical simplification to interpret endless amounts of data regarding the functional properties of each microglial cell. Still, understanding how different settings of physical activity affect these different microglial dimensions may be the key to optimizing exercise interventions for different groups of patients. The voyage towards this knowledge has just begun.

Future exercise research methods

Several novel technological tools offer promising opportunities to study different aspects of physical activity in future studies. For example, relatively cheap and accurate modern smartwatches offer new possibilities to objectively measure different aspects of

physical activity, such as pulse, duration, distance moved and number of steps walked. Until today, the majority of studies relied on subjective measurements, such as self-reported physical activity. Some studies suggest a significant discrepancy between self-reported physical activity and more subjective measurements, such as data registered by accelerometers [413-416]. Importantly, objective measurements may be better predictors [401,403]. Thus, research based on measurements from accelerometers in large populations is warranted. The increased general use of portable technologies (e.g. smartwatches) makes it possible to reliably analyze peoples' exercise behavior over a long time period. However, this technique is not without concerns [417], and consensus on how to best use these methods needs to be established.

Personalized interventions and individual-based research

Large variations within exercise intervention groups represent a major limitation to statistically reveal medically relevant outcomes [405]. In fact, the same exercise intervention is likely to result in slightly different outcomes for each person [407]. This is likely due to variance due to interfering environmental and genetic factors that cannot be standardized in the same way as in animal studies. Recent research revealed that genetics account for up to 50 % of all differences in physical activity levels [401,418,419] and fitness [405,420] among people. Adding to that, twin studies suggest that the positive effects on cognition and depression linked to exercise or fitness are explained by genetic predisposition [421,422]. However, the inverse association between exercise and use of anti-depressants did not depend on genetics [423], and low fitness still predicted depression even after adjustments for depressed siblings in a study following over one million Swedish men for up to 40 years [330].

The optimistic interpretation of the above-mentioned impact of the genetics on fitness is that the remaining 50 % of the variation seems to be explained by other factors, such as the environment, which people can influence [419,424]. To overcome the genetic issue, personalized interventions and individual-based research certainly have something to offer [405]. Indeed, when a training program was adapted to the genetic predispositions of the participants, better results were achieved [407]. Performing more individual-based exercise research will require more advanced statistical analysis. We are only at the beginning of an era wherein highly sophisticated methods for analyzing individual genomics, transcriptomics and proteomics are being developed.

Exercise in a pill

As more studies uncover the molecular effects of exercise, it is tempting to speculate about the possibility of exercise mimetics, especially for those who are unable to lead a physically active lifestyle. Excitingly, exercise-related increases in PPARδ can be induced pharmacologically [425], and this treatment doubled the time that mice were capable of running, despite no previous training [425]. Additionally, compounds that

activate AMPK improved cognition and increased the number of hippocampal neurons in some mouse models [426]. However, the positive effects on the brain seem to be transient, and in the long run, this treatment may induce neuroinflammation [427]. Moreover, lactate shows promising exercise-mimicking effects but fails to recapitulate all the effects induced in the brain following exercise [428]. Furthermore, mice overexpressing PGC-1α tended to have reduced levels of circulating pro-inflammatory cytokines but revealed no beneficial effects of PGC-1α on neurogenesis or neuronal survival following stroke [429,430]. Notwithstanding, pharmacological stimulation of neurogenesis has beneficial effects on cognition but is not as beneficial as running [175]. While there may be a future for exercise mimetics, this treatment would likely require a combination of several components, given the fact that exercise gives rise to numerous molecular effects within the body.

Shared pathways between stress and exercise

I identified stress as an interfering factor in experimental exercise settings as I observed harmful effects from forced running in mice. Furthermore, stress might be an interfering factor also in other settings. Trying to find time for physical activity might induce stress that might blunt some of the beneficial effects of exercise. Interestingly, the pathways that are activated following acute stress and acute exercise overlap [203]. In contrast, the long-term effects of stress and exercise are dissimilar [203]. To optimally benefit from using physical activity as a preventive strategy, we need to elucidate exactly what differentiates exercise from stress, both from short- and long-term perspectives. Completely avoid stress has never been possible. Thus, we need to understand how stress can be used for our good in conjunction with physical activity.

Implementation of existing knowledge in society

Scientific knowledge achieves its full value when it is made implementable in society to improve peoples' everyday life. The step towards utilizing existing knowledge about physical activity and brain health in society is not that far. We need to gain more knowledge on how to optimize such interventions in the contexts of different disorders. Additionally, I strongly believe adopting an interdisciplinary approach will be required. The effect of physical activity on brain health is not just a matter of molecular effects. Contrarily, our data on the differences between male and female skiers indicates that factors such as gender and other psychological aspects might matter. Caution must be taken, and this highlights the need to identify factors preventing certain people from being physically active. Moreover, economic aspects need to be considered to develop sustainable interventions that can not only be used during an interventional study but adapted to everyday life and maintained. Politicians and others responsible for planning and building our society and environments must collaborate with health researchers regularly. There is money to save and suffering to alleviate. This should all be done in a joint effort.

Acknowledgement

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References

1 Jellinger, K. A. Clinicopathological analysis of dementia disorders in the elderly--an update. *J Alzheimers Dis* **9**, 61-70 (2006).

2 de la Torre, J. C. Is Alzheimer's disease a neurodegenerative or a vascular disorder? Data, dogma, and dialectics. *Lancet Neurol* **3**, 184-190, doi:10.1016/S1474-4422(04)00683-0 (2004).

3 Hesdorffer, D. C. Comorbidity between neurological illness and psychiatric disorders. *CNS Spectr* **21**, 230-238, doi:10.1017/S1092852915000929 (2016).

4 Raskind, M. A. Diagnosis and treatment of depression comorbid with neurologic disorders. *Am J Med* **121**, S28-37, doi:10.1016/j.amjmed.2008.09.011 (2008).

5 Zhao, Q. F. *et al.* The prevalence of neuropsychiatric symptoms in Alzheimer's disease: Systematic review and meta-analysis. *J Affect Disord* **190**, 264-271, doi:10.1016/j.jad.2015.09.069 (2016).

6 Copeland, M. P. *et al.* Psychiatric symptomatology and prodromal Alzheimer's disease. *Alzheimer Dis Assoc Disord* **17**, 1-8, doi:10.1097/00002093-200301000-00001 (2003).

7 Perini, G. *et al.* Cognitive impairment in depression: recent advances and novel treatments. *Neuropsychiatr Dis Treat* **15**, 1249-1258, doi:10.2147/NDT.S199746 (2019).

8 Wang, X. *et al.* Comorbidity burden of patients with Parkinson's disease and Parkinsonism between 2003 and 2012: A multicentre, nationwide, retrospective study in China. *Sci Rep* **7**, 1671, doi:10.1038/s41598-017-01795-0 (2017).

9 Collaborators, G. B. D. D. I. I. a. P. Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet* **388**, 1545-1602, doi:10.1016/S0140-6736(16)31678-6 (2016).

10 Spychala, M. S., Honarpisheh, P. & McCullough, L. D. Sex differences in neuroinflammation and neuroprotection in ischemic stroke. *J Neurosci Res* **95**, 462-471, doi:10.1002/jnr.23962 (2017).

11 You, S. *et al.* Efficacy and safety of intravenous recombinant tissue plasminogen activator in mild ischaemic stroke: a meta-analysis. *Stroke Vasc Neurol* **3**, 22-27, doi:10.1136/svn-2017-000106 (2018).

12 Ringleb, P. A., Schellinger, P. D., Schranz, C. & Hacke, W. Thrombolytic therapy within 3 to 6 hours after onset of ischemic stroke: useful or harmful? *Stroke* **33**, 1437-1441, doi:10.1161/01.str.0000015555.21285.db (2002).

13 Powers, W. J. *et al.* 2018 Guidelines for the Early Management of Patients With Acute Ischemic Stroke: A Guideline for Healthcare Professionals From the American Heart Association/American Stroke Association. *Stroke* **49**, e46-e110, doi:10.1161/STR.0000000000000158 (2018).

14 Leng, T. & Xiong, Z. G. Treatment for ischemic stroke: From thrombolysis to thrombectomy and remaining challenges. *Brain Circ* **5**, 8-11, doi:10.4103/bc.bc_36_18 (2019).

15 Lambrinos, A. *et al.* Mechanical Thrombectomy in Acute Ischemic Stroke: A Systematic Review. *Can J Neurol Sci* **43**, 455-460, doi:10.1017/cjn.2016.30 (2016).

16 Go, A. S. *et al.* Heart disease and stroke statistics--2014 update: a report from the American Heart Association. *Circulation* **129**, e28-e292, doi:10.1161/01.cir.0000441139.02102.80 (2014).

17 Dirnagl, U., Iadecola, C. & Moskowitz, M. A. Pathobiology of ischaemic stroke: an integrated view. *Trends Neurosci* **22**, 391-397, doi:10.1016/s0166-2236(99)01401-0 (1999).

18 Olai, H. *et al.* Meta-analysis of targeted temperature management in animal models of cardiac arrest. *Intensive Care Med Exp* **8**, 3, doi:10.1186/s40635-019-0291-9 (2020).

19 Lackland, D. T. *et al.* Factors influencing the decline in stroke mortality: a statement from the American Heart Association/American Stroke Association. *Stroke* **45**, 315-353, doi:10.1161/01.str.0000437068.30550.cf (2014).

20 Collaborators, G. B. D. S. Global, regional, and national burden of stroke, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Neurol* **18**, 439-458, doi:10.1016/S1474-4422(19)30034-1 (2019).

21 Olsson, T. *et al.* Gene deletion of cystatin C aggravates brain damage following focal ischemia but mitigates the neuronal injury after global ischemia in the mouse. *Neuroscience* **128**, 65-71, doi:10.1016/j.neuroscience.2004.06.024 (2004).

22 Alzheimer's, A. 2016 Alzheimer's disease facts and figures. *Alzheimers Dement* **12**, 459-509, doi:10.1016/j.jalz.2016.03.001 (2016).

23 Rizzi, L., Rosset, I. & Roriz-Cruz, M. Global epidemiology of dementia: Alzheimer's and vascular types. *Biomed Res Int* **2014**, 908915, doi:10.1155/2014/908915 (2014).

24 Podcasy, J. L. & Epperson, C. N. Considering sex and gender in Alzheimer disease and other dementias. *Dialogues Clin Neurosci* **18**, 437-446 (2016).

25 O'Brien, J. T. & Thomas, A. Vascular dementia. *Lancet* **386**, 1698-1706, doi:10.1016/S0140-6736(15)00463-8 (2015).

26 Roman, G. C. Vascular dementia may be the most common form of dementia in the elderly. *J Neurol Sci* **203-204**, 7-10, doi:10.1016/s0022-510x(02)00252-6 (2002).

27 Lane, C. A., Hardy, J. & Schott, J. M. Alzheimer's disease. *Eur J Neurol* **25**, 59-70, doi:10.1111/ene.13439 (2018).

28 Niu, H., Alvarez-Alvarez, I., Guillen-Grima, F. & Aguinaga-Ontoso, I. Prevalence and incidence of Alzheimer's disease in Europe: A meta-analysis. *Neurologia* **32**, 523-532, doi:10.1016/j.nrl.2016.02.016 (2017).

29 Solfrizzi, V. *et al.* Relationships of Dietary Patterns, Foods, and Micro- and Macronutrients with Alzheimer's Disease and Late-Life Cognitive Disorders: A Systematic Review. *J Alzheimers Dis* **59**, 815-849, doi:10.3233/JAD-170248 (2017).

30 Evans, D. A. *et al.* Education and other measures of socioeconomic status and risk of incident Alzheimer disease in a defined population of older persons. *Arch Neurol* **54**, 1399-1405, doi:10.1001/archneur.1997.00550230066019 (1997).

31 Khachaturian, A. S. *et al.* Antihypertensive medication use and incident Alzheimer disease: the Cache County Study. *Arch Neurol* **63**, 686-692, doi:10.1001/archneur.63.5.noc60013 (2006).

32 Zissimopoulos, J. M., Barthold, D., Brinton, R. D. & Joyce, G. Sex and Race Differences in the Association Between Statin Use and the Incidence of Alzheimer Disease. *JAMA Neurol* **74**, 225-232, doi:10.1001/jamaneurol.2016.3783 (2017).

33 Karch, C. M. & Goate, A. M. Alzheimer's disease risk genes and mechanisms of disease pathogenesis. *Biol Psychiatry* **77**, 43-51, doi:10.1016/j.biopsych.2014.05.006 (2015).

34 Hane, F. T., Lee, B. Y. & Leonenko, Z. Recent Progress in Alzheimer's Disease Research, Part 1: Pathology. *J Alzheimers Dis* **57**, 1-28, doi:10.3233/JAD-160882 (2017).

35 Braak, H. & Braak, E. Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathol* **82**, 239-259, doi:10.1007/bf00308809 (1991).

36 Masters, C. L. *et al.* Alzheimer's disease. *Nat Rev Dis Primers* **1**, 15056, doi:10.1038/nrdp.2015.56 (2015).

37 Olsson, B. *et al.* CSF and blood biomarkers for the diagnosis of Alzheimer's disease: a systematic review and meta-analysis. *Lancet Neurol* **15**, 673-684, doi:10.1016/S1474-4422(16)00070-3 (2016).

38 Tysnes, O. B. & Storstein, A. Epidemiology of Parkinson's disease. *J Neural Transm (Vienna)* **124**, 901-905, doi:10.1007/s00702-017-1686-y (2017).

39 Surmeier, D. J. & Schumacker, P. T. Calcium, bioenergetics, and neuronal vulnerability in Parkinson's disease. *J Biol Chem* **288**, 10736-10741, doi:10.1074/jbc.R112.410530 (2013).

40 Buddhala, C. *et al.* Dopaminergic, serotonergic, and noradrenergic deficits in Parkinson disease. *Ann Clin Transl Neurol* **2**, 949-959, doi:10.1002/acn3.246 (2015).

41 Braak, H. *et al.* Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol Aging* **24**, 197-211, doi:10.1016/s0197-4580(02)00065-9 (2003).

42 Lill, C. M. Genetics of Parkinson's disease. *Mol Cell Probes* **30**, 386-396, doi:10.1016/j.mcp.2016.11.001 (2016).

43 Kalia, L. V. & Lang, A. E. Parkinson's disease. *Lancet* **386**, 896-912, doi:10.1016/S0140-6736(14)61393-3 (2015).

44 Jesulola, E., Micalos, P. & Baguley, I. J. Understanding the pathophysiology of depression: From monoamines to the neurogenesis hypothesis model - are we there yet? *Behav Brain Res* **341**, 79-90, doi:10.1016/j.bbr.2017.12.025 (2018).

45 Kuehner, C. Why is depression more common among women than among men? *Lancet Psychiatry* **4**, 146-158, doi:10.1016/S2215-0366(16)30263-2 (2017).

46 Vigo, D., Thornicroft, G. & Atun, R. Estimating the true global burden of mental illness. *Lancet Psychiatry* **3**, 171-178, doi:10.1016/S2215-0366(15)00505-2 (2016).

47 Disease, G. B. D., Injury, I. & Prevalence, C. Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet* **388**, 1545-1602, doi:10.1016/S0140-6736(16)31678-6 (2016).

48 Wang, A. K. & Miller, B. J. Meta-analysis of Cerebrospinal Fluid Cytokine and Tryptophan Catabolite Alterations in Psychiatric Patients: Comparisons Between Schizophrenia, Bipolar Disorder, and Depression. *Schizophr Bull* **44**, 75-83, doi:10.1093/schbul/sbx035 (2018).

49 Howard, D. M. *et al.* Genome-wide meta-analysis of depression identifies 102 independent variants and highlights the importance of the prefrontal brain regions. *Nat Neurosci* **22**, 343-352, doi:10.1038/s41593-018-0326-7 (2019).

50 Rush, A. J. *et al.* Acute and longer-term outcomes in depressed outpatients requiring one or several treatment steps: a STAR*D report. *Am J Psychiatry* **163**, 1905-1917, doi:10.1176/ajp.2006.163.11.1905 (2006).

51 Rush, A. J. Limitations in efficacy of antidepressant monotherapy. *J Clin Psychiatry* **68 Suppl 10**, 8-10 (2007).

52 Cowen, P. J. Backing into the future: pharmacological approaches to the management of resistant depression. *Psychol Med* **47**, 2569-2577, doi:10.1017/S003329171700068X (2017).

53 Wang, S. M. *et al.* Addressing the Side Effects of Contemporary Antidepressant Drugs: A Comprehensive Review. *Chonnam Med J* **54**, 101-112, doi:10.4068/cmj.2018.54.2.101 (2018).

54 Mittelbronn, M., Dietz, K., Schluesener, H. J. & Meyermann, R. Local distribution of microglia in the normal adult human central nervous system differs by up to one order of magnitude. *Acta Neuropathol* **101**, 249-255, doi:10.1007/s004010000284 (2001).

55 Lawson, L. J., Perry, V. H., Dri, P. & Gordon, S. Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. *Neuroscience* **39**, 151-170, doi:10.1016/0306-4522(90)90229-w (1990).

56 Yang, T. T. *et al.* Differential distribution and activation of microglia in the brain of male C57BL/6J mice. *Brain Struct Funct* **218**, 1051-1060, doi:10.1007/s00429-012-0446-x (2013).

57 Kettenmann, H., Hanisch, U. K., Noda, M. & Verkhratsky, A. Physiology of microglia. *Physiol Rev* **91**, 461-553, doi:10.1152/physrev.00011.2010 (2011).

58 von Bernhardi, R., Heredia, F., Salgado, N. & Munoz, P. Microglia Function in the Normal Brain. *Adv Exp Med Biol* **949**, 67-92, doi:10.1007/978-3-319-40764-7_4 (2016).

59 Prinz, M. & Priller, J. Microglia and brain macrophages in the molecular age: from origin to neuropsychiatric disease. *Nat Rev Neurosci* **15**, 300-312, doi:10.1038/nrn3722 (2014).

60 Reu, P. *et al.* The Lifespan and Turnover of Microglia in the Human Brain. *Cell Rep* **20**, 779-784, doi:10.1016/j.celrep.2017.07.004 (2017).

61 Davalos, D. *et al.* ATP mediates rapid microglial response to local brain injury in vivo. *Nat Neurosci* **8**, 752-758, doi:10.1038/nn1472 (2005).

62 Carbonell, W. S., Murase, S., Horwitz, A. F. & Mandell, J. W. Migration of perilesional microglia after focal brain injury and modulation by CC chemokine receptor 5: an in situ time-lapse confocal imaging study. *J Neurosci* **25**, 7040-7047, doi:10.1523/JNEUROSCI.5171-04.2005 (2005).

63 Biber, K., Neumann, H., Inoue, K. & Boddeke, H. W. Neuronal 'On' and 'Off' signals control microglia. *Trends Neurosci* **30**, 596-602, doi:10.1016/j.tins.2007.08.007 (2007).

64 Jiao, F. Z. *et al.* Histone Deacetylase 2 Inhibitor CAY10683 Alleviates Lipopolysaccharide Induced Neuroinflammation Through Attenuating TLR4/NF-kappaB Signaling Pathway. *Neurochem Res* **43**, 1161-1170, doi:10.1007/s11064-018-2532-9 (2018).

65 von Bernhardi, R., Eugenin-von Bernhardi, L. & Eugenin, J. Microglial cell dysregulation in brain aging and neurodegeneration. *Front Aging Neurosci* **7**, 124, doi:10.3389/fnagi.2015.00124 (2015).

66 Minghetti, L. & Levi, G. Microglia as effector cells in brain damage and repair: focus on prostanoids and nitric oxide. *Prog Neurobiol* **54**, 99-125, doi:10.1016/s0301-0082(97)00052-x (1998).

67 Solito, E. & Sastre, M. Microglia function in Alzheimer's disease. *Front Pharmacol* **3**, 14, doi:10.3389/fphar.2012.00014 (2012).

68 Tang, Y. & Le, W. Differential Roles of M1 and M2 Microglia in Neurodegenerative Diseases. *Mol Neurobiol* **53**, 1181-1194, doi:10.1007/s12035-014-9070-5 (2016).

69 Smith, A. M. & Dragunow, M. The human side of microglia. *Trends Neurosci* **37**, 125-135, doi:10.1016/j.tins.2013.12.001 (2014).

70 Ransohoff, R. M. A polarizing question: do M1 and M2 microglia exist? *Nat Neurosci* **19**, 987-991, doi:10.1038/nn.4338 (2016).

71 Verdonk, F. *et al.* Phenotypic clustering: a novel method for microglial morphology analysis. *J Neuroinflammation* **13**, 153, doi:10.1186/s12974-016-0614-7 (2016).

72 Cherry, J. D., Olschowka, J. A. & O'Banion, M. K. Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. *J Neuroinflammation* **11**, 98, doi:10.1186/1742-2094-11-98 (2014).

73 Franco, R. & Fernandez-Suarez, D. Alternatively activated microglia and macrophages in the central nervous system. *Prog Neurobiol* **131**, 65-86, doi:10.1016/j.pneurobio.2015.05.003 (2015).

74 Orihuela, R., McPherson, C. A. & Harry, G. J. Microglial M1/M2 polarization and metabolic states. *Br J Pharmacol* **173**, 649-665, doi:10.1111/bph.13139 (2016).

75 Walker, D. G. & Lue, L. F. Immune phenotypes of microglia in human neurodegenerative disease: challenges to detecting microglial polarization in human brains. *Alzheimers Res Ther* **7**, 56, doi:10.1186/s13195-015-0139-9 (2015).

76 Ghosh, M., Xu, Y. & Pearse, D. D. Cyclic AMP is a key regulator of M1 to M2a phenotypic conversion of microglia in the presence of Th2 cytokines. *J Neuroinflammation* **13**, 9, doi:10.1186/s12974-015-0463-9 (2016).

77 Lively, S. & Schlichter, L. C. Microglia Responses to Pro-inflammatory Stimuli (LPS, IFNgamma+TNFalpha) and Reprogramming by Resolving Cytokines (IL-4, IL-10). *Front Cell Neurosci* **12**, 215, doi:10.3389/fncel.2018.00215 (2018).

78 Liu, H. C. *et al.* N9 microglial cells polarized by LPS and IL4 show differential responses to secondary environmental stimuli. *Cell Immunol* **278**, 84-90, doi:10.1016/j.cellimm.2012.06.001 (2012).

79 Stout, R. D. *et al.* Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. *J Immunol* **175**, 342-349, doi:10.4049/jimmunol.175.1.342 (2005).

80 Van den Bossche, J. *et al.* Mitochondrial Dysfunction Prevents Repolarization of Inflammatory Macrophages. *Cell Rep* **17**, 684-696, doi:10.1016/j.celrep.2016.09.008 (2016).

81 Tarique, A. A. *et al.* Phenotypic, functional, and plasticity features of classical and alternatively activated human macrophages. *Am J Respir Cell Mol Biol* **53**, 676-688, doi:10.1165/rcmb.2015-0012OC (2015).

82 Boche, D., Perry, V. H. & Nicoll, J. A. Review: activation patterns of microglia and their identification in the human brain. *Neuropathol Appl Neurobiol* **39**, 3-18, doi:10.1111/nan.12011 (2013).

83 Dubbelaar, M. L., Kracht, L., Eggen, B. J. L. & Boddeke, E. The Kaleidoscope of Microglial Phenotypes. *Front Immunol* **9**, 1753, doi:10.3389/fimmu.2018.01753 (2018).

84 Colombo, E. & Farina, C. Astrocytes: Key Regulators of Neuroinflammation. *Trends Immunol* **37**, 608-620, doi:10.1016/j.it.2016.06.006 (2016).

85 Liddelow, S. A. *et al.* Neurotoxic reactive astrocytes are induced by activated microglia. *Nature* **541**, 481-487, doi:10.1038/nature21029 (2017).

86 Tracey, K. J. Neurons Are the Inflammatory Problem. *Cell* **173**, 1066-1068, doi:10.1016/j.cell.2018.05.005 (2018).

87 Rustenhoven, J., Jansson, D., Smyth, L. C. & Dragunow, M. Brain Pericytes As Mediators of Neuroinflammation. *Trends Pharmacol Sci* **38**, 291-304, doi:10.1016/j.tips.2016.12.001 (2017).

88 Ozen, I. *et al.* Brain pericytes acquire a microglial phenotype after stroke. *Acta Neuropathol* **128**, 381-396, doi:10.1007/s00401-014-1295-x (2014).

89 Peferoen, L., Kipp, M., van der Valk, P., van Noort, J. M. & Amor, S. Oligodendrocyte-microglia cross-talk in the central nervous system. *Immunology* **141**, 302-313, doi:10.1111/imm.12163 (2014).

90 Prinz, M. & Priller, J. The role of peripheral immune cells in the CNS in steady state and disease. *Nat Neurosci* **20**, 136-144, doi:10.1038/nn.4475 (2017).

91 Becher, B., Spath, S. & Goverman, J. Cytokine networks in neuroinflammation. *Nat Rev Immunol* **17**, 49-59, doi:10.1038/nri.2016.123 (2017).

92 Mestas, J. & Hughes, C. C. Of mice and not men: differences between mouse and human immunology. *J Immunol* **172**, 2731-2738, doi:10.4049/jimmunol.172.5.2731 (2004).

93 Grist, J. J. *et al.* Induced CNS expression of CXCL1 augments neurologic disease in a murine model of multiple sclerosis via enhanced neutrophil recruitment. *Eur J Immunol* **48**, 1199-1210, doi:10.1002/eji.201747442 (2018).

94 Tsai, H. H. *et al.* The chemokine receptor CXCR2 controls positioning of oligodendrocyte precursors in developing spinal cord by arresting their migration. *Cell* **110**, 373-383, doi:10.1016/s0092-8674(02)00838-3 (2002).

95 Shaftel, S. S., Griffin, W. S. & O'Banion, M. K. The role of interleukin-1 in neuroinflammation and Alzheimer disease: an evolving perspective. *J Neuroinflammation* **5**, 7, doi:10.1186/1742-2094-5-7 (2008).

96 Petitto, J. M., Meola, D. & Huang, Z. Interleukin-2 and the brain: dissecting central versus peripheral contributions using unique mouse models. *Methods Mol Biol* **934**, 301-311, doi:10.1007/978-1-62703-071-7_15 (2012).

97 Lee, K. S. *et al.* Peripheral cytokines and chemokines in Alzheimer's disease. *Dement Geriatr Cogn Disord* **28**, 281-287, doi:10.1159/000245156 (2009).

98 Sawada, M., Suzumura, A., Itoh, Y. & Marunouchi, T. Production of interleukin-5 by mouse astrocytes and microglia in culture. *Neurosci Lett* **155**, 175-178, doi:10.1016/0304-3940(93)90701-l (1993).

99 Liva, S. M. & de Vellis, J. IL-5 induces proliferation and activation of microglia via an unknown receptor. *Neurochem Res* **26**, 629-637, doi:10.1023/a:1010983119125 (2001).

100 Pripp, A. H. & Stanisic, M. The correlation between pro- and anti-inflammatory cytokines in chronic subdural hematoma patients assessed with factor analysis. *PLoS One* **9**, e90149, doi:10.1371/journal.pone.0090149 (2014).

101 Funk, J. A. *et al.* Voluntary exercise protects hippocampal neurons from trimethyltin injury: possible role of interleukin-6 to modulate tumor necrosis factor receptor-mediated neurotoxicity. *Brain Behav Immun* **25**, 1063-1077, doi:10.1016/j.bbi.2011.03.012 (2011).

102 Nybo, L., Nielsen, B., Pedersen, B. K., Moller, K. & Secher, N. H. Interleukin-6 release from the human brain during prolonged exercise. *J Physiol* **542**, 991-995, doi:10.1113/jphysiol.2002.022285 (2002).

103 Erta, M., Quintana, A. & Hidalgo, J. Interleukin-6, a major cytokine in the central nervous system. *Int J Biol Sci* **8**, 1254-1266, doi:10.7150/ijbs.4679 (2012).

104 Bobbo, V. C. D. *et al.* Interleukin-6 Expression by Hypothalamic Microglia in Multiple Inflammatory Contexts: A Systematic Review. *Biomed Res Int* **2019**, 1365210, doi:10.1155/2019/1365210 (2019).

105 Gresa-Arribas, N. *et al.* Modelling neuroinflammation in vitro: a tool to test the potential neuroprotective effect of anti-inflammatory agents. *PLoS One* **7**, e45227, doi:10.1371/journal.pone.0045227 (2012).

106 Chang, H. D. & Radbruch, A. The pro- and anti-inflammatory potential of IL-12: the dual role of Th1 cells. *Expert Rev Clin Immunol* **3**, 709-719, doi:10.1586/1744666X.3.5.709 (2007).

107 Liu, F. T. & Hsu, D. K. The role of galectin-3 in promotion of the inflammatory response. *Drug News Perspect* **20**, 455-460, doi:10.1358/dnp.2007.20.7.1149628 (2007).

108 Shin, T. The pleiotropic effects of galectin-3 in neuroinflammation: a review. *Acta Histochem* **115**, 407-411, doi:10.1016/j.acthis.2012.11.010 (2013).

109 Rubinstein, N., Ilarregui, J. M., Toscano, M. A. & Rabinovich, G. A. The role of galectins in the initiation, amplification and resolution of the inflammatory response. *Tissue Antigens* **64**, 1-12, doi:10.1111/j.0001-2815.2004.00278.x (2004).

110 Chen, H. L., Liao, F., Lin, T. N. & Liu, F. T. Galectins and neuroinflammation. *Adv Neurobiol* **9**, 517-542, doi:10.1007/978-1-4939-1154-7_24 (2014).

111 Venkatraman, A. *et al.* Galectin-3: an emerging biomarker in stroke and cerebrovascular diseases. *Eur J Neurol* **25**, 238-246, doi:10.1111/ene.13496 (2018).

112 Jeon, S. B. *et al.* Galectin-3 exerts cytokine-like regulatory actions through the JAK-STAT pathway. *J Immunol* **185**, 7037-7046, doi:10.4049/jimmunol.1000154 (2010).

113 Wesley, U. V., Vemuganti, R., Ayvaci, E. R. & Dempsey, R. J. Galectin-3 enhances angiogenic and migratory potential of microglial cells via modulation of integrin linked kinase signaling. *Brain Res* **1496**, 1-9, doi:10.1016/j.brainres.2012.12.008 (2013).

114 Reichert, F. & Rotshenker, S. Galectin-3 (MAC-2) Controls Microglia Phenotype Whether Amoeboid and Phagocytic or Branched and Non-phagocytic by Regulating the Cytoskeleton. *Front Cell Neurosci* **13**, 90, doi:10.3389/fncel.2019.00090 (2019).

115 Macrez, R. *et al.* Stroke and the immune system: from pathophysiology to new therapeutic strategies. *Lancet Neurol* **10**, 471-480, doi:10.1016/S1474-4422(11)70066-7 (2011).

116 Banwell, V., Sena, E. S. & Macleod, M. R. Systematic review and stratified meta-analysis of the efficacy of interleukin-1 receptor antagonist in animal models of stroke. *J Stroke Cerebrovasc Dis* **18**, 269-276, doi:10.1016/j.jstrokecerebrovasdis.2008.11.009 (2009).

117 Hallenbeck, J. M. The many faces of tumor necrosis factor in stroke. *Nat Med* **8**, 1363-1368, doi:10.1038/nm1202-1363 (2002).

118 Jayaraj, R. L., Azimullah, S., Beiram, R., Jalal, F. Y. & Rosenberg, G. A. Neuroinflammation: friend and foe for ischemic stroke. *J Neuroinflammation* **16**, 142, doi:10.1186/s12974-019-1516-2 (2019).

119 Sochocka, M., Zwolinska, K. & Leszek, J. The Infectious Etiology of Alzheimer's Disease. *Curr Neuropharmacol* **15**, 996-1009, doi:10.2174/1570159X15666170313122937 (2017).

120 Hopperton, K. E., Mohammad, D., Trepanier, M. O., Giuliano, V. & Bazinet, R. P. Markers of microglia in post-mortem brain samples from patients with Alzheimer's disease: a systematic review. *Mol Psychiatry* **23**, 177-198, doi:10.1038/mp.2017.246 (2018).

121 Grathwohl, S. A. *et al.* Formation and maintenance of Alzheimer's disease beta-amyloid plaques in the absence of microglia. *Nat Neurosci* **12**, 1361-1363, doi:10.1038/nn.2432 (2009).

122 Taipa, R., Sousa, A. L., Melo Pires, M. & Sousa, N. Does the Interplay Between Aging and Neuroinflammation Modulate Alzheimer's Disease Clinical Phenotypes? A Clinico-Pathological Perspective. *J Alzheimers Dis* **53**, 403-417, doi:10.3233/JAD-160121 (2016).

123 Jansen, I. E. *et al.* Genome-wide meta-analysis identifies new loci and functional pathways influencing Alzheimer's disease risk. **51**, 404-413, doi:10.1038/s41588-018-0311-9 (2019).

124 Tansey, K. E., Cameron, D. & Hill, M. J. Genetic risk for Alzheimer's disease is concentrated in specific macrophage and microglial transcriptional networks. *Genome Med* **10**, 14, doi:10.1186/s13073-018-0523-8 (2018).

125 Jonsson, T. *et al.* Variant of TREM2 associated with the risk of Alzheimer's disease. *N Engl J Med* **368**, 107-116, doi:10.1056/NEJMoa1211103 (2013).

126 Boza-Serrano, A. *et al.* Galectin-3, a novel endogenous TREM2 ligand, detrimentally regulates inflammatory response in Alzheimer's disease. *Acta Neuropathol* **138**, 251-273, doi:10.1007/s00401-019-02013-z (2019).

127 Condello, C., Yuan, P., Schain, A. & Grutzendler, J. Microglia constitute a barrier that prevents neurotoxic protofibrillar Abeta42 hotspots around plaques. *Nat Commun* **6**, 6176, doi:10.1038/ncomms7176 (2015).

128 Pan, X. D. *et al.* Microglial phagocytosis induced by fibrillar beta-amyloid is attenuated by oligomeric beta-amyloid: implications for Alzheimer's disease. *Mol Neurodegener* **6**, 45, doi:10.1186/1750-1326-6-45 (2011).

129 Heneka, M. T. *et al.* Neuroinflammation in Alzheimer's disease. *Lancet Neurol* **14**, 388-405, doi:10.1016/S1474-4422(15)70016-5 (2015).

130 McGeer, P. L., Itagaki, S., Boyes, B. E. & McGeer, E. G. Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. *Neurology* **38**, 1285-1291, doi:10.1212/wnl.38.8.1285 (1988).

131 Gerhard, A. *et al.* In vivo imaging of microglial activation with [11C](R)-PK11195 PET in idiopathic Parkinson's disease. *Neurobiol Dis* **21**, 404-412, doi:10.1016/j.nbd.2005.08.002 (2006).

132 Boza-Serrano, A. *et al.* The role of Galectin-3 in alpha-synuclein-induced microglial activation. *Acta Neuropathol Commun* **2**, 156, doi:10.1186/s40478-014-0156-0 (2014).

133 Setiawan, E. *et al.* Association of translocator protein total distribution volume with duration of untreated major depressive disorder: a cross-sectional study. *Lancet Psychiatry* **5**, 339-347, doi:10.1016/S2215-0366(18)30048-8 (2018).

134 Shelton, R. C. *et al.* Altered expression of genes involved in inflammation and apoptosis in frontal cortex in major depression. *Mol Psychiatry* **16**, 751-762, doi:10.1038/mp.2010.52 (2011).

135 Barnes, J., Mondelli, V. & Pariante, C. M. Genetic Contributions of Inflammation to Depression. *Neuropsychopharmacology* **42**, 81-98, doi:10.1038/npp.2016.169 (2017).

136 Dahl, J. *et al.* The plasma levels of various cytokines are increased during ongoing depression and are reduced to normal levels after recovery. *Psychoneuroendocrinology* **45**, 77-86, doi:10.1016/j.psyneuen.2014.03.019 (2014).

137 Pinto, E. F. & Andrade, C. Interferon-Related Depression: A Primer on Mechanisms, Treatment, and Prevention of a Common Clinical Problem. *Curr Neuropharmacol* **14**, 743-748, doi:10.2174/1570159x14666160106155129 (2016).

138 Caspersen, C. J., Powell, K. E. & Christenson, G. M. Physical activity, exercise, and physical fitness: definitions and distinctions for health-related research. *Public Health Rep* **100**, 126-131 (1985).

139 Petersen, A. M. & Pedersen, B. K. The anti-inflammatory effect of exercise. *J Appl Physiol (1985)* **98**, 1154-1162, doi:10.1152/japplphysiol.00164.2004 (2005).

140 Raschke, S. & Eckel, J. Adipo-myokines: two sides of the same coin--mediators of inflammation and mediators of exercise. *Mediators Inflamm* **2013**, 320724, doi:10.1155/2013/320724 (2013).

141 Petersen, A. M. & Pedersen, B. K. The role of IL-6 in mediating the anti-inflammatory effects of exercise. *J Physiol Pharmacol* **57 Suppl 10**, 43-51 (2006).

142 Steensberg, A., Fischer, C. P., Keller, C., Moller, K. & Pedersen, B. K. IL-6 enhances plasma IL-1ra, IL-10, and cortisol in humans. *Am J Physiol Endocrinol Metab* **285**, E433-437, doi:10.1152/ajpendo.00074.2003 (2003).

143 Karstoft, K. & Pedersen, B. K. Skeletal muscle as a gene regulatory endocrine organ. *Curr Opin Clin Nutr Metab Care* **19**, 270-275, doi:10.1097/MCO.0000000000000283 (2016).

144 Pedersen, B. K. Physical activity and muscle-brain crosstalk. *Nat Rev Endocrinol* **15**, 383-392, doi:10.1038/s41574-019-0174-x (2019).

145 Cervenka, I., Agudelo, L. Z. & Ruas, J. L. Kynurenines: Tryptophan's metabolites in exercise, inflammation, and mental health. *Science* **357**, doi:10.1126/science.aaf9794 (2017).

146 Agudelo, L. Z. *et al.* Skeletal muscle PGC-1alpha1 modulates kynurenine metabolism and mediates resilience to stress-induced depression. *Cell* **159**, 33-45, doi:10.1016/j.cell.2014.07.051 (2014).

147 Schlittler, M. *et al.* Endurance exercise increases skeletal muscle kynurenine aminotransferases and plasma kynurenic acid in humans. *Am J Physiol Cell Physiol* **310**, C836-840, doi:10.1152/ajpcell.00053.2016 (2016).

148 Millischer, V., Erhardt, S., Ekblom, O., Forsell, Y. & Lavebratt, C. Twelve-week physical exercise does not have a long-lasting effect on kynurenines in plasma of depressed patients. *Neuropsychiatr Dis Treat* **13**, 967-972, doi:10.2147/NDT.S131746 (2017).

149 Karstoft, K. & Pedersen, B. K. Exercise and type 2 diabetes: focus on metabolism and inflammation. *Immunol Cell Biol* **94**, 146-150, doi:10.1038/icb.2015.101 (2016).

150 Pedersen, B. K. & Febbraio, M. A. Muscles, exercise and obesity: skeletal muscle as a secretory organ. *Nat Rev Endocrinol* **8**, 457-465, doi:10.1038/nrendo.2012.49 (2012).

151 Walsh, N. P. *et al.* Position statement. Part one: Immune function and exercise. *Exerc Immunol Rev* **17**, 6-63 (2011).

152 Paulsen, G. *et al.* Delayed leukocytosis and cytokine response to high-force eccentric exercise. *Med Sci Sports Exerc* **37**, 1877-1883, doi:10.1249/01.mss.0000177064.65927.98 (2005).

153 Koh, Y. & Park, J. Cell adhesion molecules and exercise. *J Inflamm Res* **11**, 297-306, doi:10.2147/JIR.S170262 (2018).

154 Rada, I., Deldicque, L., Francaux, M. & Zbinden-Foncea, H. Toll like receptor expression induced by exercise in obesity and metabolic syndrome: A systematic review. *Exerc Immunol Rev* **24**, 60-71 (2018).

155 Sardeli, A. V. *et al.* Effect of resistance training on inflammatory markers of older adults: A meta-analysis. *Exp Gerontol* **111**, 188-196, doi:10.1016/j.exger.2018.07.021 (2018).

156 Monteiro-Junior, R. S. *et al.* Effect of Exercise on Inflammatory Profile of Older Persons: Systematic Review and Meta-Analyses. *J Phys Act Health* **15**, 64-71, doi:10.1123/jpah.2016-0735 (2018).

157 Fedewa, M. V., Hathaway, E. D. & Ward-Ritacco, C. L. Effect of exercise training on C reactive protein: a systematic review and meta-analysis of randomised and non-randomised controlled trials. *Br J Sports Med* **51**, 670-676, doi:10.1136/bjsports-2016-095999 (2017).

158 Suzuki, K. *et al.* Circulating cytokines and hormones with immunosuppressive but neutrophil-priming potentials rise after endurance exercise in humans. *Eur J Appl Physiol* **81**, 281-287, doi:10.1007/s004210050044 (2000).

159 Smith, L. L. *et al.* Cytokines and cell adhesion molecules associated with high-intensity eccentric exercise. *Eur J Appl Physiol* **82**, 61-67, doi:10.1007/s004210050652 (2000).

160 Suzuki, K. *et al.* Systemic inflammatory response to exhaustive exercise. Cytokine kinetics. *Exerc Immunol Rev* **8**, 6-48 (2002).

161 Windsor, M. T. *et al.* Cytokine Responses to Acute Exercise in Healthy Older Adults: The Effect of Cardiorespiratory Fitness. *Front Physiol* **9**, 203, doi:10.3389/fphys.2018.00203 (2018).

162 Ferreira, G. A. *et al.* The Effects of Acute and Chronic Sprint-Interval Training on Cytokine Responses Are Independent of Prior Caffeine Intake. *Front Physiol* **9**, 671, doi:10.3389/fphys.2018.00671 (2018).

163 Brown, M., McClean, C. M., Davison, G. W., Brown, J. C. W. & Murphy, M. H. The acute effects of walking exercise intensity on systemic cytokines and oxidative stress. *Eur J Appl Physiol* **118**, 2111-2120, doi:10.1007/s00421-018-3930-z (2018).

164 Tibana, R. A. *et al.* Two Consecutive Days of Crossfit Training Affects Pro and Anti-inflammatory Cytokines and Osteoprotegerin without Impairments in Muscle Power. *Front Physiol* **7**, 260, doi:10.3389/fphys.2016.00260 (2016).

165 Nielsen, H. G., Oktedalen, O., Opstad, P. K. & Lyberg, T. Plasma Cytokine Profiles in Long-Term Strenuous Exercise. *J Sports Med (Hindawi Publ Corp)* **2016**, 7186137, doi:10.1155/2016/7186137 (2016).

166 Stigger, F. S., Zago Marcolino, M. A., Portela, K. M. & Plentz, R. D. M. Effects of Exercise on Inflammatory, Oxidative, and Neurotrophic Biomarkers on Cognitively Impaired Individuals Diagnosed With Dementia or Mild Cognitive Impairment: A Systematic Review and Meta-Analysis. *J Gerontol A Biol Sci Med Sci* **74**, 616-624, doi:10.1093/gerona/gly173 (2019).

167 Benatti, F. B. & Pedersen, B. K. Exercise as an anti-inflammatory therapy for rheumatic diseases-myokine regulation. *Nat Rev Rheumatol* **11**, 86-97, doi:10.1038/nrrheum.2014.193 (2015).

168 Horsburgh, S., Robson-Ansley, P., Adams, R. & Smith, C. Exercise and inflammation-related epigenetic modifications: focus on DNA methylation. *Exerc Immunol Rev* **21**, 26-41 (2015).

169 Starzak, D. E., Semple, S. J., Smith, L. L. & McKune, A. J. Differing cytokine responses by ethnic groups to a bout of exercise-induced muscle damage: a preliminary report. *J Sports Med Phys Fitness* **56**, 665-677 (2016).

170 Lin, T. W. & Kuo, Y. M. Exercise benefits brain function: the monoamine connection. *Brain Sci* **3**, 39-53, doi:10.3390/brainsci3010039 (2013).

171 Szuhany, K. L., Bugatti, M. & Otto, M. W. A meta-analytic review of the effects of exercise on brain-derived neurotrophic factor. *J Psychiatr Res* **60**, 56-64, doi:10.1016/j.jpsychires.2014.10.003 (2015).

172 Dinoff, A., Herrmann, N., Swardfager, W. & Lanctot, K. L. The effect of acute exercise on blood concentrations of brain-derived neurotrophic factor in healthy adults: a meta-analysis. *Eur J Neurosci* **46**, 1635-1646, doi:10.1111/ejn.13603 (2017).

173 Mackay, C. P., Kuys, S. S. & Brauer, S. G. The Effect of Aerobic Exercise on Brain-Derived Neurotrophic Factor in People with Neurological Disorders: A Systematic Review and Meta-Analysis. *Neural Plast* **2017**, 4716197, doi:10.1155/2017/4716197 (2017).

174 Tsai, C. L., Pai, M. C., Ukropec, J. & Ukropcova, B. Distinctive Effects of Aerobic and Resistance Exercise Modes on Neurocognitive and Biochemical Changes in Individuals with Mild Cognitive Impairment. *Curr Alzheimer Res* **16**, 316-332, doi:10.2174/1567205016666190228125429 (2019).

175 Choi, S. H. *et al.* Combined adult neurogenesis and BDNF mimic exercise effects on cognition in an Alzheimer's mouse model. *Science* **361**, doi:10.1126/science.aan8821 (2018).

176 Kurebayashi, Y. & Otaki, J. Does Physical Exercise Increase Brain-Derived Neurotrophic Factor in Major Depressive Disorder? A Meta-Analysis. *Psychiatr Danub* **30**, 129-135, doi:10.24869/psyd.2018.129 (2018).

177 Dinoff, A., Herrmann, N., Swardfager, W., Gallagher, D. & Lanctot, K. L. The effect of exercise on resting concentrations of peripheral brain-derived neurotrophic factor (BDNF) in major depressive disorder: A meta-analysis. *J Psychiatr Res* **105**, 123-131, doi:10.1016/j.jpsychires.2018.08.021 (2018).

178 Lopez-Cancio, E. *et al.* Reported Prestroke Physical Activity Is Associated with Vascular Endothelial Growth Factor Expression and Good Outcomes after Stroke. *J Stroke Cerebrovasc Dis* **26**, 425-430, doi:10.1016/j.jstrokecerebrovasdis.2016.10.004 (2017).

179 Loprinzi, P. D. Does brain-derived neurotrophic factor mediate the effects of exercise on memory? *Phys Sportsmed* **47**, 395-405, doi:10.1080/00913847.2019.1610255 (2019).

180 Heijnen, S., Hommel, B., Kibele, A. & Colzato, L. S. Neuromodulation of Aerobic Exercise-A Review. *Front Psychol* **6**, 1890, doi:10.3389/fpsyg.2015.01890 (2015).

181 Raichlen, D. A., Foster, A. D., Gerdeman, G. L., Seillier, A. & Giuffrida, A. Wired to run: exercise-induced endocannabinoid signaling in humans and cursorial mammals with implications for the 'runner's high'. *J Exp Biol* **215**, 1331-1336, doi:10.1242/jeb.063677 (2012).

182 Kondo, M., Nakamura, Y., Ishida, Y. & Shimada, S. The 5-HT3 receptor is essential for exercise-induced hippocampal neurogenesis and antidepressant effects. *Mol Psychiatry* **20**, 1428-1437, doi:10.1038/mp.2014.153 (2015).

183 Robinson, A. M., Buttolph, T., Green, J. T. & Bucci, D. J. Physical exercise affects attentional orienting behavior through noradrenergic mechanisms. *Behav Neurosci* **129**, 361-367, doi:10.1037/bne0000054 (2015).

184 Segal, S. K., Cotman, C. W. & Cahill, L. F. Exercise-induced noradrenergic activation enhances memory consolidation in both normal aging and patients with amnestic mild cognitive impairment. *J Alzheimers Dis* **32**, 1011-1018, doi:10.3233/JAD-2012-121078 (2012).

185 Stranahan, A. M., Lee, K. & Mattson, M. P. Central mechanisms of HPA axis regulation by voluntary exercise. *Neuromolecular Med* **10**, 118-127, doi:10.1007/s12017-008-8027-0 (2008).

186 Mazzeo, R. S. Catecholamine responses to acute and chronic exercise. *Med Sci Sports Exerc* **23**, 839-845 (1991).

187 Seifert, T. & Secher, N. H. Sympathetic influence on cerebral blood flow and metabolism during exercise in humans. *Prog Neurobiol* **95**, 406-426, doi:10.1016/j.pneurobio.2011.09.008 (2011).

188 Purkayastha, S., Saxena, A., Eubank, W. L., Hoxha, B. & Raven, P. B. alpha1-Adrenergic receptor control of the cerebral vasculature in humans at rest and during exercise. *Exp Physiol* **98**, 451-461, doi:10.1113/expphysiol.2012.066118 (2013).

189 Dimitrov, S., Hulteng, E. & Hong, S. Inflammation and exercise: Inhibition of monocytic intracellular TNF production by acute exercise via beta2-adrenergic activation. *Brain Behav Immun* **61**, 60-68, doi:10.1016/j.bbi.2016.12.017 (2017).

190 Qian, L. *et al.* beta2-adrenergic receptor activation prevents rodent dopaminergic neurotoxicity by inhibiting microglia via a novel signaling pathway. *J Immunol* **186**, 4443-4454, doi:10.4049/jimmunol.1002449 (2011).

191 McNamee, E. N., Ryan, K. M., Kilroy, D. & Connor, T. J. Noradrenaline induces IL-1ra and IL-1 type II receptor expression in primary glial cells and protects against IL-1beta-induced neurotoxicity. *Eur J Pharmacol* **626**, 219-228, doi:10.1016/j.ejphar.2009.09.054 (2010).

192 Silveira, L. S. *et al.* Macrophage Polarization: Implications on Metabolic Diseases and the Role of Exercise. *Crit Rev Eukaryot Gene Expr* **26**, 115-132, doi:10.1615/CritRevEukaryotGeneExpr.2016015920 (2016).

193 Xu, H., Rajsombath, M. M., Weikop, P. & Selkoe, D. J. Enriched environment enhances beta-adrenergic signaling to prevent microglia inflammation by amyloid-beta. *EMBO Mol Med* **10**, doi:10.15252/emmm.201808931 (2018).

194 Kong, Y., Ruan, L., Qian, L., Liu, X. & Le, Y. Norepinephrine promotes microglia to uptake and degrade amyloid beta peptide through upregulation of mouse formyl peptide

receptor 2 and induction of insulin-degrading enzyme. *J Neurosci* **30**, 11848-11857, doi:10.1523/JNEUROSCI.2985-10.2010 (2010).

195 Duclos, M. & Tabarin, A. Exercise and the Hypothalamo-Pituitary-Adrenal Axis. *Front Horm Res* **47**, 12-26, doi:10.1159/000445149 (2016).

196 Anderson, T. & Wideman, L. Exercise and the Cortisol Awakening Response: A Systematic Review. *Sports Med Open* **3**, 37, doi:10.1186/s40798-017-0102-3 (2017).

197 Cadegiani, F. A. & Kater, C. E. Hypothalamic-Pituitary-Adrenal (HPA) Axis Functioning in Overtraining Syndrome: Findings from Endocrine and Metabolic Responses on Overtraining Syndrome (EROS)-EROS-HPA Axis. *Sports Med Open* **3**, 45, doi:10.1186/s40798-017-0113-0 (2017).

198 Hare, B. D., Beierle, J. A., Toufexis, D. J., Hammack, S. E. & Falls, W. A. Exercise-associated changes in the corticosterone response to acute restraint stress: evidence for increased adrenal sensitivity and reduced corticosterone response duration. *Neuropsychopharmacology* **39**, 1262-1269, doi:10.1038/npp.2013.329 (2014).

199 Zschucke, E., Renneberg, B., Dimeo, F., Wustenberg, T. & Strohle, A. The stress-buffering effect of acute exercise: Evidence for HPA axis negative feedback. *Psychoneuroendocrinology* **51**, 414-425, doi:10.1016/j.psyneuen.2014.10.019 (2015).

200 Leasure, J. L. & Jones, M. Forced and voluntary exercise differentially affect brain and behavior. *Neuroscience* **156**, 456-465, doi:10.1016/j.neuroscience.2008.07.041 (2008).

201 Yanagita, S., Amemiya, S., Suzuki, S. & Kita, I. Effects of spontaneous and forced running on activation of hypothalamic corticotropin-releasing hormone neurons in rats. *Life Sci* **80**, 356-363, doi:10.1016/j.lfs.2006.09.027 (2007).

202 Pietrelli, A. *et al.* Lifelong Aerobic Exercise Reduces the Stress Response in Rats. *Neuroscience* **376**, 94-107, doi:10.1016/j.neuroscience.2018.02.019 (2018).

203 Chen, C. *et al.* The exercise-glucocorticoid paradox: How exercise is beneficial to cognition, mood, and the brain while increasing glucocorticoid levels. *Front Neuroendocrinol* **44**, 83-102, doi:10.1016/j.yfrne.2016.12.001 (2017).

204 Kohman, R. A., Bhattacharya, T. K., Wojcik, E. & Rhodes, J. S. Exercise reduces activation of microglia isolated from hippocampus and brain of aged mice. *J Neuroinflammation* **10**, 114, doi:10.1186/1742-2094-10-114 (2013).

205 Jiang, T. *et al.* Physical Exercise Improves Cognitive Function Together with Microglia Phenotype Modulation and Remyelination in Chronic Cerebral Hypoperfusion. *Front Cell Neurosci* **11**, 404, doi:10.3389/fncel.2017.00404 (2017).

206 Kohman, R. A., DeYoung, E. K., Bhattacharya, T. K., Peterson, L. N. & Rhodes, J. S. Wheel running attenuates microglia proliferation and increases expression of a proneurogenic phenotype in the hippocampus of aged mice. *Brain Behav Immun* **26**, 803-810, doi:10.1016/j.bbi.2011.10.006 (2012).

207 Ang, E. T., Wong, P. T., Moochhala, S. & Ng, Y. K. Cytokine changes in the horizontal diagonal band of Broca in the septum after running and stroke: a correlation

to glial activation. *Neuroscience* **129**, 337-347, doi:10.1016/j.neuroscience.2004.06.087 (2004).

208 Zhang, Q. *et al.* Proinflammatory cytokines correlate with early exercise attenuating anxiety-like behavior after cerebral ischemia. *Brain Behav* 7, e00854, doi:10.1002/brb3.854 (2017).

209 Kang, E. B. *et al.* Neuroprotective Effects of Endurance Exercise Against High-Fat Diet-Induced Hippocampal Neuroinflammation. *J Neuroendocrinol* **28**, doi:10.1111/jne.12385 (2016).

210 Sung, Y. H. *et al.* Treadmill exercise ameliorates dopaminergic neuronal loss through suppressing microglial activation in Parkinson's disease mice. *Life Sci* **91**, 1309-1316, doi:10.1016/j.lfs.2012.10.003 (2012).

211 Yoo, D. Y. *et al.* Treadmill exercise is associated with reduction of reactive microgliosis and pro-inflammatory cytokine levels in the hippocampus of type 2 diabetic rats. *Neurol Res* **37**, 732-738, doi:10.1179/1743132815Y.0000000015 (2015).

212 Olah, M. *et al.* Enhanced hippocampal neurogenesis in the absence of microglia T cell interaction and microglia activation in the murine running wheel model. *Glia* **57**, 1046-1061, doi:10.1002/glia.20828 (2009).

213 Lu, Y. *et al.* Treadmill Exercise Exerts Neuroprotection and Regulates Microglial Polarization and Oxidative Stress in a Streptozotocin-Induced Rat Model of Sporadic Alzheimer's Disease. *J Alzheimers Dis* **56**, 1469-1484, doi:10.3233/JAD-160869 (2017).

214 Ziegler-Waldkirch, S. *et al.* Seed-induced Abeta deposition is modulated by microglia under environmental enrichment in a mouse model of Alzheimer's disease. *EMBO J* **37**, 167-182, doi:10.15252/embj.201797021 (2018).

215 Lima, C. B. *et al.* Spreading depression features and Iba1 immunoreactivity in the cerebral cortex of developing rats submitted to treadmill exercise after treatment with monosodium glutamate. *Int J Dev Neurosci* **33**, 98-105, doi:10.1016/j.ijdevneu.2013.12.008 (2014).

216 Rodriguez, J. J., Noristani, H. N. & Verkhratsky, A. Microglial response to Alzheimer's disease is differentially modulated by voluntary wheel running and enriched environments. *Brain Struct Funct* **220**, 941-953, doi:10.1007/s00429-013-0693-5 (2015).

217 Soch, A. *et al.* Effects of exercise on adolescent and adult hypothalamic and hippocampal neuroinflammation. *Hippocampus* **26**, 1435-1446, doi:10.1002/hipo.22620 (2016).

218 Ma, Y., He, M. & Qiang, L. Exercise Therapy Downregulates the Overexpression of TLR4, TLR2, MyD88 and NF-kappaB after Cerebral Ischemia in Rats. *Int J Mol Sci* **14**, 3718-3733, doi:10.3390/ijms14023718 (2013).

219 Christian, F., Smith, E. L. & Carmody, R. J. The Regulation of NF-kappaB Subunits by Phosphorylation. *Cells* **5**, doi:10.3390/cells5010012 (2016).

220 Marinho, R. *et al.* Endurance training prevents inflammation and apoptosis in hypothalamic neurons of obese mice. *J Cell Physiol* **234**, 880-890, doi:10.1002/jcp.26909 (2018).

221 Liu, W. *et al.* Swimming exercise ameliorates depression-like behavior in chronically stressed rats: relevant to proinflammatory cytokines and IDO activation. *Behav Brain Res* **242**, 110-116, doi:10.1016/j.bbr.2012.12.041 (2013).

222 Nichol, K. E. *et al.* Exercise alters the immune profile in Tg2576 Alzheimer mice toward a response coincident with improved cognitive performance and decreased amyloid. *J Neuroinflammation* **5**, 13, doi:10.1186/1742-2094-5-13 (2008).

223 Pervaiz, N. & Hoffman-Goetz, L. Immune cell inflammatory cytokine responses differ between central and systemic compartments in response to acute exercise in mice. *Exerc Immunol Rev* **18**, 142-157 (2012).

224 Kilic, M., Ulusoy, O., Cirrik, S., Hindistan, I. E. & Ozkaya, Y. G. Effect of exercise intensity on cerebrospinal fluid interleukin-6 concentration during recovery from exhaustive exercise in rats. *Acta Physiol Hung* **101**, 21-31, doi:10.1556/APhysiol.100.2013.019 (2014).

225 Steensberg, A., Dalsgaard, M. K., Secher, N. H. & Pedersen, B. K. Cerebrospinal fluid IL-6, HSP72, and TNF-alpha in exercising humans. *Brain Behav Immun* **20**, 585-589, doi:10.1016/j.bbi.2006.03.002 (2006).

226 Dalsgaard, M. K. *et al.* The CSF and arterial to internal jugular venous hormonal differences during exercise in humans. *Exp Physiol* **89**, 271-277, doi:10.1113/expphysiol.2003.026922 (2004).

227 Packer, N., Pervaiz, N. & Hoffman-Goetz, L. Does exercise protect from cognitive decline by altering brain cytokine and apoptotic protein levels? A systematic review of the literature. *Exerc Immunol Rev* **16**, 138-162 (2010).

228 Agarwal, D., Dange, R. B., Vila, J., Otamendi, A. J. & Francis, J. Detraining differentially preserved beneficial effects of exercise on hypertension: effects on blood pressure, cardiac function, brain inflammatory cytokines and oxidative stress. *PLoS One* **7**, e52569, doi:10.1371/journal.pone.0052569 (2012).

229 Mota, B. C. *et al.* Exercise pre-conditioning reduces brain inflammation and protects against toxicity induced by traumatic brain injury: behavioral and neurochemical approach. *Neurotox Res* **21**, 175-184, doi:10.1007/s12640-011-9257-8 (2012).

230 Kim, S. Y. *et al.* Effects of exercise on cyclooxygenase-2 expression and nuclear factor-kappaB DNA binding in human peripheral blood mononuclear cells. *Ann N Y Acad Sci* **1171**, 464-471, doi:10.1111/j.1749-6632.2009.04915.x (2009).

231 Demers, L. M., Harrison, T. S., Halbert, D. R. & Santen, R. J. Effect of prolonged exercise on plasma prostaglandin levels. *Prostaglandins Med* **6**, 413-418, doi:10.1016/0161-4630(81)90073-2 (1981).

232 Kruger, K., Bredehoft, J., Mooren, F. C. & Rummel, C. Different effects of strength and endurance exercise training on COX-2 and mPGES expression in mouse brain are

independent of peripheral inflammation. *J Appl Physiol (1985)* **121**, 248-254, doi:10.1152/japplphysiol.00284.2016 (2016).

233 Lovatel, G. A. *et al.* Time-dependent effects of treadmill exercise on aversive memory and cyclooxygenase pathway function. *Neurobiol Learn Mem* **98**, 182-187, doi:10.1016/j.nlm.2012.06.002 (2012).

234 Lee, Y. Y. *et al.* Exercise suppresses COX-2 pro-inflammatory pathway in vestibular migraine. *Brain Res Bull* **116**, 98-105, doi:10.1016/j.brainresbull.2015.06.005 (2015).

235 Ding, Y. H. *et al.* Exercise preconditioning ameliorates inflammatory injury in ischemic rats during reperfusion. *Acta Neuropathol* **109**, 237-246, doi:10.1007/s00401-004-0943-y (2005).

236 Piao, C. S. *et al.* Late exercise reduces neuroinflammation and cognitive dysfunction after traumatic brain injury. *Neurobiol Dis* **54**, 252-263, doi:10.1016/j.nbd.2012.12.017 (2013).

237 Fahimi, A. *et al.* Physical exercise induces structural alterations in the hippocampal astrocytes: exploring the role of BDNF-TrkB signaling. *Brain Struct Funct* **222**, 1797-1808, doi:10.1007/s00429-016-1308-8 (2017).

238 Saur, L. *et al.* Physical exercise increases GFAP expression and induces morphological changes in hippocampal astrocytes. *Brain Struct Funct* **219**, 293-302, doi:10.1007/s00429-012-0500-8 (2014).

239 Tatsumi, K. *et al.* Voluntary Exercise Induces Astrocytic Structural Plasticity in the Globus Pallidus. *Front Cell Neurosci* **10**, 165, doi:10.3389/fncel.2016.00165 (2016).

240 Chen, X., Zhang, X., Liao, W. & Wan, Q. Effect of Physical and Social Components of Enriched Environment on Astrocytes Proliferation in Rats After Cerebral Ischemia/Reperfusion Injury. *Neurochem Res* **42**, 1308-1316, doi:10.1007/s11064-016-2172-x (2017).

241 He, X. F. *et al.* Voluntary Exercise Promotes Glymphatic Clearance of Amyloid Beta and Reduces the Activation of Astrocytes and Microglia in Aged Mice. *Front Mol Neurosci* **10**, 144, doi:10.3389/fnmol.2017.00144 (2017).

242 Leite, M. R., Cechella, J. L., Pinton, S., Nogueira, C. W. & Zeni, G. A diphenyl diselenide-supplemented diet and swimming exercise promote neuroprotection, reduced cell apoptosis and glial cell activation in the hypothalamus of old rats. *Exp Gerontol* **82**, 1-7, doi:10.1016/j.exger.2016.05.006 (2016).

243 Zhang, J. *et al.* Long-term treadmill exercise attenuates Abeta burdens and astrocyte activation in APP/PS1 mouse model of Alzheimer's disease. *Neurosci Lett* **666**, 70-77, doi:10.1016/j.neulet.2017.12.025 (2018).

244 Tsai, S. F., Chen, P. C., Calkins, M. J., Wu, S. Y. & Kuo, Y. M. Exercise Counteracts Aging-Related Memory Impairment: A Potential Role for the Astrocytic Metabolic Shuttle. *Front Aging Neurosci* **8**, 57, doi:10.3389/fnagi.2016.00057 (2016).

245 Tomlinson, L., Huang, P. H. & Colognato, H. Prefrontal cortex NG2 glia undergo a developmental switch in their responsiveness to exercise. *Dev Neurobiol* **78**, 687-700, doi:10.1002/dneu.22590 (2018).

246 Ahn, J. H. *et al.* Long-Term Exercise Improves Memory Deficits via Restoration of Myelin and Microvessel Damage, and Enhancement of Neurogenesis in the Aged Gerbil Hippocampus After Ischemic Stroke. *Neurorehabil Neural Repair* **30**, 894-905, doi:10.1177/1545968316638444 (2016).

247 Xiao, Q. *et al.* Exercise protects myelinated fibers of white matter in a rat model of depression. *J Comp Neurol* **526**, 537-549, doi:10.1002/cne.24350 (2018).

248 Zhang, L. *et al.* Exercise Prevents Cognitive Function Decline and Demyelination in the White Matter of APP/PS1 Transgenic AD Mice. *Curr Alzheimer Res* **14**, 645-655, doi:10.2174/1567205014666161213121353 (2017).

249 Lee, C. D., Folsom, A. R. & Blair, S. N. Physical activity and stroke risk: a meta-analysis. *Stroke* **34**, 2475-2481, doi:10.1161/01.STR.0000091843.02517.9D (2003).

250 Aberg, N. D. *et al.* Influence of Cardiovascular Fitness and Muscle Strength in Early Adulthood on Long-Term Risk of Stroke in Swedish Men. *Stroke* **46**, 1769-1776, doi:10.1161/STROKEAHA.115.009008 (2015).

251 Hallmarker, U. *et al.* Risk of Recurrent Stroke and Death After First Stroke in Long-Distance Ski Race Participants. *J Am Heart Assoc* **4**, e002469, doi:10.1161/JAHA.115.002469 (2015).

252 McGinn, A. P. *et al.* Walking speed and risk of incident ischemic stroke among postmenopausal women. *Stroke* **39**, 1233-1239, doi:10.1161/STROKEAHA.107.500850 (2008).

253 Diep, L., Kwagyan, J., Kurantsin-Mills, J., Weir, R. & Jayam-Trouth, A. Association of physical activity level and stroke outcomes in men and women: a meta-analysis. *J Womens Health (Larchmt)* **19**, 1815-1822, doi:10.1089/jwh.2009.1708 (2010).

254 Reimers, C. D., Knapp, G. & Reimers, A. K. Exercise as stroke prophylaxis. *Dtsch Arztebl Int* **106**, 715-721, doi:10.3238/arztbl.2009.0715 (2009).

255 Ellekjaer, H., Holmen, J., Ellekjaer, E. & Vatten, L. Physical activity and stroke mortality in women. Ten-year follow-up of the Nord-Trondelag health survey, 1984-1986. *Stroke* **31**, 14-18, doi:10.1161/01.str.31.1.14 (2000).

256 Kubota, Y. *et al.* Daily Total Physical Activity and Incident Stroke: The Japan Public Health Center-Based Prospective Study. *Stroke* **48**, 1730-1736, doi:10.1161/STROKEAHA.117.017560 (2017).

257 Wen, C. P. *et al.* Pre-stroke physical activity is associated with fewer post-stroke complications, lower mortality and a better long-term outcome. *Eur J Neurol* **24**, 1525-1531, doi:10.1111/ene.13463 (2017).

258 Gordon, N. F. *et al.* Physical activity and exercise recommendations for stroke survivors: an American Heart Association scientific statement from the Council on Clinical Cardiology, Subcommittee on Exercise, Cardiac Rehabilitation, and Prevention; the

Council on Cardiovascular Nursing; the Council on Nutrition, Physical Activity, and Metabolism; and the Stroke Council. *Circulation* **109**, 2031-2041, doi:10.1161/01.CIR.0000126280.65777.A4 (2004).

259 El-Tamawy, M. S., Abd-Allah, F., Ahmed, S. M., Darwish, M. H. & Khalifa, H. A. Aerobic exercises enhance cognitive functions and brain derived neurotrophic factor in ischemic stroke patients. *NeuroRehabilitation* **34**, 209-213, doi:10.3233/NRE-131020 (2014).

260 Hou, L. *et al.* Exercise and quality of life after first-ever ischaemic stroke: a two-year follow-up study. *Int J Neurosci* **128**, 540-548, doi:10.1080/00207454.2017.1400971 (2018).

261 Stoller, O., de Bruin, E. D., Knols, R. H. & Hunt, K. J. Effects of cardiovascular exercise early after stroke: systematic review and meta-analysis. *BMC Neurol* **12**, 45, doi:10.1186/1471-2377-12-45 (2012).

262 Ploughman, M. & Kelly, L. P. Four birds with one stone? Reparative, neuroplastic, cardiorespiratory, and metabolic benefits of aerobic exercise poststroke. *Curr Opin Neurol* **29**, 684-692, doi:10.1097/WCO.0000000000000383 (2016).

263 Zhao, Y. *et al.* Treadmill Exercise Promotes Neurogenesis in Ischemic Rat Brains via Caveolin-1/VEGF Signaling Pathways. *Neurochem Res* **42**, 389-397, doi:10.1007/s11064-016-2081-z (2017).

264 Lovatel, G. A. *et al.* Long-term effects of pre and post-ischemic exercise following global cerebral ischemia on astrocyte and microglia functions in hippocampus from Wistar rats. *Brain Res* **1587**, 119-126, doi:10.1016/j.brainres.2014.08.068 (2014).

265 Austin, M. W., Ploughman, M., Glynn, L. & Corbett, D. Aerobic exercise effects on neuroprotection and brain repair following stroke: a systematic review and perspective. *Neurosci Res* **87**, 8-15, doi:10.1016/j.neures.2014.06.007 (2014).

266 Zwagerman, N., Plumlee, C., Guthikonda, M. & Ding, Y. Toll-like receptor-4 and cytokine cascade in stroke after exercise. *Neurol Res* **32**, 123-126, doi:10.1179/016164109X12464612122812 (2010).

267 Sabia, S. *et al.* Physical activity, cognitive decline, and risk of dementia: 28 year follow-up of Whitehall II cohort study. *BMJ* **357**, j2709, doi:10.1136/bmj.j2709 (2017).

268 de Bruijn, R. F. *et al.* The association between physical activity and dementia in an elderly population: the Rotterdam Study. *Eur J Epidemiol* **28**, 277-283, doi:10.1007/s10654-013-9773-3 (2013).

269 Rovio, S. *et al.* Leisure-time physical activity at midlife and the risk of dementia and Alzheimer's disease. *Lancet Neurol* **4**, 705-711, doi:10.1016/S1474-4422(05)70198-8 (2005).

270 Andel, R. *et al.* Physical exercise at midlife and risk of dementia three decades later: a population-based study of Swedish twins. *J Gerontol A Biol Sci Med Sci* **63**, 62-66 (2008).

271 Hamer, M. & Chida, Y. Physical activity and risk of neurodegenerative disease: a systematic review of prospective evidence. *Psychol Med* **39**, 3-11, doi:10.1017/S0033291708003681 (2009).

272 Stephen, R., Hongisto, K., Solomon, A. & Lonnroos, E. Physical Activity and Alzheimer's Disease: A Systematic Review. *J Gerontol A Biol Sci Med Sci* **72**, 733-739, doi:10.1093/gerona/glw251 (2017).

273 Norton, S., Matthews, F. E., Barnes, D. E., Yaffe, K. & Brayne, C. Potential for primary prevention of Alzheimer's disease: an analysis of population-based data. *Lancet Neurol* **13**, 788-794, doi:10.1016/S1474-4422(14)70136-X (2014).

274 Yamada, M. *et al.* Association between dementia and midlife risk factors: the Radiation Effects Research Foundation Adult Health Study. *J Am Geriatr Soc* **51**, 410-414 (2003).

275 Vemuri, P. *et al.* Evaluation of Amyloid Protective Factors and Alzheimer Disease Neurodegeneration Protective Factors in Elderly Individuals. *JAMA Neurol* **74**, 718-726, doi:10.1001/jamaneurol.2017.0244 (2017).

276 Steen Jensen, C. *et al.* Cerebrospinal Fluid Amyloid Beta and Tau Concentrations Are Not Modulated by 16 Weeks of Moderate- to High-Intensity Physical Exercise in Patients with Alzheimer Disease. *Dement Geriatr Cogn Disord* **42**, 146-158, doi:10.1159/000449408 (2016).

277 Groot, C. *et al.* The effect of physical activity on cognitive function in patients with dementia: A meta-analysis of randomized control trials. *Ageing Res Rev* **25**, 13-23, doi:10.1016/j.arr.2015.11.005 (2016).

278 Ryan, S. M. & Kelly, A. M. Exercise as a pro-cognitive, pro-neurogenic and anti-inflammatory intervention in transgenic mouse models of Alzheimer's disease. *Ageing Res Rev* **27**, 77-92, doi:10.1016/j.arr.2016.03.007 (2016).

279 Zhao, G., Liu, H. L., Zhang, H. & Tong, X. J. Treadmill exercise enhances synaptic plasticity, but does not alter beta-amyloid deposition in hippocampi of aged APP/PS1 transgenic mice. *Neuroscience* **298**, 357-366, doi:10.1016/j.neuroscience.2015.04.038 (2015).

280 Bo, H. *et al.* Exercise-induced neuroprotection of hippocampus in APP/PS1 transgenic mice via upregulation of mitochondrial 8-oxoguanine DNA glycosylase. *Oxid Med Cell Longev* **2014**, 834502, doi:10.1155/2014/834502 (2014).

281 Ke, H. C., Huang, H. J., Liang, K. C. & Hsieh-Li, H. M. Selective improvement of cognitive function in adult and aged APP/PS1 transgenic mice by continuous non-shock treadmill exercise. *Brain Res* **1403**, 1-11, doi:10.1016/j.brainres.2011.05.056 (2011).

282 Souza, L. C. *et al.* Neuroprotective effect of physical exercise in a mouse model of Alzheimer's disease induced by beta-amyloid(1)(-)(4)(0) peptide. *Neurotox Res* **24**, 148-163, doi:10.1007/s12640-012-9373-0 (2013).

283 Lin, T. W. *et al.* Running exercise delays neurodegeneration in amygdala and hippocampus of Alzheimer's disease (APP/PS1) transgenic mice. *Neurobiol Learn Mem* **118**, 189-197, doi:10.1016/j.nlm.2014.12.005 (2015).

284 Souza, L. C. *et al.* Swimming exercise prevents behavioural disturbances induced by an intracerebroventricular injection of amyloid-beta1-42 peptide through modulation of cytokine/NF-kappaB pathway and indoleamine-2,3-dioxygenase in mouse brain. *Behav Brain Res* **331**, 1-13, doi:10.1016/j.bbr.2017.05.024 (2017).

285 Hainsworth, A. H. *et al.* Translational models for vascular cognitive impairment: a review including larger species. *BMC Med* **15**, 16, doi:10.1186/s12916-017-0793-9 (2017).

286 Trigiani, L. J., Royea, J., Tong, X. K. & Hamel, E. Comparative benefits of simvastatin and exercise in a mouse model of vascular cognitive impairment and dementia. *FASEB J* **33**, 13280-13293, doi:10.1096/fj.201901002R (2019).

287 Choi, D. H., Lee, K. H. & Lee, J. Effect of exercise-induced neurogenesis on cognitive function deficit in a rat model of vascular dementia. *Mol Med Rep* **13**, 2981-2990, doi:10.3892/mmr.2016.4891 (2016).

288 Nigam, S. M. *et al.* Exercise and BDNF reduce Abeta production by enhancing alpha-secretase processing of APP. *J Neurochem* **142**, 286-296, doi:10.1111/jnc.14034 (2017).

289 Liu, H. L., Zhao, G., Zhang, H. & Shi, L. D. Long-term treadmill exercise inhibits the progression of Alzheimer's disease-like neuropathology in the hippocampus of APP/PS1 transgenic mice. *Behav Brain Res* **256**, 261-272, doi:10.1016/j.bbr.2013.08.008 (2013).

290 Moore, K. M. *et al.* A spectrum of exercise training reduces soluble Abeta in a dose-dependent manner in a mouse model of Alzheimer's disease. *Neurobiol Dis* **85**, 218-224, doi:10.1016/j.nbd.2015.11.004 (2016).

291 Revilla, S. *et al.* Physical exercise improves synaptic dysfunction and recovers the loss of survival factors in 3xTg-AD mouse brain. *Neuropharmacology* **81**, 55-63, doi:10.1016/j.neuropharm.2014.01.037 (2014).

292 Xu, Z. Q. *et al.* Aerobic exercise combined with antioxidative treatment does not counteract moderate- or mid-stage Alzheimer-like pathophysiology of APP/PS1 mice. *CNS Neurosci Ther* **19**, 795-803, doi:10.1111/cns.12139 (2013).

293 Gratuze, M., Julien, J., Morin, F., Marette, A. & Planel, E. Differential effects of voluntary treadmill exercise and caloric restriction on tau pathogenesis in a mouse model of Alzheimer's disease-like tau pathology fed with Western diet. *Prog Neuropsychopharmacol Biol Psychiatry* **79**, 452-461, doi:10.1016/j.pnpbp.2017.08.001 (2017).

294 Yuede, C. M. *et al.* Effects of voluntary and forced exercise on plaque deposition, hippocampal volume, and behavior in the Tg2576 mouse model of Alzheimer's disease. *Neurobiol Dis* **35**, 426-432, doi:10.1016/j.nbd.2009.06.002 (2009).

295 Huttenrauch, M. *et al.* Physical activity delays hippocampal neurodegeneration and rescues memory deficits in an Alzheimer disease mouse model. *Transl Psychiatry* **6**, e800, doi:10.1038/tp.2016.65 (2016).

296 Jiang, L. *et al.* Effect of running exercise on the number of the neurons in the hippocampus of young transgenic APP/PS1 mice. *Brain Res* **1692**, 56-65, doi:10.1016/j.brainres.2018.04.033 (2018).

297 Zhang, Y. *et al.* Effects of exercise on capillaries in the white matter of transgenic AD mice. *Oncotarget* **8**, 65860-65875, doi:10.18632/oncotarget.19505 (2017).

298 Muller, S. *et al.* Relationship between physical activity, cognition, and Alzheimer pathology in autosomal dominant Alzheimer's disease. *Alzheimers Dement* **14**, 1427-1437, doi:10.1016/j.jalz.2018.06.3059 (2018).

299 Brown, B. M. *et al.* Physical activity and amyloid-beta plasma and brain levels: results from the Australian Imaging, Biomarkers and Lifestyle Study of Ageing. *Mol Psychiatry* **18**, 875-881, doi:10.1038/mp.2012.107 (2013).

300 Pentikainen, H. *et al.* Cardiorespiratory fitness and brain volumes in men and women in the FINGER study. *Age Ageing* **46**, 310-313, doi:10.1093/ageing/afw191 (2017).

301 Jensen, C. S. *et al.* Patients with Alzheimer's disease who carry the APOE epsilon4 allele benefit more from physical exercise. *Alzheimers Dement (N Y)* **5**, 99-106, doi:10.1016/j.trci.2019.02.007 (2019).

302 Hoffmann, K. *et al.* Moderate-to-High Intensity Physical Exercise in Patients with Alzheimer's Disease: A Randomized Controlled Trial. *J Alzheimers Dis* **50**, 443-453, doi:10.3233/JAD-150817 (2016).

303 Sindi, S. *et al.* Baseline Telomere Length and Effects of a Multidomain Lifestyle Intervention on Cognition: The FINGER Randomized Controlled Trial. *J Alzheimers Dis* **59**, 1459-1470, doi:10.3233/JAD-170123 (2017).

304 Pentikainen, H. *et al.* Cardiorespiratory Fitness and Cognition: Longitudinal Associations in the FINGER Study. *J Alzheimers Dis* **68**, 961-968, doi:10.3233/JAD-180897 (2019).

305 Lima, L. O., Scianni, A. & Rodrigues-de-Paula, F. Progressive resistance exercise improves strength and physical performance in people with mild to moderate Parkinson's disease: a systematic review. *J Physiother* **59**, 7-13, doi:10.1016/S1836-9553(13)70141-3 (2013).

306 Yang, F. *et al.* Physical activity and risk of Parkinson's disease in the Swedish National March Cohort. *Brain* **138**, 269-275, doi:10.1093/brain/awu323 (2015).

307 Xu, Q. *et al.* Physical activities and future risk of Parkinson disease. *Neurology* **75**, 341-348, doi:10.1212/WNL.0b013e3181ea1597 (2010).

308 Shih, I. F. *et al.* Occupational and recreational physical activity and Parkinson's disease in Denmark. *Scand J Work Environ Health* **43**, 210-216, doi:10.5271/sjweh.3633 (2017).

309 Chung, C. L., Thilarajah, S. & Tan, D. Effectiveness of resistance training on muscle strength and physical function in people with Parkinson's disease: a systematic review and meta-analysis. *Clin Rehabil* **30**, 11-23, doi:10.1177/0269215515570381 (2016).

310 Shu, H. F. *et al.* Aerobic exercise for Parkinson's disease: a systematic review and meta-analysis of randomized controlled trials. *PLoS One* **9**, e100503, doi:10.1371/journal.pone.0100503 (2014).

311 Uhrbrand, A., Stenager, E., Pedersen, M. S. & Dalgas, U. Parkinson's disease and intensive exercise therapy--a systematic review and meta-analysis of randomized controlled trials. *J Neurol Sci* **353**, 9-19, doi:10.1016/j.jns.2015.04.004 (2015).

312 Flach, A. *et al.* Endurance exercise improves function in individuals with Parkinson's disease: A meta-analysis. *Neurosci Lett* **659**, 115-119, doi:10.1016/j.neulet.2017.08.076 (2017).

313 Shen, X., Wong-Yu, I. S. & Mak, M. K. Effects of Exercise on Falls, Balance, and Gait Ability in Parkinson's Disease: A Meta-analysis. *Neurorehabil Neural Repair* **30**, 512-527, doi:10.1177/1545968315613447 (2016).

314 Koo, J. H. & Cho, J. Y. Treadmill Exercise Attenuates alpha-Synuclein Levels by Promoting Mitochondrial Function and Autophagy Possibly via SIRT1 in the Chronic MPTP/P-Induced Mouse Model of Parkinson's Disease. *Neurotox Res* **32**, 473-486, doi:10.1007/s12640-017-9770-5 (2017).

315 Zhou, W., Barkow, J. C. & Freed, C. R. Running wheel exercise reduces alpha-synuclein aggregation and improves motor and cognitive function in a transgenic mouse model of Parkinson's disease. *PLoS One* **12**, e0190160, doi:10.1371/journal.pone.0190160 (2017).

316 Jang, Y. *et al.* Neuroprotective effects of endurance exercise against neuroinflammation in MPTP-induced Parkinson's disease mice. *Brain Res* **1655**, 186-193, doi:10.1016/j.brainres.2016.10.029 (2017).

317 Jang, Y. C. *et al.* Association of exercise-induced autophagy upregulation and apoptosis suppression with neuroprotection against pharmacologically induced Parkinson's disease. *J Exerc Nutrition Biochem* **22**, 1-8, doi:10.20463/jenb.2018.0001 (2018).

318 Koo, J. H. *et al.* Treadmill exercise produces neuroprotective effects in a murine model of Parkinson's disease by regulating the TLR2/MyD88/NF-kappaB signaling pathway. *Neuroscience* **356**, 102-113, doi:10.1016/j.neuroscience.2017.05.016 (2017).

319 Jang, Y., Kwon, I., Song, W., Cosio-Lima, L. M. & Lee, Y. Endurance Exercise Mediates Neuroprotection Against MPTP-mediated Parkinson's Disease via Enhanced Neurogenesis, Antioxidant Capacity, and Autophagy. *Neuroscience* **379**, 292-301, doi:10.1016/j.neuroscience.2018.03.015 (2018).

320 Tillerson, J. L., Caudle, W. M., Reveron, M. E. & Miller, G. W. Exercise induces behavioral recovery and attenuates neurochemical deficits in rodent models of Parkinson's disease. *Neuroscience* **119**, 899-911, doi:10.1016/s0306-4522(03)00096-4 (2003).

321 Chen, Y. H. *et al.* Exercise Ameliorates Motor Deficits and Improves Dopaminergic Functions in the Rat Hemi-Parkinson's Model. *Sci Rep* **8**, 3973, doi:10.1038/s41598-018-22462-y (2018).

322 Churchill, M. J. *et al.* Exercise in an animal model of Parkinson's disease: Motor recovery but not restoration of the nigrostriatal pathway. *Neuroscience* **359**, 224-247, doi:10.1016/j.neuroscience.2017.07.031 (2017).

323 Hwang, D. J. *et al.* Neuroprotective effect of treadmill exercise possibly via regulation of lysosomal degradation molecules in mice with pharmacologically induced Parkinson's disease. *J Physiol Sci* **68**, 707-716, doi:10.1007/s12576-017-0586-0 (2018).

324 Cadet, P. *et al.* Cyclic exercise induces anti-inflammatory signal molecule increases in the plasma of Parkinson's patients. *Int J Mol Med* **12**, 485-492 (2003).

325 Scalzo, P., Kummer, A., Cardoso, F. & Teixeira, A. L. Serum levels of interleukin-6 are elevated in patients with Parkinson's disease and correlate with physical performance. *Neurosci Lett* **468**, 56-58, doi:10.1016/j.neulet.2009.10.062 (2010).

326 Sacheli, M. A. *et al.* Habitual exercisers versus sedentary subjects with Parkinson's Disease: Multimodal PET and fMRI study. *Mov Disord* **33**, 1945-1950, doi:10.1002/mds.27498 (2018).

327 Sunwoo, M. K. *et al.* Premorbid exercise engagement and motor reserve in Parkinson's disease. *Parkinsonism Relat Disord* **34**, 49-53, doi:10.1016/j.parkreldis.2016.10.023 (2017).

328 Bennett, D. A. *et al.* Education modifies the relation of AD pathology to level of cognitive function in older persons. *Neurology* **60**, 1909-1915, doi:10.1212/01.wnl.0000069923.64550.9f (2003).

329 Schuch, F. B. *et al.* Physical Activity and Incident Depression: A Meta-Analysis of Prospective Cohort Studies. *Am J Psychiatry*, appiajp201817111194, doi:10.1176/appi.ajp.2018.17111194 (2018).

330 Aberg, M. A. *et al.* Cardiovascular fitness in males at age 18 and risk of serious depression in adulthood: Swedish prospective population-based study. *Br J Psychiatry* **201**, 352-359, doi:10.1192/bjp.bp.111.103416 (2012).

331 Mammen, G. & Faulkner, G. Physical activity and the prevention of depression: a systematic review of prospective studies. *Am J Prev Med* **45**, 649-657, doi:10.1016/j.amepre.2013.08.001 (2013).

332 Pedersen, B. K. & Saltin, B. Exercise as medicine - evidence for prescribing exercise as therapy in 26 different chronic diseases. *Scand J Med Sci Sports* **25 Suppl 3**, 1-72, doi:10.1111/sms.12581 (2015).

333 Vancampfort, D. *et al.* What are the factors that influence physical activity participation in individuals with depression? A review of physical activity correlates from 59 studies. *Psychiatr Danub* **27**, 210-224 (2015).

334 Busch, A. M. *et al.* Preferences for Exercise as a Treatment for Depression. *Ment Health Phys Act* **10**, 68-72, doi:10.1016/j.mhpa.2015.12.004 (2016).

335 Sui, X. *et al.* Prospective study of cardiorespiratory fitness and depressive symptoms in women and men. *J Psychiatr Res* **43**, 546-552, doi:10.1016/j.jpsychires.2008.08.002 (2009).

336 Hennings, A. *et al.* Exercise affects symptom severity but not biological measures in depression and somatization - results on IL-6, neopterin, tryptophan, kynurenine and 5-HIAA. *Psychiatry Res* **210**, 925-933, doi:10.1016/j.psychres.2013.09.018 (2013).

337 Wegner, M. *et al.* Effects of exercise on anxiety and depression disorders: review of meta-analyses and neurobiological mechanisms. *CNS Neurol Disord Drug Targets* **13**, 1002-1014 (2014).

338 Khanzada, F. J., Soomro, N. & Khan, S. Z. Association of Physical Exercise on Anxiety and Depression Amongst Adults. *J Coll Physicians Surg Pak* **25**, 546-548, doi:07.2015/JCPSP.546548 (2015).

339 Stanton, R., Reaburn, P. & Happell, B. The Effect of Acute Exercise on Affect and Arousal in Inpatient Mental Health Consumers. *J Nerv Ment Dis* **204**, 658-664, doi:10.1097/NMD.0000000000000510 (2016).

340 Danielsson, L., Noras, A. M., Waern, M. & Carlsson, J. Exercise in the treatment of major depression: a systematic review grading the quality of evidence. *Physiother Theory Pract* **29**, 573-585, doi:10.3109/09593985.2013.774452 (2013).

341 Kvam, S., Kleppe, C. L., Nordhus, I. H. & Hovland, A. Exercise as a treatment for depression: A meta-analysis. *J Affect Disord* **202**, 67-86, doi:10.1016/j.jad.2016.03.063 (2016).

342 Firth, J. *et al.* Motivating factors and barriers towards exercise in severe mental illness: a systematic review and meta-analysis. *Psychol Med* **46**, 2869-2881, doi:10.1017/S0033291716001732 (2016).

343 Hallberg, L. *et al.* Exercise-induced release of cytokines in patients with major depressive disorder. *J Affect Disord* **126**, 262-267, doi:10.1016/j.jad.2010.02.133 (2010).

344 Callaghan, C. K., Rouine, J. & O'Mara, S. M. Exercise prevents IFN-alpha-induced mood and cognitive dysfunction and increases BDNF expression in the rat. *Physiol Behav* **179**, 377-383, doi:10.1016/j.physbeh.2017.07.018 (2017).

345 Eyre, H. A., Papps, E. & Baune, B. T. Treating depression and depression-like behavior with physical activity: an immune perspective. *Front Psychiatry* **4**, 3, doi:10.3389/fpsyt.2013.00003 (2013).

346 Mikkelsen, K., Stojanovska, L., Polenakovic, M., Bosevski, M. & Apostolopoulos, V. Exercise and mental health. *Maturitas* **106**, 48-56, doi:10.1016/j.maturitas.2017.09.003 (2017).

347 Svensson, M., Lexell, J. & Deierborg, T. Effects of Physical Exercise on Neuroinflammation, Neuroplasticity, Neurodegeneration, and Behavior: What We Can Learn From Animal Models in Clinical Settings. *Neurorehabil Neural Repair* **29**, 577-589, doi:10.1177/1545968314562108 (2015).

348 Lopez-Duran, N. L., Kovacs, M. & George, C. J. Hypothalamic-pituitary-adrenal axis dysregulation in depressed children and adolescents: a meta-analysis. *Psychoneuroendocrinology* **34**, 1272-1283, doi:10.1016/j.psyneuen.2009.03.016 (2009).

349 Phillips, C. Brain-Derived Neurotrophic Factor, Depression, and Physical Activity: Making the Neuroplastic Connection. *Neural Plast* **2017**, 7260130, doi:10.1155/2017/7260130 (2017).

350 Lee, H. J. & Baek, S. S. Role of exercise on molecular mechanisms in the regulation of antidepressant effects. *J Exerc Rehabil* **13**, 617-620, doi:10.12965/jer.1735188.594 (2017).

351 Sun, L., Sun, Q. & Qi, J. Adult hippocampal neurogenesis: an important target associated with antidepressant effects of exercise. *Rev Neurosci* **28**, 693-703, doi:10.1515/revneuro-2016-0076 (2017).

352 Helgadottir, B., Forsell, Y., Hallgren, M., Moller, J. & Ekblom, O. Long-term effects of exercise at different intensity levels on depression: A randomized controlled trial. *Prev Med* **105**, 37-46, doi:10.1016/j.ypmed.2017.08.008 (2017).

353 Noh, J. W. *et al.* Relationship between the intensity of physical activity and depressive symptoms among Korean adults: analysis of Korea Health Panel data. *J Phys Ther Sci* **27**, 1233-1237, doi:10.1589/jpts.27.1233 (2015).

354 Balchin, R., Linde, J., Blackhurst, D., Rauch, H. L. & Schonbachler, G. Sweating away depression? The impact of intensive exercise on depression. *J Affect Disord* **200**, 218-221, doi:10.1016/j.jad.2016.04.030 (2016).

355 Harvey, S. B. *et al.* Exercise and the Prevention of Depression: Results of the HUNT Cohort Study. *Am J Psychiatry* **175**, 28-36, doi:10.1176/appi.ajp.2017.16111223 (2018).

356 Mikkelsen, S. S. *et al.* A cohort study of leisure time physical activity and depression. *Prev Med* **51**, 471-475, doi:10.1016/j.ypmed.2010.09.008 (2010).

357 Huang, Y. Q. *et al.* Effects of Voluntary Wheel-Running Types on Hippocampal Neurogenesis and Spatial Cognition in Middle-Aged Mice. *Front Cell Neurosci* **12**, 177, doi:10.3389/fncel.2018.00177 (2018).

358 Enette, L., Vogel, T., Fanon, J. L. & Lang, P. O. Effect of Interval and Continuous Aerobic Training on Basal Serum and Plasma Brain-Derived Neurotrophic Factor Values in Seniors: A Systematic Review of Intervention Studies. *Rejuvenation Res* **20**, 473-483, doi:10.1089/rej.2016.1886 (2017).

359 Barry, J. C. *et al.* Short-term exercise training reduces anti-inflammatory action of interleukin-10 in adults with obesity. *Cytokine* **111**, 460-469, doi:10.1016/j.cyto.2018.05.035 (2018).

360 Morais, V. A. C. *et al.* A single session of moderate intensity walking increases brain-derived neurotrophic factor (BDNF) in the chronic post-stroke patients. *Top Stroke Rehabil* **25**, 1-5, doi:10.1080/10749357.2017.1373500 (2018).

361 Fry, A. C., Schilling, B. K., Weiss, L. W. & Chiu, L. Z. beta2-Adrenergic receptor downregulation and performance decrements during high-intensity resistance exercise overtraining. *J Appl Physiol (1985)* **101**, 1664-1672, doi:10.1152/japplphysiol.01599.2005 (2006).

362 Xing, Y. *et al.* The beneficial role of early exercise training following stroke and possible mechanisms. *Life Sci* **198**, 32-37, doi:10.1016/j.lfs.2018.02.018 (2018).

363 Pin-Barre, C., Constans, A., Brisswalter, J., Pellegrino, C. & Laurin, J. Effects of High-Versus Moderate-Intensity Training on Neuroplasticity and Functional Recovery After Focal Ischemia. *Stroke* **48**, 2855-2864, doi:10.1161/STROKEAHA.117.017962 (2017).

364 Meyer, J. D., Koltyn, K. F., Stegner, A. J., Kim, J. S. & Cook, D. B. Influence of Exercise Intensity for Improving Depressed Mood in Depression: A Dose-Response Study. *Behav Ther* **47**, 527-537, doi:10.1016/j.beth.2016.04.003 (2016).

365 Griesbach, G. S., Tio, D. L., Vincelli, J., McArthur, D. L. & Taylor, A. N. Differential effects of voluntary and forced exercise on stress responses after traumatic brain injury. *J Neurotrauma* **29**, 1426-1433, doi:10.1089/neu.2011.2229 (2012).

366 Kitraki, E., Karandrea, D. & Kittas, C. Long-lasting effects of stress on glucocorticoid receptor gene expression in the rat brain. *Neuroendocrinology* **69**, 331-338, doi:10.1159/000054435 (1999).

367 Cohen, S. *et al.* Chronic stress, glucocorticoid receptor resistance, inflammation, and disease risk. *Proc Natl Acad Sci U S A* **109**, 5995-5999, doi:10.1073/pnas.1118355109 (2012).

368 Scanzano, A. & Cosentino, M. Adrenergic regulation of innate immunity: a review. *Front Pharmacol* **6**, 171, doi:10.3389/fphar.2015.00171 (2015).

369 Justice, N. J. The relationship between stress and Alzheimer's disease. *Neurobiol Stress* **8**, 127-133, doi:10.1016/j.ynstr.2018.04.002 (2018).

370 Dong, H. *et al.* Corticosterone and related receptor expression are associated with increased beta-amyloid plaques in isolated Tg2576 mice. *Neuroscience* **155**, 154-163, doi:10.1016/j.neuroscience.2008.05.017 (2008).

371 Espinosa-Garcia, C. *et al.* Stress primes microglial polarization after global ischemia: Therapeutic potential of progesterone. *Brain Behav Immun* **66**, 177-192, doi:10.1016/j.bbi.2017.06.012 (2017).

372 Sierra, A., Gottfried-Blackmore, A., Milner, T. A., McEwen, B. S. & Bulloch, K. Steroid hormone receptor expression and function in microglia. *Glia* **56**, 659-674, doi:10.1002/glia.20644 (2008).

373 Dey, A., Hao, S., Erion, J. R., Wosiski-Kuhn, M. & Stranahan, A. M. Glucocorticoid sensitization of microglia in a genetic mouse model of obesity and diabetes. *J Neuroimmunol* **269**, 20-27, doi:10.1016/j.jneuroim.2014.01.013 (2014).

374 Frank, M. G., Hershman, S. A., Weber, M. D., Watkins, L. R. & Maier, S. F. Chronic exposure to exogenous glucocorticoids primes microglia to pro-inflammatory stimuli and induces NLRP3 mRNA in the hippocampus. *Psychoneuroendocrinology* **40**, 191-200, doi:10.1016/j.psyneuen.2013.11.006 (2014).

375 Deacon, R. M. Shallow water (paddling) variants of water maze tests in mice. *J Vis Exp*, doi:10.3791/2608 (2013).

376 Olsson, T., Wieloch, T. & Smith, M. L. Brain damage in a mouse model of global cerebral ischemia. Effect of NMDA receptor blockade. *Brain Res* **982**, 260-269, doi:10.1016/s0006-8993(03)03014-2 (2003).

377 Lambertsen, K. L. *et al.* Differences in origin of reactive microglia in bone marrow chimeric mouse and rat after transient global ischemia. *J Neuropathol Exp Neurol* **70**, 481-494, doi:10.1097/NEN.0b013e31821db3aa (2011).

378 Colnot, C., Fowlis, D., Ripoche, M. A., Bouchaert, I. & Poirier, F. Embryonic implantation in galectin 1/galectin 3 double mutant mice. *Dev Dyn* **211**, 306-313, doi:10.1002/(SICI)1097-0177(199804)211:4<306::AID-AJA2>3.0.CO;2-L (1998).

379 Oakley, H. *et al.* Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. *J Neurosci* **26**, 10129-10140, doi:10.1523/JNEUROSCI.1202-06.2006 (2006).

380 Jawhar, S., Trawicka, A., Jenneckens, C., Bayer, T. A. & Wirths, O. Motor deficits, neuron loss, and reduced anxiety coinciding with axonal degeneration and intraneuronal Abeta aggregation in the 5XFAD mouse model of Alzheimer's disease. *Neurobiol Aging* **33**, 196 e129-140, doi:10.1016/j.neurobiolaging.2010.05.027 (2012).

381 O'Leary, T. P., Robertson, A., Chipman, P. H., Rafuse, V. F. & Brown, R. E. Motor function deficits in the 12 month-old female 5xFAD mouse model of Alzheimer's disease. *Behav Brain Res* **337**, 256-263, doi:10.1016/j.bbr.2017.09.009 (2018).

382 Berry, A. *et al.* Social deprivation stress is a triggering factor for the emergence of anxiety- and depression-like behaviours and leads to reduced brain BDNF levels in C57BL/6J mice. *Psychoneuroendocrinology* **37**, 762-772, doi:10.1016/j.psyneuen.2011.09.007 (2012).

383 Kim, C., Fahrenbruch, C. E., Cobb, L. A. & Eisenberg, M. S. Out-of-hospital cardiac arrest in men and women. *Circulation* **104**, 2699-2703, doi:10.1161/hc4701.099784 (2001).

384 Darweesh, S. K. *et al.* Trajectories of prediagnostic functioning in Parkinson's disease. *Brain* **140**, 429-441, doi:10.1093/brain/aww291 (2017).

385 Farahmand, B. Y. *et al.* Mortality amongst participants in Vasaloppet: a classical long-distance ski race in Sweden. *J Intern Med* **253**, 276-283 (2003).

386 Carlsson, S., Olsson, L., Farahmand, B. Y., Hallmarker, U. & Ahlbom, A. [Skiers in the long-distance ski race invest in their health]. *Lakartidningen* **104**, 670-671 (2007).

387 Westfall, J. & Yarkoni, T. Statistically Controlling for Confounding Constructs Is Harder than You Think. *PLoS One* **11**, e0152719, doi:10.1371/journal.pone.0152719 (2016).

388 Schneider, F., Baldauf, K., Wetzel, W. & Reymann, K. G. Behavioral and EEG changes in male 5xFAD mice. *Physiol Behav* **135**, 25-33, doi:10.1016/j.physbeh.2014.05.041 (2014).

389 Peters, O. M. *et al.* Chronic administration of Dimebon does not ameliorate amyloid-beta pathology in 5xFAD transgenic mice. *J Alzheimers Dis* **36**, 589-596, doi:10.3233/JAD-130071 (2013).

390 Sartori, A. C., Vance, D. E., Slater, L. Z. & Crowe, M. The impact of inflammation on cognitive function in older adults: implications for healthcare practice and research. *J Neurosci Nurs* **44**, 206-217, doi:10.1097/JNN.0b013e3182527690 (2012).

391 Newcombe, E. A. *et al.* Inflammation: the link between comorbidities, genetics, and Alzheimer's disease. *J Neuroinflammation* **15**, 276, doi:10.1186/s12974-018-1313-3 (2018).

392 Do, K. *et al.* The effects of exercise on hypothalamic neurodegeneration of Alzheimer's disease mouse model. *PLoS One* **13**, e0190205, doi:10.1371/journal.pone.0190205 (2018).

393 Hase, Y. *et al.* Effects of environmental enrichment on white matter glial responses in a mouse model of chronic cerebral hypoperfusion. *J Neuroinflammation* **14**, 81, doi:10.1186/s12974-017-0850-5 (2017).

394 Robison, L. S. *et al.* Long-term voluntary wheel running does not alter vascular amyloid burden but reduces neuroinflammation in the Tg-SwDI mouse model of cerebral amyloid angiopathy. *J Neuroinflammation* **16**, 144, doi:10.1186/s12974-019-1534-0 (2019).

395 Kelly, A. M. Exercise-Induced Modulation of Neuroinflammation in Models of Alzheimer's Disease. *Brain Plast* **4**, 81-94, doi:10.3233/BPL-180074 (2018).

396 Schmidt, A. *et al.* Meta-analysis of the efficacy of different training strategies in animal models of ischemic stroke. *Stroke* **45**, 239-247, doi:10.1161/STROKEAHA.113.002048 (2014).

397 Egan, K. J. *et al.* Exercise reduces infarct volume and facilitates neurobehavioral recovery: results from a systematic review and meta-analysis of exercise in experimental models of focal ischemia. *Neurorehabil Neural Repair* **28**, 800-812, doi:10.1177/1545968314521694 (2014).

398 Sim, Y. J., Kim, S. S., Kim, J. Y., Shin, M. S. & Kim, C. J. Treadmill exercise improves short-term memory by suppressing ischemia-induced apoptosis of neuronal cells in gerbils. *Neurosci Lett* **372**, 256-261, doi:10.1016/j.neulet.2004.09.060 (2004).

399 Intlekofer, K. A. & Cotman, C. W. Exercise counteracts declining hippocampal function in aging and Alzheimer's disease. *Neurobiol Dis* **57**, 47-55, doi:10.1016/j.nbd.2012.06.011 (2013).

400 Nieman, P. Psychosocial aspects of physical activity. *Paediatr Child Health* **7**, 309-312 (2002).

401 Choi, K. W. *et al.* Assessment of Bidirectional Relationships Between Physical Activity and Depression Among Adults: A 2-Sample Mendelian Randomization Study. *JAMA Psychiatry*, doi:10.1001/jamapsychiatry.2018.4175 (2019).

402 Tikkanen, E., Gustafsson, S. & Ingelsson, E. Associations of Fitness, Physical Activity, Strength, and Genetic Risk With Cardiovascular Disease: Longitudinal Analyses in the UK Biobank Study. *Circulation* **137**, 2583-2591, doi:10.1161/CIRCULATIONAHA.117.032432 (2018).

403 Lee, D. C. *et al.* Comparisons of leisure-time physical activity and cardiorespiratory fitness as predictors of all-cause mortality in men and women. *Br J Sports Med* **45**, 504-510, doi:10.1136/bjsm.2009.066209 (2011).

404 Voss, M. W. *et al.* Fitness, but not physical activity, is related to functional integrity of brain networks associated with aging. *Neuroimage* **131**, 113-125, doi:10.1016/j.neuroimage.2015.10.044 (2016).

405 Mullers, P., Taubert, M. & Muller, N. G. Physical Exercise as Personalized Medicine for Dementia Prevention? *Front Physiol* **10**, 672, doi:10.3389/fphys.2019.00672 (2019).

406 Laukkanen, J. A. & Kujala, U. M. Low Cardiorespiratory Fitness Is a Risk Factor for Death: Exercise Intervention May Lower Mortality? *J Am Coll Cardiol* **72**, 2293-2296, doi:10.1016/j.jacc.2018.06.081 (2018).

407 Jones, N. *et al.* A genetic-based algorithm for personalized resistance training. *Biol Sport* **33**, 117-126, doi:10.5604/20831862.1198210 (2016).

408 Mygind, E., Wulff, K., Rosenkilde Larsen, M. & Helge, J. W. Prediction of Performance in Vasaloppet through Long Lasting Ski-Ergometer and Rollerski Tests in Cross-Country Skiers. *International Journal of Sports and Exercise Medicine* **1** (2015).

409 Craft, B. B., Carroll, H. A. & Lustyk, M. K. Gender Differences in Exercise Habits and Quality of Life Reports: Assessing the Moderating Effects of Reasons for Exercise. *Int J Lib Arts Soc Sci* **2**, 65-76 (2014).

410 Call, J. B. & Shafer, K. Gendered Manifestations of Depression and Help Seeking Among Men. *Am J Mens Health* **12**, 41-51, doi:10.1177/1557988315623993 (2018).

411 Aberg, M. A. *et al.* Cardiovascular fitness in early adulthood and future suicidal behaviour in men followed for up to 42 years. *Psychol Med* **44**, 779-788, doi:10.1017/S0033291713001207 (2014).

412 Nyberg, J., Gustavsson, S., Aberg, M. A. I., Kuhn, H. G. & Waern, M. Late-adolescent risk factors for suicide and self-harm in middle-aged men: explorative prospective population-based study. *Br J Psychiatry*, 1-7, doi:10.1192/bjp.2019.243 (2019).

413 Janz, K. F. Physical activity in epidemiology: moving from questionnaire to objective measurement. *Br J Sports Med* **40**, 191-192, doi:10.1136/bjsm.2005.023036 (2006).

414 Waller, K. *et al.* Self-reported Fitness and Objectively Measured Physical Activity Profile Among Older Adults: A Twin Study. *J Gerontol A Biol Sci Med Sci* **74**, 1965-1972, doi:10.1093/gerona/gly263 (2019).

415 Prince, S. A. *et al.* A comparison of direct versus self-report measures for assessing physical activity in adults: a systematic review. *Int J Behav Nutr Phys Act* **5**, 56, doi:10.1186/1479-5868-5-56 (2008).

416 Shiroma, E. J. *et al.* Comparison of Self-Reported and Accelerometer-Assessed Physical Activity in Older Women. *PLoS One* **10**, e0145950, doi:10.1371/journal.pone.0145950 (2015).

417 Sievanen, H. & Kujala, U. M. Accelerometry-Simple, but challenging. *Scand J Med Sci Sports* **27**, 574-578, doi:10.1111/sms.12887 (2017).

418 Doherty, A. *et al.* GWAS identifies 14 loci for device-measured physical activity and sleep duration. *Nat Commun* **9**, 5257, doi:10.1038/s41467-018-07743-4 (2018).

419 Zhang, X. & Speakman, J. R. Genetic Factors Associated With Human Physical Activity: Are Your Genes Too Tight To Prevent You Exercising? *Endocrinology* **160**, 840-852, doi:10.1210/en.2018-00873 (2019).

420 Bouchard, C. *et al.* Genomic predictors of the maximal O(2) uptake response to standardized exercise training programs. *J Appl Physiol (1985)* **110**, 1160-1170, doi:10.1152/japplphysiol.00973.2010 (2011).

421 Iso-Markku, P. *et al.* Objectively measured physical activity profile and cognition in Finnish elderly twins. *Alzheimers Dement (N Y)* **4**, 263-271, doi:10.1016/j.trci.2018.06.007 (2018).

422 De Moor, M. H., Boomsma, D. I., Stubbe, J. H., Willemsen, G. & de Geus, E. J. Testing causality in the association between regular exercise and symptoms of anxiety and depression. *Arch Gen Psychiatry* **65**, 897-905, doi:10.1001/archpsyc.65.8.897 (2008).

423 Waller, K., Kaprio, J., Korhonen, T., Tuulio-Henriksson, A. & Kujala, U. M. Persistent leisure-time physical activity in adulthood and use of antidepressants: A follow-up study among twins. *J Affect Disord* **200**, 172-177, doi:10.1016/j.jad.2016.04.036 (2016).

424 Aaltonen, S. *et al.* Genetic architecture of motives for leisure-time physical activity: a twin study. *Scand J Med Sci Sports* **27**, 1431-1441, doi:10.1111/sms.12779 (2017).

425 Fan, W. *et al.* PPARdelta Promotes Running Endurance by Preserving Glucose. *Cell Metab* **25**, 1186-1193 e1184, doi:10.1016/j.cmet.2017.04.006 (2017).

426 Guerrieri, D., Moon, H. Y. & van Praag, H. Exercise in a Pill: The Latest on Exercise-Mimetics. *Brain Plast* **2**, 153-169, doi:10.3233/BPL-160043 (2017).

427 Guerrieri, D. & van Praag, H. Exercise-mimetic AICAR transiently benefits brain function. *Oncotarget* **6**, 18293-18313, doi:10.18632/oncotarget.4715 (2015).

428 E, L., Lu, J., Selfridge, J. E., Burns, J. M. & Swerdlow, R. H. Lactate administration reproduces specific brain and liver exercise-related changes. *J Neurochem* **127**, 91-100, doi:10.1111/jnc.12394 (2013).

429 Karlsson, L. *et al.* Constitutive PGC-1alpha Overexpression in Skeletal Muscle Does Not Improve Morphological Outcome in Mouse Models of Brain Irradiation or Cortical Stroke. *Neuroscience* **384**, 314-328, doi:10.1016/j.neuroscience.2018.05.036 (2018).

430 Karlsson, L. *et al.* Constitutive PGC-1alpha overexpression in skeletal muscle does not protect from age-dependent decline in neurogenesis. *Sci Rep* **9**, 12320, doi:10.1038/s41598-019-48795-w (2019).

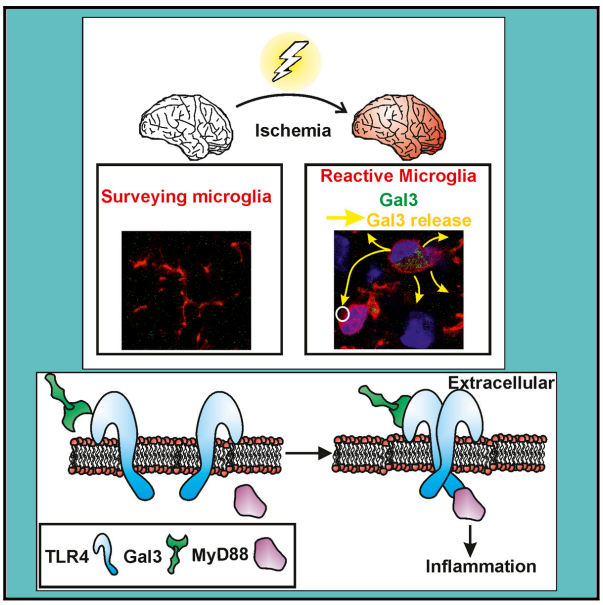
Paper I

Article

Cell Reports

Microglia-Secreted Galectin-3 Acts as a Toll-like Receptor 4 Ligand and Contributes to Microglial Activation

Graphical Abstract



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In Brief

In this publication, Burguillos et al. demonstrate how galectin-3 (Gal3) released from reactive microglia cells can activate other surrounding immune cells in a paracrine manner by binding to and activating Toll-like receptor 4 (TLR4). This finding could explain the propagation of the inflammatory response once the initial stimulus is gone.

Highlights

- Gal3 acts as an endogenous TLR4 ligand with a Kd value around 1 μM
- Gal3 can initiate a TLR4-dependent inflammatory response in microglia
- Gal3 is required for complete activation of TLR4 upon LPS treatment
- Gal3-TLR4 interaction is confirmed in vivo and in stroke patients

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Microglia-Secreted Galectin-3 Acts as a Toll-like Receptor 4 Ligand and Contributes to Microglial Activation

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SUMMARY

Inflammatory response induced by microglia plays a critical role in the demise of neuronal populations in neuroinflammatory diseases. Although the role of toll-like receptor 4 (TLR4) in microglia's inflammatory response is fully acknowledged, little is known about endogenous ligands that trigger TLR4 activation. Here, we report that galectin-3 (Gal3) released by microglia acts as an endogenous paracrine TLR4 ligand. Gal3-TLR4 interaction was further confirmed in a murine neuroinflammatory model (intranigral lipopolysaccharide [LPS] injection) and in human stroke subjects. Depletion of Gal3 exerted neuroprotective and anti-inflammatory effects following global brain ischemia and in the neuroinflammatory LPS model. These results suggest that Gal3-dependent-TLR4 activation could contribute to sustained microglia activation, prolonging the inflammatory response in the brain.

INTRODUCTION

The inflammatory response driven by microglia is a key element in brain ischemia (Lambertsen et al., 2012) and in neurodegenerative disorders (Burguillos et al., 2011; Saijo and Glass, 2011). Toll-like receptors (TLRs), like other pattern recognition receptors (PRRs), are critical for the response to inflammatory agents (Hennessy et al., 2010). Since its discovery in 1996, the TLR family member TLR4 has attracted particular attention in several inflammatory diseases, including CNS pathologies (Buchanan et al., 2010; Lemaitre et al., 1996). Pharmacological inhibition of TLR4 and transgenic mice lacking the TLR4 gene exhibit neuroprotection in conditions of experimental stroke (Caso et al., 2007; Hyakkoku et al., 2010; Suzuki et al., 2012). Despite extensive research, only very few endogenous ligands for TLR4 have been described so far (Chen and Nuñez, 2010).

Galectins represent a protein family with at least 15 members that have significant sequence similarity in their carbohydrate-recognition domain (CRD) and bind to β-galactosides with varying affinities and specificities (Barondes et al., 1994; Leffler et al., 2004). Galectins are classified into three subgroups (1) proto, (2) chimera, and (3) tandem repeat based on their molecular architecture. The proto-type and tandem-repeat-type families comprise proteins with one and two CRDs on a single polypeptide chain, respectively (Kasai and Hirabayashi, 1996).

Galectin-3 (Gal3) is the only known member of the chimera-type family comprising a C-terminal CRD and N-terminal non-CRD for carbohydrate binding and increased self-association, respectively (Lepur et al., 2012). Gal3 is known to be involved in the inflammatory response, and its expression is increased in microglial cells upon various neuroinflammatory stimuli as, for instance, the process of ischemic injury (Lalancette-Hébert et al., 2012; Satoh et al., 2011a, b; Wesley et al., 2013). Gal3 can be found in the cytoplasm, nucleus, and membranes (Shimura et al., 2004) and can be released into the extracellular

space upon certain stimuli such as lipopolysaccharide (LPS) (Li et al., 2008) and interferon γ (IFN-γ) (Jeon et al., 2010). The different subcellular localizations of Gal3 together with its possible posttranslational modifications are likely to affect the function of Gal3 and explain why rather contradictory effects have been reported, e.g., pro- versus anti-apoptotic (Nakahara et al., 2005) and pro- versus anti-inflammatory (Jeon et al., 2010; MacKinnon et al., 2008). As an example of this duality of function, it has been reported that Gal3 deficiency aggravates the neuronal damage in the adult mouse brain following transient focal brain ischemia, due to a reduced signaling of insulin-like growth factor receptor in microglia (Lalancette-Hébert et al., 2012), whereas in a transgenic mouse model of amyotrophic lateral sclerosis (ALS), the lack of Gal3 increases the inflammatory response (Lerman et al., 2012). In contrast, in a model of global brain ischemia, microglial Gal3 was suggested to contribute to neuronal death in the CA1 subregion of the hippocampus (Satoh et al., 2011a, b) as well as contribute to the inflammation and severity in experimental autoimmune encephalitis (Jiang et al., 2009).

Previous studies have focused on the relationship between Gal3 and members of the TLR family such as TLR2. For example, in differentiated macrophages, Gal3 can form a complex with TLR2 and thereby improves the inflammatory response against *C. Albicans* (Jouault et al., 2006). In addition, it has been suggested that Gal3 can act as co-receptor, presenting the *Toxoplasma gondii* glycosylphosphatidylinositols (GPIs) to TLR2 and TLR4 on macrophages (Debierre-Grockiego et al., 2010). Furthermore, an interaction between Gal3 and LPS, a known TLR4 ligand, has been reported as well (Li et al., 2008; Mey et al., 1996). Gal3 and TLR4 are both considered to be independent actors in the initiation and progression of the inflammatory response after brain ischemia. In this study, we demonstrate that Gal3 can act as an endogenous ligand for TLR4. We show that Gal3 can induce, per se, a TLR4-dependent inflammatory response as well as contribute to the full activation of this receptor upon binding to other proinflammatory stimuli, such as LPS.

RESULTS

Gal3 Affects Downstream TLR-Signaling Pathways in Microglia

We first set out to determine the effect of Gal3 on the TLR-mediated signaling pathways. To achieve this, we took advantage of an array that monitors the expression of 84 genes involved in the TLRs intracellular signaling pathways. BV2 microglia cells were exposed to endotoxin-free (as confirmed by *Limulus* amebocyte lysate assay) soluble Gal3 (referred henceforth as sGal3) for 6 hr. In addition, because Gal3 can be rapidly internalized by cells and thereby activate intracellular signaling pathways, we used a so-called ''immobilized form'' of Gal3 (referred to as iGal3) that only can interact with proteins on the cell surface (e.g., receptors). Due to Gal3's high hydrophobicity of its N terminus part, it can bind to plastic, allowing the exposure of both domains: its CRD and also its N-terminal site (Sörme et al., 2002). Cell culture wells were coated overnight at 4°C with 100 μg/ml of Gal3 and washed three times with PBS to remove unbound Gal3. BV2 microglial cells were then seeded in these Gal3-coated plastic wells for 6 hr before harvesting them. Cells seeded on non-coated wells for 6 hr were used as a negative control. LPS (1 μg/ml) added to the cell culture medium for 6 hr was used as a positive control for TLR4 activation.

Thus, BV2 microglia cells were treated with sGal3, iGal3, or LPS and gene expression of the TLRs-signaling pathway investigated. As shown in Figure 1, sGal3 or iGal3 treatment results in statistically significant changes in gene expression as compared to untreated cells. Both induction and repression in gene expression can be observed after either of these treatments. Remarkably, there was significant overlap in microglial gene expression related to TLR4 signaling in responses to either Gal3 or LPS (Figures S1A and S1B).

Gal3 Binds to TLR4 through Its CRD

Next, we explored the possibility of a direct physical interaction between Gal3 and TLR4. Using confocal microscopy, Gal3 and TLR4 were found to be colocalized in BV2 cells 1 hr after adding sGal3 (Figure 2A). Under these conditions, TLR4 was immunoprecipitated and Gal3 was found to be part of the resulting immune complexes (Figure 2B).

Gal3 interaction with glycoproteins is complex, and the initial binding of the CRD often triggers a subsequent self-association of Gal3, sometimes resulting in crosslinking and precipitation (Lepur et al., 2012). This self-association also involves the canonical carbohydrate recognition site in the CRD but also the N-terminal non-CRD domain of Gal3, which makes it much more efficient, and is also required for most biological effects of Gal3.

The apparent affinity of the interaction between TLR4 and Gal3 was determined using microscale thermophoresis (MST). In MST, the thermophoretic mobility of a fluorescently labeled molecule in an infrared-laser-induced microscopic temperature gradient is recorded, yielding a fluorescence time trace from which a normalized fluorescence value (F_{norm}) is derived. Changes in the thermophoretic mobility of the molecule upon ligand binding manifest as shifts in the F_{norm} values and are used to quantify the affinity of the interactions (Seidel et al., 2013). Accordingly, binding of Gal3 to fluorophore-tagged TLR4 (at a constant concentration of about 120 nM) produced a clear shift in the recorded fluorescence time traces (Figure S2B) with increased F_{norm} values for the Gal3-TLR4 complex. The minimal and maximal F_{norm} values for the unbound and fully bound state of TLR4, respectively, were used to calculate the fraction of TLR4 bound at each Gal3 concentration. The resulting saturation binding curve (Figure 2C) shows that 50% of TLR4 is bound at about 1.5 μM Gal3.

The presence of lactose, a competitive inhibitor of both Gal3 carbohydrate binding and self-association, completely abolished the interaction (purple data points in Figure 2C). Further evidence for the involvement of the Gal3 canonical carbohydrate-binding site was the fact that a mutant, Gal3 R186S, showed interaction with TLR4 at a much-higher concentration with 50% bound at about 45 μM. This mutant reduces affinity of Gal3 for many glycoproteins and for the disaccharide LacNAc, which is the most common minimal galectin-binding moiety in glycoproteins (Lepur et al., 2012; Salomonsson et al., 2010a). The Gal3 CRD, lacking the N-terminal domain, also bound

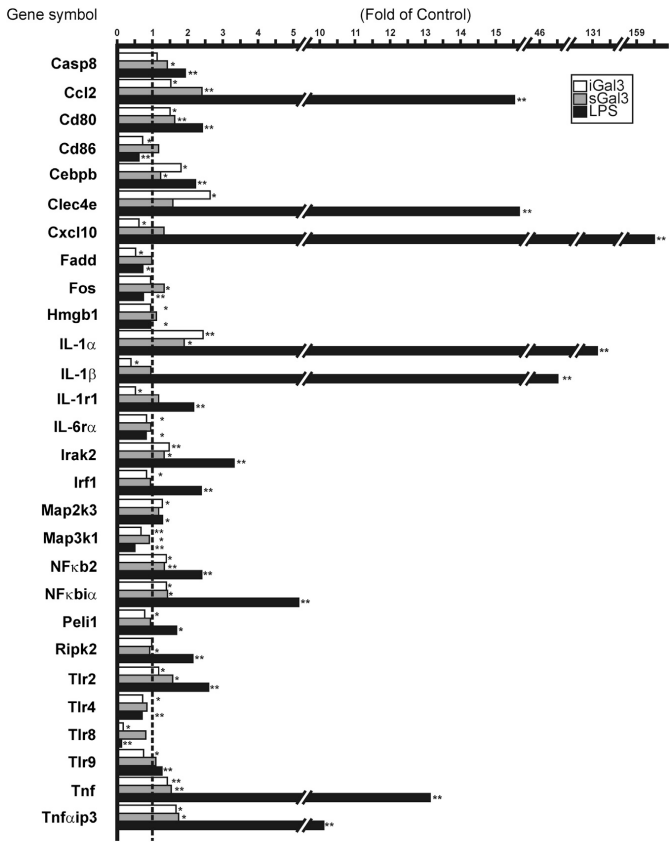

Figure 1. Expression Analysis of Genes Related to the TLR Family after Treatment with Gal3 and LPS

Gene expression array analysis of mRNA related to TLR activation in BV2 microglial cells upon sGal3 (1 μM), iGal3 (100 μg/ml coated well), and LPS (1 μg/ml) treatment for 6 hr. Data are representative of three independent experiments and expressed as mean (n = 3). *$p < 0.05$; **$p < 0.01$. χ^2 analysis revealed similar up- or downregulation of Gal3 compared to LPS (*$p < 0.05$). See also Figure S1.

TLR4 at about equal concentrations as intact Gal3 (red curve in Figure 2C).

To gain further evidence for Gal3-TLR4 interaction, we used fluorescence anisotropy as a separate independent technique. In this technique, the interaction of Gal3 with a fluorescein-tagged saccharide probe is inhibited by increasing concentrations of TLR4 and quantitatively analyzed, as has been done for many other inhibitors before (Lepur et al., 2012; Salomonsson et al., 2010b). The data are presented in the form of percent Gal3 bound to TLR4 to make them more easily comparable to the previous experiment (Figure 2D). This again demonstrated that TLR4 binds both Gal3 and Gal3 CRD, with 50% of Gal3 bound by about 1 μM TLR4, and also shows that TLR4 competes for the canonical carbohydrate-binding site of Gal3.

The data also provided insight into TLR4-induced self-association of Gal3. The slope of the binding curve in Figure 2C, where fixed TLR4 is titrated with a range of Gal3 concentrations, was consistent with a Hill coefficient of above 2 for intact Gal3 but was about 1 for the CRD. In Figure 2D, where fixed Gal3 is titrated with a range of TLR4 concentrations, the Hill coefficient for intact Gal3 was about 0.4, whereas for the CRD, it was again about 1 (Table S1). This indicates that intact Gal3 binds with apparent positive cooperativity and/or in an event with stoichiometry of greater than two Gal3 per TLR4, whereas the CRD binds in simple 1:1 interactions to one or more independent sites on TLR4.

Addition of Gal3 concentration >~1 μM to fluorescent TLR4 at 120 nM caused precipitation, as measured by removal of fluorescence by centrifugation of the samples before loading into capillaries that are used for the MST measurements (Figure S2A). This observation probably also explains the gradual fluorescence increase in un-centrifuged samples (Figure S2A) and the wavy line shapes of the fluorescence time traces recorded in the MST experiment (Figure S2B). However, the aggregation did not prevent obtaining highly reproducible binding curves that could be used for quantitative analysis of the interaction (Figure 2C). The different methods, hence, demonstrate that Gal3 interacts directly with TLR4 at physiologically relevant concentrations and also at the Gal3 concentrations (1 μM) used in the cell experiment here.

All galectin family members have in common a canonical CRD with high-sequence homology. Galectin-1 and galectin-4 were chosen as examples of the proto and tandem repeat families, respectively, and they also bind to TLR4 in MST experiments but with lower apparent affinities of about 8 and 14 μM, respectively, and Hill coefficients of about 1, indicating a lower cooperativity (Figure S2F).

TLR4 Contributes to Gal3 Proinflammatory Response

Contradictory reports suggest that Gal3 can play both proinflammatory and anti-inflammatory roles. Gal3 has been shown to elicit a proinflammatory (M1) response per se (Jeon et al., 2010) or amplify a pre-existent proinflammatory reaction (Devillers et al., 2013) in macrophages. Similarly, we have recently demonstrated that Gal3 is involved in the proinflammatory

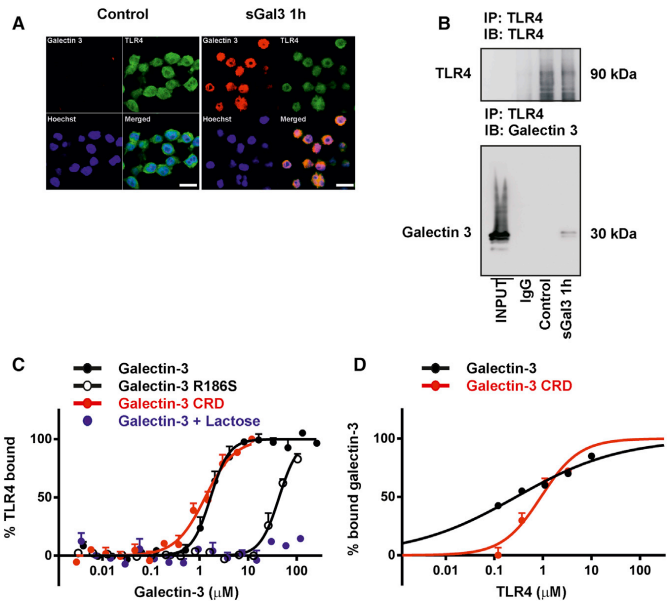

Figure 2. Gal3 Acts as a Ligand to TLR4

(A) Colocalization of Gal3 and TLR4 in BV2 cells after 1 hr exposure with sGal3 protein.

(B) Immunoblot showing the presence of Gal3 in an immune complex formed after pull-down of TLR4 in BV2 microglial cell line after being treated with 1 μM of soluble Gal3 for 1 hr.

(C) Microscale thermophoresis was used to analyze the direct binding of TLR4 to Gal3, the Gal3 CRD, Gal3 R186S, and Gal3 in the presence of inhibitory lactose (40 mM). Whereas the concentration of fluorescently labeled TLR4 was kept constant, the non-labeled proteins were titrated (x axis), and the minimal and maximal F_{norm} values of the unbound and bound state of TLR4, respectively, were used to calculate percent TLR4 bound to Gal3 (y axis).

(D) Fluorescence anisotropy was used to analyze the potency of TLR4 (x axis) to inhibit binding of Gal3 (0.2 μM) proteins to a fluorescent saccharide probe (0.02 μM).

The measured values were used to calculate the percent of Gal3 bound to TLR4 (y axis). The scale bar for (A) represents 15 μm. Data points in (C) and (D) are averaged from two to six measurements for each of the different conditions and binding curves obtained by non-linear regression to the Hill equation, with EC_{50} and Hill coefficient as variables and minimum (0%) and maximum (100%) constrained; numerical results are given in Table S1. Values are expressed as mean ± SE. See also Figure S2 and Table S1.

response triggered by α-synuclein in microglial cells (Boza-Serrano et al., 2014). Other studies have, however, suggested that Gal3 is involved in the alternative activation of macrophages and microglia (Hoyos et al., 2014; MacKinnon et al., 2008). In order to clarify the effect of Gal3 per se on microglial cells, BV2 cells were treated with sGal3 and several phenotypical markers were analyzed, including the expression of inducible nitric oxide synthase (iNOS) (M1 phenotype), CD206, TGF-β, Ym 1/2, arginase-1 activity (M2 phenotype), and CD45 (phosphatase that can inhibit the proinflammatory response; Starossom et al., 2012). We observed that sGal3 treatment induced iNOS expression (Figures 3B and 3C) and an overall trend to decrease the different M2 markers, although only arginase activity and CD206 expression reached statistical significance (Figures S3A and S3B). These data support the view that Gal3 stimulates a proinflammatory M1 phenotype in microglia.

The similarities between the changes in gene expression induced by Gal3 and LPS, which acts as a TLR4 ligand (Figure 1), and the physical interaction between Gal3 and TLR4 made us think that Gal3 could be inducing a TLR4-dependent inflammatory response. To explore this possibility, the expression of TLR4 was silenced in BV2 microglial cells using small interfering RNA (siRNA) (Figure 3A). Interestingly, silencing of TLR4 in BV2 cells leads to a reduction in the iNOS protein expression upon LPS, sGal3, and iGal3 treatments (Figures 3B–3E), suggesting that these stimuli share a common TLR4-dependent signaling pathway. The silencing of MyD88, a downstream protein triggered by activation of TLR4, shows as well a decrease in iNOS expression upon sGal3 treatment in BV2 cells (Figures S3C and S3D). To validate the TLR4 dependency of the Gal3 response, the release of several cytokines (i.e., TNF-α and interleukins [IL-1β, IL-4, IL-5, IL-10, and IL-12]) were investigated in primary microglia cultures derived from wild-type and TLR4 knockout mice upon sGal3 and iGal3 treatment. The release of the above-mentioned cytokines was found to be increased upon both types of Gal3 treatment in wild-type microglia (Figures 3F–3K). In contrast, the increases in cytokines released upon Gal3 treatments were abrogated in primary microglial cells originating from TLR4 knockout mice (Figures 3F–3K), demonstrating that TLR4 is essential for Gal3-induced cytokine release. In the case of IL-10 and TNF-α, we observed that their decrease is not complete in TLR4 knockout mice, which suggests also that Gal3 may be interacting also with other receptors other than TLR4 such as for example TLR2 (Jouault et al., 2006).

Gal3 Promotes Caspase-3/7 and Caspase-8 Activities in the Absence of Cell Death

We recently uncovered that the orderly activation of caspase-8 and caspase-3/7 contributes to the activation of microglia by several proinflammatory stimuli including LPS (Burguillos et al., 2011; Venero et al., 2011). Because both Gal3 and LPS can act as TLR4 ligands, we next examined whether Gal3 induces the activation of these caspases. Indeed, both sGal3 (Figure 4A) and iGal3 treatments (Figure 4B) induced DEVDase activity (caspase-3/7 activation) and IETDase activity (caspase-8 activation)

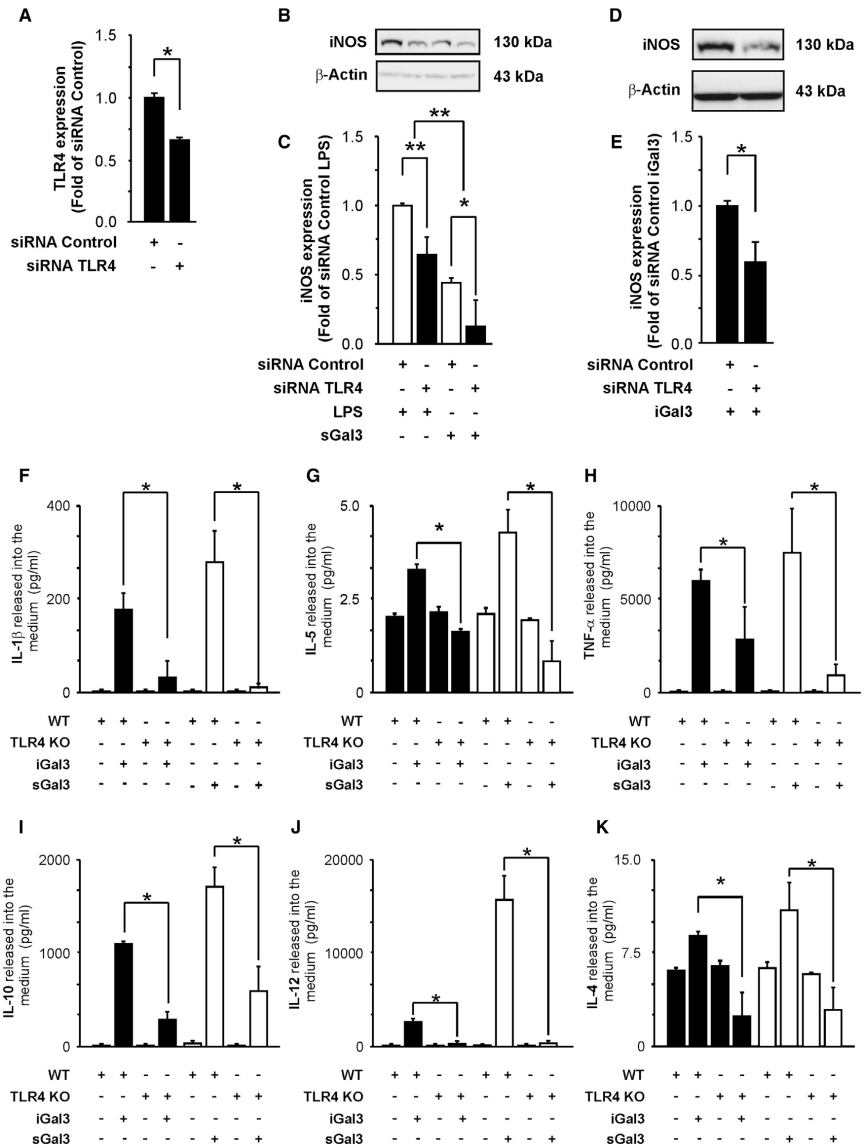

Figure 3. Gal3-Induced Inflammatory Response Is Dependent on TLR4

(A) TLR4 mRNA levels are downregulated after its knockdown.

(B–K) Knocking down TLR4 decreases iNOS expression after LPS, sGal3 (B and C), and iGal3 (D and E) treatment for 6 hr. Cytokine profile in wild-type (WT) primary microglia versus TLR4 knockout (TLR4 KO) primary microglia cells after 24 hr treatment with sGal3 and iGal3 (F–K).

Data are expressed as mean ± SD (A–E; n = 4) and mean ± SEM (F–K; n = 4). *p < 0.05; **p < 0.01. See also Figure S3.

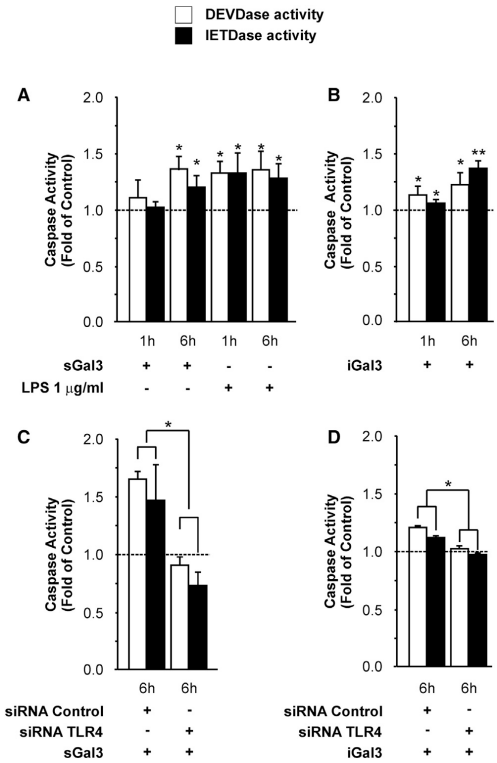

Figure 4. Gal3 Treatment Induces Caspase-3/7 and Caspase-8 Activities in a TLR4-Dependent Manner

(A and B) Analysis of caspase 3/7 (DEVDase) and caspase 8 (IETDase) activities at 1 hr and 6 hr treatment with 1 μM of sGal3, LPS (1 μg/ml; A), and iGal3 (B) in BV2 microglial cells. LPS treatment was used as a positive control for caspase 3/7 and 8 activation under inflammatory conditions.

(C and D) The increase of DEVDase and IETDase activity after sGal3 and iGal3 treatment is abolished when TLR4 is knocked down.

Data are expressed as mean ± SEM (n = 3). *p < 0.05; **p < 0.01. See also Figure S4.

as early as 6 hr after sGal3 and 1 hr after iGal3 treatment. In accordance with the TLR4 dependency of Gal3 response, silencing of TLR4 expression using siRNA abrogated the increase of both caspase-3/7 and casapase-8 activities after either sGal3 (Figure 4C) or iGal3 (Figure 4D) treatment.

We previously demonstrated that the TLR4-dependent activation of these caspases during microglia activation did not lead to cell death (Burguillos et al., 2011). We confirm here the absence of apoptotic cell death upon Gal3 treatment using a panel of methods (Figures S4A–S4D). Some reports indicated that Gal3 can affect the cell cycle (Lin et al., 2002). However, we did not find any alteration in the cell cycle after Gal3 treatment (Figures S4E and S4F).

Released Gal3 Is Essential for Full Microglial Response upon LPS Stimulation

Several proinflammatory stimuli, including LPS, have been shown to induce the release of Gal3 in macrophages and glial cells (Jeon et al., 2010; Li et al., 2008). This urged us to investigate whether endogenous Gal3 could play a paracrine role in the response triggered by an inflammatory stimulus. In culture, we observed a time- and dose-dependent release of Gal3 protein from BV2 microglia cells in response to LPS exposure (Figure S5A). After LPS treatment, Gal3 and TLR4 were also found to colocalize in BV2 cells (Figure S5B). Furthermore, the amount of Gal3 found to co-immunoprecipitate with TLR4 was directly proportional to the dose of LPS used (Figure 5A).

To study the contribution of Gal3 in the response of microglia cells to a LPS stimulus, we decided to inhibit it through two different approaches: (1) Gal3 expression was suppressed using siRNAs in BV2 cells (Figure S5C) and (2) a Gal3 blocking antibody was used to neutralize the effects of released Gal3. We observed that both methods prevented LPS-induced iNOS expression at 6 hr and 24 hr (Figures 5B–5D). To validate Gal3 effect over the inflammatory response upon LPS stimulus, the release of several proinflammatory cytokines were checked in wild-type and Gal3 knockout primary microglial cell cultures, confirming the BV2 cell data, with reduced inflammatory response in Gal3 knockout microglia (Figure 5E).

We also analyzed the effect of Gal3 inhibition in terms of IETDase and DEVDase activities in response to LPS treatment; the Gal3 siRNA knockdown has an inhibitory effect on both activities, especially at 24 hr (Figures S5D and S5E). Collectively, these results demonstrate that Gal3 indeed contributes to the response of microglia cells to LPS stimulus.

In Vivo Interaction between Gal3 and TLR4 and Its Contribution to the Inflammatory Response Induced by LPS

At this point, we wanted to validate our in vitro observation in vivo and explore whether Gal3-TLR4 interactions could be observed in the brains of mice 24 hr after LPS injection into the substantia nigra, an established model of neuroinflammation (Castaño et al., 2002; Herrera et al., 2000). First, we established an in vivo rat brain microdialysis approach to detect released Gal3 in the ventral mesencephalon in response to intranigral LPS injection. We discovered that Gal3 is released in the substantia nigra 24 hr after LPS injection (Figure 6A). We further used TLR4, Iba-1, and Gal3 immunohistochemistry and observed colocalization of the three markers in several cells in the same region after LPS injection in mice (Figure 6B). We confirmed colocalization of Gal3 and TLR4 in microglial cells by using double heterozygous Cx3cr1GFP/+Ccr2RFP/+ mice, where GFP is expressed only in microglial cells and RFP in monocytes (Figure S6F). We performed fluorescence resonance energy transfer (FRET) analysis, using TLR4-FITC as a donor and Gal3 Texas Red as an acceptor, and an interaction between TLR4 and Gal3 proteins was demonstrated at 24 hr following injection of LPS in the substantia nigra (Figure 6C). Our in vitro investigations suggest that the absence of Gal3 is associated with reduced inflammation upon LPS stimulus. We decided to compare the neuroinflammatory response after intranigral LPS injection in wild-type and Gal3

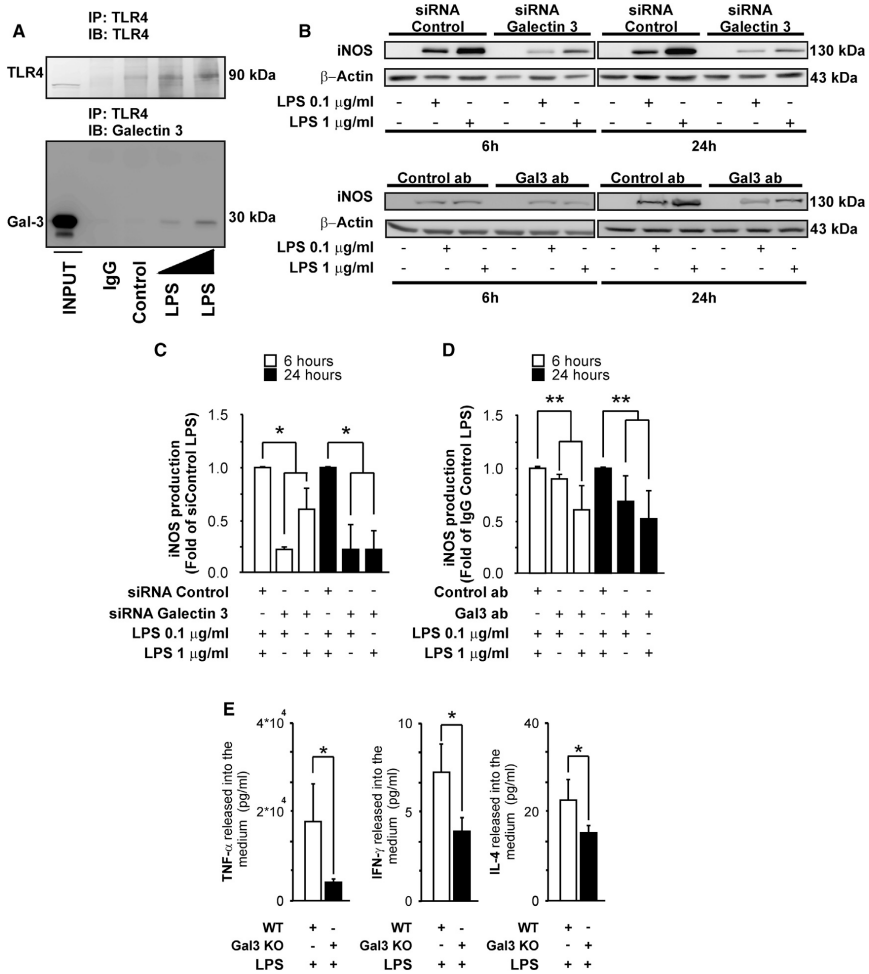

Figure 5. Released Gal3 Enhances the Inflammatory Response after LPS Treatment in a Dose- and Time-Dependent Manner In Vitro

(A) Immunoblot showing the presence of Gal3 in immune complexes formed after pull-down of TLR4 in BV2 microglial cell line after being treated with LPS (0.1 μg/ml and 1 μg/ml) for 24 hr.

(B–D) Reduced iNOS expression upon LPS treatment for 6 hr and 24 hr in BV2 cells transfected with siRNA-targeting Gal3 as compared to BV2 cells transfected with siRNA control and after co-treatment of LPS with a neutralizing antibody against Gal3 as compared to the same amount of the same isotype of IgG as a negative control.

(E) Cytokine profile in WT primary microglia versus Gal3 knockout (Gal3 KO) primary microglia cells after 12 hr treatment with LPS 0.1 μg/ml (E). a

Data are expressed as mean ± SEM (n = 4). *p < 0.05; **p < 0.01. See also Figure S5.

knockout mice. We found a significant decrease in the expression of proinflammatory markers IL-1β and IL-6, (Figure 6D) which is consistent with the reduced numbers of reactive microglia/macrophages (Figures 6E and 6F) and proliferating microglia (Iba1 and BrdU double-positive cells; Figures S6A and S6B) in the Gal3 knockout mice as compared to wild-type mice after LPS treatment. As a consequence of this ameliorated inflammatory response in Gal3 knockout mice, there was a clear neuroprotection of the dopaminergic system 7 days after LPS injection (sham WT animals: 1,467 ± 304, LPS WT animals: 711 ± 128,

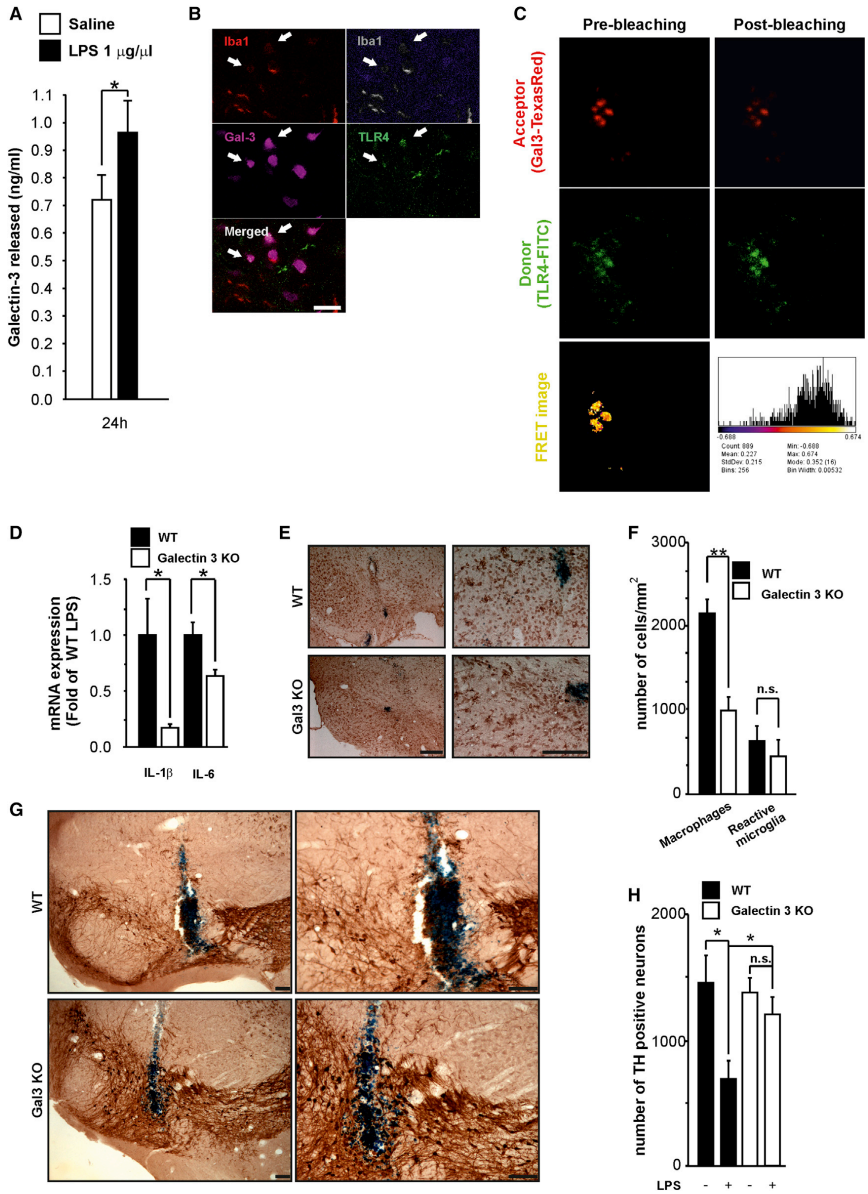

Figure 6. Gal3 Colocalizes with TLR4 and Contributes to the Inflammatory Response Induced by LPS Injection in the Substantia Nigra

(A) Measurement of Gal3 release 24 hr after LPS injection compared to saline injection in the substantia nigra.

(B) Increased expression and colocalization of Gal3 (purple), Iba1 (in red and also using range indicator filter in gray), and TLR4 (green) 24 hr upon LPS injection in the substantia nigra.

(legend continued on next page)

and LPS Gal3 KO: 1,213 ± 130; Figures 6G and 6H), with a clear decrease in the number (Figure S6C) and M1 polarization phenotype (measured as CD16/32 expression; Figures S6D and S6E) of the microglial population.

Gal3 Contribution to the Inflammatory Response Induced in Global Brain Ischemia Model

Our next step was to assess the importance of Gal3 in a mouse model of global cerebral ischemia that mimics the brain damage caused by cardiac arrest. For this reason, wild-type and Gal3 knockout mice were used, and we found an increase of the survival of the hippocampal neurons in the mice lacking Gal3 (3,100 NeuN-positive neurons in sham; 1,415 ± 774 in wild-type and 1,868 ± 658 in ischemia-treated animals; Figures 7A and 7B). The increase in the neuronal survival in the Gal3 knockout mice was linked to a lower inflammatory response in terms of hippocampal Iba1 protein expression (Figures 7C and 7D). Mice lacking Gal3 showed a lower body weight reduction (Figure 7E) following ischemia. Also, mice lacking Gal3 show a tendency (although not statistically significant) of ameliorated memory deficits in the hippocampal-dependent Y-maze test 1 week after ischemia (Figure 7F).

Gal3 Interacts with TLR4 in Human Brain Tissue

The expression of Gal3 and TLR4 was also investigated in postmortem brain tissue from patients who had suffered and died from cardiac arrest. High expression of both Gal3 and TLR4 was observed in the ischemia-damaged brain tissue as compared to age-matched controls (Figures S7A–S7D). Both markers were found to be present, suggesting colocalization (Figure S7D). Finally, FRET signal between Gal3 and TLR4 could also be detected in cells in human stroke brain (Figures S7E–S7G).

DISCUSSION

In this study, we show that, under conditions of acute brain inflammation, there is release of endogenous Gal3, which subsequently binds to and stimulates microglial TLR4, thus eliciting a proinflammatory M1 response in the brain. Furthermore, released Gal3 appears necessary to elicit a full-blown activation of microglia in response to proinflammatory stimuli such as LPS.

In ischemia/stroke, microglial cells are highly activated around the site of a brain injury, where they typically express high levels of Gal3 (Inácio et al., 2011; Lalancette-Hébert et al., 2012), a protein known to be a potent immunomodulator in neuroinflammatory disorders. The inflammatory role of Gal3 in brain ischemia appears to be diverse, conceivably depending on the specific neuroinflammatory conditions. This is most likely due to several factors such as the type of ischemic insult, its timing, and the subcellular localization of Gal3, as well as the immunological status of the individual. In neuroinflammatory models of ALS, Gal3 can induce an anti-inflammatory response (Lerman et al., 2012), whereas in experimental autoimmune encephalomyelitis, Gal3 can exacerbate the disease by reinforcing the inflammatory response (Jiang et al., 2009).

Other members of the galectin family of proteins, despite significant sequence homologies and shared functional capabilities, exert diverse and even sometimes opposite effects on several biological processes. For instance, galectin-1 and galectin-9 illustrate the variety of effects of the galectin family during the inflammatory response. Indeed, galectin-1 can deactivate "classically activated microglia" through binding to the CD45 phosphatase, increasing the microglial surface's retention time of this glycoprotein and increasing its inhibitory function over the inflammatory response (Starossom et al., 2012). In contrast, galectin-9, acting as a ligand for Tim-3, can trigger a proinflammatory response in naive resting immune cells (such as dendritic cells) and synergizes with the TLR-signaling pathway (Anderson et al., 2007). Here, we show that Gal3 acts as a ligand for TLR4 under the described conditions and at a given time of cellular activation/differentiation. This is driven by CRD-mediated engagement of Gal3 to TLR4-attached carbohydrates (Figure 2). Our data indicate that the CRDs of the other galectin subclasses are capable of binding to TLR4, albeit with lower apparent affinities (Figure S2F; Table S1). The fine specificity that varies between different galectins has been already thoroughly discussed (Carlsson et al., 2007; Salomonsson et al., 2010a; Stowell et al., 2008). This paper focuses on the role of Gal3 in the TLR4-mediated microglial activation. The biological effect of other galectins as TLR4 ligands should be addressed in future studies.

TLR4 is considered to be a key player in the innate inflammatory response, but most of the studies performed in the field of TLR4 are based on LPS administration. Although great advances have been achieved using LPS as TLR4 ligand, its physiological relevance is more related to sepsis than to sterile inflammation (Chen and Nuñez, 2010). To support this, we observe a quantitative difference between LPS and Gal3 in the gene expression profile, most likely because of the low LPS Kd value toward TLR4 (range of nM; Akashi et al., 2003) as compared with Gal3 (range of μM). The results show not only a quantitative but also qualitative difference in the gene expression response after LPS or Gal3 treatment (Figures 1 and S1), which suggests a different TLR4 downstream response depending on the stimulus. In the past years, considerable efforts have been made to identify endogenous ligands that can activate TLR4. As a result, some proteins—i.e., heat shock protein (HSP)-70 and high mobility group box 1 (HMGB1)—and glycosaminoglycans such as hyaluronan

(C) FRET of Gal3 and TLR4 after 24 hr treatment of LPS in substantia nigra.
(D) Comparison of IL-1β and IL-6 mRNA expression by qPCR between WT LPS-injected mice versus LPS-injected Gal3 knockout mice.
(E and F) Comparison of the levels of macrophages (i.e., Iba-1+ cells with amoeboid morphology) and reactive microglia (i.e., Iba-1+ cells with thick processes) directly in the vicinity of the injection site in WT mice and in Gal3 knockout mice.
(G and H) Quantification of TH^+ dopaminergic neurons in the nigra 7 days after LPS injection in WT and Gal3 knockout mice.
Data are expressed as mean ± SEM in (D; n = 4), mean ± SD in (F; n = 4), and mean ± SD in (H; n = 4). White arrows show colocalization of the three markers. The percent of FRET is represented as a color bar besides the FRET picture. The scale bar for (B) and (E) represents 27 μm. *$p < 0.05$. White arrows in (B) represent colocalization of the three markers. See also Figure S6.

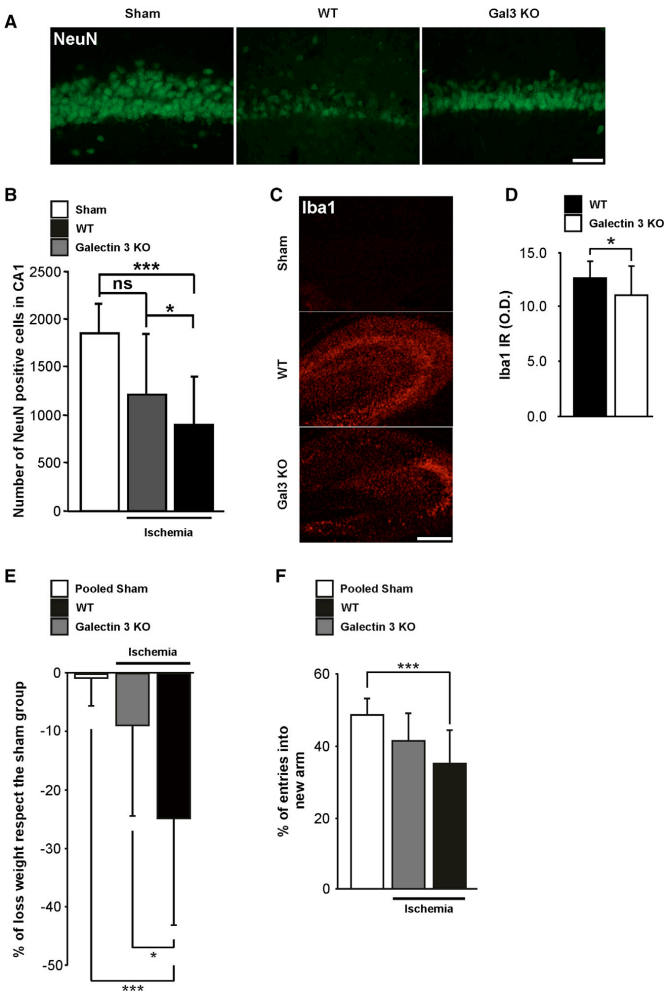

Figure 7. Gal3 Deficiency Ameliorates Microglial Activity, Neuronal Cell Death, and Memory Impairment following Global Brain Ischemia in Mice

(A and B) Representative picture (A) and quantification (B) of viable NeuN+ pyramidal neurons in hippocampal CA1 (A) subregion in sham, Gal3 knockout, and WT mice 8 days following global brain ischemia (B).

(C and D) Reduced inflammatory response in hippocampus measured by Iba1 immunoreactivity in Gal3 knockout mice compared to WT mice.

(E and F) Body weight (E) and memory impairment assessed by the Y-maze behavioral test (F) measurements.

Values are expressed as mean ± SEM (n = 4) in (B), (D), (E), and (F). The scale bar for (A) represents 50 μm and for (C) represents 372 μm. See also Figure S7.

have been shown to be TLR4 ligands, as reviewed in Chen and Nuñez (2010).

In summary, we demonstrate that (1) Gal3 can be actively released into the extracellular compartment by activated microglial cells, (2) Gal3 binds directly to TLR4 at physiological concentrations, (3) Gal3 itself activates TLR4 and is capable of activating surrounding microglia, (4) Gal3 amplifies the typical TLR4-dependent proinflammatory response, including caspase-mediated inflammation (Burguillos et al., 2011; Venero et al., 2011), and (5) TLR4/Gal3 interaction occurs in the brain of stroke patients as evidenced by FRET analysis. The discovery that Gal3 can act as a TLR4 ligand brings further importance to the elevated production and release of Gal3 by microglia under ischemia/stroke condition. These findings indicate that Gal3 can play a decisive role in the expansion and enforcement of the inflammatory response and might potentially contribute to the long-term inflammatory response. New therapies specifically targeting Gal3 released from microglia could counteract some of the deleterious effects resulting from ischemia/stroke.

EXPERIMENTAL PROCEDURES

Cell Lines, Transfection, and Reagents

Murine microglial BV2 cell line was cultured as described (Bocchini et al., 1992). Cells were maintained in 10% FCS in DMEM and reduced to 2%–5% FCS while performing the experiments. Transfection of BV2 cells was carried out using Lipofectamine 2000 (Invitrogen) following the manufacturer's recommendation. LPS (from *Escherichia coli*, serotype 026:B6) and staurosporine were purchased from Sigma-Aldrich. Recombinant Gal3 production and Gal3R186S mutant were prepared as described (Salomonsson et al., 2010a). The purity of Gal3 and mutants proteins were determined by the *Limulus* amebocyte lysate assay (Charles River Laboratories), and only endotoxin-free proteins were used. The recombinant proteins used for MST and fluorescence anisotropy were obtained from R&D Systems, and the catalog numbers are provided in the Supplemental Experimental Procedures. Non-targeting control, TLR4, and Gal3 siRNAs were obtained from Dharmacon. A complete list of siRNA sequences, primers, and antibodies are provided in the Supplemental Experimental Procedures. In order to study the effect of the released Gal3 over the sustained inflammatory response, cells were treated with 3 μg/ml of anti-Gal3 antibody or IgG as a negative control, together with LPS for 24 hr, and the inflammatory response checked.

Animals and Surgery

Gal3-null mutant mice (Colnot et al., 1998; C57BL/6 background) were obtained from Dr. K. Sävman/Gothenburg University and housed and bred at Lund University and the Center of Production and Animal Experimentation.

The Gal3 −/− and +/+ genotyping was performed as described in Doverhag et al. (2010).

Double heterozygous Cx3cr1GFP/+Ccr2RFP/+ mice were generated as previously described in (Mizutani et al., 2012) from CX3CR1-GFP knockin and CCR2-red fluorescent protein (RFP) knockin reporter mice (Jung et al., 2000; Saederup et al., 2010).

Male albino rats weighing 270–320 g were used for Gal3 microdialysis after LPS injection.

Animals had free access to food and water. Experiments were performed in accordance with the Guidelines of the European Union Council (86/609/EU), following Spanish and Swedish regulations and approved by the Ethical Committee for Animal Research (ethical permit numbers M303-09 and N248/13).

In order to model the brain damage following cardiac arrest with successful cardiopulmonary resuscitation, global ischemia was induced in mice (Olsson et al., 2003; Deierborg et al., 2008). In brief, mice were first anesthetized with 5% isoflurane in oxygen. Thereafter, the anesthesia was maintained at 2% isoflurane (IsobaVet; Schering-Plough Animal Health). A small cut parallel to the trachea was made. The common carotid arteries were isolated and encircled with a thin silk thread to allow occlusion with a micro-aneurysm clip. Ischemia was induced for 13 min. The wound was then sealed with a few absorbable stitches before the anesthesia was discontinued. During the surgery, the body temperature was monitored and controlled by a heating pad and infrared lamp to keep the mice normothermic. The body temperature of the mouse was maintained around 37.5°C during the whole procedure. Mice were housed in an incubator at 34°C overnight in order to maintain normothermia. Sham mice were subjected to the same surgical protocol, except occlusion to the common carotid arteries. The person performing the surgery was blinded to the genotype of the animals.

Intranigral LPS injections (2 μg in 1 μl sterile saline) were made 1.2 mm posterior, 1.2 mm lateral, and 5.0 mm ventral to the lambda.

Twenty-four hours later, mice were transcardially perfused under deep anesthesia with 4% paraformaldehyde/PBS (pH 7.4). Brains were removed, cryoprotected in sucrose, and frozen in isopentane at −15°C, and serial coronal sections (25 μm sections) covering the substantia nigra were cut and further processed for immunohistochemistry.

Statistical Analysis

The differences between control and experimental groups were evaluated with one-way ANOVA with a Bonferroni's post hoc analysis. χ^2 test was used to analyze the up/downregulation of the genes presented in Figure 1. Mann-Whitney test was used to analyze the NeuN-positive cells in Figure 7B. $p < 0.05$ was considered as statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.02.012.

AUTHOR CONTRIBUTIONS

M.A.B. performed all the experiments except as otherwise noted. T.S, H.L., and A.A. determined the k_D between Gal1/Gal3/Gal4-TLR4 by MST and FA. qPCR was performed by A.G.-Q. E.K., M.A.B., M. Svensson, J.L.V., M. Santiago, M.J.O.-M., and T.D. performed the surgery and dissecting of the animal brains. M. Santiago and N.V. performed the microdialysis. A.M.O. and K.B. performed and analyzed the experiments in the Cx3cr1GFP/+Ccr2RFP/+ double-heterozygous mice. M.A.B., T.D., A.B.-S., and M. Svensson performed primary cell culture experiments and cytokine analysis. E.S. and H.L. produced and prepared the protein. B.J. collaborated in the confocal imaging analysis. M. Svensson and T.D. performed the behavioral tests. E.E. did the neuropathology analysis of the individuals with stroke and control cases. A.P. prepared tissue and participated in the morphological assessment of human brain specimens. L.A. was involved in the study design. M.A.B., J.L.V., B.J., and T.D. designed the study and analyzed and interpreted the data. All authors discussed the results and commented on or edited the manuscript. The first draft of the paper was written by M.A.B. and T.D. B.J., and J.L.V. discussed the results and commented on or edited the manuscript.

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REFERENCES

Akashi, S., Saitoh, S., Wakabayashi, Y., Kikuchi, T., Takamura, N., Nagai, Y., Kusumoto, Y., Fukase, K., Kusumoto, S., Adachi, Y., et al. (2003). Lipopolysaccharide interaction with cell surface Toll-like receptor 4-MD-2: higher affinity than that with MD-2 or CD14. J. Exp. Med. *198*, 1035–1042.

Anderson, A.C., Anderson, D.E., Bregoli, L., Hastings, W.D., Kassam, N., Lei, C., Chandwaskar, R., Karman, J., Su, E.W., Hirashima, M., et al. (2007). Promotion of tissue inflammation by the immune receptor Tim-3 expressed on innate immune cells. Science *318*, 1141–1143.

Barondes, S.H., Castronovo, V., Cooper, D.N., Cummings, R.D., Drickamer, K., Feizi, T., Gitt, M.A., Hirabayashi, J., Hughes, C., Kasai, K., et al. (1994). Galectins: a family of animal beta-galactoside-binding lectins. Cell *76*, 597–598.

Bocchini, V., Mazzolla, R., Barluzzi, R., Blasi, E., Sick, P., and Kettenmann, H. (1992). An immortalized cell line expresses properties of activated microglial cells. J. Neurosci. Res. *31*, 616–621.

Boza-Serrano, A., Reyes, J.F., Rey, N.L., Leffler, H., Bousset, L., Nilsson, U., Brundin, P., Venero, J., Burguillos, M., and Deierborg, T. (2014). The role of Galectin-3 in α-synuclein-induced microglial activation. Acta Neuropathol Commun *2*, 156.

Buchanan, M.M., Hutchinson, M., Watkins, L.R., and Yin, H. (2010). Toll-like receptor 4 in CNS pathologies. J. Neurochem. *114*, 13–27.

Burguillos, M.A., Deierborg, T., Kavanagh, E., Persson, A., Hajji, N., Garcia-Quintanilla, A., Cano, J., Brundin, P., Englund, E., Venero, J.L., and Joseph, B. (2011). Caspase signalling controls microglia activation and neurotoxicity. Nature *472*, 319–324.

Carlsson, S., Oberg, C.T., Carlsson, M.C., Sundin, A., Nilsson, U.J., Smith, D., Cummings, R.D., Almkvist, J., Karlsson, A., and Leffler, H. (2007). Affinity of galectin-8 and its carbohydrate recognition domains for ligands in solution and at the cell surface. Glycobiology *17*, 663–676.

Caso, J.R., Pradillo, J.M., Hurtado, O., Lorenzo, P., Moro, M.A., and Lizasoain, I. (2007). Toll-like receptor 4 is involved in brain damage and inflammation after experimental stroke. Circulation *115*, 1599–1608.

Castaño, A., Herrera, A.J., Cano, J., and Machado, A. (2002). The degenerative effect of a single intranigral injection of LPS on the dopaminergic system is

prevented by dexamethasone, and not mimicked by rh-TNF-alpha, IL-1beta and IFN-gamma. J. Neurochem. *81*, 150–157.

Chen, G.Y., and Nuñez, G. (2010). Sterile inflammation: sensing and reacting to damage. Nat. Rev. Immunol. *10*, 826–837.

Colnot, C., Fowlis, D., Ripoche, M.-A., Bouchaert, I., and Poirier, F. (1998). Embryonic implantation in galectin 1/galectin 3 double mutant mice. Dev. Dyn. *211*, 306–313.

Debierre-Grockiego, F., Niehus, S., Coddeville, B., Elass, E., Poirier, F., Weingart, R., Schmidt, R.R., Mazurier, J., Guérardel, Y., and Schwarz, R.T. (2010). Binding of Toxoplasma gondii glycosylphosphatidylinositols to galectin-3 is required for their recognition by macrophages. J. Biol. Chem. *285*, 32744–32750.

Devillers, A., Courjol, F., Fradin, C., Coste, A., Poulain, D., Pipy, B., Bernardes, E.S., and Jouault, T. (2013). Deficient beta-mannosylation of Candida albicans phospholipomannan affects the proinflammatory response in macrophages. PLoS ONE *8*, e84771.

Doverhag, C., Hedtjärn, M., Poirier, F., Mallard, C., Hagberg, H., Karlsson, A., and Sävman, K. (2010). Galectin-3 contributes to neonatal hypoxic-ischemic brain injury. Neurobiol. Dis. *38*, 36–46.

Hennessy, E.J., Parker, A.E., and O'Neill, L.A. (2010). Targeting Toll-like receptors: emerging therapeutics? Nat. Rev. Drug Discov. *9*, 293–307.

Herrera, A.J., Castaño, A., Venero, J.L., Cano, J., and Machado, A. (2000). The single intranigral injection of LPS as a new model for studying the selective effects of inflammatory reactions on dopaminergic system. Neurobiol. Dis. *7*, 429–447.

Hoyos, H.C., Rinaldi, M., Mendez-Huergo, S.P., Marder, M., Rabinovich, G.A., Pasquini, J.M., and Pasquini, L.A. (2014). Galectin-3 controls the response of microglial cells to limit cuprizone-induced demyelination. Neurobiol. Dis. *62*, 441–455.

Hyakkoku, K., Hamanaka, J., Tsuruma, K., Shimazawa, M., Tanaka, H., Uematsu, S., Akira, S., Inagaki, N., Nagai, H., and Hara, H. (2010). Toll-like receptor 4 (TLR4), but not TLR3 or TLR9, knock-out mice have neuroprotective effects against focal cerebral ischemia. Neuroscience *171*, 258–267.

Inácio, A.R., Ruscher, K., Leng, L., Bucala, R., and Deierborg, T. (2011). Macrophage migration inhibitory factor promotes cell death and aggravates neurologic deficits after experimental stroke. J. Cereb. Blood Flow Metab. *31*, 1093–1106.

Jeon, S.B., Yoon, H.J., Chang, C.Y., Koh, H.S., Jeon, S.H., and Park, E.J. (2010). Galectin-3 exerts cytokine-like regulatory actions through the JAK-STAT pathway. J. Immunol. *185*, 7037–7046.

Jiang, H.R., Al Rasebi, Z., Mensah-Brown, E., Shahin, A., Xu, D., Goodyear, C.S., Fukada, S.Y., Liu, F.T., Liew, F.Y., and Lukic, M.L. (2009). Galectin-3 deficiency reduces the severity of experimental autoimmune encephalomyelitis. J. Immunol. *182*, 1167–1173.

Jouault, T., El Abed-El Behi, M., Martínez-Esparza, M., Breuilh, L., Trinel, P.A., Chamaillard, M., Trottein, F., and Poulain, D. (2006). Specific recognition of Candida albicans by macrophages requires galectin-3 to discriminate Saccharomyces cerevisiae and needs association with TLR2 for signaling. J. Immunol. *177*, 4679–4687.

Jung, S., Aliberti, J., Graemmel, P., Sunshine, M.J., Kreutzberg, G.W., Sher, A., and Littman, D.R. (2000). Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. Mol. Cell. Biol. *20*, 4106–4114.

Kasai, K., and Hirabayashi, J. (1996). Galectins: a family of animal lectins that decipher glycocodes. J. Biochem. *119*, 1–8.

Lalancette-Hébert, M., Swarup, V., Beaulieu, J.M., Bohacek, I., Abdelhamid, E., Weng, Y.C., Sato, S., and Kriz, J. (2012). Galectin-3 is required for resident microglia activation and proliferation in response to ischemic injury. J. Neurosci. *32*, 10383–10395.

Lambertsen, K.L., Biber, K., and Finsen, B. (2012). Inflammatory cytokines in experimental and human stroke. J. Cereb. Blood Flow Metab. *32*, 1677–1698.

Leffler, H., Carlsson, S., Hedlund, M., Qian, Y., and Poirier, F. (2004). Introduction to galectins. Glycoconj. J. *19*, 433–440.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., and Hoffmann, J.A. (1996). The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell *86*, 973–983.

Lepur, A., Salomonsson, E., Nilsson, U.J., and Leffler, H. (2012). Ligand induced galectin-3 protein self-association. J. Biol. Chem. *287*, 21751–21756.

Lerman, B.J., Hoffman, E.P., Sutherland, M.L., Bouri, K., Hsu, D.K., Liu, F.T., Rothstein, J.D., and Knoblach, S.M. (2012). Deletion of galectin-3 exacerbates microglial activation and accelerates disease progression and demise in a SOD1(G93A) mouse model of amyotrophic lateral sclerosis. Brain Behav *2*, 563–575.

Li, Y., Komai-Koma, M., Gilchrist, D.S., Hsu, D.K., Liu, F.T., Springall, T., and Xu, D. (2008). Galectin-3 is a negative regulator of lipopolysaccharide-mediated inflammation. J. Immunol. *181*, 2781–2789.

Lin, H.M., Pestell, R.G., Raz, A., and Kim, H.R. (2002). Galectin-3 enhances cyclin D(1) promoter activity through SP1 and a cAMP-responsive element in human breast epithelial cells. Oncogene *21*, 8001–8010.

MacKinnon, A.C., Farnworth, S.L., Hodkinson, P.S., Henderson, N.C., Atkinson, K.M., Leffler, H., Nilsson, U.J., Haslett, C., Forbes, S.J., and Sethi, T. (2008). Regulation of alternative macrophage activation by galectin-3. J. Immunol. *180*, 2650–2658.

Mey, A., Leffler, H., Hmama, Z., Normier, G., and Revillard, J.P. (1996). The animal lectin galectin-3 interacts with bacterial lipopolysaccharides via two independent sites. J. Immunol. *156*, 1572–1577.

Mizutani, M., Pino, P.A., Saederup, N., Charo, I.F., Ransohoff, R.M., and Cardona, A.E. (2012). The fractalkine receptor but not CCR2 is present on microglia from embryonic development throughout adulthood. J. Immunol. *188*, 29–36.

Nakahara, S., Oka, N., and Raz, A. (2005). On the role of galectin-3 in cancer apoptosis. Apoptosis *10*, 267–275.

Olsson, T., Wieloch, T., and Smith, M.L. (2003). Brain damage in a mouse model of global cerebral ischemia. Effect of NMDA receptor blockade. Brain Res. *982*, 260–269.

Deierborg, T., Wieloch, T., Diano, S., Warden, C.H., Horvath, T.L., and Mattiasson, G. (2008). Overexpression of UCP2 protects thalamic neurons following global ischemia in the mouse. J. Cereb. Blood Flow Metab. *28*, 1186–1195.

Saederup, N., Cardona, A.E., Croft, K., Mizutani, M., Cotleur, A.C., Tsou, C.L., Ransohoff, R.M., and Charo, I.F. (2010). Selective chemokine receptor usage by central nervous system myeloid cells in CCR2-red fluorescent protein knock-in mice. PLoS ONE *5*, e13693.

Saijo, K., and Glass, C.K. (2011). Microglial cell origin and phenotypes in health and disease. Nat. Rev. Immunol. *11*, 775–787.

Salomonsson, E., Carlsson, M.C., Osla, V., Hendus-Altenburger, R., Kahl-Knutson, B., Oberg, C.T., Sundin, A., Nilsson, R., Nordberg-Karlsson, E., Nilsson, U.J., et al. (2010a). Mutational tuning of galectin-3 specificity and biological function. J. Biol. Chem. *285*, 35079–35091.

Salomonsson, E., Larumbe, A., Tejler, J., Tullberg, E., Rydberg, H., Sundin, A., Khabut, A., Frejd, T., Lobsanov, Y.D., Rini, J.M., et al. (2010b). Monovalent interactions of galectin-1. Biochemistry *49*, 9518–9532.

Satoh, K., Niwa, M., Binh, N.H., Nakashima, M., Kobayashi, K., Takamatsu, M., and Hara, A. (2011a). Increase of galectin-3 expression in microglia by hyperthermia in delayed neuronal death of hippocampal CA1 following transient forebrain ischemia. Neurosci. Lett. *504*, 199–203.

Satoh, K., Niwa, M., Goda, W., Binh, N.H., Nakashima, M., Takamatsu, M., and Hara, A. (2011b). Galectin-3 expression in delayed neuronal death of hippocampal CA1 following transient forebrain ischemia, and its inhibition by hypothermia. Brain Res. *1382*, 266–274.

Seidel, S.A., Dijkman, P.M., Lea, W.A., van den Bogaart, G., Jerabek-Willemsen, M., Lazic, A., Joseph, J.S., Srinivasan, P., Baaske, P., Simeonov, A., et al. (2013). Microscale thermophoresis quantifies biomolecular interactions under previously challenging conditions. Methods *59*, 301–315.

Shimura, T., Takenaka, Y., Tsutsumi, S., Hogan, V., Kikuchi, A., and Raz, A. (2004). Galectin-3, a novel binding partner of beta-catenin. Cancer Res. *64*, 6363–6367.

Sörme, P., Qian, Y., Nyholm, P.G., Leffler, H., and Nilsson, U.J. (2002). Low micromolar inhibitors of galectin-3 based on 3′-derivatization of N-acetyllactosamine. ChemBioChem *3*, 183–189.

Starossom, S.C., Mascanfroni, I.D., Imitola, J., Cao, L., Raddassi, K., Hernandez, S.F., Bassil, R., Croci, D.O., Cerliani, J.P., Delacour, D., et al. (2012). Galectin-1 deactivates classically activated microglia and protects from inflammation-induced neurodegeneration. Immunity *37*, 249–263.

Stowell, S.R., Arthur, C.M., Mehta, P., Slanina, K.A., Blixt, O., Leffler, H., Smith, D.F., and Cummings, R.D. (2008). Galectin-1, -2, and -3 exhibit differential recognition of sialylated glycans and blood group antigens. J. Biol. Chem. *283*, 10109–10123.

Suzuki, Y., Hattori, K., Hamanaka, J., Murase, T., Egashira, Y., Mishiro, K., Ishiguro, M., Tsuruma, K., Hirose, Y., Tanaka, H., et al. (2012). Pharmacological inhibition of TLR4-NOX4 signal protects against neuronal death in transient focal ischemia. Sci Rep *2*, 896.

Venero, J.L., Burguillos, M.A., Brundin, P., and Joseph, B. (2011). The executioners sing a new song: killer caspases activate microglia. Cell Death Differ. *18*, 1679–1691.

Wesley, U.V., Vemuganti, R., Ayvaci, E.R., and Dempsey, R.J. (2013). Galectin-3 enhances angiogenic and migratory potential of microglial cells via modulation of integrin linked kinase signaling. Brain Res. *1496*, 1–9.

Cell Reports

Supplemental Information

Microglia-Secreted Galectin-3 Acts as a Toll-like Receptor 4 Ligand and Contributes to Microglial Activation

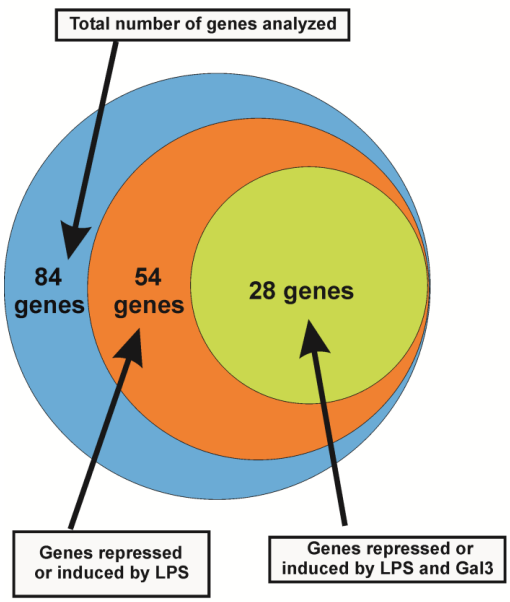
Miguel Angel Burguillos, Martina Svensson, Tim Schulte, Antonio Boza-Serrano, Albert Garcia-Quintanilla, Edel Kavanagh, Martiniano Santiago, Nikenza Viceconte, Maria Jose Oliva-Martin, Ahmed Mohamed Osman, Emma Salomonsson, Lahouari Amar, Annette Persson, Klas Blomgren, Adnane Achour, Elisabet Englund, Hakon Leffler, Jose Luis Venero, Bertrand Joseph, and Tomas Deierborg

Supplemental figures

Figure S1 related to Figure 1. Gene expression after LPS and galectin-3 treatment in BV2 cells.

Panel A represents the number of genes that change their expression after LPS or/and galectin-3 treatment. Panel B represents the genes that change significantly with LPS treatment only and their fold of increase/decrease respect to control (red, reduction; green increase).

A



B

List of genes only affected by LPS

Cd14	4,18
Csf3	491,43
Hras1	0,85
Agfg1	1,59
Ifnb1	5,44
Il6	576,37
Irf3	0,64
Lta	31,70
Ly96	1,64
Nfkb1	2,30
Nfkbib	2,10
Nfkbil1	1,84
Nr2c2	0,77
Eif2ak2	3,17
Ptgs2	11,30
Rel	1,35
Rela	1,42
Tbk1	1,66
Ticam1	2,72
Ticam2	0,37
Tirap	0,85
Tlr3	21,12
Tlr6	1,71
Tnfrsf1a	1,29
Tollip	1,53
Ube2n	0,57

Figure S2 related to Figure 2. Specific binding of galectin-3 to TLR-4 leads to the formation of aggregates by MST and analysis of galectin-3 ligand interaction by fluorescence anisotropy. Determination of the apparent affinity of the interaction between TLR4 and galection-1 and galectin-4 was using MST. Capillaries for MST were loaded with samples that were either centrifuged or not centrifuged before loading. Fluorescence was determined using the MST instrument. Without centrifugation, increased fluorescence intensities were observed at high galectin-3 concentrations. After centrifugation, a galectin-3 concentration dependent loss of fluorescence was observed, possibly due to the precipitation of galectin-3/TLR4 aggregates (A). Normalized raw fluorescence time traces from an MST experiment are exemplified for a single dataset from 16 capillaries with labelled TLR4 and different concentrations of galectin-3. The MST signal is due to several subsequent processes separated by their respective timescales and dependence of the IR laser pulse and are described in detail in (Seidel et al., 2013). An initial equilibrium phase produces a horizontal line at F_{norm} = 1. After 5 s the IR laser is turned on and a sharp drop in fluorescence is observed (called 'T-jump', lasts a few 100 ms), followed by the thermophoretic molecule motion ('thermophoresis', last for several seconds). After 30 s laser-on time, the laser is switched off and an inverse T-jump is observed, followed by back-diffusion of the molecules. The experimental F_{norm} values used for the galectin-3-TLR4 interaction were derived from analysis of both the temperature jump and thermophoretic effects. The top curves are those from capillaries with the highest galectin-3 concentrations. Their wavy and bumpy appearance may be due to formation of galectin-3/TLR-4 aggregates, but did not prevent calculation of reproducible binding curves (B). Structure of the fluorescein tagged TDG-amine probe used (C). Steady state fluorescence anisotropy measured (Y-axis) when 20 nM probe was mixed with increasing concentrations of full galectin-3 (black symbols) or galectin-3 CRD (red symbols) (D). Lines show best fit model curves as described in (Salomonsson et al., 2010b). The anisotropy was 35 mA for the free of the TDG-amine probe, and 108 mA or 102 mA for the complex of probe

with galectin-3 or galectin-3 CRD, respectively, as estimated from binding curves. Steady state anisotropy of a fixed concentration of galectin (0.2 μM) and probe (20 nM) (marked by symbols X) with increasing concentration of TLR4 (round symbols) for full galectin-3 (black symbols) or galectin-3 CRD (red symbols) (E). MicroScale Thermophoresis was also used to analyse the direct binding of TLR4 to galectin-1 and galectin-4 (F). The measurements were analyzed in the same way as previously described for the TLR4/galectin-3 binding curve (Figure 2) that is shown for comparison.

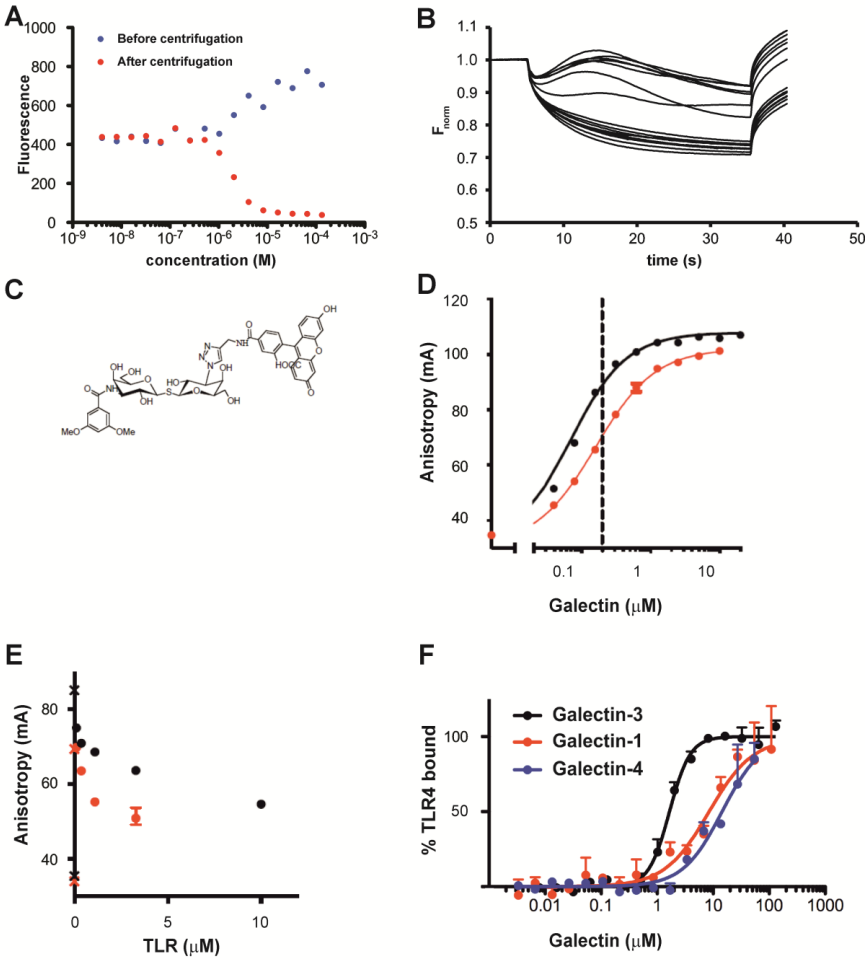

A
Before centrifugation
After centrifugation
Fluorescence
concentration (M)
B
F_{norm}
time (s)
C
D
Anisotropy (mA)
Galectin (μM)
E
Anisotropy (mA)
TLR (μM)
F
Galectin-3
Galectin-1
Galectin-4
% TLR4 bound
Galectin (μM)

Table S1 related to Figure 2. Interaction of galectin-3 protein with TLR4 based on the data in Figure 2C and D and Figure S2F. (SE represents standard error).

	MST (Fig. 2C & Figure S2F)		FA (Fig. 2d)	
Galectin	EC50 μM ± SE	Hill-coef. ± SE	EC50 μM ± SE	Hill-coef. ± SE
Galectin-3	1.7 ± 0.1	2.3 ± 0.3	0.3 ± 0.06	0.4 ± 0.05
Galectin-3 CRD	1.3 ± 0.3	1.4 ± 0.4	0.9 ± 0.3	1.1 ± 0.4
Galectin-3 R186S	43.5 ± 1.9	2.2 ± 0.2		
Galectin-1	8.0 ± 3.0	1.1 ± 0.3		
Galectin-4	14.0 ± 6.0	1.2 ± 0.4		

Figure S3 related to Figure 3. Galectin-3 stimulates a pro-inflammatory M1 phenotype in microglia and dependent of MyD88. Galectin-3 stimulates a pro-inflammatory M1 phenotype in microglia. Reduced arginase activity after sGal3 (1μM) and LPS (1 μg/ml) treatment for 24 h (A). mRNA expression of several markers after 24 h treatment with sGal3 (1μM) (B). Knockdown of MyD88 in BV2 (C) decreases iNOS expression upon galectin-3 treatment after 6 hours. Data are expressed as Mean +/- SD (n=3) for A, B and C and n=4 in D. * $P < 0.05$ ** $P < 0.01$**** $P < 0.0001$

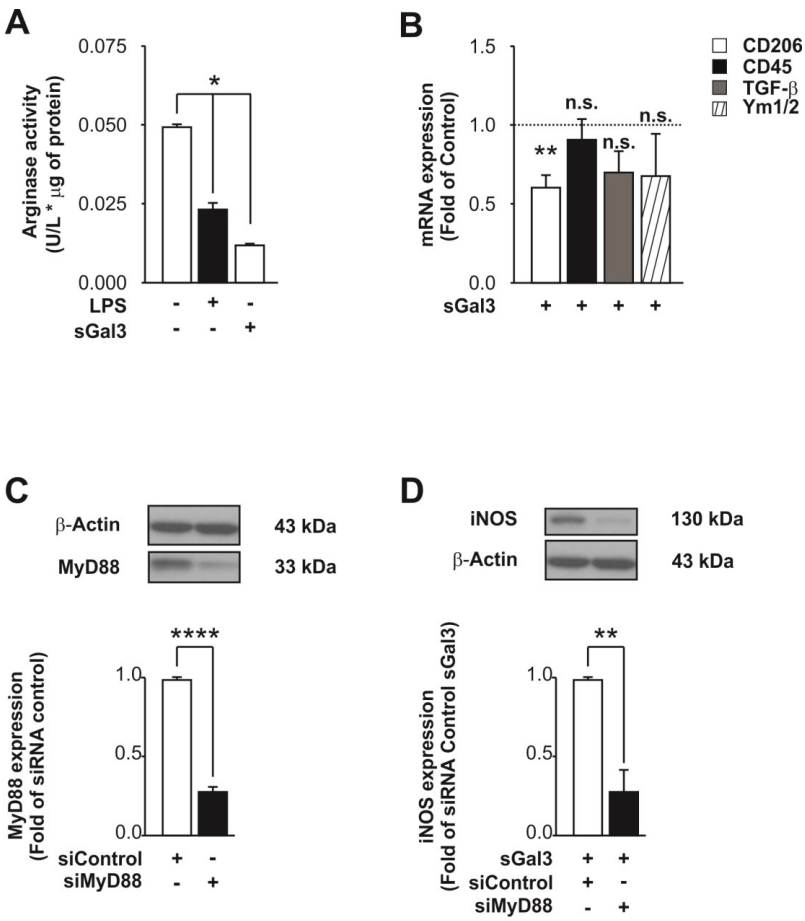


Figure S4 related to Figure 4. The induction of caspase activity after galectin-3 treatment does not correspond to an increase in cell death. Quantification of condensed nuclei (A), loss of the mitochondrial potential (B) and appearance of cleaved PARP (C and D) after 24 h treatment with sGal3. LPS was used as a control for activation of microglia and staurosporine (STS) as a positive control for cell death. Analysis of the cell cycle 6 h later of sGal3 and LPS treatments (E and F). Data are expressed as Mean +/- SD (n=4). * $P < 0.05$

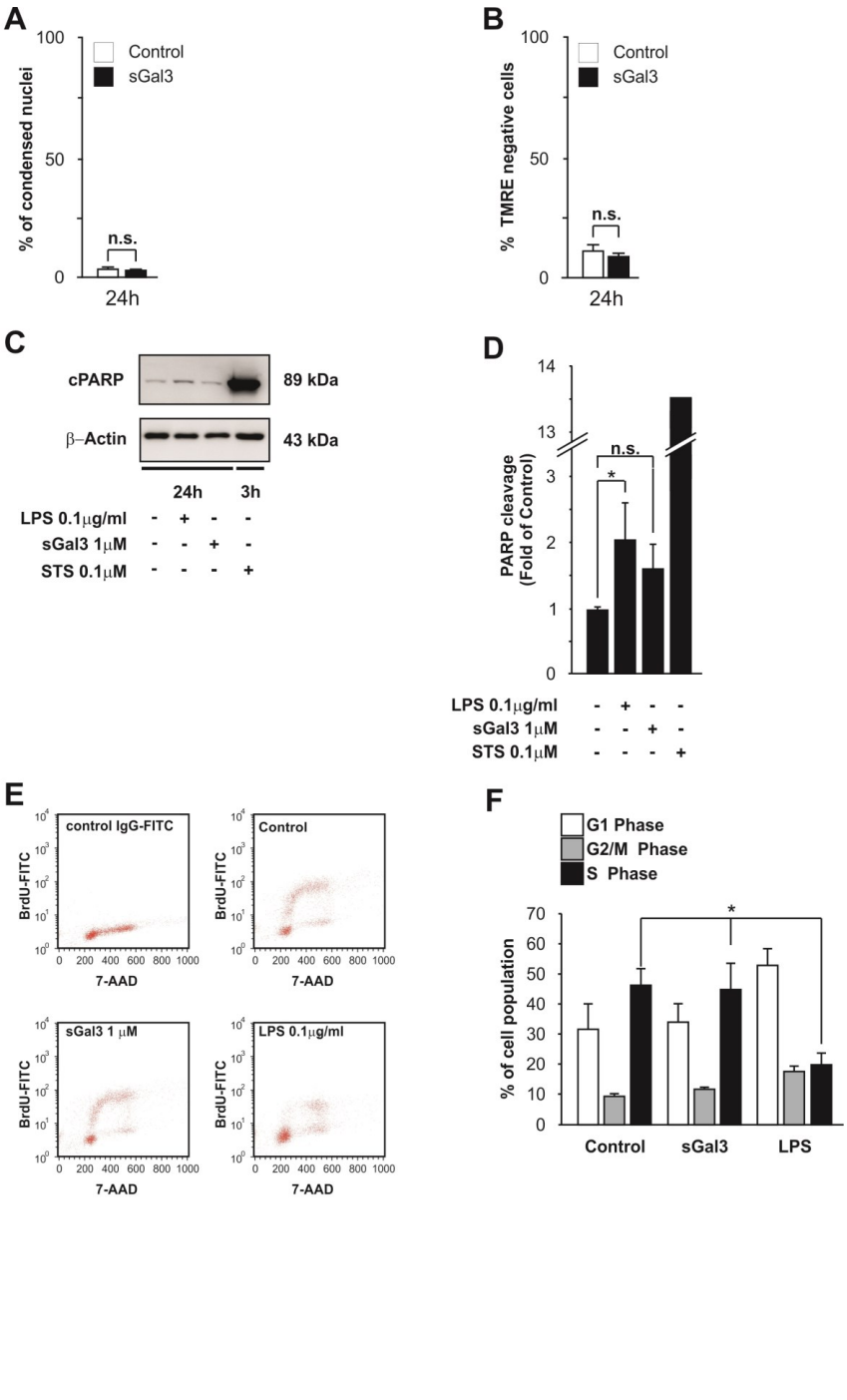

A
100
50
0
% of condensed nuclei
Control
sGal3
n.s.
24h
B
100
50
0
% TMRE negative cells
Control
sGal3
n.s.
24h
C
cPARP
89 kDa
β–Actin
43 kDa
24h
3h
LPS 0.1μg/ml - + - -
sGal3 1μM - - + -
STS 0.1μM - - - +
D
14
13
3
2
1
0
PARP cleavage (Fold of Control)
n.s.
*
LPS 0.1μg/ml - + - -
sGal3 1μM - - + -
STS 0.1μM - - - +
E
control IgG-FITC
Control
sGal3 1 μM
LPS 0.1μg/ml
BrdU-FITC
7-AAD
0 200 400 600 800 1000
F
G1 Phase
G2/M Phase
S Phase
70
60
50
40
30
20
10
0
% of cell population
*
Control
sGal3
LPS

Figure S5 related to Figure 5. Galectin-3 released from microglia affect caspase 3/7 and 8 activity upon LPS treatment in BV2 cells.

Quantification of galectin-3 released upon LPS treatment (A). Immunofluorescence of TLR4 and galectin-3 showing co-localization after 24 h treatment with LPS 1 µg/ml (B). Immunoblot with quantification showing successful galectin-3 knockdown in BV2 cells (C). Comparison of DEVDase and IETDase activity after 6 and 24 h after LPS treatment in cells treated with siRNA against galectin-3 *vs* siRNA negative control (D), and cells incubated with neutralization antibody against galectin-3 or isotype negative control antibody (E). Data are expressed as Mean +/- SD (n=4).

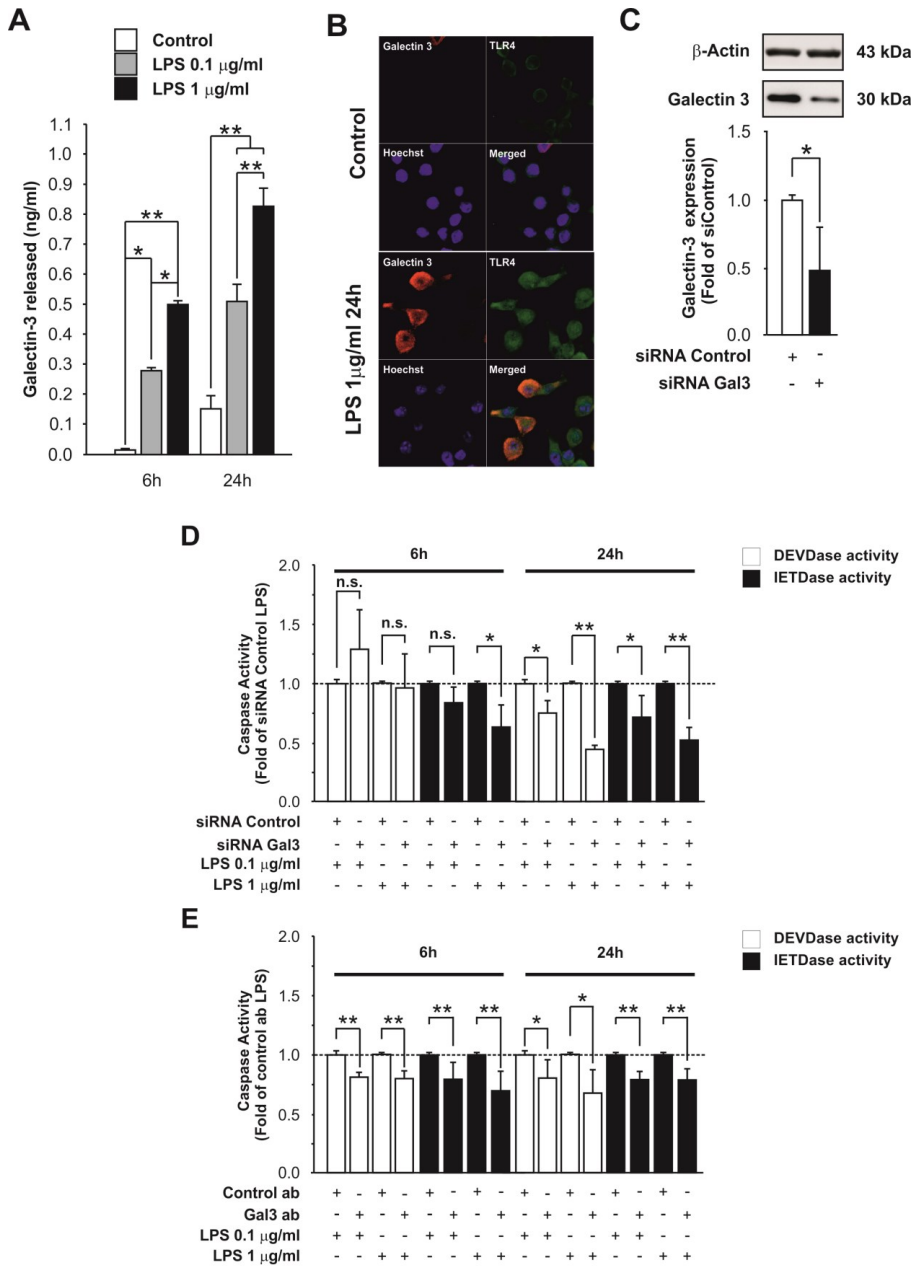

A
Control
LPS 0.1 μg/ml
LPS 1 μg/ml
Galectin-3 released (ng/ml)
6h
24h
B
Control
LPS 1μg/ml 24h
Galectin 3
TLR4
Hoechst
Merged
C
β-Actin
43 kDa
Galectin 3
30 kDa
Galectin-3 expression (Fold of siControl)
siRNA Control
siRNA Gal3
D
6h
24h
DEVDase activity
IETDase activity
Caspase Activity (Fold of siRNA Control LPS)
n.s.
siRNA Control
siRNA Gal3
LPS 0.1 μg/ml
LPS 1 μg/ml
E
DEVDase activity
IETDase activity
Caspase Activity (Fold of control ab LPS)
Control ab
Gal3 ab
LPS 0.1 μg/ml
LPS 1 μg/ml

Figure S6 related to Figure 6. Lack of galectin-3 the proliferation and reactivity of microglial in mouse substantia nigra after LPS injections.

Quantification of proliferative (BrdU+, red) microglia (Iba1+, green) in the substantia nigra 24 h after LPS injection in wild type and galectin-3 knockout mice (A and B). Illustration of Iba-1 immunostaining 7 days after treatment (C). Confocal analysis and quantification of Iba1/CD16-positive cells 7 days after LPS treatment (D and E). Confocal analysis of galectin-3 and TLR-4 in double heterozygous Cx3cr1GFP/+Ccr2RFP/+ mice (GFP is expressed only in microglia and RFP is expressed only in monocytes) (F). Data are expressed as Mean +/- SD (n=4) in B and E. Scale bar for panel C represents 30 μm and for panel D 56 μm.

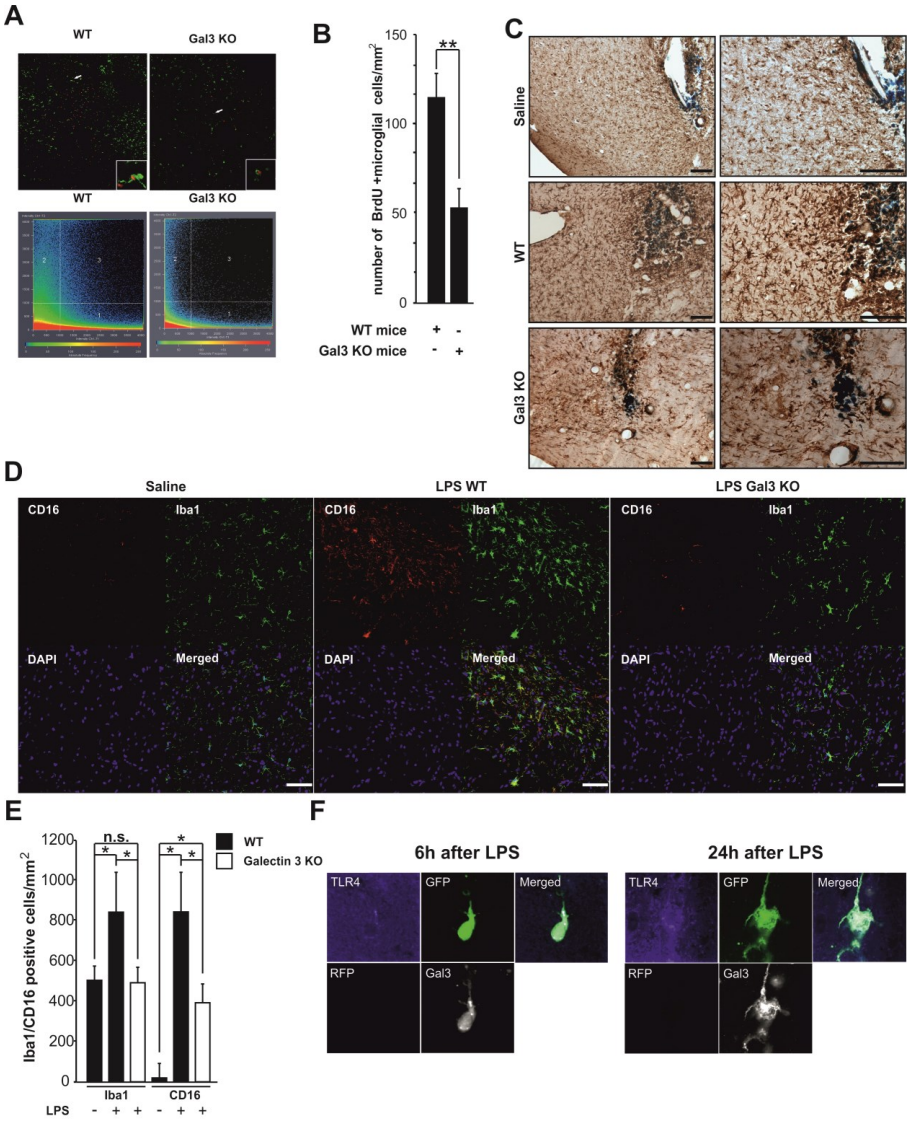
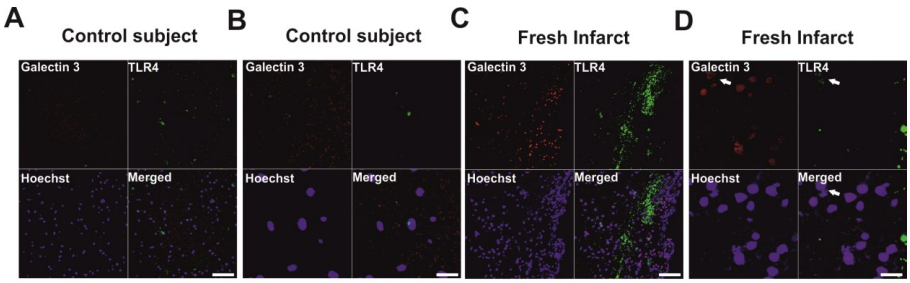
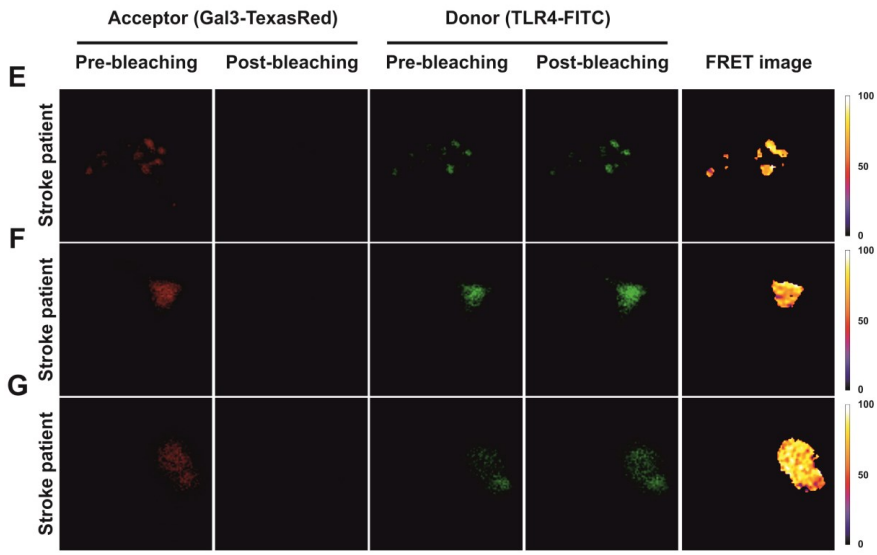

A
WT
Gal3 KO
WT
Gal3 KO
B
number of BrdU +microglial cells/mm2
150
100
50
0
**
WT mice + -
Gal3 KO mice - +
C
Saline
WT
Gal3 KO
D
Saline
LPS WT
LPS Gal3 KO
CD16
Iba1
DAPI
Merged
E
Iba1/CD16 positive cells/mm2
1200
1000
800
600
400
200
0
n.s.
*
WT
Galectin 3 KO
Iba1
CD16
LPS - + + - + +
F
6h after LPS
24h after LPS
TLR4
GFP
Merged
RFP
Gal3

Figure S7 related to Figure 7. Galectin-3 interacts with TLR4 in human brain tissue.

The expression of galectin-3 and TLR4 was also investigated in post-mortem brain tissue from patients who had suffered and died from cardiac arrest. High expression of both galectin-3 and TLR4 was observed in the ischemia-damaged brain tissue as compared to age-matched controls (Panels A-D). Both markers were found to be present, suggesting that they might be colocalized (D). Finally, FRET signal between galectin-3 and TLR4 could also be detected in cells in human stroke brain (Panels E-G).


A
Control subject
B
Control subject
C
Fresh Infarct
D
Fresh Infarct
Galectin 3
TLR4
Hoechst
Merged


Acceptor (Gal3-TexasRed)
Donor (TLR4-FITC)
Pre-bleaching
Post-bleaching
Pre-bleaching
Post-bleaching
FRET image
E
F
G
Stroke patient
Stroke patient
Stroke patient
100
50
0

Supplemental Experimental Procedures

Immunohistochemistry

Sections were incubated with the indicated primary antibodies. After three washes, sections were incubated with biotinylated horse anti-mouse or goat anti-rabbit IgG (Vector) followed by an incubation with ExtrAvidin-Peroxidase solution (Sigma) and for immunofluorescence by a fluorescein isothiocyanate (FITC)-conjugated anti-rabbit and Texas Red anti-mouse antibody (Vector). The peroxidase was visualized with a standard diaminobenzidine/hydrogen reaction for 5min. For paraffin-embedded human tissue material, sections (5µm) were mounted on capillary glass slides (DAKO). Sections were microwaved pre-treated in 10mM citrate buffer pH 6.0 for 10min at 800W for antigen retrieval. An automated immunostainer (TechMateTM 500 Plus, DAKO) was used for the staining procedure using DAKO ChemMate Kit Peroxidase/3-3diaminobenzidine. The primary antibodies used are listed in the supplementary antibody list.

Immunoprecipitation, Immunoblot and RT-PCR analysis

Physical interaction between proteins was determined by immunoprecipitation analysis of BV2 cells. 1.2 mg of protein per sample was immunoprecipitated. We follow the method described in (Qiu et al., 2002) with slight modifications. Cells are lysed after treatment, homogenized with one pulse of 30 s sonication, and pelleted by centrifugation in buffer containing 20 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 µM *p*-amidinophenylmethanesulfonyl fluoride hydrochloride, 50 mM NaF, and 10% glycerol. The supernatants are pre-cleared with protein G-sepharose (GE Healthcare) for 4 h at 4°C. Later on, the cleared

supernatants are incubated with 2.5 μg of anti-TLR4 (Santa Cruz Biotech.) antibody and normal rabbit IgG (R&D systems) as a negative control overnight. The next day, the lysates are incubated with protein G-sepharose for 4 h at 4°C. The immunoprecipitates were washed four times with buffer containing 50 mM Tris-HCl, pH 7.5, 0.1% SDS, 1% NP-40, and 62.5 mM NaCl and subsequently dissolved in denaturing sample buffer.

All cell extracts were processed for immunoblotting with a SDS-polyacrylamide gel electrophoresis as described previously (Joseph et al., 2002). Rabbit polyclonal antibody directed against iNOS (Santa Cruz Biotech) mouse monoclonal antibody β-actin (Sigma Aldrich), rat polyclonal antibody directed against galectin-3 (from Dr. Hakon Leffler), rabbit polyclonal antibody directed against TLR4 (Santa Cruz Biotech) and rabbit polyclonal against Cleaved PARP (Cell Signalling) were employed.

Mouse monoclonal antibody β-actin (Sigma Aldrich), was used to verify equal loading of the gel. Secondary horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were from Vector labs. Secondary horseradish peroxidase-conjugated anti-rat was from GE Healthcare UK.

RT-PCR was performed to measure the gene expression of intracellular TLR4 signalling pathways. mRNA was extracted using the RNeasy Mini kit from Qiagen. Using the Maxima™ First Strand cDNA Synthesis Kit for RT-PCR (Fermentas, Sweden), 1 μg mRNA was transformed into cDNA.

Primary microglia cell culture and cytokine release measurement

Primary cortical microglial cells were obtained from TLR4 knockout, galectin-3 knockout and wild type postnatal P 1-3 mouse brain using a previously described

protocol(Giulian and Baker, 1986) of mixed glial culture. After treatment of the primary microglia, the conditioned medium was collected and snap frozen until the cytokine analysis. The Cytokine content of the conditioned medium was analysed using the Mouse TH1/TH2 9-PlexTissue Culture Kit (Meso-scale Discovery) using manufacturer's instructions.

Gene expression array analysis.

Toll-like receptor signalling pathway was analysed using the Mouse Toll-like receptor signalling pathway PCR array (SABiosciences). mRNA was obtained after 6 h treatment with either, sGal3, iGal3 and LPS (as a TLR-4 agonist positive control). We synthesized cDNA from 1 μg of mRNA using RT^2 First Strand Kit from Qiagen.

Y-maze spatial memory test

In order to examine any deficits in hippocampus-dependent spatial memory, a Y-maze test was performed 5 days after ischemia. For this purpose we used a Y-maze arena (21x4 cm/arm) with visual cues put in 5 groups of 2 cues each around the maze. The mice were first trained for 2 trial sessions (one arm of the maze was closed) of 5 minutes day 4 and day 5. The real probe test was performed 2 hours after the last trial session in order to test the long-term memory to study how much the third arm; "new arm" was explored. A mouse with intact memory will notice that the two other arms has been explored before and will spend more time in the new arm and enter this arm more frequently compared to the two other arms. The movements of the mice was recorded with a camera and later analysed with SMART software system (PanLab, Spain).

Immunohistochemistry data analysis

Stereological analysis of TH-immunopositive neurons in SN was performed using a CAST Grid Stereology System (Olympus). Thus, a bounded region of the SN with a length of 150 microns in the anterior-posterior axis centered at the point of injection was used for analysis (3.5mm with respect to bregma). For each animal, three sections (sampling fraction 1:3) were systematically sampled along the anterior-posterior axis from a random starting point, following stereological criteria (Gundersen et al., 1988).

The number of TH-positive neurons in the SN was estimated using a fractionator sampling design (Gundersen et al., 1988). Counts were made at regular predetermined intervals (x = 150 µm and y = 200 µm) within each section. An unbiased counting frame of known area (40 × 25 µm = 1000 μm^2) was superimposed on the tissue section image under a 100× oil immersion objective. Therefore, the area sampling fraction was 1000/ (150 × 200) = 0.033. The entire z-dimension of each section was sampled; hence, the section thickness sampling fraction was 1. In all animals, 25-µm sections, each 100 µm apart, were analyzed; thus, the fraction of sections sampled was 20/100 = 0.20. The number of neurons in the SN was estimated by multiplying the number of neurons counted by the reciprocals of the area sampling fraction and the fraction of section sampled.

Hippocampal cell death analysis following global brain ischemia was performed in the ischemia-sensitive hippocampal subregion Cornu Ammonis 1 (CA1). Viable pyramidal neurons were identified by NeuN-positive cells with typical round homogenous immunoreactivity. The quantitative analysis was made by performing a double-blind manual assessment of viable neurons using x40 objective (Nikon Eclipse 80i). 30 µm thick coronal brain sections were quantified at three different

bregma levels (-1.46, -1.94 and -2.46), to give a total number of viable NeuN-positive cells. The entire *z*-dimension of each section was used to estimate the total number of cells. Each hippocampus contributed to one individual CA1 cell count value.

Human brain

Human brain tissue was obtained from patients who suffered and died from cardiac arrest and from age-matched controls for this study. The regions investigated were the central/periventricular white matter, within the border zones. The patients with dissimilar duration of disease exhibited different degrees of severity of brain disease, reflecting different stages of the ischemic-degenerative process. All sections were stained with haematoxylin and eosin and with antibodies against galectin-3 and TLR4. They were microscopically reviewed for verification of pathology. Before the investigation, the entire collection of brain sections, were subjected to a neuropathological whole-brain analysis for clinical diagnostic purposes, according to routine procedures at the Department of Pathology, Division of Neuropathology, Lund University Hospital. The project procedures involving human brain tissue were approved by the Regional Ethical Review Board in Lund (Sweden), Dnr 196/2010).

Brain microdialysis

Microdialysis in the substantia nigra (SN) was performed with an I-shaped cannula (Santiago and Westerink, 1990). The exposed tip of the dialysis membrane was 2mm. The dialysis tube (ID: 0.215 mm; OD: 0.315 mm) was prepared from polyarylethersulfone/ polyvinylpyrrolidone (Theralite 2100, Gambro, Hechingen, Germany). The probe was stereotaxically implanted into both SN with coordinates from bregma and dura; 0.6 mm posterior, lateral 2.8 mm and ventral 6.0 mm.

The perfusion experiments were carried out 24 h after implantation of the probe. The SN was perfused at a constant flow rate of 2.0 µl/min, using a microperfusion pump (model 22, Harvard Apparatus, South Natick, MA, U.S.A.), with a Ringer's solution containing NaCl, 140 mM; KCl, 4.0 mM; CaCl2, 1.2 mM; and MgCl2, 1.0mM. After 24 h of the LPS injection, the dialysate was collected for four hours and frozen for later analysis of galectin-3. The value of galectin-3 release induced by LPS injection was compared with saline injection for the same time point.

Caspase activity assays

Changes in caspase activity in microglia were measured using a luciferase based assay from Promega known as Caspase-Glo® (G890 for caspase 3/7 and G8201 for Caspase 8). Equal volumes of cells and kit component were mixed and incubated for 1 h at room temperature. The plate was analysed using a Luminometer and the value obtained was normalized with the number of cells used.

Quantification of condensed nuclei

After 24h and 48h of treatment with soluble galectin-3, the cells were fixed with 4% paraformaldehyde for 10 min and stained with 0.1µg/ml Hoechst for 15 minutes and washed 3 times with PBS. Cells were analysed by fluorescence microscopy. A minimum of 400 cells were counted per treatment.

Determination of the apparent affinity between galectin-3 and TLR4

Protein interaction studies were performed using MicroScale Thermophoresis (MST). (Duhr and Braun, 2006; Seidel et al., 2013; Wienken et al., 2010). For labeling, TLR4 (1478-TR; R&D systems, Minneapolis, USA) and the fluorescent dye NT-547-NHS were mixed at concentrations of 7 µM and 20 µM, respectively, in a total volume of

200 μL and purified according to the supplied protocol of the Monolith NT Protein Labeling Kit Green-NHS (NanoTemper Technologies, Munich, Germany). Before filling into capillaries, samples were premixed to give volumes of 20 μL. The unlabeled binding partners, galectin-3 and galectin-3 R186S , galectin-1 (1152-GF-050/CF, R&D systems, Minneapolis, USA) and galectin-4 (1227-GA-050/CF, R&D systems, Minneapolis, USA) were titrated in 1:1 dilutions using highest final concentrations of 130, and 104, 109 and 109 μM, respectively. Labeled TLR4 was added to give a final concentration of 120 nM.. For the lactose inhibition assay, the highest final concentration of galectin-3 was 120 μM, lactose and labeled TLR4 were added to give concentrations of 40 μM and 110 nM, respectively. All measurements were performed in 50 mM Tris–HCl buffer, pH 7.6 containing 150 mM NaCl, 10 mM MgCl2 and 0.05% Tween-20 (MST-buffer) using hydrophilic capillaries (NanoTemper Technologies) in a Monolith NT.115 system (Nano-Temper Technologies) using 50% LED. The galectin-1/TLR4 interaction was measured in MST-buffer supplemented with 5 mM TCEP as reducing agent. The IR-laser power was set to 40 %, the laser on and off times were set at 30 s and 5 s, respectively. The signal output of the thermophoretic analysis is represented as normalized fluorescence values (F_{norm}). These values were used to calculate the fraction of TLR4 bound to the galectin molecules-3 and plotted against the concentrations of galectin-3 on a logarithmic scale. Binding curves were averaged from two measurements for the galectin-3/TLR4/lactose, and galectin-3 CRD/TLR4 and galectin-4/TLR4 interactions, and from three measurements for the galectin-3/TLR4, galectin-3 R186S/TLR4 and galectin-1/TLR4 interactions, respectively. Assuming that the free concentration of the ligand was not altered by complex formation, the binding data were fit using the Hill equation in Prism (GraphPad, USA).

Fluorescence anisotropy

The interactions of TLR4 with galectin-3 proteins were also tested by analyzing its potency to inhibit galectin binding to a fluorescein tagged saccharide probe (TDG-amine (Salomonsson et al., 2010b), supplementary Figure 2c) using fluorescence anisotropy, in the same way as we have used extensively before for both small molecules and glycoprotein inhibitors (Lepur et al., 2012; Salomonsson et al., 2010a; Salomonsson et al., 2010b). In brief, a fixed concentration of galectin-3 was mixed with a fixed concentration of a fluorescein tagged saccharide probe and a range of concentrations of TLR4. Steady state anisotropy was measured using a PheraStar plate reader (BMG, Offenburg, Germany). This instrument uses excitation with plane polarized light (at 485 nm in this case), splitting of the emission (in this case at 520 nm) and simultaneous detection of its two planes of polarization by two photomultipliers. The instrument software (PHERAstar Mars version 2.10 R3) was used for initial gaining and calibration, using fluorescein as a standard set to anisotropy 23 mA. The anisotropy of the TDG-amine probe was 35 mA, and 108 mA or 102 mA for the complex of probe with galectin-3 or galectin-3 CRD, respectively, as estimated from binding curves of a fixed concentration of probe (0.02 μM) with increasing concentrations of either protein (Figure S2D). Then the same concentration of probe was mixed with a fixed concentration of galectin-3 (0.2 μM, indicated by dotted vertical line in supplementary Figure 2E) and a range of concentrations of TLR4. The measured values of anisotropy were used to calculate concentrations of free and bound probe, which are linearly related to the anisotropy value (Lakowicz, 2006). Since the total concentration of galectin, probe and TLR4 are known, the concentration of all components, bound and unbound, can be calculated by solving the system of mass action equations given by the two binding

pairs galectin-probe and galectin-TLR4 as given in detail in (Salomonsson et al., 2010b). There was no evidence for direct interaction between the probe and TLR4 when tested alone without galectin-3, and, hence, this interaction pair was ignored in the calculations. All measurements were made in half-area plates (black, Costar) in a total reaction volume of 40 μl.

Quantification of Mitochondrial Activity

The loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) was quantified using tetramethylrhodamine, ethyl ester (TMRE) staining (Invitrogen). Mitochondria with an intact ψ_m are labelled with the potential-dependent dye TMRE (25 nM). Cells were incubated with TMRE for 30 min at 37°C. The samples were passed through FACSCalibur flow cytometer (Becton Dickinson) and the data obtained were analyzed using Cell Quest software.

Cell cycle analysis

For the measurement of the different cell cycle phases in microglia, FITC BrdU Flow Kit from BD Pharmingen (Cat no. 559619) was used. A BrdU pulse (final concentration 10 μM) was given to the microglial cells during the last 15 minutes of the treatment. Samples were prepared following the manufacturer's recommendations, and analysed using a FACSCalibur flow cytometer (Becton Dickinson) using the Cell Quest software.

Quantification of arginase activity

Arginase activity was measured at 24h after treatment with galectin-3 and used LPS as a positive control. QuantiChrom^{TM} Arginase Assay Kit (DARG-200) was used to measure arginase activity, which was then normalized with the protein content of each sample.

Measurement of galectin-3 release

The amount of galectin-3 released after LPS treatment in the media was analysed using the galectin-3 kit from BG-Medicine. 10^4 BV2 cells were seeded per well in a 96 well plate for each treatment and time point.

Cell proliferation assay

Mice were injected twice with a BrdU solution (10 mg/ml) (12 hours after surgery and then 1 hour before sacrifice) at a dose of 50 mg/kg. BrdU positive cells were quantified by immunofluorescence using a BrdU antibody. Using a Zeiss LSM 7 Duo, 25 pictures at 40x were taken per each substantia nigra and then we quantified the whole area for BrdU positive cells.

Quantification of microglial population in animal models

Macrophages and reactive microglial cells were counted in LPS-injected mice detected by Iba1 immunohistochemistry based on morphological features or CD16/32 expression, a marker of M1-polarized microglia. In the former case, for each animal, eight sections covering the entire antero-posterior ventral mesencephalon were analyzed. For each section, eight photographs were taken at 20x magnification (four for each substantia nigra) and microglial cells were counted.

For quantification of M1 polarized microglia, five sections (sampling fraction 1:3) were systematically sampled along the anterior-posterior axis from a starting point coinciding with the injection point, following stereological criteria and photographs were taken in the substantia nigra. The number of Iba1 (microglia) and double Iba1/CD16/32 (M1 polarized microglia) were counted with the aid of a computer-assisted software CAST-Grid de Olympus.

Acceptor photobleaching-Fluorescent resonance energy transfer (FRET)

Acceptor photobleaching Fluorescent resonance energy transfer (FRET) measurements were performed on a Zeiss LSM 7 DUO TCS SP inverted confocal scanning laser microscope using a Plan-Apochromat 40x/1.3 Oil DIC objective. A detailed description of the FRET technique can be found elsewhere (Kenworthy, 2001; Wouters et al., 2001). To determine FRET, we quantified the quenching of donor fluorescence (TLR4-FITC) by performing acceptor photobleaching (Galectin-3-Texas Red) using a HeNe 2mW 594 nm laser. The sections were double stained against TLR4 and galectin-3 and then labeled with FITC conjugated secondary goat anti rabbit and also with Texas Red conjugated secondary goat anti rat antibodies (Vector labs). Pictures were taken using 488 and 594 nm lasers and collected separately. The acceptor, Texas Red, was then irreversibly photobleached in a selected adequate region by continuous excitation with 2000 interactions of 594 nm laser. Thereafter, the residual Texas Red and FITC image was obtained, and identical regions, at the plasma membrane on individual cells, were outlined in the photobleached area and processed with ImageJ software using the plugin AccPbFRET (Roszik et al., 2008). Ratios between FITC intensities of the plasma membrane region, after and before photobleaching, were calculated to quantify

FRET. The FRET images presented here have been processed using a correction factor set for donor bleaching that was established with the donor only (FITC).

Primers list including the sequence:

Name	Forward sequence (5´⟶3´)	Reverse sequence (5´⟶3´)
TLR4	ATGGAAAAGCCTCGAATCCT	CTCTCGGTCCATAGCAGAGC
G3PDH	TGCACCACCAACTGCTTAGC	GGCATGGAACTGTGGTCATGAG
CD206	CAAGGAAGGTTGGCATTTGT	CCTTTCAGTCCTTTGCAAGC
TGF-β	TGCGCTTGCAGAGATTAAAA	CGTCAAAAGACAGCCACTCA
Ym1/2	CAGGGTAATGAGTGGGTTGG	CAGGCACCTCCTAAATTGT
CD45	TCATGGCACACGATGTGAAGA	AGCCCGAGTGCCTTCCT

Recombinant protein list including company name and catalogue number:

Recombinant protein	Catalog number	Company
Human Galectin-3, carrier free	1154-GA-050/CF	R & D
Human Galectin-1, carrier free	1152-GA-050/CF	R & D
Human Galectin-4, carrier free	1127-GA-050/CF	R & D
Human TLR4, carrier free	1478-TR-50	R & D

Antibody list: including the company name, catalogue number and technique where it was used.

Name (purpose)	Company	Catalog number
Mouse Anti β-Actin (WB)	Sigma-Aldrich	A-3582
Rat Anti BrdU (IHC)	AbD Serotec	OBT 0030
Rabbit Anti Cleaved PARP (WB)	Cell Signaling	9544
Mouse Anti CD16/32 (IHC)	BD Pharmingen	553141
Mouse anti-Galectin 3 (Neutralization)	BD Pharmingen	556904
Rat anti-Galectin 3 (IF and WB)	Hakon Leffler's lab	
Goat anti-Galectin 3 (IHC)	R & D Systems	AF1197
Mouse IgG κ1 isotype control (Neutralization)	BD Pharmingen	556648
Mouse Anti-Iba1 (IHC)	Millipore	MABN92
Rabbit Anti-Iba1 (IHC)	Wako	019-19741
Rabbit IgG Control (IP)	R & D	AB-105-C
Rabbit Anti-MyD88 (WB)	Cell Signaling	4283
Mouse Anti-NeuN (IHC)	Chemicon	MAB377
Mouse Anti-RFP (IHC)	Abcam	ab65856
Rabbit Anti-TH (IHC)	Sigma-Aldrich	T8700
Rabbit Anti-TLR4 (IP)	Santa Cruz Biotech.	sc-10741
Rabbit Anti-TLR4 (IHC)	Abcam	ab13556
Rabbit Anti-iNOS (WB)	Santa Cruz Biotech.	sc-650

SMART Pool Dharmacon siRNA sequences

Name	Catalog number	Sequence
siControl	D-001810-01	UGGUUACAUGUCGACUAA
siTLR4(1)	J-047487-05	GCAUAGAGGUAGUUCCUAA
siTLR4(2)	J-047487-06	GAGUUCAGGUUAACAUAUA
siTLR4(3)	J-047487-07	GGAAUUGUAUCGCCUUCUU
siTLR4(4)	J-047487-08	UGACGAACCUAGUACAUGU
siLGal3S3(1)	J-041097-09	GAGAGAUACCCAUCGCUUU
siLGal3S3(2)	J-041097-10	ACUUCAAGGUUGCGGUCAA
siLGal3S3(3)	J-041097-11	ACAGUGAAACCCAACGCAA
siLGal3S3(4)	J-041097-12	GGAUGAAGAACCUCCGGGA
siMyD88(1)	J-063057-08	GUUAGACCGUGAGGAUAUA
siMyD88(2)	J-063057-07	GCCUAUCGCUGUUCUUGAA
siMyD88(3)	J-063057-06	UGCCAGAAAUACUUAGGUA
siMyD88(4)	J-063057-05	CGACUGAUUCCUAUUAAAU

Supplemental References

Duhr, S., and Braun, D. (2006). Why molecules move along a temperature gradient. Proc Natl Acad Sci U S A *103*, 19678-19682.

Giulian, D., and Baker, T.J. (1986). Characterization of ameboid microglia isolated from developing mammalian brain. J Neurosci *6*, 2163-2178.

Gundersen, H.J., Bendtsen, T.F., Korbo, L., Marcussen, N., Moller, A., Nielsen, K., Nyengaard, J.R., Pakkenberg, B., Sorensen, F.B., Vesterby, A., *et al.* (1988). Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. APMIS : acta pathologica, microbiologica, et immunologica Scandinavica *96*, 379-394.

Joseph, B., Marchetti, P., Formstecher, P., Kroemer, G., Lewensohn, R., and Zhivotovsky, B. (2002). Mitochondrial dysfunction is an essential step for killing of non-small cell lung carcinomas resistant to conventional treatment. Oncogene *21*, 65-77.

Kenworthy, A.K. (2001). Imaging protein-protein interactions using fluorescence resonance energy transfer microscopy. Methods *24*, 289-296.

Lakowicz, J.R. (2006). Principles of Fluorescence Spectroscopy 3rd ed. , Vol XXVI.

Lepur, A., Salomonsson, E., Nilsson, U.J., and Leffler, H. (2012). Ligand induced galectin-3 protein self-association. J Biol Chem *287*, 21751-21756.

Qiu, J., Whalen, M.J., Lowenstein, P., Fiskum, G., Fahy, B., Darwish, R., Aarabi, B., Yuan, J., and Moskowitz, M.A. (2002). Upregulation of the Fas receptor death-inducing signaling complex after traumatic brain injury in mice and humans. J Neurosci *22*, 3504-3511.

Roszik, J., Szollosi, J., and Vereb, G. (2008). AccPbFRET: an ImageJ plugin for semi-automatic, fully corrected analysis of acceptor photobleaching FRET images. BMC bioinformatics *9*, 346.

Salomonsson, E., Carlsson, M.C., Osla, V., Hendus-Altenburger, R., Kahl-Knutson, B., Oberg, C.T., Sundin, A., Nilsson, R., Nordberg-Karlsson, E., Nilsson, U.J., *et al.* (2010a). Mutational tuning of galectin-3 specificity and biological function. J Biol Chem *285*, 35079-35091.

Salomonsson, E., Larumbe, A., Tejler, J., Tullberg, E., Rydberg, H., Sundin, A., Khabut, A., Frejd, T., Lobsanov, Y.D., Rini, J.M., *et al.* (2010b). Monovalent interactions of galectin-1. Biochemistry *49*, 9518-9532.

Santiago, M., and Westerink, B.H. (1990). Role of adenylate cyclase in the modulation of the release of dopamine: a microdialysis study in the striatum of the rat. Journal of neurochemistry *55*, 169-174.

Seidel, S.A., Dijkman, P.M., Lea, W.A., van den Bogaart, G., Jerabek-Willemsen, M., Lazic, A., Joseph, J.S., Srinivasan, P., Baaske, P., Simeonov, A., *et al.* (2013). Microscale thermophoresis quantifies biomolecular interactions under previously challenging conditions. Methods *59*, 301-315.

Wienken, C.J., Baaske, P., Rothbauer, U., Braun, D., and Duhr, S. (2010). Protein-binding assays in biological liquids using microscale thermophoresis. Nature communications *1*, 100.

Wouters, F.S., Verveer, P.J., and Bastiaens, P.I. (2001). Imaging biochemistry inside cells. Trends in cell biology *11*, 203-211.

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Forced treadmill exercise can induce stress and increase neuronal damage in a mouse model of global cerebral ischemia

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ABSTRACT

Physical exercise is known to be a beneficial factor by increasing the cellular stress tolerance. In ischemic stroke, physical exercise is suggested to both limit the brain injury and facilitate behavioral recovery. In this study we investigated the effect of physical exercise on brain damage following global cerebral ischemia in mice. We aimed to study the effects of 4.5 weeks of forced treadmill running prior to ischemia on neuronal damage, neuroinflammation and its effect on general stress by measuring corticosterone in feces. We subjected C57bl/6 mice (n = 63) to either treadmill running or a sedentary program prior to induction of global ischemia. Anxious, depressive, and cognitive behaviors were analyzed. Stress levels were analyzed using a corticosterone ELISA. Inflammatory and neurological outcomes were analyzed using immunohistochemistry, multiplex electrochemoluminescence ELISA and Western blot. To our surprise, we found that forced treadmill running induced a stress response, with increased anxiety in the Open Field test and increased levels of corticosterone. In accordance, mice subjected to forced exercise prior to ischemia developed larger neuronal damage in the hippocampus and showed higher cytokine levels in the brain and blood compared to non-exercised mice. The extent of neuronal damage correlated with increased corticosterone levels. To compare forced treadmill with voluntary wheel running, we used a different set of mice that exercised freely on running wheels. These mice did not show any anxiety or increased corticosterone levels. Altogether, our results indicate that exercise pre-conditioning may not be beneficial if the animals are forced to run as it can induce a detrimental stress response.

1. Introduction

Physical exercise is regarded as a promising treatment and complement to pharmacological treatments in several different neurological diseases (Svensson et al., 2014). Exercise can affect both the adrenergic and corticosteroid systems involved in stress response as well as the microglia function and other cells and signaling molecules involved in inflammatory processes (Svensson et al., 2014).

In the central nervous system (CNS), microglial cells are the major source of pro-inflammatory cytokines (Kim and de Vellis, 2005; Carson et al., 2006). In response to neuronal injury, such as stroke, resident microglia are immediately activated and start to proliferate and produce pro-inflammatory cytokines (Banati et al., 1993; Barone et al., 1997). It has been shown in both patients and animal models that cerebral ischemia leads to an inflammatory response, systemically as well as in the brain (Lambertsen et al., 2012). Neuroinflammatory response can further aggravate the neuronal damage and administration of the pro-inflammatory cytokine IL-1β after induction of cerebral ischemia in animal models can exacerbate the brain injury (Yamasaki et al., 1995). Microglial cells or their inflammatory mediator molecules may therefore be suitable targets for treating or preventing deleterious neuroinflammation following brain ischemia (Yenari et al., 2010). Several studies have shown that the levels of pro-inflammatory

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cytokines decrease, and the levels of anti-inflammatory cytokines increase following physical exercise (Mota et al., 2012; Piao et al., 2013). Indeed, treadmill running in animals has been shown to reduce the microgliosis and inhibit the release of pro-inflammatory cytokines, as well as reducing lesion size and cell death in the hippocampus and prevent short-term memory disturbances following cerebral ischemia (Austin et al., 2014; Lovatel et al., 2014; Sim et al, 2004, 2005).

Out-of-hospital cardiac arrest is a severe complication that can lead to global cerebral ischemia with pronounced neuronal damage. The survival rate is very low, below 10%, and patients that survive are often affected by severe neurological injury and lifelong cognitive and motor disabilities (Sasson et al., 2010). In an experimental setting, global cerebral ischemia can be modeled in mice by transient occlusion of the common carotid arteries (Olsson et al., 2003). Global cerebral ischemia results in neuronal cell death in vulnerable brain regions such as hippocampus (Kirino and Sano, 1984; Back et al., 2004) evolving during the first week after the ischemic insult (Bottiger et al., 1998). By altering detrimental neuroinflammatory reactions, physical exercises could make the brain more resistant to ischemic injuries.

On the negative side, exercise can also induce a chronic stress response, which may result in detrimental effects in the event of a brain injury. For example, forced running exercise in rodents can lead to anxiety and increase the levels of the stress hormone corticosterone in serum (Leasure and Jones, 2008; Brown et al., 2007). It has been shown that stress can evoke a pro-inflammatory response in the brain, with increased expression of NLRP3 inflammasome involved in the cleavage and secretion of pro-inflammatory IL-1β (Gadek-Michalska et al., 2013; Frank et al., 2014). Therefore, the overall positive effect of physical exercise in experimental models could potentially be masked by the stress response.

To the best of our knowledge, the effects of treadmill exercise pre-conditioning on stress and neuroinflammatory responses following global cerebral ischemia have not previously been studied in mice. Therefore, the aim of this study was to investigate the effect of pre-conditioning forced treadmill running on stress response, neuroinflammation, neuronal damage and behavioral alterations following global cerebral ischemia. An additional aim was to compare the stress response after forced running with the response after voluntary running.

2. Material and methods

2.1. Animals

All proceedings and animal treatment were in accordance with the guidelines and requirements of the government committee on animal experimentation at Lund University. We used 63 male C57Bl/6 mice, aged 8–10 weeks, weighing 22–27 g that were obtained from Charles River. The mice were housed in standard laboratory cages (3–7 animals per cage), with sawdust bedding and free access to water and food. They were allowed to acclimatize for at least 5 days before testing. The holding room had a 12:12 h light-dark cycle. The mice were weighed at the day of exercise introduction, and thereafter once every second week until the induction of global cerebral ischemia. Thereafter the mice were weighed 3–4 days as well as 10–12 days after ischemia. When the experiment was initiated, the mice were assigned to different groups ensuring an even distribution in body weight and age.

2.2. Experimental outline

An overview of the experimental outline can be seen in Fig. 1. Briefly, some of the animals were subjected to treadmill running exercise for 4.5 weeks. During the third week of exercise their anxious and motor behavior was assessed once with an Open Field test. After 4.5 weeks of exercise, some of the animals were subjected to ischemia. Behavioral tests were then conducted 5–15 days after ischemia, after which, the animals were sacrificed and samples were collected (15–16 days after ischemia).

2.3. Running exercise

2.3.1. Treadmill running exercise

Originally, the mice were divided into two different treatment groups, one subjected to exercise and one that was not subjected to any exercise (sedentary). Exercise consisted of 30 min treadmill running at a speed of 25 cm/s with no inclination of the treadmill (5-lane treadmill, Harvard Apparatus, Panlab). The mice were exercised 3 days/week for 4.5 weeks of which the first week was an introduction week with a lower speed and duration of the exercise. On the first day of the introduction, the mice were subjected to 10 min of walking/running at a speed increasing from 5 to 18 cm/s. The second day of the introduction consisted of 20 min of running with speeds up to 25 cm/s. During the third and last day of the introduction, the duration was increased to 30 min, with a speed of 25 cm/s. To motivate the mice to run the researcher pushed them with a small stick if needed. If the mouse refused to run it could be motivated by a transient and light electric stimulation from the grid at the beginning of the treadmill platform. After this, if the mouse persisted in its refusal to run, it was removed from the treadmill and re-introduced to it at a later time point. In this study, all mice without exceptions had to be motivated at least some times by pushing them with the small stick at each day of exercise. Four of our best running mice even had to be motivated with a light electric stimulation at up to three different days. Despite this, several mice refused to run after repeated trials. These mice were excluded from the exercise procedure, and referred to as “bad runners”. Exercise took place in a room separated from the housing room to which all mice were transferred and kept during the exercise procedures in order to minimize environmental confounders among the mice not subjected to the exercise protocol.

2.3.2. Voluntary wheel running

To investigate the effect on stress response resulting from the enforcement to run compared to voluntary running exercise, our main study was complemented with mice subjected to voluntary wheel running. For this, 10 male C57Bl/6 mice at an age of 12 month were used. These mice had the same housing conditions as the mice in the main study, except that they were single caged. Six of these

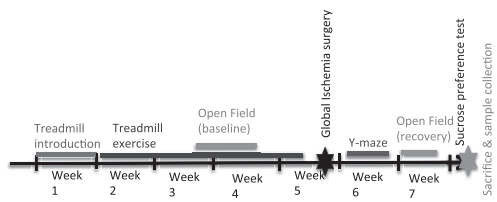

Fig. 1. Experimental Design. Mice in the exercise groups were introduced to treadmill running for a week and thereafter subjected to 3.5 weeks of exercise. During this time an Open Field test was conducted. After 3.5 weeks of exercise some of the exercised and non-exercised mice were subjected to global brain ischemia. The mice were allowed to recover for 3–4 days before Y-maze test was conducted. Then, a new Open Field test was performed followed by a sucrose preference test the night before the mice were sacrificed.

mice had access to low-profile wireless running wheel for mouse (med associates) in their home cages during 6.5 weeks. The running wheels had a wireless connection to a software measuring the distance each mouse ran every day, assuring that all mice with access to running wheels actually run. The remaining four mice were sedentary controls with no access to running wheels in their homecage. After 6.5 weeks of this running intervention, an Open Field test was performed (as described in 2.5.1) and feces samples were collected (as described in 2.6) in order to compare anxiety and corticosterone levels with the sedentary control group.

2.4. Induction of global cerebral ischemia

In order to model cardiac arrest patients with successful cardiopulmonary resuscitation, global ischemia was induced to the mice as previously described (Olsson et al., 2003; Burguillos et al., 2015; Lambertsen et al., 2011; Deierborg et al., 2008). Briefly, the mice were first anesthetized with 5% isofluorane in oxygen. Thereafter, the anesthesia was maintained using 2% isofluorane. A small cut parallel to the trachea was made. The common carotid arteries were then isolated and encircled with a thin silk thread to allow bilateral occlusion using a micro aneurysm clip. Ischemia was induced for 13 min. The wound was then sealed with absorbable stitches before the anesthesia was discontinued. During the surgery, the body temperature was monitored and controlled using a heating pad and an infrared lamp to keep the mice normothermic. The body temperature was maintained around 37.5 °C during the whole procedure. Mice with body temperatures below 35.5 °C a few hours after the intervention were put in an incubator at 34 °C overnight in order to recover. Mice in the sham groups were subjected to the same surgical protocol, except that no occlusion to the common carotid arteries was made. The person performing the surgery was blinded to the animals exercise regimen.

2.5. Behavioral tests

2.5.1. Open Field test

In order to evaluate the locomotion and anxiety levels of the mice subjected to forced treadmill exercise, an Open Field test was conducted before and after induction of ischemia. The test before the induction of ischemia was performed during the third week of exercise. The test was then repeated 12–13 days after the induction of ischemia. The mice were put in an Open Filed arena (Panlab, Barcelona, Spain, 45 cm by 45 cm) and allowed to freely explore it for 10 min. An automated SMART system (Panlab, Barcelona, Spain) was used to measure the velocity of movements, distance moved and time spent in the center and periphery of the box. Spending more time in the periphery was regarded as a sign of anxiety. The box was cleaned first with ethanol and then with water before each mouse was introduced to the Open Field arena. Furthermore, anxious behavior in the Open Field test of mice subjected to voluntary wheel running with age-matched sedentary controls were also analyzed to test if voluntary running results in anxious behavior.

2.5.2. Y-maze spatial memory test

In order to examine any defects in hippocampus-dependent spatial memory, a Y-maze test was performed 5–7 days after induction of ischemia. This test did not show any significant differences between groups. The setup of the test is further described in Supplementary material 1.

2.5.3. Sucrose preference test

Anhedonic behavior of the mice was assessed using a sucrose preference test during the night between day14–15 or 15–16. This test did not show any significant differences between groups. The setup of the test is further described in Supplementary material 1.

2.6. Fecal corticosterone levels

Fecal samples were collected from the Open Field box after conducting the Open Field test performed 12–13 days following induction of ischemia in order to measure the stress levels of the mice. Since anxiety in itself can increase the frequency of defecation, fecal samples from mice that did not defecated during the Open Field test were collected by putting those mice in individual cages until they had defecated. This was done to prevent potential bias that could arise if only the corticosterone levels from those mice that indeed defecated during the test would have been analyzed. Corticosterone levels measured in feces is likely to reflect the level of corticosterone that was found in serum several hours earlier (Kalliokoski et al., 2010). To compare the effect of forced running on the corticosterone levels without ischemic insult as a confounding factor, mice only subjected to sham surgery were used for this test. The feces were stored at −80 °C until analysis. Corticosterone was then extracted and analyzed using a corticosterone ELISA kit (Enzo Life Sciences) as described by Touma et al. (Touma et al., 2003), except that feces was homogenized in 1 ml of 80% Methanol per 100 mg sample. Feces samples from mice subjected to voluntary wheel running with age-matched sedentary controls were also analyzed to investigate if running exercise per see results in corticosterone increase.

2.7. Euthanization and sample collection

The mice were first anesthetized with isofluorane 15–16 days after ischemia. Thereafter, the mice were euthanized and blood samples were collected through cardiac puncture. Blood samples were kept in room temperature for 25 min and then stored on ice a few hours until the samples were centrifuged at 1300 g in 4 °C for 10 min and the serum supernatants were collected and stored at −80 °C until analysis. The brains were dissected out and snap frozen in crushed dry ice and stored at −80 °C until analysis.

2.8. Immunohistochemistry

The brains where sectioned with a Leica CM3050 S Cryostat (Leica Microsystems Nussloch GmbH, Germany). The hippocampus was extracted from −1.20 to −2.20 (according to K. Franklin, G. Paxinos, 'The Mouse Brain: in Stereotaxic Coordinates', Academic Press, USA). The brain sections were 30 μm thick and collected into 6 series, with 3 series mounted onto microscope slides and 3 series stored in tubes for preparations of brain homogenates. All sliced samples were stored at −80 °C until used. Sections used for immunohistochemical stainings were fixated with 4% of PFA for 10 min prior to staining procedure.

Viable neurons were stained on hippocampal sections using Mouse-anti-NeuN antibody (1:200, Anti-NeuN, MAB377, ©EMD Millipore Corporation, Billerica, MA, USA). 4′,6-diamidino-2-phenylindole (DAPI) (1:1000, Sigma Aldrich) was used to stain cell nuclei. The number of NeuN positive cells with viable cell morphology was counted in the hippocampal subregions CA1, CA2 and CA3 of each hemisphere from two coronal subsequential sections (bregma −1.2 and 1.4) from each mouse using an epifluorescence microscope (Nikon Eclipse 80i microscope, Europe).

Inflammation and microglial activation was visualized using polyclonal rabbit-anti-Iba1 (1:500, Wako, Japan) and monoclonal rat-anti-galectin-3 (M3/38, 1:300 made in-house, provided by Professor Hakon Leffler) antibodies. To evaluate the overall number and activity of microglia in the hippocampus pictures were captured of the 3 subsequential sections of hippocampus (bregma-

1.2 mm, bregma-1.4 mm and bregma-1.6 mm) from each mouse, using the epifluorescence microscope. The extent and intensity of the Iba1 staining was evaluated using ImageJ. The number of galectin-3 positive cells in the hippocampus was counted in the same sections using the epifluorescence microscope.

2.9. Cytokine levels in brain tissue

2.9.1. Brain homogenization protocol

Eight brain slices at the level of the hippocampus from each mouse were homogenized in 100 μl complete lysis buffer (Mesoscale Discovery, Rockville, USA) containing protease inhibitor. The tissue was mechanically dissociated using a syringe plunger. The homogenates were then incubated 20 min on ice and tip-sonicated with 5 pulses for 2–3 s. Samples were thereafter centrifuged at 10,000 g in 4 °C for 20 min before the supernatants were collected. Protein concentrations were determined using the Bradford method and samples were stored at −80 °C until use.

2.9.2. Multiplex cytokine ELISA

The concentrations of different cytokines in the collected serum and homogenized brain samples (25 μl/sample) were measured in 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 SECTOR Imager 6000 (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 concentrations were normalized to the different protein concentrations measured in the Bradford assay of each homogenized sample.

2.10. Western blot

Eight brain slices at the level of hippocampus from each mouse were homogenized in 160 μl of RIPA buffer (R0278, Sigma-Aldrich) containing protease inhibitor (1% protease inhibitor cocktail, P8340, Sigma-Aldrich) Samples were sonicated twice for 3 s before they were centrifuged at 8000 g for 10 min and the supernatants were collected. Briefly, proteins were loaded on 4–20% Mini-Protean TGX Precast Gels (Bio-Rad) then transferred to Nitrocellulose membranes (Bio-Rad) using Trans-Blot Turbo System (Bio-Rad). The membranes were then blocked with 10% Casein (Sigma-Aldrich) diluted in PBS (tablets, Sigma-Aldrich). After blocking, the membranes were incubated with primary antibodies against NLRP3 (mAb, Adipogen 1:4000) and galectin-3 (M3/38, made in-house, provided by Professor Hakon Leffler, 1:500) at 4 °C over night. The membranes were then incubated with peroxidase secondary antibody (Vector Labs) and the blots were developed using Clarity Western ECL Substrate (Bio-Rad). Protein levels were normalized to beta-actin.

2.11. Exclusions and protocol violations

Unfortunately, the forced treadmill exercise induced stress in the animals to such an extent that severe fighting behavior was induced in their home cages. Therefore, several mice ($n = 16$) had to be sacrificed during this exercise paradigm, which explains the uneven distribution of mice in the different groups. During the ischemic surgery, several mice ($n = 7$) also died, as expected. The number of mice in each group during the behavioral tests also decreased due to the expected mortality caused by evolution of secondary neuronal damage during the two weeks following induction of global cerebral ischemia. Furthermore, for the analysis of cytokines, all samples with lack of signal in the SECTOR Imager 6000 plate reader were excluded. This altogether explains why the number of samples in each group varies for each experiment. Thus, the variations in number of samples between the experiments are not due to exclusion of outliers or conscious selection of subgroups.

2.12. Statistical analyses

All statistical analyses were performed in SPSS version 22.0. ANOVA were performed followed by Fisher's post hoc test for all comparisons between groups regarding the immunohistochemical evaluations, Western Blots, behavioral tests and cytokine levels. Two-tailed T-tests were used to compare the levels of corticosterone and anxiety in the two different groups (exercising and non-exercising) during the exercise program. For cytokines IFNγ, IL-6 and IL-10, as well as for galectin-3 and NLRP3, the levels were converted to a logarithmic scale before analysis. The correlations between immunohistochemical data, behavioral data and cytokine and corticosterone levels were analyzed using Pearson's correlation coefficients (r). R-values above 0.7 were regarded as very strong relationships and R-values between 0.4 and 0.69 were regarded as moderate to strong relationships. For survival analysis following ischemic surgery, Kaplan-Meier curves and subsequent log rank tests were performed in order to compare groups. Survival during the ischemic surgery procedure for the exercised and non-exercised groups was evaluated by Fisher's exact test. P-values below 0.05 were considered as statistical significant.

3. Results

3.1. Mice subjected to forced treadmill exercise are more anxious

The results from the Open Field tests conducted before the induction of cerebral ischemia can be seen in Fig. 2. At baseline, there were no significant differences in distance moved between mice subjected to exercise and those that were not (Fig. 2a, 3902 ± 962 cm and 3496 ± 502 cm respectively, T-test, $p = 0.067$). However, exercised mice spent more time close to the walls (Fig. 2b, 81.3 ± 9.6% of time and 69.0 ± 13.6% of time respectively, T-test, $p = 0.002$) of the Open Field box compared to those who were not subjected to any exercise. This suggests that treadmill exercise by itself could have an impact on anxious behavior. After ischemia, there was no difference between the group subjected to exercise and the group that had not been exercising (data not shown). However, there was a difference between the sham group and the groups subjected to ischemia in the behavior in the Open Field test (see Fig. 1S in Supplementary material 2).

3.2. Treadmill exercise prior to ischemia aggravates the neuronal damage in hippocampus

Our model of global brain ischemia is known to be dependent on the patency and lack of posterior communicating artery (Zhen and Dore, 2007; Murakami et al., 1998). Due to heterogeneity in the anatomy of the cerebral vascularization among the mice the experimental induction of ischemia might cause neuronal damage in one or both hemispheres, and the respective hemispheres can therefore be analyzed separately (Olsson et al., 2003; Burguillos et al., 2015). The global brain ischemia caused neuronal damage in the hippocampus both in the left and right hemispheres (Fig. 3a,b 1232 ± 209 and 1210 ± 220 viable-appearing neurons in sham compared to 652 ± 487 and 795 ± 297 living neurons in ischemic mice, ANOVA $p = 0.015$ and $p = 0.001$ respectively, Fisher's post hoc test, $p = 0.036$ and $p = 0.03$, respectively). Following ischemia, fewer NeuN positive neurons were detected in the right hippocampus of exercised mice compared to those who did not exercise (Fig. 3, Fisher's post hoc test, 411 ± 263 and 795 ± 297 living neurons respectively, $p = 0.039$). No difference in NeuN positive cells

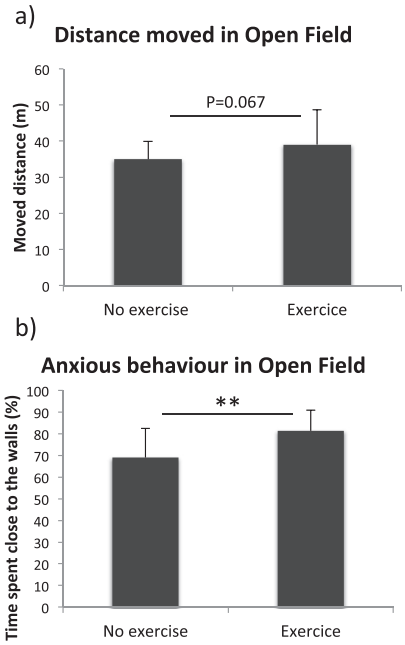

Fig. 2. Locomotor and anxious behavior in the Open Field test during the exercise program. The distance moved (a) and the anxious behavior (b), measured by time spent close to the walls, in the Open Field test during the exercise program. Bars represent the mean values for each group with error bars indicating the SD. * represents $p < 0.05$ and ** $p < 0.01$ in T-test. For non-exercised mice $n = 17$ and for exercised mice $n = 12$.

could be found in the left hemispheres between the experimental groups. The mortality during the surgical procedure did not differ between exercising or sedentary mice (Fisher's exact test, $p = 0.13$, in the ischemic groups 4/12 mice died in the exercising group and 1/17 mice died in the sedentary group). Furthermore, the mortality after ischemia did not differ between exercising or sedentary mice (Log Rank test, $p = 0.103$, in the sham group, no mice died, and in the ischemic groups 2/8 mice died in the exercising group and 7/16 mice died in the sedentary group).

3.3. The increased neuronal damage in the hippocampus cannot be explained by increased microglial activity in the same region

We found no differences in microglial activity between the exercising and non-exercising mice in Iba1 (optical density were 13.7 ± 8.4 and 14.8 ± 8.6 for exercising and non-exercising mice respectively, $p = 0.81$) or galectin-3 (87 ± 74 and 42 ± 35 positive cells for exercising and non-exercising mice respectively, $p = 0.13$) immunohistochemical stainings following ischemia that could explain the differences in neuronal survival in the hippocampus.

3.4. Forced treadmill exercise and ischemia induces a stress response that correlates with the degree of neuronal damage in hippocampus

Our behavioral data (Fig. 2) suggest that the mice in the treadmill training groups might be stressed due to the forced exercise. We therefore measured the levels of corticosterone in the feces, which is an established method to measure the stress levels in mice (Kalliokoski et al., 2010; Siswanto et al., 2008). The corticosterone levels in feces collected during the Open Field test are shown in Fig. 4. To compare the effect of forced running on the corticosterone levels without ischemic insult as a confounding factor, mice only subjected to sham surgery are presented in this figure. In these sham groups, corticosterone levels were elevated in mice subjected to forced treadmill running with 61% compared to mice which had not been subjected to any exercise (Fig. 4a, 394 ± 107 pg/ml and 244 ± 73 pg/ml respectively, T-test, $p = 0.016$). These data suggest that the mice that were exposed to forced treadmill training were profoundly stressed the weeks before the ischemic insult, which can be related to the increased brain damage seen in this group. Indeed, by correlating the corticosterone levels to the number of viable neurons in the hippocampus we found a negative correlation (Fig. 4b, Pearson's $R = -0.567$, $p = 0.004$). During the exercise program, there was no significant difference in corticosterone levels between mice shown to be the most unwilling to run and mice that were more willing to run (520 ± 208 pg/ml and 365 ± 147 pg/ml respectively, T-test, $p = 0.08$, for the mice most unwilling to run $n = 6$ and for the mice more willing to run $n = 12$). There was no difference in the levels of corticosterone in feces between those that had been exercising and those that had not been exercising in the group subjected to ischemia (data not shown). However, both groups subjected to ischemia displayed high corticosterone levels, which is most probably due to the effect of global ischemia that has been previously shown (de la Tremblaye et al., 2014).

3.5. Ischemia leads to increased levels of NLRP3 and galectin-3 in the brain

The levels of galectin-3 and NLRP3 in the brain of the different groups were analyzed with Western blots, and can be seen in Figs. 5a and 6a respectively. The levels of NLRP3 and galectin-3 did not significantly differ between exercised and non-exercised mice following ischemia. For galectin-3, no differences were detected in the ANOVA ($p = 0.054$). However, a difference in galectin-3 was found in Fisher's post hoc test comparing non-exercised ischemic mice to sham ($p = 0.038$, Fig. 5a). The number of galectin-3 positively stained microglia also correlated negatively with the number of viable NeuN positive neurons in hippocampus (Fig. 5b, Pearson's $R = -0.721$, $p = 0.001$). Ischemia induced an increase in NLRP3 (Fig. 6a and b, ANOVA $p = 0.027$, Fisher's post hoc test $p = 0.048$) in non-exercised mice compared to sham. The levels of NLRP3 correlated with the levels of corticosterone found in feces (Fig. 6c, Pearson's $R = 0.475$, $p = 0.046$) following ischemia. Collectively, this data support our microglial Iba1 immunohistochemistry data, showing increased levels of inflammatory proteins after ischemia, but no significant effect of treadmill exercise prior to ischemia.

3.6. Global cerebral ischemia induces a cytokine response that is more pronounced in mice subjected to forced exercise for several cytokines

We measured several pro- and anti-inflammatory cytokines in the blood and the brain after global ischemia. The levels of four of the cytokines for which significant differences could be observed between different groups are presented in Fig. 7. The levels of the other cytokines measured in the collected serum and brain tissue from the different groups can be seen in Supplementary material 2, Tables 1 and 2, respectively. Ischemic mice had elevated IL-1β levels in the brain compared to sham (Fig. 7a, 0.040 ± 0.023 and

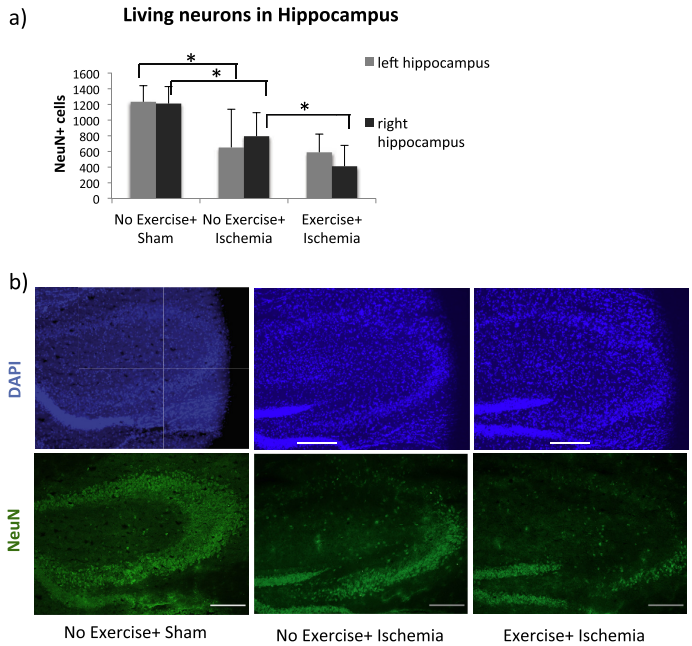

Fig. 3. Neuronal damage in hippocampus following global ischemia. The number of NeuN positive neurons in the left and right hippocampus for exercised and non-exercised groups compared to sham (a). Bars represent the mean values for each group, with error bars indicating the SD. * represents $p < 0.05$ in Fisher's post hoc test. Representative images of NeuN immunohistochemical staining with DAPI background staining in the right hippocampus from each group (b) at 10× magnification. Scale bars represent 200 μm. For sham mice n = 5, non-exercised mice n = 6 and for exercised mice n = 6.

0.014 ± 0.002 pg/mg protein respectively, ANOVA $p = 0.045$, Fisher's post hoc test, $p = 0.002$). No significant difference between non-exercised and exercised mice following ischemia was seen for this specific cytokine.

Strikingly, mice that had been subjected to exercise prior to ischemia showed much higher levels of IFNγ in the brain compared to non-exercised mice with ischemia (Fig. 7c, $8.93*10^{-3} \pm 8.1*10^{-3}$ and $1.64*10^{-3} \pm 0.71*10^{-3}$ pg/mg protein respectively, Fisher's post hoc test, $p = 0.008$). For the IL-10 levels in the brain, no significant difference was seen between exercised and non-exercised mice after ischemia (Fig. 7b, 0.39 ± 0.10 and 0.31 ± 0.09 pg/mg protein respectively, $p = 0.10$). No difference was seen in the ANOVA ($p = 0.084$) for the serum levels of IL-10. However, higher levels of IL-10 were observed in serum from exercised mice compared to non-exercised mice following ischemia in Fisher's post hoc test (Fig. 7d, 5.68 ± 0.55 and 4.68 ± 1.08 pg/ml respectively, $p = 0.042$).

The concentration of IL-10 in serum correlated negatively with the immunoreactivity of the Iba1 staining in the hippocampus (Fig. 8a, Pearson $R = -0.535$, $p = 0.022$). The amount of surviving NeuN positive cells in the hippocampus showed a negative correlation with the levels of several different pro-inflammatory cytokines such as IL-1β (Fig. 8b, Pearson $R = -0.641$, $p = 0.006$) in the brain. The corticosterone levels also correlated with the levels of IL-1β (Fig. 8c, Pearson $R = 0.61$, $p = 0.006$) and IL-10 (Fig. 8d, Pearson $R = 0.594$, $p = 0.007$) in the brain. In summary, our data suggest an expected, negative association between pro-inflammatory cytokines and neuronal survival (Fig. 8b) and also a positive correlation between cytokine levels in the brain and corticosterone levels in feces indicating that the stress caused by the forced exercise affects the neuroinflammation in the injured brain.

3.7. Global ischemia does not induce cognitive dysfunctions or anhedonic behavior

There were no differences between the different groups in the Y-maze test and the sucrose preference test (See Supplementary Material 2, Table 3).

3.8. Voluntary wheel running does not induce a stress response

Mice subjected to voluntary wheel running did not display increased anxious behavior compared to their age-matched sedentary controls (Fig. 9a, 74.2 ± 12.8% of time and 72.6 ± 12.0% of time respectively, T-test, $p = 0.86$). As can be seen in Fig. 9b, the corticosterone levels in feces from wheel running mice did not differ from their age-matched sedentary controls. Taken together, we found that running exercise per se does not cause a stress response with increased anxiety and corticosterone levels.

4. Discussion

In the present study, we investigated the potential preventive effect of physical exercise prior to global cerebral ischemia in mice by subjecting one group of mice to a four-week long treadmill running program prior to induction of global cerebral ischemia. Our main findings show that forced treadmill exercise induced a stress

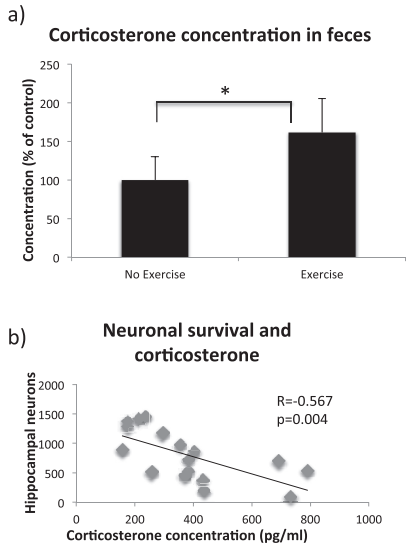

Fig. 4. Corticosterone levels during the Open Field test. The levels of corticosterone in feces (a). Bars represent the mean values for each sham group expressed as percentage of non-exercised control, with error bars indicating the SD. * represents $p < 0.05$ in T-test. For non-exercised mice $n = 10$ and for exercised mice $n = 3$. The correlation between the corticosterone concentration following ischemia and the number of surviving neurons in the left hippocampus (b). Each dot corresponds to the measured values from one mouse.

response that can counteract the anti-inflammatory effects of exercise, and lead to increased neuronal damage in a mouse model of global cerebral ischemia. In the Open Field test during the exercise period prior to ischemic onset, we observed that exercised mice were significantly more anxious, spending more time close to the walls of the Open Field arena, compared to the non-exercised group. These results confirm our observations during the running procedure, where the mice gave the impression of being anxious and stressed during and after the training. Indeed, we lost several mice ($n = 16$) during the training period due to fighting between the mice that were housed together. Also, many of the mice showed an increasing unwillingness to run and were therefore excluded from the exercise group. Mice that were most unwilling to run also seemed to have higher levels of the stress hormone corticosterone in feces collected during the Open Field test conducted during this exercise period, even though this difference was not statistically significant (data not shown). Importantly, we found that the forced running itself significantly increased the corticosterone levels. These stress levels represent the status of the mice the weeks before they were subjected to ischemia, suggesting that stress prior to an ischemic insult is detrimental. Indeed, the potential of stress hormones such as corticosterone to potentiate the neuronal damage in hippocampus following global cerebral ischemia was shown already in the 1980s (Sapolsky and Pulsinelli, 1985). Examination of the brain samples from our mice also showed that exercised mice had fewer surviving neurons in the hippocampus compared to non-exercised mice following ischemia. Furthermore, higher corticosterone levels correlated with fewer surviving neurons, indicating that the reduced neuronal survival in the exercised mice may be due to the increased stress caused by the enforcement to run. This conclusion was further strengthened by the fact that this increase in corticosterone levels where not observed in mice subjected to voluntary wheel running when compared to age-matched sedentary littermate controls.

When it comes to the effect of exercise on the level of inflammation following ischemia, our results demonstrate the complexity of the immune response following exercise and cerebral ischemia. Galectin-3 is a pro-inflammatory immunomodulator expressed by activated microglia. It has been suggested to sustain microglial activation through binding and activating the toll-like receptor 4 (Burguillos et al., 2015). Interestingly, we found a strong negative correlation between galectin-3 positive cells in the hippocampus and the number of viable hippocampal neurons (NeuN), suggesting that the presence of galectin-3 positive microglia is associated with increased neuronal cell death.

Previous studies in rats have shown that running exercise can reduce microgliosis if the rats are exercised after ischemic induction (Lovatel et al., 2014). However, no microglial effect of exercise prior to ischemic induction was observed. In our study, following ischemia, exercised mice had a subtle increase in the levels of the anti-inflammatory cytokine IL-10 in serum compared to non-exercised mice. Increased levels of IL-10 is a well-known anti-inflammatory effect of exercise (Pedersen and Febbraio, 2008) and can provide neuroprotection after cerebral ischemia (Ooboshi et al., 2005). Though, we did not find an altered level of IL-10 in the brain.

However, ischemic exercised mice had a 5 times elevation of the pro-inflammatory IFNγ in the brain compared to non-exercised counterparts. As IFNγ is a canonical cytokine known to induce a pro-inflammatory phenotype in microglia (Prajeeth et al., 2014; Papageorgiou et al., 2016), it is tempting to speculate that this could in part explain the increased neuronal damage seen in the exercised mice compared to the corresponding non-exercised mice following ischemic insult. The effect of exercise on the levels of IFNγ has not yet been elucidated, several studies have shown that exercise can decrease as well as increases the levels of IFNγ (Tuon et al., 2015; Nichol et al., 2008). However, in mice, IFNγ has been shown to increase in response to stress (Fuertig et al., 2015). This suggests the possibility that the elevated levels of IFNγ observed in our mice may be caused by the stress associated with the exercise rather than the exercise per se.

Our data confirm the complex relationship between different cytokines, where functions are dependent on the inflammatory context and possible interplay between the different cytokines, and where the effect of pro-inflammatory cytokines can be counterbalanced by high levels of anti-inflammatory cytokines. It is also possible that exercise and stress can alter the inflammatory interplay between CNS and the periphery. The connection between the various cytokines involved in neuroinflammation and their importance in the context of physical exercise remains to be elucidated. A limitation of this study is that we measured the cytokines fairly late, and only at one time-point in the process, which made it impossible to determine their temporal dynamics.

We further show that high levels of pro-inflammatory cytokines, such as IL-1β, in the brain is correlated with significantly lower number surviving neurons in hippocampus following ischemia, suggesting that these cytokines are related to a negative impact on neuronal survival. This negative impact of pro-inflammatory cytokines on neuronal survival has been shown in ischemic rats previously (Li et al., 2014). In our study, mice with high levels of corticosterone also had higher levels of cytokines, such as IL-1β and IL-10, in the brain. These results are in accordance with other studies where it has been shown that stress can evoke a pro-inflammatory response in the brain, with increased expression of NLRP3 inflammasome involved in the cleavage and secretion of pro-inflammatory IL-1β (Gadek-Michalska et al., 2013; Frank et al., 2014). Recently, it has been shown that a stress-induced increase in

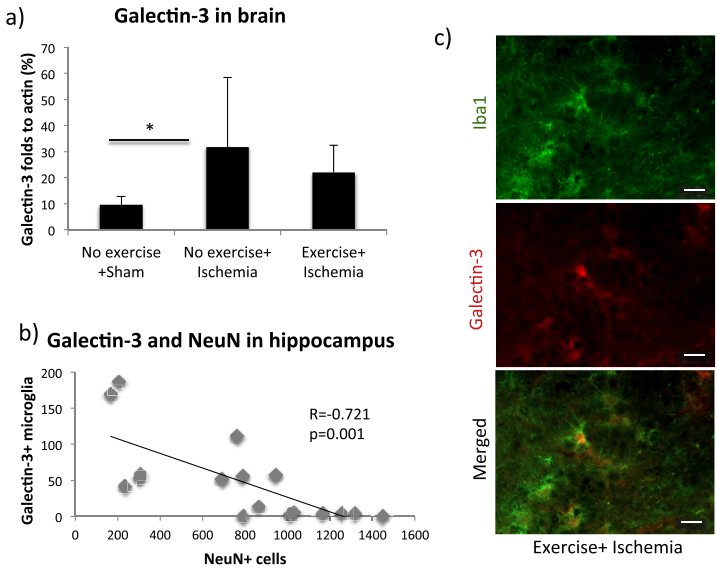

Fig. 5. Inflammation in hippocampus and whole brain following global brain ischemia. The levels of galectin-3 in the brain in exercised and non-exercised groups compared to sham. Bars represent the mean values for each group, with error bars indicating the SD. * represents $p < 0.05$ in Fisher's post hoc test. For sham mice $n = 5$, non-exercised mice $n = 9$ and for exercised mice $n = 5$. The correlation between the number of galectin-3 positive cells and the number of surviving neurons in the right hippocampus following ischemia (b). Each dot corresponds to the measured values from one mouse. Representative images of galectin-3 and Iba1 staining in the hippocampus (c) at 40× magnification. Scale bars represent 20 μm.

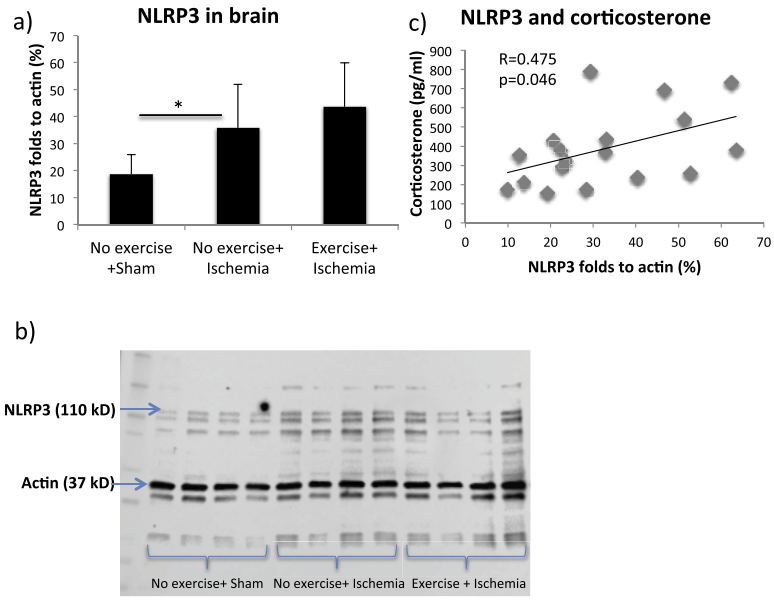

Fig. 6. Levels of NLRP3 in brain following global ischemia. The levels of NLRP3 in the brain in exercised and non-exercised groups compared to sham (a). Bars represent the mean values for each group, with error bars indicating the SD. * represents $p < 0.05$ in Fisher's post hoc test. For sham mice $n = 5$, non-exercised mice $n = 9$ and for exercised mice $n = 5$. A picture of the blot with the bands of interest indicated (b). The correlation between the levels of NLRP3 and corticosterone concentration following ischemia (c). Each dot corresponds to the measured values from one mouse.

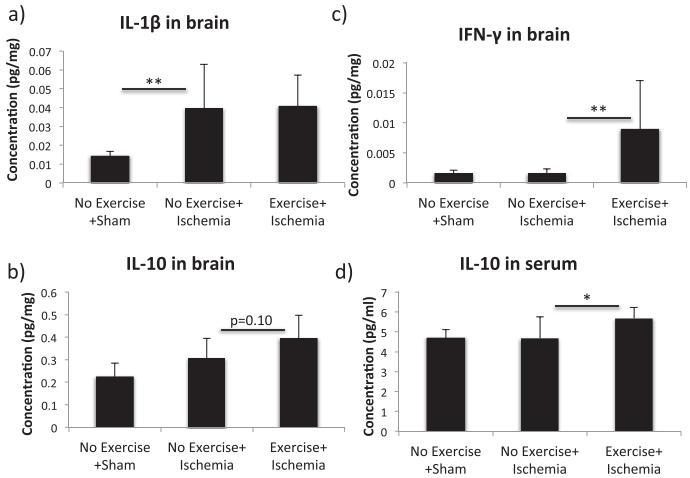

Fig. 7. Cytokine levels in serum and brain following global ischemia. Levels of different cytokines in the brain (a–c) and serum (d) for exercised and non-exercised groups compared to sham. Bars represent the mean values for each group, with error bars indicating the SD. * represents $p < 0.05$, ** represents $p < 0.01$ and *** represents $p < 0.001$ in Fisher's post hoc test. For the sham group n = 5, n = 4, n = 5 and n = 4 respectively, for the non-exercised mice with ischemia n = 9, n = 9, n = 9 and n = 8 respectively and for exercised mice with ischemia n = 6, n = 5, n = 6 and n = 6 respectively.

corticosterone levels lowers the threshold for microglia to release pro-inflammatory cytokines (Dey et al., 2014). Increased stress has even been suggested to exacerbate neuronal cell death by increasing neuroinflammation, which in turn is neurotoxic (de Pablos et al., 2014). Indeed, several studies have shown that stress increase the vulnerability to inflammation resulting in increased microglial activation and neuronal damage in different brain regions such as hippocampus (Espinosa-Oliva et al., 2011), substantia nigra (de Pablos et al., 2014) and prefrontal cortex (de Pablos et al., 2006). Furthermore, the same studies also showed that these effects of stress were dependent on glucocorticoid receptor signaling. In our study we did not detect any differences between exercised and non-exercised mice in the levels of IL-1β and NLRP3 in the brain, suggesting that the link between stress and the

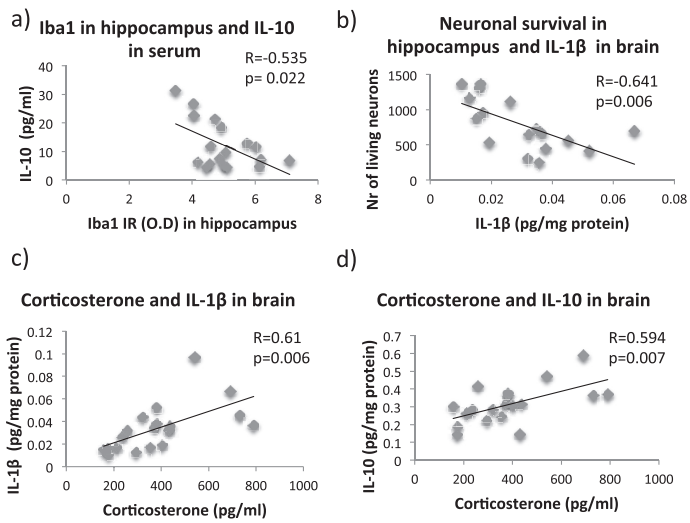

Fig. 8. Correlations between cytokines, microglial activation, neuronal survival and corticosterone. Correlations between immunoreactivity of Iba1 in the hippocampus and IL-10 in serum (a), neuronal survival and IL-1β in the brain (b), corticosterone levels after ischemia and IL-1β in the brain (c) and corticosterone levels after ischemia and IL-10 in the brain (d). Each dot corresponds to the measured values from one mouse.

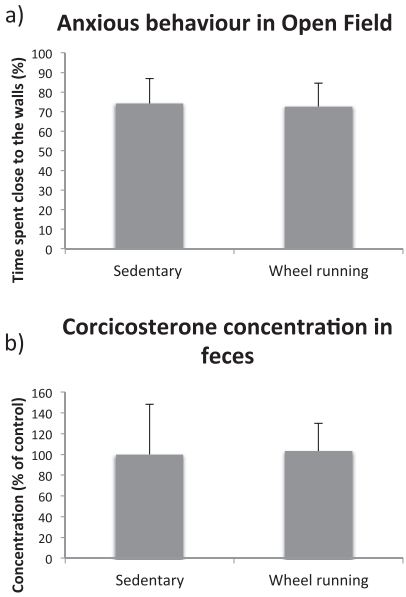

Fig. 9. Anxious behavior and fecal corticosterone levels after voluntary wheel running. Anxious behavior in the Open Field test in mice subjected to voluntary wheel running (a). Bars represent the mean values for each group, with error bars indicating the SD. For sedentary control mice n = 4 and for wheel running mice n = 4. The levels of corticosterone in feces in mice subjected to voluntary wheel running(b). Bars represent the mean values for each group expressed as percentage of the sedentary control group, with error bars indicating the SD. For sedentary control mice n = 4 and for wheel running mice n = 6.

inflammatory response may also involve other mechanisms. However, one possible confounding factor is that the corticosterone samples were collected more than a week prior to collecting the samples used for measuring cytokine levels, NLRP3 and assessing brain damage.

The forced treadmill running clearly induced a stress response that could explain the negative effects on the brains of the mice in our study. The reason why many studies, including ours, are based on forced treadmill running as a method for studying the effects of exercise is that this method can be standardized compared to, for example, voluntary wheel running. With treadmills, the intensity, duration and timing of the exercise can be standardized so that all mice in the same exercise group are subjected to exactly the same amount of exercise. This is not possible when using voluntary wheel running as the wheel is placed in the home cages, allowing the mice to run as much, fast and often as they want. However, one advantage of using voluntary wheel running is that it does not include the stressful element of forced exercise, and that the mice can choose to run during the night, which in itself is less stressful as they are nocturnal animals. However, for the voluntary wheel running, we choose to cage the mice separately in order to better control the distance run by each mouse. It has been shown in other study that social isolation per see could be very stressful to the mice, as it is used in standardized stress protocols (de Pablos et al., 2014; Espinosa-Oliva et al., 2011). However, our single caged mice did not display any signs of anxiety in the Open Field test compared to the behavior we use to observe in our mice. Our treadmill study is not alone in demonstrating that forced exercise can have negative effects due to stress. Indeed, other studies have shown that forced treadmill running causes anxiety and stress in form of increased levels of corticosterone, effects that were not observed in animals subjected to voluntary wheel running (Leasure and Jones, 2008; Ke et al., 2011; Griesbach et al., 2012). Interestingly, another study showed that it might not be the enforcement to run per se that induces stress, but rather the fact that being pushed if not running (Greenwood et al., 2013). In this study, there were no differences in stress induction between voluntary wheel running and motorized, forced wheel running. However, the animals subjected to forced treadmill running, being pushed by foot shocks when not running, were more stressed. In fact, forced treadmill running is also used as a model to induce and study stress responses in mice (Hong et al., 2015). Furthermore, Zheng and coauthors showed that following experimental stroke in rats, forced treadmill running leads to reduced motor recovery compared to rats subjected to voluntary wheel running (Ke et al., 2011). It has also been shown that forced and voluntary exercise can have different effects on inflammatory response, where forced exercise increases and voluntary exercise decreases levels of pro-inflammatory cytokines after injury in mice (Cook et al., 2013). Moreover, it has been shown that voluntary running leads to higher levels of BDNF compared to forced running (Uysal et al., 2015; Griesbach et al., 2014). However, there are also studies comparing the effect of forced versus voluntary running showing that both regimens can be of equal beneficial effect when it comes to improving cognition, neuronal survival and BDNF levels in different models of brain injury (Lin et al., 2015a, 2015b).

Furthermore, it has been shown that forced exercise can activate a stress response similar to restraint stress, reducing the ability to cope with an oxidative challenge. For example, forced running has been shown to increase the cardiac infarct size in rats (Mancardi et al., 2009), and failed to improve recovery after experimental stroke in rats (Auriat et al., 2006). However, several studies have shown beneficial effects of forced exercise on the recovery from cerebral hypoperfusion (Cechetti et al., 2012) as well as experimental stroke (Park et al., 2012).

5. Conclusions

This study shows that forced treadmill running induces stress, which can cause increased neuronal damage following global cerebral ischemia in mice. We detected 5-fold increased IFNγ levels in ischemic mice that had been subjected to treadmill running, even though we found a small increase in serum IL-10 levels. This stress response with increased anxiety and corticosterone levels were not seen in mice exposed to voluntary wheel running. It is therefore tempting to conclude that running exercise can have beneficial neuroinflammatory effects, but that the stress induced by the enforcement to run is disadvantageous and exceeds the beneficial effects. Our study highlights the importance of taking into account the stress that the enforcement of running exercise can induce, which can counteract the otherwise positive effects of physical exercise. Hence, it is important to monitor chronic stress in experimental animals to be able to infer the right conclusions about the effects of physical exercise.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ynstr.2016.09.002.

References

Auriat, A.M., et al., 2006. Forced exercise does not improve recovery after hemorrhagic stroke in rats. Brain Res. 1109 (1), 183–191.
Austin, M.W., et al., 2014. Aerobic exercise effects on neuroprotection and brain repair following stroke: a systematic review and perspective. Neurosci. Res. 87, 8–15.
Back, T., Hemmen, T., Schuler, O.G., 2004. Lesion evolution in cerebral ischemia. J. Neurol. 251 (4), 388–397.
Banati, R.B., et al., 1993. Cytotoxicity of microglia. Glia 7 (1), 111–118.
Barone, F.C., et al., 1997. Tumor necrosis factor-alpha. A mediator of focal ischemic brain injury. Stroke 28 (6), 1233–1244.
Bottiger, B.W., et al., 1998. Neuronal stress response and neuronal cell damage after cardiocirculatory arrest in rats. J. Cereb. Blood Flow Metab. 18 (10), 1077–1087.
Brown, D.A., et al., 2007. Short-term treadmill running in the rat: what kind of stressor is it?, 1985 J. Appl. Physiol. 103 (6), 1979–1985.
Burguillos, M.A., et al., 2015. Microglia-secreted Galectin-3 acts as a toll-like receptor 4 ligand and contributes to microglial activation. Cell Rep. 10 (9), 1626–1638.
Carson, M.J., Thrash, J.C., Walter, B., 2006. The cellular response in neuroinflammation: the role of leukocytes, microglia and astrocytes in neuronal death and survival. Clin. Neurosci. Res. 6 (5), 237–245.
Cechetti, F., et al., 2012. Forced treadmill exercise prevents oxidative stress and memory deficits following chronic cerebral hypoperfusion in the rat. Neurobiol. Learn Mem. 97 (1), 90–96.
Cook, M.D., et al., 2013. Forced treadmill exercise training exacerbates inflammation and causes mortality while voluntary wheel training is protective in a mouse model of colitis. Brain Behav. Immun. 33, 46–56.
Deierborg, T., et al., 2008. Overexpression of UCP2 protects thalamic neurons following global ischemia in the mouse. J. Cereb. Blood Flow Metab. 28 (6), 1186–1195.
Dey, A., et al., 2014. Glucocorticoid sensitization of microglia in a genetic mouse model of obesity and diabetes. J. Neuroimmunol. 269 (1–2), 20–27.
Espinosa-Oliva, A.M., et al., 2011. Stress is critical for LPS-induced activation of microglia and damage in the rat hippocampus. Neurobiol. Aging 32 (1), 85–102.
Frank, M.G., et al., 2014. Chronic exposure to exogenous glucocorticoids primes microglia to pro-inflammatory stimuli and induces NLRP3 mRNA in the hippocampus. Psychoneuroendocrinology 40, 191–200.
Fuertig, R., et al., 2015. Mouse chronic social stress increases blood and brain kynurenine pathway activity and fear behaviour: both effects are reversed by inhibition of indoleamine 2,3-dioxygenase. Brain Behav. Immun. 54, 59–72.
Gadek-Michalska, A., et al., 2013. Cytokines, prostaglandins and nitric oxide in the regulation of stress-response systems. Pharmacol. Rep. 65 (6), 1655–1662.
Greenwood, B.N., et al., 2013. Exercise-induced stress resistance is independent of exercise controllability and the medial prefrontal cortex. Eur. J. Neurosci. 37 (3), 469–478.
Griesbach, G.S., et al., 2012. Differential effects of voluntary and forced exercise on stress responses after traumatic brain injury. J. Neurotrauma 29 (7), 1426–1433.
Griesbach, G.S., et al., 2014. Recovery of stress response coincides with responsiveness to voluntary exercise after traumatic brain injury. J. Neurotrauma 31 (7), 674–682.
Hong, S.S., et al., 2015. The traditional drug Gongjin-Dan ameliorates chronic fatigue in a forced-stress mouse exercise model. J. Ethnopharmacol. 168, 268–278.
Kalliokoski, O., et al., 2010. Distribution and time course of corticosterone excretion in faeces and urine of female mice with varying systemic concentrations. Gen. Comp. Endocrinol. 168 (3), 450–454.
Ke, Z., et al., 2011. The effects of voluntary, involuntary, and forced exercises on brain-derived neurotrophic factor and motor function recovery: a rat brain ischemia model. PLoS One 6 (2), e16643.
Kim, S.U., de Vellis, J., 2005. Microglia in health and disease. J. Neurosci. Res. 81 (3), 302–313.
Kirino, T., Sano, K., 1984. Selective vulnerability in the gerbil hippocampus following transient ischemia. Acta Neuropathol. 62 (3), 201–208.
Lambertsen, K., et al., 2011. Differences in origin of reactive microglia in bone marrow chimeric mouse and rat after transient global ischemia. J. Neuropathol. Exp. Neurol. 70 (6), 481–494.
Lambertsen, K.L., Biber, K., Finsen, B., 2012. Inflammatory cytokines in experimental and human stroke. J. Cereb. Blood Flow Metab. 32 (9), 1677–1698.
Leasure, J.L., Jones, M., 2008. Forced and voluntary exercise differentially affect brain and behavior. Neuroscience 156 (3), 456–465.
Li, S.J., et al., 2014. The role of TNF-alpha, IL-6, IL-10, and GDNF in neuronal apoptosis in neonatal rat with hypoxic-ischemic encephalopathy. Eur. Rev. Med. Pharmacol. Sci. 18 (6), 905–909.
Lin, Y., et al., 2015. Involuntary, forced and voluntary exercises are equally capable of inducing hippocampal plasticity and the recovery of cognitive function after stroke. Neurol. Res. 37 (10), 893–901.
Lin, Y., et al., 2015. Involuntary, forced and voluntary exercises equally attenuate neurocognitive deficits in vascular dementia by the BDNF-pCREB mediated pathway. Neurochem. Res. 40 (9), 1839–1848.
Lovatel, G.A., et al., 2014. Long-term effects of pre and post-ischemic exercise following global cerebral ischemia on astrocyte and microglia functions in hippocampus from Wistar rats. Brain Res. 1587, 119–126.
Mancardi, D., et al., 2009. Omega 3 has a beneficial effect on ischemia/reperfusion injury, but cannot reverse the effect of stressful forced exercise. Nutr. Metab. Cardiovasc Dis. 19 (1), 20–26.
Mota, B.C., et al., 2012. Exercise pre-conditioning reduces brain inflammation and protects against toxicity induced by traumatic brain injury: behavioral and neurochemical approach. Neurotox. Res. 21 (2), 175–184.
Murakami, K., et al., 1998. The development of a new mouse model of global ischemia: focus on the relationships between ischemia duration, anesthesia, cerebral vasculature, and neuronal injury following global ischemia in mice. Brain Res. 780 (2), 304–310.
Nichol, K.E., et al., 2008. Exercise alters the immune profile in Tg2576 Alzheimer mice toward a response coincident with improved cognitive performance and decreased amyloid. J. Neuroinflamm. 5, 13.
Olsson, T., Wieloch, T., Smith, M.L., 2003. Brain damage in a mouse model of global cerebral ischemia. Effect of NMDA receptor blockade. Brain Res. 982 (2), 260–269.
Ooboshi, H., et al., 2005. Postischemic gene transfer of interleukin-10 protects against both focal and global brain ischemia. Circulation 111 (7), 913–919.
de Pablos, R.M., et al., 2006. Stress increases vulnerability to inflammation in the rat prefrontal cortex. J. Neurosci. 26 (21), 5709–5719.
de Pablos, R.M., et al., 2014. Chronic stress enhances microglia activation and exacerbates death of nigral dopaminergic neurons under conditions of inflammation. J. Neuroinflamm. 11, 34.
Papageorgiou, I.E., et al., 2016. TLR4-activated microglia require IFN-gamma to induce severe neuronal dysfunction and death in situ. Proc. Natl. Acad. Sci. U. S. A. 113 (1), 212–217.
Park, S., et al., 2012. Forced exercise enhances functional recovery after focal cerebral ischemia in spontaneously hypertensive rats. Brain Sci. 2 (4), 483–503.
Pedersen, B.K., Febbraio, M.A., 2008. Muscle as an endocrine organ: focus on muscle-derived interleukin-6. Physiol. Rev. 88 (4), 1379–1406.
Piao, C.S., et al., 2013. Late exercise reduces neuroinflammation and cognitive dysfunction after traumatic brain injury. Neurobiol. Dis. 54, 252–263.
Prajeeth, C.K., et al., 2014. Effector molecules released by Th1 but not Th17 cells drive an M1 response in microglia. Brain Behav. Immun. 37, 248–259.
Sapolsky, R.M., Pulsinelli, W.A., 1985. Glucocorticoids potentiate ischemic injury to neurons: therapeutic implications. Science 229 (4720), 1397–1400.
Sasson, C., et al., 2010. Predictors of survival from out-of-hospital cardiac arrest: a systematic review and meta-analysis. Circ. Cardiovasc Qual. Outcomes 3 (1), 63–81.
Sim, Y.J., et al., 2004. Treadmill exercise improves short-term memory by suppressing ischemia-induced apoptosis of neuronal cells in gerbils. Neurosci. Lett. 372 (3), 256–261.
Sim, Y.J., et al., 2005. Long-term treadmill exercise overcomes ischemia-induced apoptotic neuronal cell death in gerbils. Physiol. Behav. 84 (5), 733–738.
Siswanto, H., et al., 2008. Corticosterone concentrations in blood and excretion in faeces after ACTH administration in male Sprague-Dawley rats. In Vivo 22 (4), 435–440.
Svensson, M., Lexell, J., Deierborg, T., 2014. Effects of physical exercise on neuroinflammation, neuroplasticity, neurodegeneration, and behavior: what we can learn from animal models in clinical settings. Neurorehabil Neural Repair 29 (6), 577–589.
Touma, C., et al., 2003. Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. Gen. Comp. Endocrinol. 130 (3), 267–278.
de la Tremblaye, P.B., et al., 2014. Evidence of lasting dysregulation of neuroendocrine and HPA axis function following global cerebral ischemia in male rats and the effect of Antalarmin on plasma corticosterone level. Horm. Behav. 65 (3), 273–284.
Tuon, T., et al., 2015. Physical training regulates mitochondrial parameters and neuroinflammatory mechanisms in an experimental model of Parkinson's disease. Oxid. Med. Cell Longev. 2015, 261809.
Uysal, N., et al., 2015. Effects of voluntary and involuntary exercise on cognitive functions, and VEGF and BDNF levels in adolescent rats. Biotech. Histochem. 90 (1), 55–68.
Yamasaki, Y., et al., 1995. Interleukin-1 as a pathogenetic mediator of ischemic brain damage in rats. Stroke 26 (4), 676–680 discussion 681.
Yenari, M.A., Kauppinen, T.M., Swanson, R.A., 2010. Microglial activation in stroke: therapeutic targets. Neurotherapeutics 7 (4), 378–391.
Zhen, G., Dore, S., 2007. Optimized protocol to reduce variable outcomes for the bilateral common carotid artery occlusion model in mice. J. Neurosci. Methods 166 (1), 73–80.

Supplementary Method descriptions

2.5.2 Y-maze spatial memory test

In order to examine any defects in hippocampus-dependent spatial memory, a Y-maze test was performed 5-7 days after induction of ischemia. For this purpose, a Y-maze arena (21 cm*4 cm/arm) in the shape of a Y was used[1]. Some bedding material from the home cage of the mouse was put in the arms of the maze for the mouse to feel more relaxed and thus increase the likelihood of it to explore the maze. The bedding material was removed and the maze was cleaned with ethanol followed by water before the next mouse was introduced to the maze to prevent odor bias. Five groups of 2 visual cues were added around the maze. The mice were first trained for 2 trial sessions of 5 minutes each. For the trial sessions, one arm of the maze was closed so that the mouse could only explore 2 arms, the “home arm” and the “familiar arm”. At the start of each session, the mouse was put into the home arm facing the walls and then left alone to freely explore the two arms. The first trial session was performed on the afternoon of day 5 or 6 after ischemia and the second trial was performed on day 6 or 7. The real probe test was performed 2 hours after the second and last trial session in order to test the long-term memory. During this probe test, the mouse was put in the home arm facing the walls, as performed during the trial session. During the probe test, the third arm, called “new arm” was open to explore. A mouse with intact memory will notice that the two other arms had been explored previously, and will spend more time in the new arm and enter this arm more frequently compared to the two other arms. However, if a mouse has memory deficits it may not notice that it has explored the two other arms before and will therefore not spend more time in the new arm compared to the other two arms. The movements of the mice were monitored with a camera and later analyzed using the SMART software system to detect the

number of entrances into and time spent in each arm.

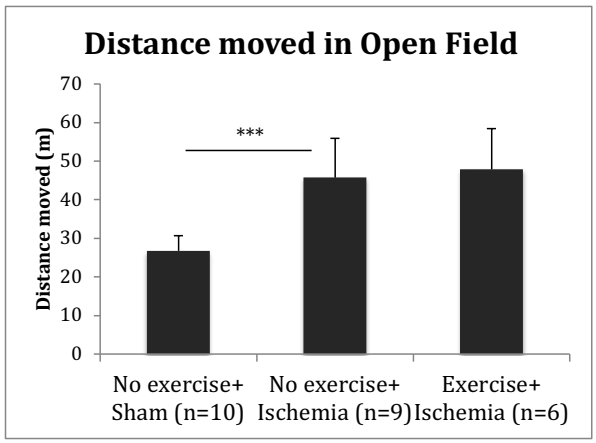
2.5.3 Sucrose preference test

Anhedonic behavior of the mice was assessed using a sucrose preference test during the night between day14-15 or 15-16. In this test, the mice were introduced to a sucrose solution in their home cages one night before the actual test. A bottle containing 2% sucrose solution was put in the place of the regular water bottle. The regular water bottle was put in the opposite corner of the cage, allowing the mice a choice of water or sucrose. The day before the test, the mice were deprived of drinking solutions for 5 hours prior to starting the test. In the evening, the mice were individually caged with access to nesting material and food. Each mouse also had a bottle of tap water and one bottle of sucrose solution to choose from during the night. The 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*

1. Albani, S.H., D.G. McHail, and T.C. Dumas, *Developmental studies of the hippocampus and hippocampal-dependent behaviors: insights from interdisciplinary studies and tips for new investigators.* Neurosci Biobehav Rev, 2014. **43**: p. 183-90.

Supplementary Data- cytokine levels and non-significant behavioral data

a)



b)

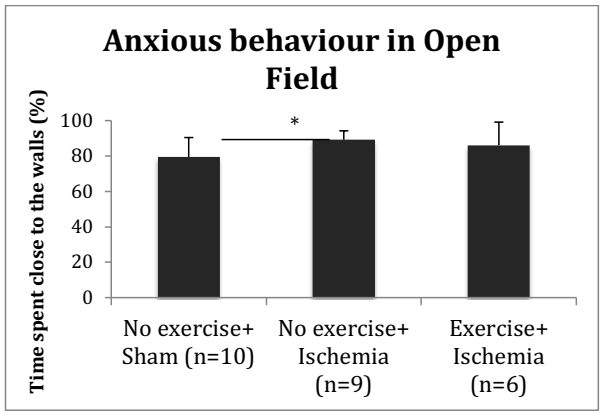


Figure 1S. Locomotor and anxious behavior in the Open Field test after ischemia. The distance moved (a) and the anxious behavior (b), measured by the time spent close to the walls, in the Open Field test after induction of ischemia. Bars represent the mean values for each group with error bars indicating SD. * represents $p<0.05$ and *** $p<0.001$ in Fishers post-hoc test.

Table 1 - Cytokine concentrations in serum

Cytokine	**Sham** Mean concentration (pg/ml) ± SD	**No exercise+ Ischemia** Mean concentration (pg/ml) ± SD	**Exercise+ Ischemia** Mean concentration (pg/ml) ± SD	T-test Sham vs. No exercise+ Ischemia	T-test No exercise+ Ischemia vs. exercise+ Ischemia
Serum IL-1 β	0.09±0.02	0.09±0.04	0.11±0.04		
Serum IL-2	0.07±0.05	0.11±0.06	0.09±0.06		
Serum IL-4	Below detection	Below detection	Below detection		
Serum IL-5	1.84±0.44	1.95±0.35	1.30±0.12	p<0.001	
Serum IL-6	0.65±0.47	5.21±4.46	8.29±6.17	p=0.003	
Serum IL-10	4.68±0.44	4.68±1.08	5.68±0.55		p=0.042
serum IL-12p70	Below detection	Below detection	Below detection		
Serum IFNγ	0.25±0.14	0.21±0.08	0.20±0.02		
Serum KC/GRO	40.5±6.4	41.4±9.2	46.4±6.9		
Serum TNFα	4.3±0.27	4.47±0.89	4.73±0.28		

Table 1. The mean concentration± SD for all the cytokines in each group for serum samples. p values are shown for those cytokines and groups that were analyzed with Fisher´s post hoc test

Table 2 - Cytokine concentrations in the brain

Cytokine	**Sham** Mean concentration (pg/mg of protein) ± SD	**No exercise+ Ischemia** Mean concentration (pg/mg of protein) ± SD	**Exercise+ Ischemia** Mean concentration (pg/mg of protein) ± SD	T-test Sham vs. No exercise+ Ischemia	T-test No exercise+ Ischemia vs. exercise+ Ischemia
Brain IL-1 β	0.014±0.002	0.040±0.023	0.041±0.017	p=0.002	
Brain IL-2	0.029±0.007	0.036±0.007	0.042±0.021		
Brain IL-4	Below detection	Below detection	Below detection		
Brain IL-5	0.007±0.007	0.009±0.007	0.019±0.017		
Brain IL-6	0.57±0.1	1.86±1.4	2.24±1.2	p=0.003	
Brain IL-10	0.22±0.06	0.31±0.09	0.39±0.10		p=0.10
Brain IL-12p70	2.11±0.69	1.92±0.28	2.53±0.88		
Brain IFNγ	$1.56*10^{-3}$± $0.59*10^{-3}$	$1.64*10^{-3}$± $0.71*10^{-3}$	$8.93*10^{-3}$ ± $8.1*10^{-3}$		p=0.008
Brain KC/GRO	0.68±0.2	1.37±0.7	1.61±0.6		
Brain TNFα	0.041±0.009	0.091±0.039	0.101±0.041	p=0.015	

Table 2. The mean concentration± SD for all the cytokines in each group for brain samples. p values are shown for those cytokines and groups that were analyzed with Fisher´s post hoc test.

Table 3 - Results from Y-maze and sucrose preference behavioural tests

Behavioral test	**Sham** Mean value ± SD	**No exercise+ Ischemia** Mean value ± SD	**Exercise+ Ischemia** Mean value ± SD	T-test Sham vs. No exercise+ Ischemia	T-test No exercise+ Ischemia vs. exercise+ Ischemia
Y-maze (time in new arm in s)	28.6±4.8	30.2±13.0	32.1±11.3	p=0.73	p=0.77
Sucrose preference test (sucrose preference index in %)	61±6	62±4	66±6	p=0.56	p=31

Table 3. The non-significant results of the Y-maze and sucrose preference behavioural tests for the different groups. For Y-maze, the readout is presented as time spent in seconds in the new arm of the maze. For sucrose preference test, the readout is presented as the sucrose preference index in %. p values for the tests and groups analyzed with Fisher´s post hoc test.

Paper III

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Long distance ski racing is associated with lower long-term incidence of depression in a population based, large-scale study

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ABSTRACT

Physical activity has been proposed to be beneficial for prevention of depression, although the importance of exercise intensity, sex-specific mechanisms, and duration of the effects need to be clarified. Using an observational study design, following 395,369 individuals up to 21 years we studied whether participation in an ultralong-distance cross-country ski race was associated with lower risk of developing depression. Skiers (participants in the race) and matched non-skiers from the general population (non-participants in the race) were studied after participation (same year for non-participation) in the race using the Swedish population and patient registries. The risk of depression in skiers (n = 197,685, median age 36 years, 38% women) was significantly lower, to nearly half of that in non-skiers (adjusted hazard ratio, HR 0.53) over the follow-up period. Further, a higher fitness level (measured as the finishing time to complete the race, a proxy for higher exercise dose) was associated with lower incidence of depression in men (adjusted HR 0.65), but not in women. Our results support the recommendations of engaging in physical activity as a preventive strategy decreasing the risk for depression in both men and women. Furthermore, the exercise could reduce risk for depression in a dose-dependent matter, in particular in males.

1. Introduction

Depressive disorders are highly prevalent in most societies all around the world. Globally, depression constitutes one of the largest burdens of disability (Collaborators, 2016). The lifetime prevalence depression is estimated to be 5–10% (Kessler et al., 2009) and together with other mental disorders accounting for 21–32% of the total years lived with disability (Vigo et al., 2016). Even though several pharmacological treatment strategies targeting depression, including selective serotonin reuptake inhibitors, have become available over the past century, many patients still suffer from side-effects such as sexual dysfunction and gastrointestinal problems (Wang et al., 2018; Souery et al., 2007) or lack of effects (Rush, 2007). Numerous studies have pointed out physical activity as a promising strategy to reduce the burden of depressive disorders (Schuch et al., 2018; Pedersen and Saltin, 2015). Interestingly, interventional studies have shown that exercise can reduce symptoms of depression (Hennings et al., 2013; Wegner et al., 2014; Khanzada et al., 2015; Stanton et al., 2016). However, there are also studies, showing no additional effect of exercise compared to antidepressant medication alone (Danielsson et al., 2013; Kvam et al., 2016) or cognitive behavioral therapy alone (Bernard et al., 2018).

Furthermore, several studies have proposed exercise to have protective effects when it comes to development of depression (Sui et al., 2009; Jonsdottir et al., 2010; Aberg et al., 2012; Mammen and Faulkner, 2013; McPhie and Rawana, 2015; Schuch et al., 2018). A recent meta-analysis of prospective cohort studies, demonstrated that physical activity was protective against development of depression across all ages and also across different geographical regions (Schuch et al., 2018). Conversely, it has been pointed out that the association between higher level of physical activity and subsequent lower incidence of depression might be due to the fact of reverse

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causation. Individuals already having undiagnosed depression tend to be less engaged in physical activities as a consequence of symptoms such as reduced mood (Vancampfort et al., 2015; Busch et al., 2016). Indeed, exercise has to the large part been proposed as a treatment for individuals suffering from mild to moderate depression and not for more severe cases, where failure of adherence to exercise programs due to depressive symptoms might be an important limitation (Vancampfort et al., 2015; Firth et al., 2016).

To date, many studies investigating the effects of physical activity are conducted on small study populations and only have a few years of follow-up time (Khanzada et al., 2015; Schuch et al., 2018). This might increase the risk of biased results if several participants reporting low physical activity at baseline, due to yet undiagnosed depression, indeed get diagnosed within the next few years. Therefore, studies taking these potential barriers to engage in exercise in the initial phase of a yet undiagnosed depressive disorder into account are of great importance.

Further, the impact of fitness level or exercise dose on risk of depression has not been thoroughly investigated and existing studies so far show inconsistent results (Noh et al., 2015; Balchin et al., 2016; Harvey et al., 2018; Helgadottir et al., 2017). Some studies show that only exercise above a certain intensity have beneficial effects (Noh et al., 2015; Balchin et al., 2016), whereas others reveal no impact of intensity levels (Harvey et al., 2018). There are even studies in which the most beneficial effects are seen in exercise of lower intensities (Helgadottir et al., 2017). However, none of these studies analyze the impact of exercise intensity on incident depression in both men and women separately. Depression affects women more and it has been shown that the effect of physical activity on mental disorders might differ between men and women (Mikkelsen et al., 2010). Hence, investigating also the effects of physical activity also in women in a large-scale study with long-term follow up is needed.

To minimize the impact of any possible symptoms of undiagnosed mental illnesses on study results, it is important to follow a large number of subjects over a long time after initial exposure. Increasing the time of follow-up and excluding those who develop mental disorders within the first years after baseline are two ways of decreasing the risk for this type of bias. The aim of this study was to investigate the association between ultralong-distance ski racing and subsequent diagnosis of depression in a large scale, population-based cohort, in a long-term perspective. In addition, we also aimed to investigate the impact of fitness level as a measurement of exercise dosage on depression in men and women separately. As our treated cases, we used participants in the world's largest long-distance cross-country ski race (Vasaloppet). We matched the participants in the ski race with non-skiers from the general population, to include a total of 395,369 subjects in the study. Anderson et al. revealed that 93% of 5000 endurance skiers answering an online questionnaire reported freedom of depression (Anderson et al., 2017). To the best of our knowledge, no prospective studies have investigated the effect of skiing on development of depression. We hypothesized that there would be a lower risk of developing depression in the participants of the ski race compared to the general population that would also manifest long-term after participation.

2. Methods

2.1. Study design

This observational study was approved by the Ethical Review Board in Uppsala, Sweden, Dnr 2010/305. The cases in the study population comprise all Swedes who took part in the world's largest long-distance (30 to 90 km), cross-country ski race (Vasaloppet) between 1989 and 2010 (n = 197,685), together with frequency-matched, individuals from the general population (n = 197,684) (Supplementary Materials, Fig. 1). Frequency matching was done by Statistics Sweden to draw non-skier controls from the population registry according to age group (five-year intervals), sex, region of residency, and year of participation in ski race as previously described (Hallmarker et al., 2018). Individuals participating in Vasaloppet were considered physically active and were denominated skiers. According to previous studies, on average, Vasaloppet skiers smoke less, have a healthier diet, and lower mortality than the general Swedish population (Farahmand et al., 2003; Carlsson et al., 2007). The majority of Vasaloppet skiers exercise for at least 4 h a week (Carlsson et al., 2007) and on average they have higher leisure time physical activity than the general Swedish population (Farahmand et al., 2003). In general, Vasaloppet skiers have higher fitness levels, with VO2max 45–80 ml/kg per minute, compared to around 35 ml/kg per minute in the general population (Hallmarker et al., 2016). To reduce bias due to inability to participate in the race because of poor physical health, all individuals (skiers and non-skiers) with severe disease were excluded as previously described (*e.g.* cancer, chronic neurologic disease, dementia, heart- and lung disease) (Hallmarker et al., 2016). Participants with dementia (all cause, Alzheimer's disease (AD), vascular dementia (VaD), Parkinson's disease dementia, Lewy body dementia, senile dementia), Parkinson's disease, meningitis/encephalitis, epilepsy, psychiatric disorders (depressive episode, schizophrenia, bipolar disorder, anxiety disorders and mental disorders due to the use of alcohol) were additionally excluded from the study (see Supplementary Material Table 1). If skiers participated in several races, the first race was the only one considered and set as baseline. If a non-skier participated in the ski race after baseline it contributed with data for the skiers population from the time of participation in the ski race until the end of follow-up period.

The participants were monitored for participation as well as finishing time in the race in three categories with finishing time of 100–150, 150–200 and above 200% of the winning finishing time for each sex respectively as previously described (Hallmarker et al., 2015). The finishing time analysis was used as a measurement of physical fitness and a proxy for the more extreme doses of exercise during their preparation before the race, as previously explained (Andersen et al., 2013). Information on date of birth, sex, and education level were derived from Swedish registries (Swedish National Patient Registry for diagnoses and Statistics Sweden for socio-economic data) (Hallmarker et al., 2018). The total study cohort (n = 395,369) was followed in the Swedish National Patient Registry (described below) throughout 2010.

2.2. The Swedish national patient registry

Data on psychiatric and somatic diagnoses were retrieved from the Swedish National Patient Registry. Since 1987 it provides information on all primary and secondary diagnoses in patients attending hospital-based care in Sweden. The register covers 99% of all hospital-based diagnoses, both somatic and psychiatric. Primary care diagnoses are not included in the registry, but they are imported into the patient registry as soon as the patient becomes an inpatient. Since 2001, this registry also covers out-hospital diagnoses made in hospital clinics closely related to primary care. Depressive disorders were defined according to the International Classification of Diseases (ICD), tenth revision (ICD10, Socialstyrelsen) or ninth revision (ICD9, Socialstyrelsen). Diagnoses included are depressive episode (F32, F33, F34, F38, F399/296B, 296X, 29620, 29800).

2.3. Statistical analyses

We used R statistical software package for analyses. P-values <0.05 were considered statistically significant. Demographic data are presented as median and interquartile range (IQR) or numbers (n) and percent (%). Numeric group differences were estimated with Mann-Whitney U test and categorical with Pearson's χ^2 test. Cox regression models were used to compare risk of depression for Vasaloppet skiers vs non-skiers. Risk of depressive disorders are presented as hazard ratios

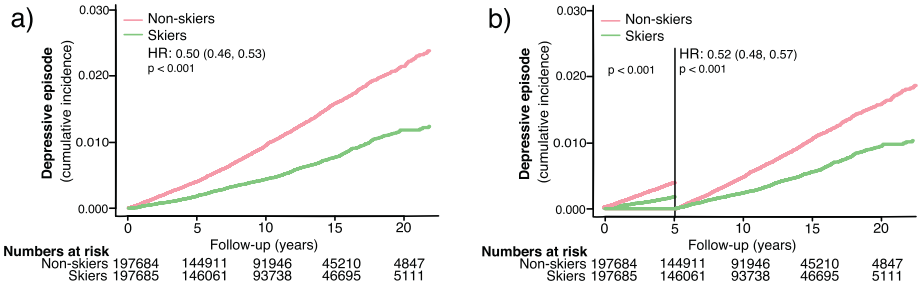

Fig. 1. The risk of developing depressive episodes in Vasaloppet skiers compared to non-skiers (a) and the risk of developing depressive episodes more than 5 years after completing the ski race (b). HR represents hazard ratios from an unadjusted cox regression.

(HR) with 95% confidence intervals (CI). Numbers at risk were derived from survival tables specifying number of individuals entering each five-year interval, as presented in the graph. The time variable was calculated as years between participation in the ski race (and the same year for the matched non-skier) and event or censoring. The event was defined as date of first registered depression diagnosis in the Patient Registry. Censoring appeared when subjects died or at time of register outtake/end of follow-up. Information on date of death for deceased study individuals was available through the Causes of Death Register (CDR), held at the National Board of Health and Welfare. Schoenfeld residuals were modeled graphically to assess the proportionality assumption. Sex was suggested to be a possible effect modifier, therefore men and women were also analyzed separately. The impact of finishing time (fitness level) was assessed by trichotomizing the finishing time to 100–150%, 150–200% and above 200% of the winning finishing time for men and women separately. Adjustments were done for age, sex and education in the adjusted cox model. In sensitivity analyses, all individuals who developed depression within five years of inclusion were excluded.

3. Results

3.1. Ski race participation is associated with lower incidence of depression

Demographic data comparing the Vasaloppet skiers and non-skiers are presented in Table 1. A total of 395,369 individuals were followed over 3975,881 person years. After a median follow-up of 10 (IQR 5–15) years, a total of 3075 individuals were newly diagnosed with depression. The occurrence of comorbidity with other psychiatric disorders did not differ between Vasaloppet skiers and non-skiers (Supplementary Table 2). Participation in the long-distance ski race was associated with a lower risk of developing depression in the follow-up compared to non-skiers (unadjusted HR 0.50, 95% CI 0.46–0.53, Table 2, Fig. 1a). Skiers had higher education than non-skiers (Table 1), but adjustments for age, sex and education, did not alter the results (adjusted cox model, Table 2). The effect remained even when individuals that developed depression within five years of the ski race (baseline) were excluded (unadjusted HR 0.52, 95% CI 0.48–0.57, Table 2, Fig. 1b). Taken together, ski race participation was associated with a relative risk reduction of 50% for developing depression.

Table 2
Association between physical activity and incident mental disorders, based on participation in a long-distance ski race (skiers) compared to non-skiers.

Depressive episode	Unadjusted model	Adjusted model*
Physical activity	HR (95% CI)	HR (95% CI)
Nr events	3075	3040
Non-skiers (Reference)	1	1
Skiers	0.50 (0.46–0.53)	0.53 (0.49–0.58)
Excluding psychiatric diagnoses <5 years		
Nr events	2029	2003
Non-skiers (Reference)	1	1
Skiers	0.52 (0.48–0.57)	0.56 (0.51–0.62)

HR: hazard ratio, CI: confidence interval.
Cox regression models showing HR for risk of depressive disorders.
* Model adjusted for age, sex, and education.

3.2. Both male and female Vasaloppet skiers have lower incidence of depression

The association between ski race participation and lower incidence of depression was seen in both men and women (unadjusted HR 0.52, 95% CI, 0.47–0.57 for men and unadjusted HR 0.47, 95% CI, 0.42–0.53 for women, Fig. 2a-b).

Table 1
Characteristics of the study population, presented for the whole cohort and by skiers and non-skiers separately.

	All n = 395,369	Skiers n = 197,685	Non-skiers n = 197,684
Characteristics 1989–2010	Median (IQR) or n (%)	Median (IQR) or n (%)	Median (IQR) or n (%)
Age at baseline, y	36.0 (29.0–46.0)	36.0 (29.0–46.0)	36.0 (29.0–46.0)
Women	149,796 (38)	74,897 (38)	74,899 (38)
Education:			
Primary/elementary school (≤8 y)	49,344 (13)	14,538 (7.4)	34,806 (18) ***
Secondary school/high school (9–12 y)	17,6571 (45)	76,635 (39)	99,936 (51)
Higher education/university (≥13 y)	16,6133 (42)	10,6147 (54)	59,986 (31)
Depression at follow-up	3075	1030	2045***

IQR: interquartile range, y: years, n: numbers.
*** $p < 0.001$. Group difference between skiers and non-skiers, estimated with Wilcoxon test (numeric variables) and Pearson's χ^2 test (categorical variables). Only significant differences are noted in the table.

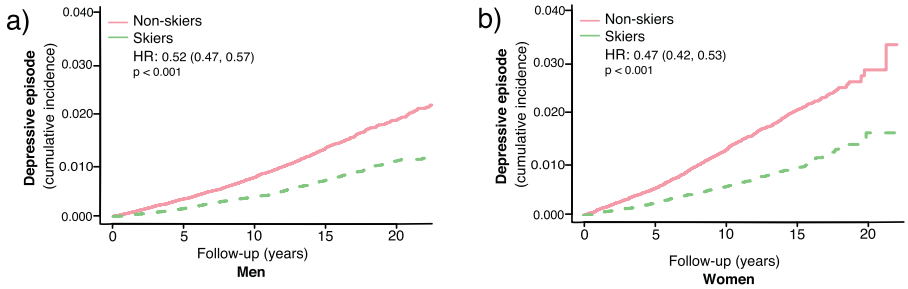

Fig. 2. The risk of developing depressive episodes in Vasaloppet skiers compared to non-skiers in men (a) and women separately (b). HR represents hazard ratios from an unadjusted cox regression.

3.3. The impact of exercise dose on incident depression is sex-specific

Men completing the race with shortest finishing time (a proxy for the effect of extreme exercise) had an even lower incidence of depression compared to slower Vasaloppet skiers (unadjusted HR 0.65, 95% CI, 0.49–0.87, Table 3, Fig. 3a). This was not the case among women, where the finishing time did not have any significant impact (unadjusted HR 1.14, 95% CI, 0.77–1.70, Table 3, Fig. 3b). Adjustments for age, sex and education, did not alter the results (adjusted Cox model, Table 3). These associations remained even when excluding cases that developed depression within the first five years (unadjusted HR 0.61, 95% CI, 0.44–0.86 for men, Table 3, Fig. 3c-d).

4. Discussion

In a large, population-based study on nearly 400,000 individuals, followed over nearly 4 million person years, we found that higher baseline participation in physical exercise was associated with a significant lower risk of the development of depression, one of the most prevalent psychiatric disorders. Our results were highly significant and showed relative risk reductions of around 50% for depression. Of high importance, our results were the same even when we excluded all cases of depression that occurred during the first five years after the baseline event. This minimizes the risk that our results were due to a selection bias towards more psychiatrically healthy individuals at baseline in the group participating in the ski race. Furthermore, our analysis of ski race finishing time (a proxy for the level of fitness) revealed a sex-specific impact of the dose of exercise on incident depression, where we found fast male Vasaloppet skiers to have lower risk of depression compared to slow male skiers.

Our study setup offered a unique possibility to study the effect of a physical active life-style on the development of depression in a very large study population over a long period of time.

We used the unique national patient registries available in Sweden, which is one of the largest in the world covering diagnoses set on the entire population since 1964.

By use of this registry, we were able to exclude all subjects (skiers and non-skiers) that had psychiatric diseases or somatic disorder that could impact physical activity prior to the baseline participation in the long-distance ski race (Hallmarker et al., 2016) (See supplementary material, Table 1). We carefully matched the Vasaloppet skiers with non-skiers based on sex, age and geographic location. Psychiatric diagnoses set after baseline for participation in the world's largest ski race were extracted. This enabled us to follow the participants up to 21 years after participation. As such, this is the largest population wide epidemiological study to date including both men and women, confirming an effect of physical exercise on the later development of depression seen in previous studies (Aberg et al., 2012; Mammen and Faulkner, 2013; Khanzada et al., 2015; Schuch et al., 2018). In addition to these studies, our study takes into consideration the impact of exercise dose and

Table 3
Association between ski race finishing time and incident mental disorders in men and women.

Depressive episode	Men	Women
Finishing time (% of winning time)	HR (95% CI)	HR (95% CI)
Unadjusted model		
+200% (Reference)	1	1
150–200%	0.94 (0.78, 1.14)	0.84 (0.67, 1.05)
100–150%	0.65 (0.49, 0.87)	1.14 (0.77, 1.70)
Adjusted model*		
+200% (Reference)	1	1
150–200%	0.91 (0.75, 1.09)	0.83 (0.66, 1.04)
100–150%	0.65 (0.49, 0.87)	1.11 (0.74, 1.66)
Excluding psychiatric diagnoses < 5 years		
Unadjusted model		
+200% (Reference)	1	1
150–200%	0.94 (0.75, 1.17)	0.83 (0.62, 1.11
100–150%	0.61 (0.44, 0.86)	1.12 (0.69, 1.82)
Adjusted model*		
+200% (Reference)	1	1
150–200%	0.89 (0.71, 1.11)	0.84 (0.63, 1.12)
100–150%	0.60 (0.43, 0.84)	1.13 (0.69, 1.86)

HR: hazard ratio, CI: confidence interval.
Cox regression models showing HR for risk of depressive disorders in men and women respectively.
* Model adjusted for age and education.

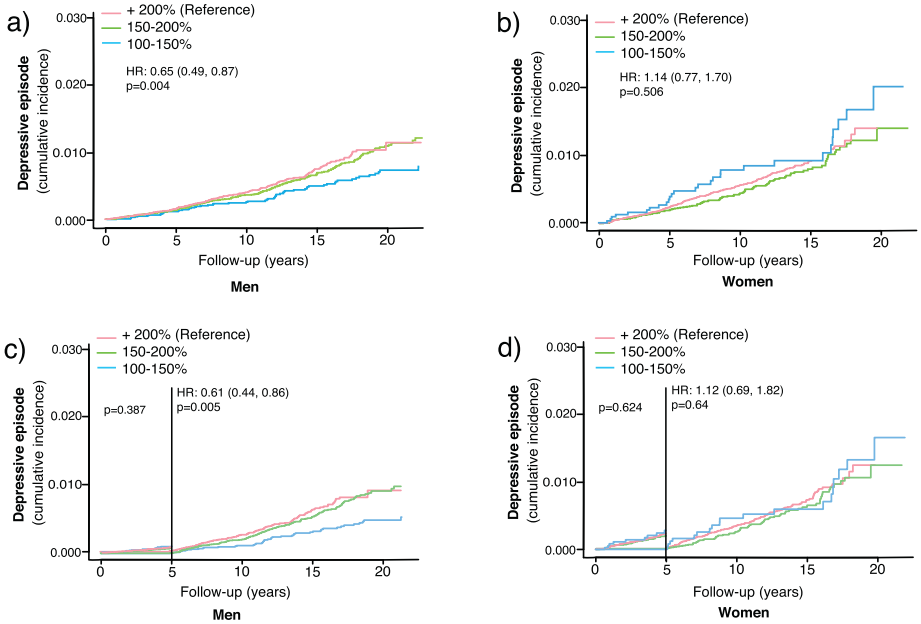

Fig. 3. The impact of ski race finishing time on the risk of developing depressive episodes in skiers in men (a) and women separately (b). The impact of ski race finishing time on the risk of developing depressive episodes more than 5 years after completing the ski race in men (c) and women (d). HR represents hazard ratios from an unadjusted cox regression for the fastest (100–150% of winning finishing time) group, using + 200% as the reference group.

differences between men and women.

Our study is important because it provides new information about the effect of physical activity on development of depressive disorders in both men and women, in addition to the discoveries made by Åberg et al. Indeed, in their study, following over 1 million men in the Swedish population for a period of up to 40 years, physical fitness was associated with a lower incidence of depression (Aberg et al., 2012). However, their study did not assess the effects of physical activity on development of depression in women. This additional analysis included in our study is of great importance, since the incidence/prevalence is significantly higher in women. Furthermore, the effect of physical activity on mental disorders may differ between men and women (Mikkelsen et al., 2010). Importantly, we found an association of physical activity on incidence of mental disorders in both men and women. Our study revealed that the impact of physical activity on the risk for depression lasts over long time, and remained even when we excluded cases during the first 5 years following inclusion in the study (Table 2). This is in line with a recent study by Choi et al., which investigated the relationship between physical activity and depression (Choi et al., 2019). They demonstrated additional evidence for objectively measured physical activity to be protective against later risk for depression.

Our study demonstrates an impact of the dose of exercise on the incidence of depression. Interestingly, this effect differed between men and women. Notably, men with higher dose of exercise had even lower incidence of depression, whereas no such tendency could be seen for women in our study. This emphasizes the importance of evaluating the effect of physical exercise in men and women separately. Our findings on the impact of exercise dose on depression are in line with other studies investigating the effect on depressive symptoms (Asztalos et al., 2010; Balchin et al., 2016; Craft et al., 2014; Helgadottir et al., 2017; Meyer et al., 2016). Indeed, higher intensities of exercise has been shown to specifically reduce depression in men (Balchin et al., 2016), whereas the intensities did not matter for women (Meyer et al., 2016). In a randomized controlled trial demonstrating that the most beneficial effects on depression was seen in the group with the lightest form of exercise, 74% of the participants where women (Helgadottir et al., 2017). The reasons for these discrepancies between men and women were not possible to investigate in our study. However, it has been shown that the reasons for having high levels of physical activity among women might be a better predictor of mental well-being than the exercise per se (Craft et al., 2014). Notably, women reporting aims such as weight loss and body toning as reasons for exercising tended to have lower quality of life compared to those reporting such as improving health as reasons (Craft et al., 2014). Interestingly, the same pattern was not detected in men. In addition, only self-reported level of physical activity and not objectively measured fitness level was shown to be related to depressive symptoms (Lindwall et al., 2012). Hence, it is tempting to speculate that psychological reasons behind the exercise results might be a confounding factor and explain at least some of these differences, although we lack this kind of data in our study. To that adds also a need of gaining more knowledge in the potential mechanisms behind the beneficial effects of exercise and how these might differ between men and women.

Even though this epidemiological study did not attempt to assess the mechanisms by which exercise may exert protective effects on the development of depression, many experimental studies have done so in the past. A large wealth of studies indicate that inflammation is involved in the pathogenesis of neuropsychiatric disorders (Hallberg et al., 2010; Shelton et al., 2011; Dahl et al., 2014; Wang and Miller, 2018). Thus, a main proposed mechanisms by which exercise might be protective might be by reducing the amount of inflammation in the body (Hallberg et al., 2010), and ultimately in the brain. Other

studies indicate that beneficial effects of exercise might be mediated by increased levels of neurotrophic factors (Callaghan et al., 2017). Exercise may also affect the regulation of the hypothalamic pituitary adrenal (HPA) axis (Svensson et al., 2015; Phillips, 2017), known to be altered in depression (Lopez-Duran et al., 2009). Further, exercise is also known to induce endorphins, proven to contribute to the perception of well-being (Mikkelsen et al., 2017). However, long-term effects of exercise on molecular mechanisms involved in depression still need to be elucidated (Millischer et al., 2017). To further address these questions, animal models are of great importance in the first phase. In addition to the above-mentioned molecular effects, exercise also have significant psychological and psychosocial effects as previously discussed by Nieman et al. (Nieman, 2002). Importantly, Nieman mention how exercise might facilitate distraction from negative thoughts and also how it contributes to the persons increased perceived self-significance as being physically active is regarded as doing something good according to the society.

Limitations of the study include that we do not have any detailed information about the physical activity in the cohort. The information we use as a proxy of physical activity is the baseline participation in the long-distance ski race (30–90 km). The race is physically demanding and requires arduous preparation, assuring that the participants in the race had led an active life-style with preparatory exercise long-term prior to the race. It is likely that the reference group of non-skiers thereby to some extent include physically active as well and this may attenuate the true association. However, the exercise habits of the participants in this classical ski race have been characterized and described as more physically active compared to the general population in (Farahmand et al., 2003; Carlsson et al., 2007). Furthermore, as with many other sports, the ski racers spend a substantial amount of time outdoors, being exposed to natural light, a factor associated with reduced risk of depression. A synergistic effect of being outdoor and exercising has been proposed in alleviating depression (Lahart et al., 2019). Natural environment has been shown to improve coping with psycho-physiological stress (Berto, 2014; Ulrich et al., 1991). However, a systematic review revealed that physical activity had a more important effect than light therapy, suggesting that the activity per se is of substantial importance (Cooney et al., 2013). Skiing is a winter activity and individuals with seasonal mood disorders might have difficult to participate. To this adds the possible limitation for those with asthma to participate in skiing. However, recent report indicate that a substantial amount of Vasaloppet skiers manage to participate in the ski race despite having asthma (Nasman et al., 2018). Further, a meta-analysis of prospective studies indicates that it was more likely that having depression predicted subsequent development of asthma than vice versa (Gao et al., 2015). We tried to reduce the impact of the above mentioned potential bias by excluding all individuals diagnosed with depression prior to baseline, and also within the first five years from baseline.

Moreover, our study is based on diagnoses found in the Swedish Patient Registry, which does not cover all diagnoses. Due to the long follow-up time we assume that many diagnoses set in primary care would have been imported to this registry. Another limitation is that our study does not isolate physical activity as a truly independent factor. For instance, it has been shown before that Vasaloppet skiers may have a healthier lifestyle including less smoking and better diet, than a control population of non-skiers (Farahmand et al., 2003; Carlsson et al., 2007). We cannot adjust for smoking, weight or alcohol consumption since that information is not available in the Swedish Patient Registry. However, when we adjust for age, sex and education in our statistical models, the results were not altered. Additionally, we demonstrate that faster Vasaloppet skiers had lower incidence of depression, implicating that the association between physical activity and lower incidence of depression can be attributable to the physical fitness level per se. We used finishing time as a proxy for fitness level/dose of exercise as previously described (Hallmarker et al., 2015). Further, it has been shown that physical performance level (work output measured in W/kg) before participation in Vasaloppet predicts the finishing time of the skier (Mygind et al., 2015). However, it should be mentioned that also other factors, such as race experience and pacing strategy affects the finishing time (Carlsson et al., 2016; Nikolaidis et al., 2018).

Last, but not least, if these findings should be implemented in healthcare, one also has to take into account the reasons preventing patients with depression from engaging in physical activity. Most researchers agree that physical activity is beneficial for this group of patients, but in order to find strategies for motivating patients in engaging in more physical activities, factors preventing them from doing so must be determined more in detail. In our study, we take this factor into account by excluding patients with psychiatric disorders prior to baseline and also cases newly diagnosed within the first five years after baseline. Nevertheless, this is important for reducing potential bias also when designing future studies.

In conclusion, our study on 395,369 individuals in the Swedish population indicates that participating in this long distance ski race is associated with a substantially lower risk of developing depression. The effect was of similar size in men and women, and persisted even when removing the first five years after baseline from the analysis. In addition, we detected an impact of exercise dose on incidence of subsequent depression that differed between men and women. Our results indicate that the effects of physical activity may be greater than previously estimated and warrants additional experimental studies characterizing the neurobiological mechanisms by which exercise impacts mental health.

Data statements

Database for the Vasaloppet cohort with disease incidence belong to Uppsala Clinical Research Center and can be made available upon request.

Declaration of Competing Interest

No author reports conflict of interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psychres.2019.112546.

References

Aberg, M.A., Waern, M., Nyberg, J., Pedersen, N.L., Bergh, Y., Aberg, N.D., Nilsson, M.,

Kuhn, H.G., Toren, K., 2012. Cardiovascular fitness in males at age 18 and risk of serious depression in adulthood: Swedish prospective population-based study. Br. J. Psychiatry 201, 352–359.
Andersen, K., Farahmand, B., Ahlbom, A., Held, C., Ljunghall, S., Michaelsson, K., Sundstrom, J., 2013. Risk of arrhythmias in 52 755 long-distance cross-country skiers: a cohort study. Eur. Heart J. 34, 3624–3631.
Anderson, P.J., Bovard, R.S., Murad, M.H., Beebe, T.J., Wang, Z., 2017. Health status and health behaviors among citizen endurance Nordic skiers in the united states. BMC Res. Notes 10, 305.
Asztalos, M., de Bourdeaudhuij, I., Cardon, G., 2010. The relationship between physical activity and mental health varies across activity intensity levels and dimensions of mental health among women and men. Public Health Nutr. 13, 1207–1214.
Balchin, R., Linde, J., Blackhurst, D., Rauch, H.L., Schonbachler, G., 2016. Sweating away depression? The impact of intensive exercise on depression. J. Affect Disord. 200, 218–221.
Bernard, P., Romain, A.J., Caudroit, J., Chevance, G., Carayol, M., Gourlan, M., Needham Dancause, K., Moullec, G., 2018. Cognitive behavior therapy combined with exercise for adults with chronic diseases: systematic review and meta-analysis. Health Psychol. 37, 433–450.
Berto, R., 2014. The role of nature in coping with psycho-physiological stress: a literature review on restorativeness. Behav. Sci. (Basel) 4, 394–409.
Busch, A.M., Ciccolo, J.T., Puspitasari, A.J., Nosrat, S., Whitworth, J.W., Stults-Kolehmainen, M., 2016. Preferences for exercise as a treatment for depression. Ment. Health Phys. Act. 10, 68–72.
Callaghan, C.K., Rouine, J., O'Mara, S.M., 2017. Exercise prevents IFN-alpha-induced mood and cognitive dysfunction and increases BDNF expression in the rat. Physiol. Behav. 179, 377–383.
Carlsson, M., Assarsson, H., Carlsson, T., 2016. The influence of sex, age, and race experience on pacing profiles during the 90 Km vasaloppet ski race. Open Access. J. Sports Med. 7, 11–19.
Carlsson, S., Olsson, L., Farahmand, B.Y., Hallmarker, U., Ahlbom, A., 2007. Skiers in the long-distance ski race invest in their health. Lakartidningen 104, 670–671.
Choi, K.W., Chen, C.Y., Stein, M.B., Klimentidis, Y.C., Wang, M.J., Koenen, K.C., Smoller, J.W., Major depressive disorder working group of the psychiatric genomics, C., 2019. Assessment of bidirectional relationships between physical activity and depression among adults: a 2-Sample mendelian randomization study. JAMA Psychiatry 76 (4), 399–408.
Collaborators, GBDDIIAP, 2016. Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990–2015: a systematic analysis for the global burden of disease study 2015. Lancet 388, 1545–1602.
Cooney, G.M., Dwan, K., Greig, C.A., Lawlor, D.A., Rimer, J., Waugh, F.R., Mcmurdo, M., Mead, G.E., 2013. Exercise for depression. Cochrane Database Syst. Rev CD004366.
CRAFT, B.B., Carroll, H.A., Lustyk, M.K., 2014. Gender differences in exercise habits and quality of life reports: assessing the moderating effects of reasons for exercise. Int. J. Lib. Arts Soc. Sci. 2, 65–76.
Dahl, J., Ormstad, H., Aass, H.C., Malt, U.F., BENDZ, L.T., Sandvik, L., Brundin, L., Andreassen, O.A., 2014. The plasma levels of various cytokines are increased during ongoing depression and are reduced to normal levels after recovery. Psychoneuroendocrinology 45, 77–86.
Danielsson, L., Noras, A.M., Waern, M., Carlsson, J., 2013. Exercise in the treatment of major depression: a systematic review grading the quality of evidence. Physiother. Theory Pract. 29, 573–585.
Farahmand, B.Y., Ahlbom, A., Ekblom, O., Ekblom, B., Hallmarker, U., Aronson, D., Brobert, G.P., 2003. Mortality amongst participants in Vasaloppet: a classical long-distance ski race in Sweden. J. Intern. Med. 253, 276–283.
Firth, J., Rosenbaum, S., Stubbs, B., Gorczynski, P., Yung, A.R., Vancampfort, D., 2016. Motivating factors and barriers towards exercise in severe mental illness: a systematic review and meta-analysis. Psychol. Med. 46, 2869–2881.
Gao, Y.H., Zhao, H.S., Zhang, F.R., Gao, Y., Shen, P., Chen, R.C., Zhang, G.J., 2015. The relationship between depression and asthma: a meta-analysis of prospective studies. PLoS One 10, e0132424.
Hallberg, L., Janelidze, S., Engstrom, G., Wisen, A.G., Westrin, A., Brundin, L., 2010. Exercise-induced release of cytokines in patients with major depressive disorder. J. Affect Disord. 126, 262–267.
Hallmarker, U., Asberg, S., Michaelsson, K., Arnlov, J., Hellberg, D., Lindback, J., Wester, P., James, S., 2015. Risk of recurrent stroke and death after first stroke in long-distance ski race participants. J. Am. Heart Assoc. 4, e002469.
Hallmarker, U., Lindback, J., Michaelsson, K., Arnlov, J., Asberg, S., Wester, P., Hellberg, D., Lagerqvist, B., James, S., 2018. Survival and incidence of cardiovascular diseases in participants in a long-distance ski race (Vasaloppet, Sweden) compared with the background population. Eur. Heart J. Qual. Care Clin. Outcomes 4, 91–97.
Hallmarker, U., Michaelsson, K., Arnlov, J., Hellberg, D., Lagerqvist, B., Lindback, J., James, S., 2016. Risk of recurrent Ischaemic events after myocardial infarction in long-distance ski race participants. Eur. J. Prev. Cardiol. 23, 282–290.
Harvey, S.B., Overland, S., Hatch, S.L., Wessely, S., Mykletun, A., Hotopf, M., 2018. Exercise and the prevention of depression: results of the hunt cohort study. Am. J. Psychiatry 175, 28–36.
Helgadottir, B., Forsell, Y., Hallgren, M., Moller, J., Ekblom, O., 2017. Long-term effects of exercise at different intensity levels on depression: a randomized controlled trial. Prev. Med. 105, 37–46.
Hennings, A., Schwarz, M.J., Riemer, S., Stapf, T.M., Selberdinger, V.B., Rief, W., 2013. Exercise affects symptom severity but not biological measures in depression and somatization - results on IL-6, neopterin, tryptophan, kynurenine and 5-HIAA. Psychiatry Res. 210, 925–933.
Jonsdottir, I.H., Rodjer, L., Hadzibajramovic, E., Borjesson, M., Ahlborg JR., G., 2010. A prospective study of leisure-time physical activity and mental health in Swedish health care workers and social insurance officers. Prev. Med. 51, 373–377.
Kessler, R.C., Aguilar-Gaxiola, S., Alonso, J., Chatterji, S., Lee, S., Ormel, J., Ustun, T.B., Wang, P.S., 2009. The global burden of mental disorders: an update from the who world mental health (WMH) surveys. Epidemiol. Psichiatr. Soc. 18, 23–33.
Khanzada, F.J., Soomro, N., Khan, S.Z., 2015. Association of physical exercise on anxiety and depression amongst adults. J. Coll. Physician. Surg. Pak. 25, 546–548.
Kvam, S., Kleppe, C.L., Nordhus, I.H., Hovland, A., 2016. Exercise as a treatment for depression: a meta-analysis. J. Affect Disord. 202, 67–86.
Lahart, I., Darcy, P., Gidlow, C., Calogiuri, G., 2019. The effects of green exercise on physical and mental wellbeing: a systematic review. Int. J. Environ. Res. Public Health 16.
Lindwall, M., Ljung, T., Hadžibajramović, E., Jonsdottir, I.H., 2012. Self-reported physical activity and aerobic fitness are differently related to mental health. Mental Health Phys. Activity 5, 28–34.
Lopez-Duran, N.L., Kovacs, M., GEORGE, C.J., 2009. Hypothalamic-pituitary-adrenal axis dysregulation in depressed children and adolescents: a meta-analysis. Psychoneuroendocrinology 34, 1272–1283.
Mammen, G., Faulkner, G., 2013. Physical activity and the prevention of depression: a systematic review of prospective studies. Am. J. Prev. Med. 45, 649–657.
Mcphie, M.L., Rawana, J.S., 2015. The effect of physical activity on depression in adolescence and emerging adulthood: a growth-curve analysis. J. Adolesc. 40, 83–92.
Meyer, J.D., Koltyn, K.F., Stegner, A.J., Kim, J.S., COOK, D.B., 2016. Influence of exercise intensity for improving depressed mood in depression: a dose-response study. Behav. Ther. 47, 527–537.
Mikkelsen, K., Stojanovska, L., Polenakovic, M., Bosevski, M., Apostolopoulos, V., 2017. Exercise and mental health. Maturitas 106, 48–56.
Mikkelsen, S.S., Tolstrup, J.S., Flachs, E.M., Mortensen, E.L., Schnohr, P., Flensborg-Madsen, T., 2010. A cohort study of leisure time physical activity and depression. Prev. Med. 51, 471–475.
Millischer, V., Erhardt, S., Ekblom, O., Forsell, Y., Lavebratt, C., 2017. Twelve-week physical exercise does not have a long-lasting effect on kynurenines in plasma of depressed patients. Neuropsychiatr. Dis. Treat. 13, 967–972.
Mygind, E., Wulff, K., Rosenkilde Larsen, M., Helge, J.W., 2015. Prediction of performance in Vasaloppet through long lasting ski-ergometer and Rollerski tests in cross-country skiers. Int. J. Sport. Exer. Med. 1, 1–7.
Nasman, A., Irewall, T., Hallmarker, U., Lindberg, A., Stenfors, N., 2018. Asthma and asthma medication are common among recreational athletes participating in endurance sport competitions. Can. Respir. J. 2018, 3238546.
Nieman, P., 2002. Psychosocial aspects of physical activity. Paediatr. Child Health 7, 309–312.
Nikolaidis, P.T., Villiger, E., Rosemann, T., Knechtle, B., 2018. The effect of aging on pacing strategies of cross-country skiers and the role of performance level. Eur. Rev. Aging Phys. Act. 15, 4.
Noh, J.W., LEE, S.A., Choi, H.J., Hong, J.H., Kim, M.H., Kwon, Y.D., 2015. Relationship between the intensity of physical activity and depressive symptoms among Korean adults: analysis of Korea health panel data. J. Phys. Ther. Sci. 27, 1233–1237.
Pedersen, B.K., Saltin, B., 2015. Exercise as medicine - evidence for prescribing exercise as therapy in 26 different chronic diseases. Scand. J. Med. Sci. Sports 25 (Suppl 3), 1–72.
Phillips, C., 2017. Brain-Derived neurotrophic factor, depression, and physical activity: making the neuroplastic connection. Neural. Plast 2017, 7260130.
Rush, A.J., 2007. Limitations in efficacy of antidepressant monotherapy. J. Clin. Psychiatry 68 (Suppl 10), 8–10.
Schuch, F.B., Vancampfort, D., Firth, J., Rosenbaum, S., Ward, P.B., Silva, E.S., Hallgren, M., Ponce de Leon, A., Dunn, A.L., deslandes, A.C., Fleck, M.P., Carvalho, A.F., Stubbs, B., 2018. Physical activity and incident depression: a meta-analysis of prospective cohort studies. Am. J. Psychiatry appiajp201817111194.
Shelton, R.C., Claiborne, J., Sidoryk-Wegrzynowicz, M., Reddy, R., Aschner, M., Lewis, D.A., Mirnics, K., 2011. Altered expression of genes involved in inflammation and apoptosis in frontal cortex in major depression. Mol. Psychiatry 16, 751–762.
Souery, D., Oswald, P., Massat, I., Bailer, U., Bollen, J., Demyttenaere, K., Kasper, S., Lecrubier, Y., Montgomery, S., Serretti, A., Zohar, J., Mendlewicz, J., Group for the study of resistant, D., 2007. Clinical factors associated with treatment resistance in major depressive disorder: results from a European multicenter study. J. Clin. Psychiatry 68, 1062–1070.
Stanton, R., Reaburn, P., Happell, B., 2016. The effect of acute exercise on affect and arousal in inpatient mental health consumers. J. Nerv. Ment. Dis. 204, 658–664.
Sui, X., Laditka, J.N., Church, T.S., Hardin, J.W., Chase, N., Davis, K., Blair, S.N., 2009. Prospective study of cardiorespiratory fitness and depressive symptoms in women and men. J. Psychiatr. Res. 43, 546–552.
Svensson, M., Lexell, J., Deierborg, T., 2015. Effects of physical exercise on neuroinflammation, neuroplasticity, neurodegeneration, and behavior: what we can learn from animal models in clinical settings. Neurorehabil. Neural. Repair. 29, 577–589.
Ulrich, S.R., Simons, R.F., Losito, B.D., Fiorito, E., Miles, M.A., Zelson, M., 1991. STRESS recovery during exposure to natural and urban environments. J. Environ. Psychol. 11, 201–230.
Vancampfort, D., Stubbs, B., Sienaert, P., Wyckaert, S., de Hert, M., Rosenbaum, S., Probst, M., 2015. What are the factors that influence physical activity participation in individuals with depression? A review of physical activity correlates from 59 studies. Psychiatr. Danub. 27, 210–224.
Vigo, D., Thornicroft, G., Atun, R., 2016. Estimating the true global burden of mental illness. Lancet Psychiatry 3, 171–178.
Wang, A.K., Miller, B.J., 2018. Meta-analysis of cerebrospinal fluid cytokine and tryptophan catabolite alterations in psychiatric patients: comparisons between schizophrenia, bipolar disorder, and depression. Schizophr. Bull. 44, 75–83.
Wang, S.M., Han, C., Bahk, W.M., Lee, S.J., Patkar, A.A., Masand, P.S., Pae, C.U., 2018. Addressing the side effects of contemporary antidepressant drugs: a comprehensive review. Chonnam. Med. J. 54, 101–112.
Wegner, M., Helmich, I., Machado, S., Nardi, A.E., Arias-Carrion, O., Budde, H., 2014. Effects of exercise on anxiety and depression disorders: review of meta- analyses and neurobiological mechanisms. CNS Neurol. Disord. Drug. Target. 13, 1002–1014.

Supplementary material

1. Extended Method description

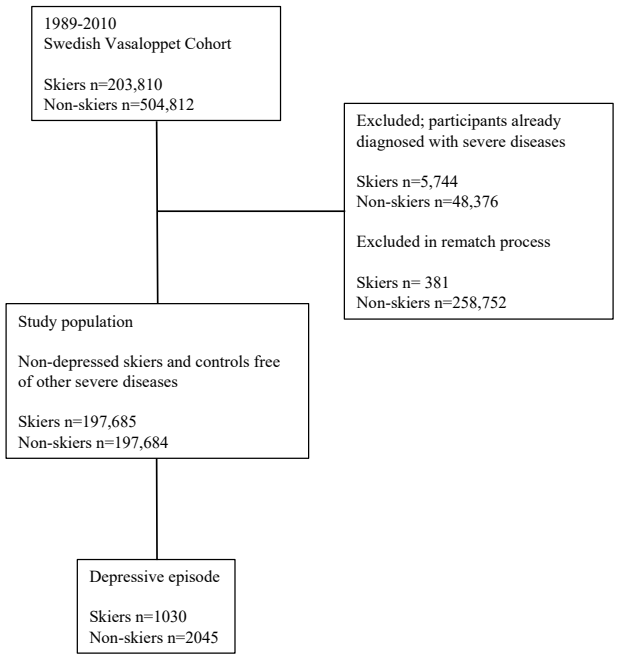
Exclusion criteria and matching procedure

In the Vasaloppet study, individuals with severe disease were excluded since such diseases are likely to hinder participation in a demanding long-distance race. A flow diagram describing numbers excluded due to severe disease can be seen in Supplementary Figure S1. The exact diagnoses have been stated previously(1). We additionally excluded participants with diagnoses listed in Table S1.

In the first matching process, a control individual from the general population was assigned for every ski race, so that skiers participating in Vasaloppet several times got several controls. We performed a re-matching procedure to get equally many skiers as non-skiers. Since we only used the index race for each skier, the non-skiers would have been older as a group if we had included one control for every time a skier participated in the race.

2. Supplementary Figure

Vasaloppet Study population



Supplementary Figure S1. Flow diagram describing the Vasaloppet Study population.

3. Supplementary Tables

Supplementary Table 1. Additional exclusion criteria

Diagnosis	ICD-9	ICD-10
Alzheimer´s disease	331A/3310, 29010	F00, G30
vascular dementia	290E, 2904, 2930	F01
all-cause dementia	290, F070, 294C, 294B, 331A, 310A, G318A	F00, F01, F02, F03, G30
Lewy body dementia	331X, G318A, 33182	F028
dementia in Parkinson disease	294B, 332A	F023
Parkinson disease	332A, 3420	G20
meningitis/encephalitis	3200, 320A, 320B, 320C, 320D, 320W 320X, 321A, 321B, 321C, 321D, 321E, 321X, 322A, 322B, 322C, 320X, 323, 3230	G00, G01, G03, G04, G05
epilepsy	345, 3450	G40
depressive episode	F399, 296B, 296X, 29620, 29800	F32, F33, F34, F38
anxiety disorders	300A, 300B, 300C, 300D, 300D, 3000, 3001, 3002, 3003	F40, F41, F42
bipolar disorder	296A, 29610 ,296C, 296D, 296E, 29600, 29610, 29620, 29630, 29688, 29699	F30, F29, F310, F311, F312, F313, F314 ,F315, F316, F317, F318, F319
schizophrenia	295, 297, 2970, 2979, 29999	F20, F21, F22, F23, F24, F25, F28, F29
mental disorders due to the use of alcohol	291, 2910, 2919	F10

Supplementary Table 2. Psychiatric disorder comorbidities

Diagnosed with depression	Skiers n (%)	Non-skiers n (%)
Total nr of depression (n)	1030	2045
Schizophrenia prior to	17 (1.65)	42 (2.05)
Schizophrenia at the same time	5 (0.49)	18 (0.88)
Schizophrenia after	23 (2.23)	42 (2.05)
Anxiety prior to	43 (4.17)	112 (5.48)
Anxiety at the same time	45 (4.37)	96 (4.69)
Anxiety after	57 (5.53)	148 (7.24)
Bipolar disorder prior to	14 (1.36)	26 (1.27)
Bipolar disorder at the same time	8 (0.78)	11 (0.54)
Bipolar disorder after	50 (4.85)	98 (4.79)

The absolute numbers (n) and percentage of psychiatric comorbidities among those diagnosed with depression displayed for skiers and non-skiers separately. The occurrence of comorbidity did not differ between skiers and non-skiers for any of the psychiatric disorders, as estimated with Pearson's χ^2 test.

References

1. Hallmarker U, Michaelsson K, Arnlov J, Hellberg D, Lagerqvist B, Lindback J, et al. Risk of recurrent ischaemic events after myocardial infarction in long-distance ski race participants. Eur J Prev Cardiol. 2016;23(3):282-90.

Paper IV

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Research Report

Delayed Clinical Manifestation of Parkinson's Disease Among Physically Active: Do Participants in a Long-Distance Ski Race Have a Motor Reserve?

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Abstract.
Background: Physical activity is associated with reduced risk of Parkinson's disease (PD). The explanations for this association are not completely elucidated. We use long-term PD-incidence data from long-distance skiers to study the relationship between exercise and PD.
Objective: We aimed to investigate if physical activity is associated with long-term lower risk of PD and if this association could be explained by physically active people being able to sustain more PD neuropathology before clinical symptoms, a motor reserve.
Methods: Using a prospective observational design, we studied whether long-distance skiers of the Swedish Vasaloppet ($n = 197{,}685$), exhibited reduced incidence of PD compared to matched individuals from the general population ($n = 197{,}684$) during 21 years of follow-up (median 10, interquartile range (IQR) 5–15 years).
Results: Vasaloppet skiers (median age 36.0 years [IQR 29.0–46.0], 38% women) had lower incidence of PD (HR: 0.71; 95 % CI 0.56–0.90) compared to non-skiers. When reducing risk for reverse causation by excluding PD cases within the first five years from race participation, there was still a trend for lower risk of PD (HR: 0.80; 95 % CI 0.62–1.03). Further, the PD prevalence converged between skiers and non-skiers after 15 years of follow-up, which is more consistent with a motor reserve in the physically active rather than neuroprotection.
Conclusions: A physical active lifestyle is associated with reduced risk for PD. This association weakens with time and might be explained by a motor reserve among the physically active.

Keywords: Physical activity, exercise, Parkinson's disease, motor reserve

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INTRODUCTION

Parkinson's disease (PD) is the most common neurodegenerative movement disorder and with an aging population more people will be afflicted [1]. The risk of PD is determined by an interaction of environmental and genetic factors [2]. Physical activity is known to be associated with lower risk of PD [3, 4] with several possible explanations. First, it could be reverse-causation, i.e., people with prodromal PD reduce their activity level. However, the protective effect of physical activity remains even if you exclude people who develop PD within 8 years, as reported by Yang and colleagues in a comprehensive study [3]. Second, it could be misdiagnosis, for example exercise could protect against vascular parkinsonism which is often misdiagnosed as PD. Only 80–90% of patients with clinical PD have the diagnosis confirmed post-mortem [5, 6]. Third, it could of course be a real protective effect on dopaminergic neurons, we could call this "brain resilience" [7]. In contrast to resilience exercise could confer a greater reserve against PD, henceforth termed motor reserve. A patient with a high motor reserve would be able to sustain a higher amount of PD brain pathology before the onset of overt symptoms. Thus, the diagnosis of PD could be delayed in those with high motor reserves (Fig. 1A).

In this report we study the risk of PD among participants in Vasaloppet, an up to 90 km annual cross-country ski race compared to age matched non-skiers. We use participation in Vasaloppet as a proxy for physical activity similar to previous studies [8]. First, we investigate whether physical activity is associated with lower risk of PD and if it can be explained by reverse causation. We then investigate whether this association is more likely to be mediated by inhibiting brain pathology (brain resilience, Fig. 1B) or by a greater motor reserve.

MATERIALS AND METHODS

Swedish national patient registry

Data on PD diagnoses were retrieved from the Swedish National Patient Registry, which since 1987 provides information on all primary and secondary diagnoses in patients attending hospital-based care in Sweden. The register covers 99% of all hospital-based diagnoses, both somatic and psychiatric, and includes hospital-based outpatient visits since 2001. Primary care diagnoses are not included in the registry. PD was defined according to the International Classification of Diseases, tenth revision (ICD10) or ninth revision (ICD9). Diagnoses included are Parkinson's disease (G20, 332A, 3420).

Vasaloppet cohort

The Vasaloppet study population comprises all Swedish participators in the world's largest long distance (30, 45, or 90 km) cross-country ski race (Vasaloppet) between 1989 and 2010 ($n = 197{,}685$), together with frequency-matched individuals from the general population ($n = 197{,}684$). Although Vasaloppet started already in 1922 it was not until 1989 that the personal number of the participants was registered which made the present study possible. Frequency matching was done from the population registry according to age group (five-year intervals), sex, region of residency, and year of participation in ski race as previously described [9]. On average,

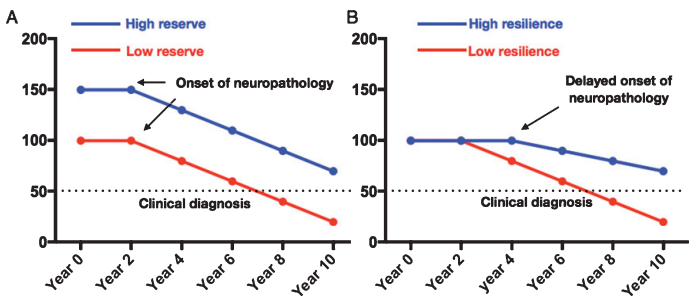

Fig. 1. Two possible mechanisms of protection. A) High cognitive/motor reserve where the brain can sustain more neuropathological damage before the onset of overt clinical symptoms. B) High brain resilience may delay onset of neuropathology and then slow the rate of decline.

Vasaloppet skiers have higher leisure time physical activity, smoke less, have a healthier diet, and lower mortality than the general Swedish population [10]. To reduce bias due to inability to participate in the race because of poor health, individuals with severe disease (e.g., cardiovascular and respiratory diseases) were excluded as previously described [11] (for more information, see the Supplementary Material including Supplementary Figure 1 and Supplementary Table 1). Information on date of birth, sex, and education level were derived from Swedish registries [9] The total study cohort ($n = 395{,}369$) was followed in the Swedish National Patient Registry throughout 2010. The Ethical Review Board in Uppsala, Sweden, approved the Vasaloppet study.

Statistical analyses

We used R statistical software package for analyses. Two-tailed p-values <0.05 were considered statistically significant. Demographic data are presented as median and interquartile range (IQR) or numbers (n) and percent (%). Numeric and categorical group differences were estimated with Mann-Whitney U test and Pearson's χ^2 test, respectively. Cox regression models were used to compare risk of PD for skiers vs. non-skiers. For the cox regression models, the time variable was calculated as years between participation in Vasaloppet (and the same year for the matched non-skier) and event or censoring. The event was PD. Censoring appeared when subjects died or at time of register outtake. Information on date of death for deceased study individuals was available through the Causes of Death Register, held at the National Board of Health and Welfare. Risk of PD is presented as hazard ratio (HR) with 95% confidence intervals (CI). We present both a crude model and an age-, sex-, and education-adjusted model. Education was categorized as noted in Table 1. We modeled Schoenfeld residuals graphically to confirm the proportionality assumption. Figure data were constructed using Kaplan Meier curves. The same time and event variables were used as in the Cox regressions, and the hazards are presented for skiers vs. non-skiers. Numbers at risk were derived from survival tables specifying number of individuals entering each five-year interval, as presented in the graph (Fig. 2). Since there is evidence suggesting that patients may have motor symptoms 5 years before the diagnosis of PD [12], we decided to set five years as a cut-off for sensitivity analyses.

RESULTS

Vasaloppet skiers have a lower cumulative incidence of PD

Demographic data for the Vasaloppet cohort is presented in Table 1. After a median follow-up of 10 years (IQR 5–15 years), 283 PD diagnoses were identified. The overall risk of developing PD was significantly lower among those who had participated in Vasaloppet compared to those who had not (Fig. 2A, Hazard ratio (HR) 0.71, confidence interval (CI) 0.56–0.9). When excluding individuals diagnosed with PD within the first five years from baseline HR rose to 0.8 (Fig. 2B, HR 0.80, CI 0.62–1.03). Adjusting for age, sex and education level did not alter the results (see Table 2).

Table 1
Characteristics of the Vasaloppet study population

	All $n = 395\ 369$	Skiers $n = 197\ 685$	Non-skiers $n = 197\ 684$
Characteristics 1989–2010	Median (IQR) or *n* (%)	Median (IQR) or *n* (%)	Median (IQR) or *n* (%)
Age at baseline, y	36.0 (29.0–46.0)	36.0 (29.0–46.0)	36.0 (29.0–46.0)
Women	149796 (38)	74897 (38)	74899 (38)
Education:			
Primary/elementary school (≤8 y)	49344 (13)	14538 (7.4)	34806 (18)***
Secondary school/high school (9–12 y)	176571 (45)	76635 (39)	99936 (51)
Higher education/university (≥13 y)	166133 (42)	106147 (54)	59986 (31)
Diagnoses at follow-up		N events	
Parkinson's Disease	283	119	164

Characteristics of the Vasaloppet study population presented for the whole cohort and by skiers and non-skiers separately. ***$p < 0.001$. Group difference between skiers and non-skiers, estimated with Mann-Whitney U test (numeric variables) and Pearson's χ^2 test (categorical variables). Only significant differences are noted in the table.

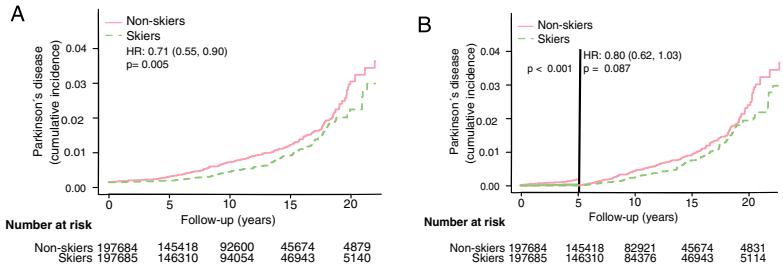

Fig. 2. Cumulative incidence of PD among skiers and non-skiers. A) Cumulative incidence of PD among all skiers and non-skiers. B) Cumulative incidence of PD among all Vasaloppet participants with exclusion of diagnoses set within the first five years after baseline. HR represents hazard ratios from an unadjusted cox regression.

Table 2
Association between physical activity and incident dementia in the Vasaloppet cohort

	PD incidence	
Physical activity	HR (95% CI)	*p*
Unadjusted model	283 events	
Non-skiers (Reference)	1	
Skiers	0.71 (0.56–0.90)	0.005
Adjusted model	275 events	
Non-skiers (Reference)	1	
Skiers	0.73 (0.57–0.93)	0.01
Excluding PD cases <5 years		
Unadjusted model	246 events	
Non-skiers (Reference)	1	
Skiers	0.80 (0.62–1.03)	0.087
Adjusted model	239 events	
Non-skiers (Reference)	1	
Skiers	0.80 (0.62–1.04)	0.099

Association between physical activity and PD incidence in the Vasaloppet cohort, based on participation in a long-distance ski race (skiers) compared to non-skiers. Cox regression models showing hazard ratio (HR) with 95% confidence interval (CI) for risk of PD. Adjusted model for age, sex, and education.

The differential of cumulative PD-incidence between participants and non-participants decrease with time

The motor reserve hypothesis predicts that the cumulative incidences would converge between skiers and non-skiers in the older age groups. We therefore broke the results down by age of subject at participation in Vasaloppet (Fig. 3). In the age group 18–39 there was barely any incidence of PD (data not shown). In age group 39–49, the incidence of PD was significantly lower among the skiers (Fig. 3A, HR 0.50, CI 0.29–0.84). Given the up to 20-year follow-up the oldest participants here could be 69 at the end of follow-up (Fig. 3A). In age-group 49–59 years, we observed no difference in PD prevalence between skiers and non-skiers (Fig. 3B, HR 0.84, CI 0.57–1.24). In age group 59–69 the prevalence of PD was lower at early follow-up period but converged at later follow-ups (Fig. 3C, HR 0.69, CI 0.45–1.05). In the oldest age group, 69–100, we observed the same pattern, with a lower PD prevalence among skiers to begin with but convergence at the end (Fig. 3D, HR 0.46, CI 0.20–1.05).

To further look at this convergence of PD-prevalence with time we specifically looked at the group with longest follow-up times, participating in the ski race 1991–2000. In this group we see a convergence in prevalence after 15+ years (Fig. 4). It should also be noted that 90 % of all events (PD-cases) come from this group.

The differential of cumulative incidence of PD between participants and non-participants in men and women separately

The incidence of PD plateaus at an earlier age among women than men [13]. The motor reserve hypothesis thus predicts a greater convergence of cumulative incidence among female skiers than male skiers. We therefore performed a subgroup analysis for men and women separately to test this aspect of the hypothesis. We observed a greater convergence in cumulative incidence towards the end of follow-up between skiers and non-skiers in women (Fig. 5B, HR 0.54, CI 0.30–0.98) compared to men (Fig. 5A, HR 0.75, CI 0.58–0.97).

DISCUSSION

In the current study, we aimed to investigate if physical exercise is correlated to the risk of PD and if

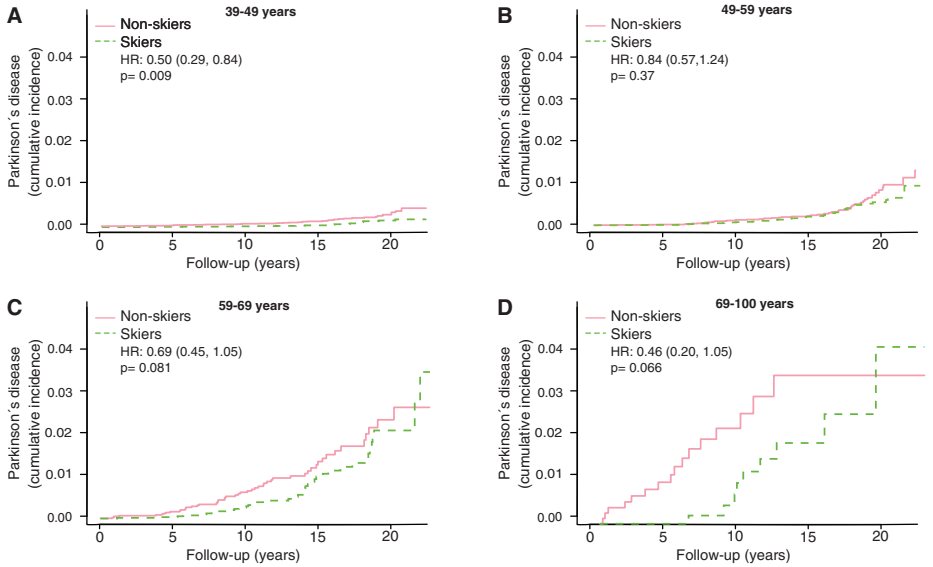

Fig. 3. Kaplan Meier plots of cumulative incidence of Parkinson's disease in subgroups with different age at participation, 39–49 years (A), 49–59 year (B), 59–69 years (C) and 69-100 years (D). HR represents hazard ratios from an unadjusted cox regression.

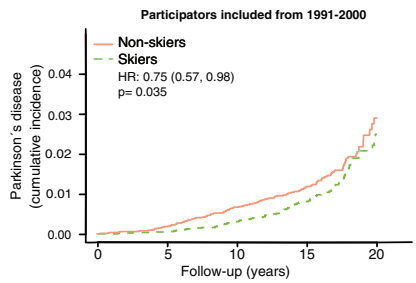

Fig. 4. Kaplan Meier plot of Parkinson's disease prevalence among those with the longest follow-up time. HR represents hazard ratios from an unadjusted cox regression.

this correlation could be explained by a motor reserve built up over years of physical activity.

We found that physical activity, as measured by participation in a long-distance ski race, was associated with lower incidence of PD. This effect was weakened when we excluded those who were diagnosed within 5 years after inclusion, indicating some degree of reverse-causation due to people with premorbid PD exercising less. The association between physical activity and lower incidence of PD in our study are in concordance with earlier studies showing a protective effect of exercise against PD [3, 4].

There are principally three possible protective mechanisms of physical activity against PD: 1) greater resilience of the neurons against the neuropathology of PD; 2) less neuropathology; and 3) a greater motor reserve, so that the brain can sustain more damage before symptoms become apparent, a phenomenon analogous to the cognitive reserve concept in Alzheimer's disease [14]. It has been shown that the negative correlation between Alzheimer's disease and education is more consistent with a greater cognitive reserve rather than greater resilience or less neuropathology [15]. In this study, we have no possibility of distinguishing between mechanisms 1 and 2, but the concept of motor reserve makes some predictions that can be examined in our material. If the sole reason for the negative correlation between PD and exercise was direct protection from neuropathology, then you would expect a lower incidence of PD at all ages and time-points. This is not what we observed. If it were instead a motor reserve protecting against PD you would expect converging cumulative incidence with longer follow-up time and older age. That is what we observed. In both the 59–69 and 69–100 age group we see an initial lower

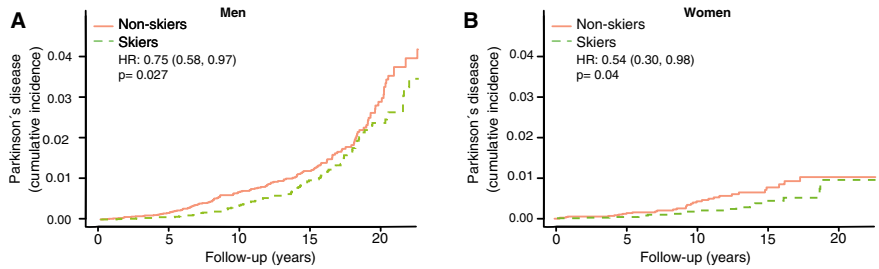

Fig. 5. Cumulative incidence of Parkinson's disease among skiers vs. non-skiers in men (A) and women (B) separately. HR represents hazard ratios from an unadjusted cox regression.

cumulative incidence of PD, but at the end of the more than 20 years of follow-up the cumulative incidence of PD converged (Fig. 3C, D). Among those who had the longest follow-up times (ski race participation between 1991–2000), we also observe a convergence of PD prevalence with time (Fig. 4). This convergence somewhat depends on the relation between PD-incidence and age. If PD-incidence always increased with age, the cumulative incidence of PD would converge between those with a low and high motor reserve as long as the incidence to prevalence ratio was declining, but the convergence would not be complete. If the incidence of PD declines after a certain age we would move towards complete convergence between the high and low motor reserve groups. The data on PD-incidence among those over 80 is not as robust as for lower ages but there seems to be an incidence decline after 79 [16]. Interestingly the incidence decline in PD with age is significantly more pronounced among women [13]. Thus, the motor reserve hypothesis also predicts a greater convergence of cumulative PD-incidence among women skiers than male skiers, which is what we observe (Fig. 5B).

Though outside the scope of our study the motor reserve hypothesis also predicts milder motor symptoms for every given level of neuropathology/neuronal death in those with a high motor reserve. It should thus be noted that PD patients with a higher premorbid exercise activity have better motor scores relative to their dopamine levels compared to sedentary peers [17]. Further, PD is often unilateral at onset with persistent asymmetries. It has been shown that patients with PD on their dominant side have better motor scores than those affected on their non-dominant side, possibly due to a greater motor reserve in the dominant hemisphere [18].

Interestingly, our cohort has previously shown that the level of physical activity can affect the risk for amyotrophic lateral sclerosis (ALS) [8], specifically a four-fold risk-increase among elite-skiers and a moderate risk-decrease in recreational skiers. In that study, the motor reserve did not seem to have the same compensatory effect in ALS. ALS is a very aggressive disease affecting upper and lower motor neurons. The primary cause of death is respiratory failure due to degeneration of respiratory muscles [19] and the median survival may be as low as 2 years [20]. Thus any motor reserve in ALS would delay the disease by months rather than years which would make it difficult to detect. This makes it more likely that the associations seen between ALS and exercise are more directly related to the neuropathology.

Our study includes limitations such as the lack of data on physical activity among the non-skiers. Thereby, the non-skier group also includes physically active individuals to some extent and this may attenuate the true association. Skiers were assumed to be physically active since it is necessary to prepare for such a demanding ski race with regular physical training, as demonstrated by a previous study [10]. We excluded individuals with severe diseases to reduce bias due to inability to participate in the ski race. However, bias due to inability to participate due to poor health is not completely eliminated since it is not possible to exclude all diagnoses that might indirectly affect participation (type I error). Furthermore, other lifestyle factors, such as diet, smoking and education differs between the skiers and non-skiers [10]. However, adjusting for education did not significantly alter our results. It is both a strength and a weakness that we do not adjust for additional confounders. It is a weakness as other factors such as smoking habits (smoking is more prevalent among non-skiers) and

diet could independently of exercise affect the risk of PD. However, smoking has been associated with lower incidence of PD [4] and could thus contribute to an underestimation of the true association between physical activity and PD (type II error). In addition, we did not compensate for possible immortal time bias. This skiing population has been shown to live longer than the control population [10], which should increase their risk of getting PD (type II error). However, adjusting for confounders can also increase the risk of type I errors, particularly when the measurement of the confounder is not exact, diet for example is difficult to retrospectively measure [21]. By not adjusting for confounders, we therefore decrease the risk of type I error at the expense of less certainty in what exact factor among the Vasaloppet skiers that decreases the risk of PD. Nevertheless the most salient differential characteristic among the skiers is their higher level of exercise [10]. Our skiing population has been characterized before and it is known that the majority exercise for at least 4 hours a week, which was not the case for the general population [10, 22]. Our data thus points to a protective effect of physical activity against PD.

Conclusion

In summary we observe a lower incidence of PD among skiers, likely mediated by physical activity. This association dissipates with time and is consistent with a greater motor reserve among the well-trained. Thus, skiers may suffer as much brain pathology but take longer to develop clinical PD than non-skiers. However, studies confirming these findings in other contexts as well as elucidating the mechanisms behind it are needed in order to draw more general conclusions.

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CONFLICTS OF INTEREST

The authors have no conflict of interest to report.

SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: http://dx.doi.org/10.3233/JPD-191762.

REFERENCES

[1] de Lau LML, Breteler MMB (2006) Epidemiology of Parkinson's disease. *Lancet Neurol* **5**, 525-535.

[2] Nalls MA, McLean CY, Rick J, Eberly S, Hutten SJ, Gwinn K, Sutherland M, Martinez M, Heutink P, Williams NM, Hardy J, Gasser T, Brice A, Price TR, Nicolas A, Keller MF, Molony C, Gibbs JR, Chen-Plotkin A, Suh E, Letson C, Fiandaca MS, Mapstone M, Federoff HJ, Noyce AJ, Morris H, Deerlin VMV, Weintraub D, Zabetian C, Hernandez DG, Lesage S, Mullins M, Conley ED, Northover CAM, Frasier M, Marek K, Day-Williams AG, Stone DJ, Ioannidis JPA, Singleton AB (2015) Diagnosis of Parkinson's disease on the basis of clinical and genetic classification: A population-based modelling study. *Lancet Neurol* **14**, 1002-1009.

[3] Yang F, Trolle Lagerros Y, Bellocco R, Adami H-O, Fang F, Pedersen NL, Wirdefeldt K (2015) Physical activity and risk of Parkinson's disease in the Swedish National March Cohort. *Brain* **138**, 269-275.

[4] Bellou V, Belbasis L, Tzoulaki I, Evangelou E, Ioannidis JPA (2016) Environmental risk factors and Parkinson's disease: An umbrella review of meta-analyses. *Parkinsonism Relat Disord* **23**, 1-9.

[5] Hughes AJ, Daniel SE, Lees AJ (2001) Improved accuracy of clinical diagnosis of Lewy body Parkinson's disease. *Neurology* **57**, 1497-1499.

[6] Tolosa E, Wenning G, Poewe W (2006) The diagnosis of Parkinson's disease. *Lancet Neurol* **5**, 75-86.

[7] Tillerson JL, Caudle WM, Reverón ME, Miller GW (2003) Exercise induces behavioral recovery and attenuates neurochemical deficits in rodent models of Parkinson's disease. *Neuroscience* **119**, 899-911.

[8] Fang F, Hållmarker U, James S, Ingre C, Michaëlsson K, Ahlbom A, Feychting M (2016) Amyotrophic lateral sclerosis among cross-country skiers in Sweden. *Eur J Epidemiol* **31**, 247-253.

[9] Hållmarker U, Lindbäck J, Michaëlsson K, Ärnlöv J, Åsberg S, Wester P, Hellberg D, Lagerqvist B, James S (2018) Survival and incidence of cardiovascular diseases in participants in a long-distance ski race (Vasaloppet, Sweden) compared with the background population. *Eur Heart J Qual Care Clin Outcomes* **4**, 91-97.

[10] Farahmand BY, Ahlbom A, Ekblom Ö, Ekblom B, Hållmarker U, Aronson D, Brobert GP (2003) Mortality amongst participants in Vasaloppet: A classical long-distance ski race in Sweden. *J Intern Med* **253**, 276-283.

[11] Hållmarker U, Michaëlsson K, Ärnlöv J, Hellberg D, Lagerqvist B, Lindbäck J, James S (2016) Risk of recurrent

ischaemic events after myocardial infarction in long-distance ski race participants. *Eur J Prev Cardiol* **23**, 282-290.

[12] Darweesh SKL, Verlinden VJA, Stricker BH, Hofman A, Koudstaal PJ, Ikram MA (2017) Trajectories of prediagnostic functioning in Parkinson's disease. *Brain* **140**, 429-441.

[13] Van Den Eeden SK, Tanner CM, Bernstein AL, Fross RD, Leimpeter A, Bloch DA, Nelson LM (2003) Incidence of Parkinson's disease: Variation by age, gender, and race/ethnicity. *Am J Epidemiol* **157**, 1015-1022.

[14] Bennett DA, Wilson RS, Schneider JA, Evans DA, Mendes de Leon CF, Arnold SE, Barnes LL, Bienias JL (2003) Education modifies the relation of AD pathology to level of cognitive function in older persons. *Neurology* **60**, 1909-1915.

[15] Stern Y, Albert S, Tang MX, Tsai WY (1999) Rate of memory decline in AD is related to education and occupation: Cognitive reserve? *Neurology* **53**, 1942-1947.

[16] Hirsch L, Jette N, Frolkis A, Steeves T, Pringsheim T (2016) The incidence of Parkinson's disease: A systematic review and meta-analysis. *Neuroepidemiology* **46**, 292-300.

[17] Sunwoo MK, Lee JE, Hong JY, Ye BS, Lee HS, Oh JS, Kim JS, Lee PH, Sohn YH (2017) Premorbid exercise engagement and motor reserve in Parkinson's disease. *Parkinsonism Relat Disord* **34**, 49-53.

[18] Ham JH, Lee JJ, Kim JS, Lee PH, Sohn YH (2015) Is dominant-side onset associated with a better motor compensation in Parkinson's disease? *Mov Disord* **30**, 1921-1925.

[19] Pattinson KTS, Turner MR (2016) A wider pathological network underlying breathlessness and respiratory failure in amyotrophic lateral sclerosis. *Eur Respir J* **47**, 1632-1634.

[20] Knibb JA, Keren N, Kulka A, Leigh PN, Martin S, Shaw CE, Tsuda M, Al-Chalabi A (2016) A clinical tool for predicting survival in ALS. *J Neurol Neurosurg Psychiatry* **87**, 1361-1367.

[21] Westfall J, Yarkoni T (2016) Statistically controlling for confounding constructs is harder than you think. *PLoS One* **11**, e0152719.

[22] Carlsson S, Olsson L, Farahmand BY, Hållmarker U, Ahlbom A (2007) Skiers in the long-distance ski race invest in their health. *Lakartidningen* **104**, 670-671.

Supplementary Material

Delayed Clinical Manifestation of Parkinson's Disease Among Physically Active: Do Participants in a Long-Distance Ski Race Have a Motor Reserve?

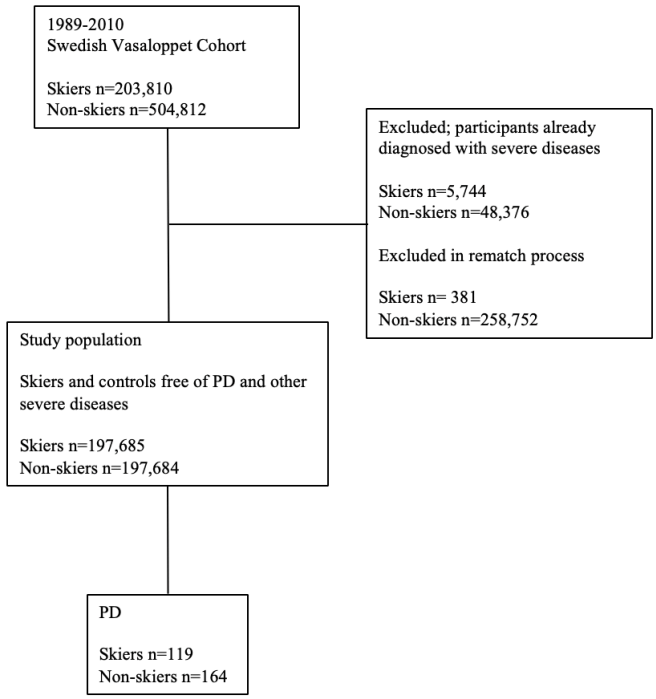
Extended Method Description

Exclusion criteria and matching procedure

In the Vasaloppet study, individuals with severe disease were excluded since such diseases are likely to hinder participation in a demanding long-distance race. A flow diagram describing numbers excluded due to severe disease can be seen in Supplementary Figure 1. The exact diagnoses have been stated previously [11]. We additionally excluded participants with diagnoses listed in Table 1.

In the first matching process, a control individual from the general population was assigned for every ski race, so that skiers participating in Vasaloppet several times got several controls. We performed a re-matching procedure to get equally many skiers as non-skiers. Since we only used the index race for each skier, the non-skiers would have been older as a group if we had included one control for every time a skier participated in the race.

Vasaloppet Study population



Supplementary Figure 1. Flow diagram describing the Vasaloppet Study population.

Supplementary Table 1. Additional exclusion criteria

Diagnosis	ICD-9	ICD-10
Alzheimer's disease	331A/3310, 29010	F00, G30
vascular dementia	290E, 2904, 2930	F01
all-cause dementia	290, F070, 294C, 294B, 331A, 310A, G318A	F00, F01, F02, F03, G30
Lewy body dementia	331X, G318A, 33182	F028
dementia in Parkinson disease	294B, 332A	F023
Parkinson disease	332A, 3420	G20
meningitis/encephalitis	3200, 320A, 320B, 320C, 320D, 320W 320X, 321A, 321B, 321C, 321D, 321E, 321X, 322A, 322B, 322C, 320X, 323, 3230	G00, G01, G03, G04, G05
epilepsy	345, 3450	G40
depressive episode	F399, 296B, 296X, 29620, 29800	F32, F33, F34, F38
anxiety disorders	300A, 300B, 300C, 300D, 300D, 3000, 3001, 3002, 3003	F40, F41, F42
bipolar disorder	296A, 29610 ,296C, 296D, 296E, 29600, 29610, 29620, 29630, 29688, 29699	F30, F29, F310, F311, F312, F313, F314, F315, F316, F317, F318, F319
schizophrenia	295, 297, 2970, 2979, 29999	F20, F21, F22, F23, F24, F25, F28, F29
mental disorders due to the use of alcohol	291, 2910, 2919	F10

Paper V

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RESEARCH Open Access

Midlife physical activity is associated with lower incidence of vascular dementia but not Alzheimer's disease

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Abstract

Background: Physical activity might reduce the risk of developing dementia. However, it is still unclear whether the protective effect differs depending on the subtype of dementia. We aimed to investigate if midlife physical activity affects the development of vascular dementia (VaD) and Alzheimer's disease (AD) differently in two large study populations with different designs.

Methods: Using a prospective observational design, we studied whether long-distance skiers of the Swedish Vasaloppet ($n = 197,685$) exhibited reduced incidence of VaD or AD compared to matched individuals from the general population ($n = 197,684$) during 21 years of follow-up (median 10, interquartile range (IQR) 5–15 years). Next, we studied the association between self-reported physical activity, stated twice 5 years apart, and incident VaD and AD in 20,639 participants in the Swedish population-based Malmo Diet and Cancer Study during 18 years of follow-up (median 15, IQR 14–17 years). Finally, we used a mouse model of AD and studied brain levels of amyloid-β, synaptic proteins, and cognitive function following 6 months of voluntary wheel running.

Results: Vasaloppet skiers (median age 36.0 years [IQR 29.0–46.0], 38% women) had lower incidence of all-cause dementia (adjusted hazard ratio (HR) 0.63, 95% CI 0.52–0.75) and VaD (adjusted HR 0.49, 95% CI 0.33–0.73), but not AD, compared to non-skiers. Further, faster skiers exhibited a reduced incidence of VaD (adjusted HR 0.38, 95% CI 0.16–0.95), but not AD or all-cause dementia compared to slower skiers. In the Malmo Diet and Cancer Study (median age 57.5 years [IQR 51.0–63.8], 60% women), higher physical activity was associated with reduced incidence of VaD (adjusted HR 0.65, 95% CI 0.49-0.87), but not AD nor all-cause dementia. These findings were also independent of *APOE*-ε4 genotype. In AD mice, voluntary running did not improve memory, amyloid-β, or synaptic proteins.

Conclusions: Our results indicate that physical activity in midlife is associated with lower incidence of VaD. Using three different study designs, we found no significant association between physical activity and subsequent development of AD.

Keywords: Physical activity, Alzheimer's disease, Vascular dementia, Exercise, Amyloid-β

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Background

Alzheimer's diseases (AD) followed by vascular dementia (VaD) are the most common types of dementia. Risk factor control is an important strategy to postpone dementia onset, and physical inactivity is regarded as one of the main modifiable risk factors that can be targeted [1, 2]. However, recent intervention trials involving physical activity report mixed results, thereby highlighting the lack of consistency within the field [3–5]. A systematic review showed that physical activity interventions improved cognition in demented persons [6], but revealed that most trials do not distinguish between pure AD and pure VaD patients. Published trials are often multidomain interventions, making it difficult to draw any conclusions regarding the effect of only physical activity. Among trials with physical activity as the only intervention, improved cognition was reported in patients with mild AD after 16 weeks of exercise [7], whereas no cognitive effects were seen in demented patients after 12 months [8]. An ongoing trial with a 2-year exercise intervention will provide further information if physical exercise can be beneficial in preventing dementia [9]. As summarized in reviews and meta-analyses, findings from previous prospective cohort studies *differ* but pooled results indicate protective effects [10–14]. Nevertheless, there are important concerns within the prevailing literature, such as possible publication bias and follow-up effects [11].

Beneficial effects are mainly found in late-life assessments with short-term follow-up [10, 13, 15, 16] and tend to become non-significant after longer follow-up [11, 15, 16]. These discrepancies may be attributable to reverse causation where cognitive dysfunction may lead to reduced physical activity. A recent population-based study on physical activity and dementia (n = 10,308) provide repeated physical activity assessments and reports that physical activity begins to decline up to 9 years before diagnosis of dementia [17], thus emphasizing the possible impact of reverse causation in studies with shorter follow-up. In this study, no association between midlife physical activity and dementia was found during 27 years of follow-up [17].

Further, the different diseases causing cognitive impairment are associated with very different underlying disease mechanisms, such as gradual accumulation of amyloid-β (Aβ) and tau in AD and arteriosclerosis and ischemia in VaD. Therefore, it is unlikely that the same preventive strategies are equally effective against different pathological mechanisms causing dementia. Literature reports variable effects of physical activity on incident VaD [12, 18] and AD [15, 18–21]. Furthermore, it is unclear whether individuals carrying the genetic risk factor *APOE*-ε4 [22] might benefit specifically from physical exercise [16, 23, 24]. Working in transgenic animal models makes it easier to study the mechanistic effects of physical activity on different molecular hallmarks of AD, such as Aβ and synaptic proteins, as well as cognitive symptoms. Indeed, several studies have been conducted to investigate the effect of physical activity on AD pathology [25]. For example, exercise resulted in improved cognition [25] as well as reduction of both Aβ soluble and insoluble Aβ species in a dose-dependent manner [26]. However, the effects are inconsistent between studies [25], since other studies show lack of effects [25, 27]. The majority of studies also investigate the effect of physical activity in a relatively short period of time [25]. Thus, additional experimental studies are needed to investigate the long-term effects of physical activity, starting in the pre-manifest stage, on AD hallmarks.

As mentioned, the setup and quality of published studies in the field are limited [11, 13]. Long follow-up periods are needed to reduce the effect of reverse causation, and large study populations are necessary to study differences between dementia subtypes. To address these limitations, we investigated if physical activity in midlife affects the development of VaD and AD in two separate large study populations with different study designs and long follow-up. Further, to study the long-term effect on AD pathology such as Aβ and synaptic proteins, we exposed transgenic AD mice to voluntary wheel running.

Materials and methods

Dementia diagnoses

Dementia diagnoses were made by physicians in clinical routine and retrieved from the Swedish National Patient Register (NPR). It started in 1964, and since 1987, it provides information on all primary and secondary diagnoses, covering 99% of all hospital-based diagnoses. Primary care diagnoses are not included. Dementia was defined as any dementia diagnosis according to the International Classification of Diseases, tenth revision or ninth revision. Diagnoses included are AD (F00, G30, 331A/3310, 29010), VaD (F01, 290E/2904), or other forms included among all-cause dementia (2900, 2901, F023, 2941/294B, 3320/332A, F028, G318A, 331/331X, 33182/331H, F020, G310, 3311/331B, F03, F070, 290, or 2942/294C). Based on this classification, AD cases include atypical and mixed cases (F002), thus also covering AD with a vascular component. In the Vasaloppet cohort, the differentiation between AD and VaD was done by the diagnosing physician in line with the available clinical diagnostic criteria and no further information on the diagnostic routine was available. In the Malmo Diet and Cancer study (MDCS), we reviewed and verified all register diagnoses in medical records as part of the research protocol. Among MDCS dementia cases (n = 1375), electronic charts provided history regarding cognitive symptoms in 92%, cognitive test results in 92%, and neuroimaging (mainly CT) in

connection to diagnosis in 99.6%, which were all reviewed by us in depth to determine the type of dementia diagnosis (see below). Further, 82% were assessed at a tertiary unit specializing in memory disorders, where CSF analyses of AD biomarkers were often part of the diagnostic work-up.

Vasaloppet cohort

Physical activity

The Vasaloppet study population comprises non-demented participators of the world's largest long-distance (30 to 90 km) cross-country ski race (Vasaloppet) between 1989 and 2010 (n = 197,685), together with frequency-matched, non-demented individuals from the general population (n = 197,684). Frequency matching was done from the population register according to age group (5-year intervals), sex, region of residency, and year of participation in ski race as previously described [28]. In the first matching process, a control individual from the general population was assigned for every ski race, so that skiers participating in Vasaloppet several times got several controls. We performed a re-matching procedure to get equally many skiers as non-skiers. Since we only used the index race for each skier, the non-skiers would have been older as a group if we had included one control for every time a skier participated in the race. The total study cohort (n = 395,369) was prospectively followed in the Swedish NPR throughout 2010. Skiers are considered to be physically active since it is necessary to undergo regular physical training in order to complete such a demanding long-distance race. For example, the majority of skiers exercise for at least 4 h a week [29]. On average, Vasaloppet skiers have higher leisure time physical activity than the general Swedish population [30]. Regarding fitness, the oxygen consumption (V_{O2MAX}) has been shown to be 45–80 ml/kg/min in skiers, compared to around 35 ml/kg/min in the general population [31].

Covariates

Information on date of birth, sex, and education level was derived from Swedish registries [28]. We categorized education as primary/elementary school (≤ 8 years), secondary school/high school (9–12 years), or higher education/university (≥ 13 years). No further data were available in this cohort.

Attrition

In addition to having higher physical activity, the average Vasaloppet skier also smokes less and has a healthier diet and lower mortality than the general Swedish population [30]. To avoid bias due to inability to participate in the race because of poor health, individuals with severe disease were excluded as previously described [31]. We additionally excluded participants with Parkinson's disease (G20, 332A, 3420), meningitis/encephalitis (G00, G01, G03, G04, G05, 3200, 320A, 320B, 320C, 320D, 320 W 320X, 321A, 321B, 321C, 321D, 321E, 321X, 322A, 322B, 322C, 320X, 323, 3230), epilepsy (G40, 345, 3450), depressive episode (F32, F33, F34, F38, F399, 296B, 296X, 29620, 29800), manic episode (F30, F29, 296A, 29610), bipolar disorder (F310, F311, F312, F313, F314, F315, F316, F317, F318, F319, 296C, 296D, 296E, 29600, 29610, 29620, 29630, 29688, 29699), anxiety disorders (F40, F41, F42, 300A, 300B, 300C, 300D, 300D, 3000, 3001, 3002, 3003), and mental disorders due to the use of alcohol (F10, 291, 2910, 2919). A flow diagram describing numbers excluded can be seen in Fig. 1a.

Malmo Diet and Cancer study (MDCS) cohort

The MDCS population is part of a large prospective population-based study, where baseline investigations were performed between 1991 and 1996. At baseline, participants responded to questionnaires and underwent a basic clinical examination. Research nurses draw blood samples and measured height, weight, and blood pressure [32]. Five years later, between 1997 and 2001, participants were invited to respond to the questionnaire again as part of a reexamination. The present study cohort (n = 20,639) consists of all participants who were non-demented at the reinvestigation and provided data on physical activity at both baseline and reinvestigation (Fig. 1b).

Physical activity assessment

Information on physical activity during leisure time was stated in both questionnaires as the form of physical activity (e.g., walking, gardening, and running) and minutes per week the activity was performed at every season (spring, summer, autumn, winter). The activity was multiplied with an activity-specific factor, where heavier activities were graded with a higher factor [33]. This generated a total physical activity score calculated as the sum of minutes per week for all four seasons multiplied with the activity-specific factor, for every activity stated. We calculated the combined physical activity score as the sum of the scores from the two time points.

Review of dementia diagnoses

The MDCS cohort was followed in the Swedish NPR throughout 2014, when all registered dementia diagnoses were extracted. A diagnostic evaluation was performed by medical doctors at the Memory Clinic at Skåne University Hospital. All register diagnoses were reviewed in medical records and evaluated based on symptom presentation, test results, and brain imaging in accordance with DSM-5 (The Diagnostic and Statistical Manual of Mental Disorder, Fifth Edition) [34]. One thousand four hundred forty-six dementia diagnoses were first identified in the register. Based on the diagnostic review process, 54 out of 1446 individuals (3.7%) did not meet criteria for dementia (e.g., reversible

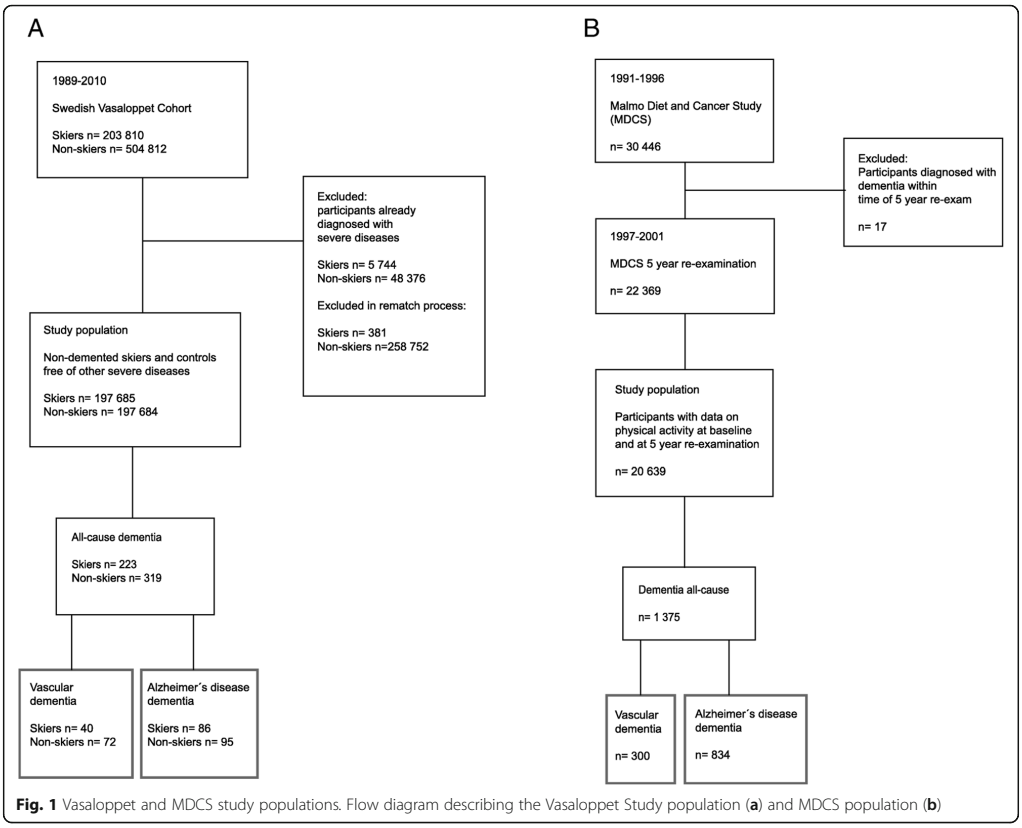

Fig. 1 Vasaloppet and MDCS study populations. Flow diagram describing the Vasaloppet Study population (**a**) and MDCS population (**b**)

disorientation, major depression, or mild cognitive impairment) and were instead regarded as non-demented participants. Further, 17 out of 1446 individuals (1.2%) received their dementia diagnosis within the time of the reinvestigation and were excluded (Fig. 1b). Among the 20,639 participants in the final study population, 1375 individuals (6.7%) fulfilled the criteria for dementia. The diagnosis was refined in 322 of 1446 cases (22%), mainly from unspecified dementia to AD with concomitant vascular disease. One hundred three participants (7%) remained classified as unspecified dementia since available medical records did not provide enough information to diagnose with further accuracy. In 172 individuals, no e-chart was available (mainly due to emigration or death before conversion to the current e-chart system) and then the last diagnosis in the register was used.

Covariates

Covariates were selected based on previous literature and availability [35]. Information on education, smoking, alcohol consumption, medication use, and work-related physical activity was self-reported and derived from the baseline questionnaire. We categorized education as primary/elementary school (≤ 8 years), secondary school/high school (9–12 years), or higher education/university (≥ 13 years). Smoking was categorized as ever smoker (current or former) or never smoker. Alcohol consumption was entered numerically as grams of alcohol per day, computed from the units of beer, wine, and liquor participants stated to have consumed during the last month. Drugs were classified according to the international Anatomical Therapeutic Chemical Classification (ATC). Blood pressure-lowering medication was defined as any drug with blood pressure-lowering effect regardless of indication and consisted of diuretics (ATC group C03), beta-blocking agents (ATC group C07), calcium channel blockers (ATC group C08), or agents acting on the renin-angiotensin system (ATC group C09). Lipid-lowering medication was defined as any drug with serum lipid-reducing effect (ATC group C10). Work activity

was stated as "what degree of physical activity is usually demanded in your work" with options (1) very light, (2) light or medium heavy, (3) heavy, or (4) very heavy. We categorized heavy or very heavy as physically heavy work. Baseline information on the prevalence of diabetes mellitus (type 1 or 2) was derived from the Swedish National Diabetes Register and the NPR. Cardiovascular disease was defined as ischemic or hemorrhagic stroke or ischemic heart disease and originates from the NPR and the Stroke register of Malmo.

Attrition

In the MDCS database, there are data on 30,446 individuals. When comparing baseline data for participants included in the present study ($n = 20{,}639$) and the remaining original cohort ($n = 9807$), included participants were younger (mean age [SD] 57.8 [7.5] years vs 58.5 [7.8] years, $p < 0.001$) and had a higher physical activity score at baseline (mean score 8292 [6746] vs 7532 [6344], $p < 0.0001$). Further, included participants were higher educated and generally healthier (e.g., had lower blood pressure, less cardiovascular disease, and less diabetes) than non-included individuals ($p < 0.0001$). Further, the incidence rate per 1000 person-years (based on time from baseline till event or end of study) differs between included participants (3.5 for any dementia, 0.8 for VaD, and 2.1 for AD) and non-participants (4.7 for any dementia, 1.5 for VaD, and 2.4 for AD). There were no differences in sex or *APOE*-ε4 carrier status. Further information on recruitment bias has been described in previous publications [36].

5xFAD mouse model

The 5xFAD strain is a mouse model co-expressing five mutations associated with familial form of AD, resulting in increased production of Aβ42. These mice have a fast development of AD pathology, showing accumulation of Aβ plaques as early as 2–3 months of age, cognitive dysfunctions already at 5 months of age, and neuronal and synaptic losses at 9 months of age [37–39]. Taken together, this makes the 5xFAD a suitable mouse model to study the effects of exercise on the development of Aβ plaque load, as well as cognitive dysfunctions seen in AD patients.

We used female 5xFAD mice ($n = 30$), aged 9–12 weeks, from Jackson Laboratories, weighing 14–20 g when starting the experiment. Mice were housed two animals/cage in standard laboratory cages with sawdust bedding and free access to water and food. They acclimatized for at least 5 days before starting the experiment. The holding room had a 12:12 h light-dark cycle. There were no differences in body weight, age, and general motor function between the groups when the experiment was initiated.

Voluntary running wheel exercise

Mice were randomly assigned to sedentary ($n = 14$) or exercising ($n = 16$) group. At 9–12 weeks of age, mice in the running group were provided with low-profile wireless running wheels for mouse (ENV-047; med-associates.com) in their home cage, allowing the mice to run as much as and whenever they wanted, during 24 weeks, until the end of the study.

Cognitive tests

Y-maze spontaneous alternation test was performed to examine any defects in working-memory after 18 weeks of running as previously described [40]. For this purpose, a Y-maze arena (21 × 4 cm/arm) was used. Mice with less than five arm entries were excluded from the analysis. Y-maze spatial memory test was performed to examine any defects in hippocampus-dependent spatial memory after 21 weeks of running as described previously [41]. To examine hippocampus-independent object memory, the mice were subjected to a novel object recognition test after 19–20 weeks of running. This test was conducted in an open field arena (30 cm × 30 cm) as described previously [42]. Both training and trial session duration was 5 min. Mice that did not explore both objects at least one time during the trial session were excluded.

Collection of samples

After 24 weeks of running, samples were collected. The mice were anesthetized with isofluorane and perfused with saline solution before the brains were dissected out. The right hemisphere was fixed in 4% paraformaldehyde in phosphate buffer for 24 h before they were stored in 30% sucrose solution at 4 °C until analysis. From the left hemisphere, the hippocampus and cortex were dissected, snap frozen on dry ice, and stored at − 80 °C until analysis.

Western blot

The hippocampus was homogenized as previously described [43] with some modifications. Briefly, we used 120 μl of TBS buffer (20 mM Tris-HCl, 137 mM NaCl, pH 7.6) containing protease and phosphatase inhibitors and 1% Triton-X100 in a dounce homogenizer. After 30-min incubation on ice, it was centrifuged at 14000*g* at 4 °C for 30 min. The supernatant was collected. Protein concentrations were determined (Pierce microplate BCA Protein Assay kit, thermofisher.com). Western blot was used as previously described [44]. The levels of the synaptic proteins PSD-95 (1:3000, MAB1596, Millipore) and synaptophysin (1:1000, Ab14692, Abcam,) were measured and normalized to beta-actin.

Immunohistochemistry

Immunohistochemistry was performed as previously described [44] with some modifications. Briefly, 30-μm sagittal sections were stained with 6E10 (1:500; BioLegend, San Diego, USA) and secondary antibody labeled with Alexa Fluor® 594 (1:500; Invitrogen, Carlsbad, CA, USA). Three sections per brain (lateral 0.84–1.2 mm) were analyzed using an epifluorescence microscope (Nikon Eclipse 80i microscope, Europe). The 6E10-positive Aβ were analyzed in dentate gyrus/CA4 in the hippocampus and cortical layer 4 and 5 in the neocortex area above/dorsally of the lateral ventricle. The immunofluorescence intensity was measured in 0.25 mm^2 within regions of interest using ImageJ.

ELISA

The concentration of Aβ species (Aβ40 and Aβ42) in the homogenized hippocampus was measured as previously described [44], with the MSD MULTI-SPOT Human (4G8) Aβ Assay (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. Aβ concentrations were normalized to total protein concentrations measured in the BCA or Bradford assay.

Statistical analyses

We used R statistical software and SPSS statistical software (v.22, Windows). Two-tailed p values < 0.05 were considered statistically significant. Demographic data are presented as median and interquartile range (IQR) or numbers (n) and percent (%). Numeric and categorical group differences were estimated with Mann-Whitney U test and Pearson's χ^2 test, respectively. Based on tertiles, participants in the MDCS were divided into three groups according to their reported physical activity in leisure time, referred to as high, intermediate, and low. Cox regression models were used to compare risk of dementia for skiers vs non-skiers in the Vasaloppet cohort and per SD increase in physical activity score (continuous variable converted to z-score) and per physical activity group (categorical variable) in the MDCS cohort. Time of event was defined as the date of first registered dementia diagnosis in the NPR. Censoring appeared when subjects died or at the time of register outtake/end of follow-up. In the Vasaloppet cohort, the time variable was calculated as years between participation in the ski race and event/censoring. In the MDCS cohort, the time variable was calculated as years between the reinvestigation and event/censoring since individuals who were diagnosed with dementia before the reinvestigation were excluded (i.e., no events occurred between baseline and reinvestigation based on the study design). Information on the date of death for deceased study individuals was available through Statistics Sweden and the Causes of Death Register, held at the National Board of Health and Welfare. In the MDCS, we also performed analyses treating death as a competing risk event, using the cmprsk (competing risk) package in R.

Risk of all-cause dementia, VaD and AD are presented as hazard ratios (HR) with 95% confidence intervals (CI). In the Vasaloppet cohort, we present both a crude model and an age-, sex-, and education-adjusted model (model 1). Education is categorized as noted in Table 1. In the MDCS cohort, adjustments were performed in a stepwise manner, where model 1 is adjusted for age, sex,

Table 1 Characteristics of the Vasaloppet study population

	All	Skiers	Non-skiers
	$n = 395{,}369$	$n = 197{,}685$	$n = 197{,}684$
Characteristics 1989–2010	Median (IQR) or n (%)	Median (IQR) or n (%)	Median (IQR) or n (%)
Age at baseline, years	36.0 (29.0–46.0)	36.0 (29.0–46.0)	36.0 (29.0–46.0)
Women	149,796 (38)	74,897 (38)	74,899 (38)
Education			
Primary/elementary school (≤ 8 years)	49,344 (13)	14,538 (7.4)	34,806 (18)***
Secondary school/high school (9–12 years)	176,571 (45)	76,635 (39)	99,936 (51)
Higher education/university (≥ 13 years)	166,133 (42)	106,147 (54)	59,986 (31)
Dementia diagnoses at follow-up	N events (incidence rate/1000 person-years)		
All-cause dementia	542 (0.14)	223 (0.11)	319 (0.16)
Vascular dementia	112 (0.03)	40 (0.02)	72 (0.04)***
Alzheimer's disease dementia	181 (0.05)	86 (0.04)	95 (0.05)

Characteristics of the Vasaloppet study population presented for the whole cohort and by skiers and non-skiers separately

***$p < 0.001$. Group difference between skiers and non-skiers, estimated with Mann-Whitney U test (numeric variables) and Pearson's χ^2 test (categorical variables). Only significant differences are noted in the table

and education and model 2 is further adjusted for smoking, systolic blood pressure, body mass index, alcohol consumption, diabetes, cardiovascular disease, blood pressure-lowering medication, lipid-lowering medication, and physically heavy work.

Overall, we performed complete case analyses, rendering fewer individuals in adjusted models. We modeled Schoenfeld residuals graphically to confirm the proportionality assumption. Figure data were constructed using Kaplan-Meier curves. The same time and event variables were used as in the Cox regressions, and the hazards are presented for skiers vs non-skiers. Numbers at risk were derived from survival tables specifying the number of individuals entering each 5-year interval, as presented in the graph (Fig. 2).

Since physical activity has been shown to be reduced up to 9 years before diagnosis [17] and beneficial effects of physical activity on dementia was shown to disappear after 4 years [15], we decided to set 5 years as a cut-off for sensitivity analyses. All individuals who developed dementia within 5 years of participation in the Vasaloppet ski race and within 5 years of the second physical activity assessment in the MDCS were excluded. In the MDCS, we performed further sensitivity analyses where we used pure AD and AD with cerebrovascular disease as separate event variables. We also added *APOE*-ε4 as a covariate in the subpopulation with available data and stratified this subpopulation on *APOE*-ε4 carrier status. Interaction statistics for *APOE*-ε4 was applied by simultaneously entering physical activity score and *APOE*-ε4 together with a variable consisting of their product in Cox regression models. In order to account for attrition bias, we also investigated if the physical activity at baseline (only one assessment) was associated with the different event variables (all-cause dementia, VaD, and AD).

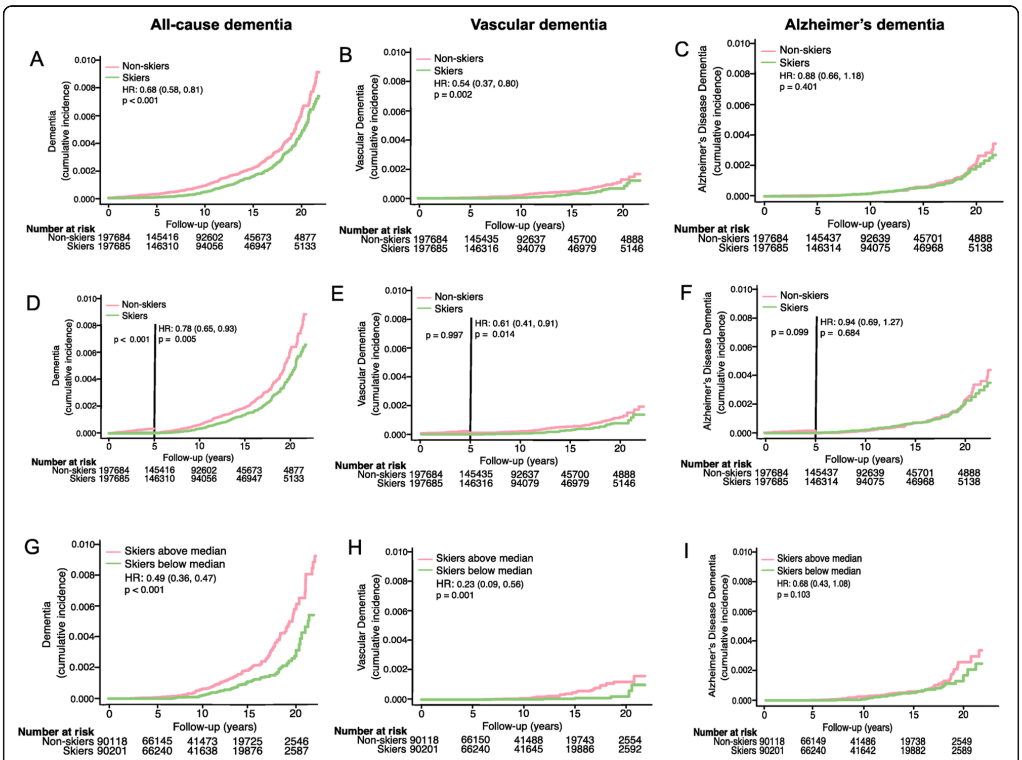

Fig. 2 The effects of physical activity on the incidence of dementia, VaD, and AD in the Vasaloppet. The risk of developing all-cause dementia (**a**), VaD (**b**), or AD (**c**). The risk of developing all-cause dementia (**d**), VaD (**e**), or AD (**f**) more than 5 years after completing Vasaloppet. The risk of developing all-cause dementia (**g**), VaD (**h**), or AD (**i**) in skiers completing the Vasaloppet at a finishing time above or below median. HR represents hazard ratios from an unadjusted Cox regression

Results

Vasaloppet skiers had a reduced risk of developing vascular dementia but not Alzheimer's dementia

Demographic data for the Vasaloppet cohort is presented in Table 1. The total number of deaths was less than 2%. After a median follow-up of 10 years (IQR 5–15 years), 542 dementia diagnoses were identified in the NPR. Out of these, 112 (21%) were diagnosed with VaD and 181 individuals (33%) with AD. Participation in the Vasaloppet ski race was associated with a lower risk of developing all-cause dementia and VaD, but there was no significant difference between skiers and non-skiers for AD (Table 2, Fig. 2a–c). Skiers had higher education than non-skiers (Table 1), but adjustments for age, gender, and education did not alter the results (model 1, Table 2). When we excluded cases that developed dementia within 5 years of the ski race (baseline), results were not altered (Table 2, Fig. 2d–f). Furthermore, faster skiers (accomplishing Vasaloppet with a finishing time below median) had a lower incidence of VaD (adjusted hazard ratio (HR) 0.38, 95% CI 0.16–0.95), but not all-cause dementia (HR 0.80, 95% CI 0.59–1.09) or AD (HR 1.17, 95% CI 0.73–1.88), compared to slower skiers (Fig. 2g–i, unadjusted HR).

Higher physical activity was associated with reduced risk of vascular dementia but not Alzheimer's dementia in the MDCS

Demographics for all participants can be seen in Table 3. Participants were followed for a median of 20 years (IQR 19–22) from baseline and 15 years (IQR 14–17 years) from the reinvestigation. Based on the diagnostic review process, 1375 individuals were diagnosed with dementia during the follow-up period. Out of these, 300 (22%) were classified as VaD and 834 (61%) were classified as AD, out of which 436 were classified as pure AD and 398 as AD with concomitant cerebrovascular disease. In age-, sex-, and education-adjusted Cox regression models (model 1), higher physical activity score, modeled linearly, reduced the risk of developing VaD (HR 0.81 per SD increase, 95% CI 0.72–0.93), but not all-cause dementia (HR 0.96 per SD increase, 95% CI 0.91–1.02) nor AD (HR 1.03 per SD increase, 95% CI 0.97–1.09). In the fully adjusted model (model 2), the results were robust for VaD (HR 0.83 per SD increase, 95% CI 0.73–0.95). There was still no significant association between physical activity score and incident all-cause dementia (HR 0.97 per SD increase, 95% CI 0.92–1.02) or AD (HR 1.03 per SD increase, 95% CI 0.97–1.10) after full adjustments (model 2). When the population was categorized based on tertiles, high physical activity decreased the risk of developing VaD, even when we adjusted for multiple confounders (Table 4). We found no significant associations between physical activity categories and incident all-cause dementia or AD (Table 4). These results were not altered when cases who developed dementia within the first 5 years of the reinvestigation were excluded (Table 4).

Table 2 Association between physical activity and incident dementia in the Vasaloppet cohort

	All-cause dementia HR (95% CI)	*p*	Vascular dementia HR (95% CI)	*p*	Alzheimer's dementia HR (95% CI)	*p*
Physical activity						
Unadjusted model	542 events		112 events		181 events	
Non-skiers (reference)	1		1		1	
Skiers	0.68 (0.58–0.81)	< 0.001	0.54 (0.37–0.80)	0.002	0.88 (0.66–1.18)	0.40
Model 1	533 events		112 events		177 events	
Non-skiers (reference)	1		1		1	
Skiers	0.63 (0.52–0.75)	< 0.001	0.49 (0.33–0.73)	< 0.001	0.74 (0.55–1.00)	0.052
Excluding dementia cases < 5 years						
Unadjusted model	483 events		104 events		169 events	
Non-skiers (reference)	1		1		1	
Skiers	0.78 (0.65–0.93)	0.005	0.61 (0.41–0.91)	0.014	0.94 (0.69–1.27)	0.68
Model 1	477 events		104 events		166 events	
Non-skiers (reference)	1		1		1	
Skiers	0.68 (0.57–0.82)	< 0.001	0.54 (0.36–0.80)	0.002	0.78 (0.57–1.07)	0.12

Association between physical activity and incident dementia in the Vasaloppet cohort, based on participation in a long-distance ski race (skiers) compared to non-skiers. Cox regression models showing hazard ratio (HR) with 95% confidence interval (CI) for risk of all-cause dementia, vascular dementia, or Alzheimer's dementia, respectively. Model 1 adjusted for age, sex, and education

Table 3 Characteristics of the MDCS population at baseline investigation (1991–1996)

	All	Low physical activity group	Intermediate physical activity group	High physical activity group
	n = 20,639	*n* = 6882	*n* = 6882	*n* = 6875
Characteristics at baseline	Median (IQR) or *n* (%)	Median (IQR) or *n* (%)	Median (IQR) or *n* (%)	Median (IQR) or *n* (%)
Age at baseline, years	57.5 (51.0–63.8)	57.0 (50.9–63.7)	57.1 (50.8–63.4)	58.3 (51.6–64.2)***
Women	12,460 (60)	4205 (61)	4335 (63)*	3920 (57)***
Education				
Primary/elementary school (≤ 8 years)	8159 (40)	3041 (44)	2515 (37)***	2603 (38)***
Secondary school/high school (9–12 years)	7449 (36)	2387 (35)	2568 (37)	2494 (36)
Higher education/university (≥ 13 years)	5001 (24)	1443 (21)	1793 (26)	1765 (26)
Smoking, ever	12,573 (61)	40,239 (62)	4151 (60)	4183 (61)
Systolic blood pressure, mmHg	140 (126–152)	140 (128–152)	140 (126–150)**	140 (126–152)
Diastolic blood pressure, mmHg	85 (80–90)	85 (80–90)	85 (80–90)**	85 (80–90)**
Body mass index, kg/m^2	25.2 (22.9–27.7)	25.6 (23.2–28.3)	25.0 (22.8–27.5)***	25.0 (22.9–27.4)***
Alcohol, g/day	7.6 (1.9–15.6)	6.8 (1.3–15.3)	7.8 (2.3–15.7)***	8.1 (2.3–15.9)***
Physically heavy work	7659 (38)	2613 (39)	2444 (36)**	2602 (38)
Physical activity score combined	13,300 (8460–19,785)	6720 (4589–8460)	13,304 (11602–15,076)***	23,320 (19790–29,050)***
Cardiovascular disease	543 (2.6)	205 (3.0)	166 (2.4)*	172 (2.5)
Diabetes mellitus	790 (3.8)	305 (4.4)	235 (3.4)**	250 (3.6)*
Blood pressure-lowering medication	3568 (17)	1323 (19)	1177 (17)**	1068 (16)***
Lipid-lowering medication	629 (3.0)	207 (3.0)	205 (3.0)	217 (3.2)
APOE-ε4 carriers[a]	3306 (30)	1146 (31)	1055 (30)	1105 (30)
Dementia diagnoses at follow-up	*N* events (incidence rate/1000 person-years)			
All-cause dementia	1375 (4.7)	455 (4.8)	460 (4.7)	460 (4.7)
Vascular dementia	300 (1.0)	112 (1.2)	101 (1.0)	87 (0.9)
Alzheimer's dementia	834 (2.9)	266 (2.8)	271 (2.8)	297 (3.0)
Age at dementia diagnosis	80.0 (75.7–83.7)	79.7 (75.8–83.2)	80.2 (75.7–84.1)	80.3 (75.8–84.1)

Characteristics of the MDCS population at baseline investigation (1991–1996) for the total cohort, and by physical activity tertiles. Blood pressure and body mass index were measured at the baseline investigation in the Malmo Diet and Cancer Study. Cardiovascular disease (coronary disease or stroke) and diabetes mellitus (type 1 or 2) were derived from hospital registries at baseline. Dementia diagnoses were derived from registries and validated in e-charts. All other data was self-reported, derived from the baseline questionnaire. Group differences between participants in the lowest physical activity group compared to intermediate and high respectively were estimated with Mann-Whitney *U* test (numeric variables) and Pearson's χ^2 test (categorical variables). Only significant differences are noted in the table

***$p < 0.001$, **$p < 0.01$, *$p < 0.05$

[a]Data on 10,971 participants (53% of the study cohort)

Further, we found no significant association between physical activity and pure AD (HR 1.06 per SD increase, 95% CI 0.98–1.14) nor between physical activity and AD with concomitant cerebrovascular disease (HR 1.04 per SD increase, 95% CI 0.92–1.17) in fully adjusted models (model 2).

Data on *APOE* genotype was available in a subpart of the MDCS cohort ($n = 10,971$), and 3306 participants (30%) were hetero- or homozygote *APOE-ε4* carriers. Three hundred five *APOE*-ε4 carriers (9.1%) were diagnosed with AD during the study period, compared to 2.6% among non-carriers and 4.6% in the total cohort (with available *APOE* data). When *APOE*-ε4 status was entered as a dichotomous covariate in the Cox regression models, the results per SD increase in physical activity score were not affected for any of the outcome variables (all-cause dementia, VaD, or AD) (data not shown). There was no significant interaction between *APOE*-ε4 and physical activity for any of the dependent variables ($p = 0.68$ for AD, $p = 0.40$ for vascular dementia, and $p = 0.32$ for all-cause dementia). When the population was stratified based on *APOE*-ε4 carrier status, physical activity did not affect the risk of developing AD among *APOE*-ε4 carriers (HR 1.00 per SD increase,

Table 4 Association between midlife physical activity and incident dementia in the MDCS cohort

	All-cause dementia HR (95% CI)	*p*	Vascular dementia HR (95% CI)	*p*	Alzheimer's dementia HR (95% CI)	*p*
Physical activity						
Model 1	1373 events		300 events		832 events	
Low (reference)	1		1		1	
Intermediate	0.99 (0.87–1.12)	0.84	0.87 (0.66–1.14)	0.30	1.01 (0.85–1.19)	0.95
High	0.90 (0.79–1.02)	0.11	0.63 (0.48–0.84)	0.002	1.04 (0.88–1.23)	0.64
Model 2	1341 events		293 events		815 events	
Low (reference)	1		1		1	
Intermediate	0.97 (0.85–1.11)	0.68	0.88 (0.67–1.16)	0.36	0.98 (0.82–1.16)	0.79
High	0.90 (0.79–1.03)	0.11	0.65 (0.49–0.87)	0.003	1.03 (0.87–1.22)	0.75
Excluding dementia cases < 5 years						
Model 1	1204 events		270 events		714 events	
Low (reference)	1		1		1	
Intermediate	1.02 (0.89–1.18)	0.75	0.92 (0.69–1.22)	0.55	1.06 (0.88–1.28)	0.54
High	0.95 (0.83–1.09)	0.47	0.65 (0.48–0.88)	0.005	1.14 (0.95–1.37)	0.16
Model 2	1172 events		263 events		697 events	
Low (reference)	1		1		1	
Intermediate	1.01 (0.88–1.17)	0.85	0.93 (0.70–1.24)	0.63	1.04 (0.86–1.25)	0.71
High	0.96 (0.83–1.10)	0.53	0.66 (0.49–0.90)	0.008	1.14 (0.95–1.37)	0.16

Association between midlife physical activity and incident dementia in the MDCS cohort, based on self-reported physical activity at two different occasions in midlife categorized as low, intermediate, or high activity group. Cox regression models showing hazard ratio (HR) with 95% confidence interval (CI) per physical activity group for risk of all-cause dementia, vascular dementia, or Alzheimer's dementia, respectively. Number of events per model is presented for transparency, since we used complete case analyses. Model 1 adjusted for age, sex, and education. Model 2 adjusted for age, sex, education, smoking, systolic blood pressure, body mass index, alcohol consumption, diabetes, cardiovascular disease, blood pressure-lowering medication, lipid-lowering medication, and physically heavy work

95% CI 0.89–1.13), nor among non-carriers (HR 1.04 per SD increase, 95% CI 0.92–1.16) in fully adjusted models (model 2).

By the end of the follow-up, 5220 individuals (25%) in the MDCS cohort were deceased, and among individuals without a dementia diagnosis, this number was 23%. In analyses treating death as a competing risk event, the association between physical activity and VaD was attenuated (fully adjusted HR 0.88 per SD increase, 95% CI 0.74–1.04 and HR 0.74 for the highest vs lowest physical activity group, 95% CI 0.56–0.98). There was still no association between physical activity and all-cause dementia (fully adjusted HR 1.00 per SD increase, 95% CI 0.95–1.05), but higher physical activity indicated a borderline increased risk of AD (fully adjusted HR 1.05 per SD increase, 95% CI 1.00–1.10), though not significant when modeled categorically (fully adjusted HR 1.13 for the highest vs lowest physical activity group, 95% CI 0.95–1.33).

Finally, to address attrition bias, we also performed analyses including all individuals who provided data on physical activity at baseline ($n = 28{,}360$), thus only assessing physical activity *once* in midlife. Still, no association was found between physical activity and incident all-cause dementia nor AD in either model 1 or 2 (all $p > 0.20$ per SD increase in physical activity score, data not shown). There was a significant association between physical activity at baseline and incident VaD in model 1 (HR per SD increase 0.87, 95% CI 0.78–0.96) and in model 2 (HR per SD increase 0.89, 95% CI 0.80–0.99).

Physical activity does not protect against Alzheimer pathology in Alzheimer's disease mice

Running did not affect the object memory ($p = 0.21$) or working memory ($p = 0.38$) (Fig. 3a). However, running mice had reduced spatial memory as they entered the new arm of the maze less frequently compared to sedentary mice ($p = 0.03$) (Fig. 3a). The levels of the synaptic proteins PSD-95 ($p = 0.09$) and synaptophysin ($p = 0.79$) in the hippocampus were not affected by running (Fig. 3b). Furthermore, the levels of amyloid-β did not differ between the running and sedentary mice, neither as measured by immunohistochemistry in the hippocampus ($p = 0.77$) or cortex ($p = 0.40$) (Fig. 3c and Additional file 1: Figure S1), nor as measured by ELISA in the hippocampus ($p = 0.46$ and $p = 0.44$ for Aβ40 and Aβ42 respectively, Fig. 3d).

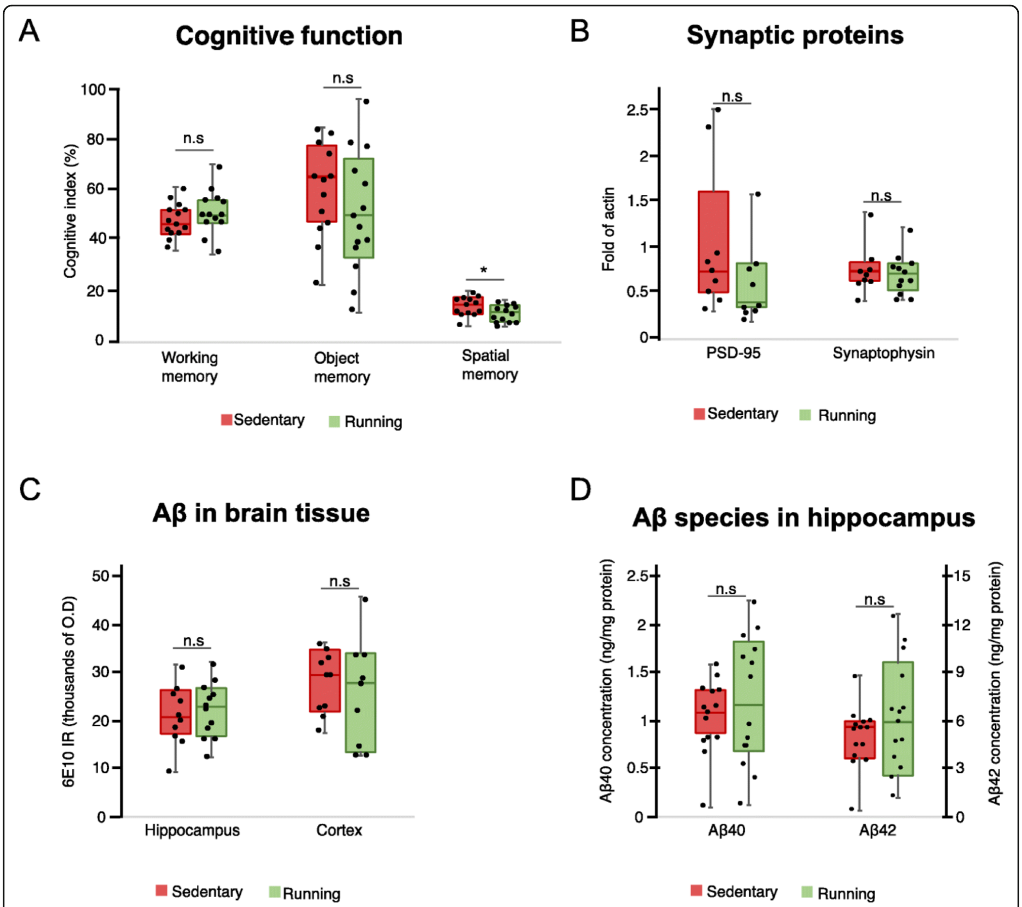

Fig. 3 The effect of running on AD pathology in the 5xFAD mouse model. The effect on cognitive function (**a**), synaptic proteins (**b**) in the hippocampus, amyloid-β levels in the cortex and hippocampus (**c**), and Aβ-species in the hippocampus (**d**). Box plot represents the median values for each group with interquartile ranges and error bars indicating the minimum and maximum. *$p < 0.05$ in Mann-Whitney *U* test. For cognitive tests, $n = 13–14$ in each group; for amyloid-β and synaptic proteins, $n = 9–14$ in each group

Discussion

Our study setup offered a unique possibility to study the effect of midlife physical activity on the development of different forms of dementia in very large study populations over long time periods. We found physical activity to be associated with lower incidence of VaD, but not AD, in both our epidemiological study populations. In addition, individuals carrying *APOE*-ε4, did not exhibit any specific beneficial protection from physical activity on the development of AD. The lack of protective effect of physical activity on the development of AD was also seen in an experimental setup subjecting AD transgenic mouse to voluntary wheel running.

The effect of physical activity on all-cause dementia differed in our study cohorts, in line with inconsistent results from previous studies [15, 17, 19, 20]. This might be due to the fact that all-cause dementia constitutes different underlying pathologies, identifying the need to differentiate between dementia subtypes. Indeed, for VaD and AD, our results were consistent in both cohorts. In line with a meta-analysis [12], we found physical activity to be associated with a lower incidence of VaD, presumably resulting from improved cerebral perfusion and reduction of cerebrovascular pathology [45]. In an attempt to use a more objective measure of physical activity, we stratified skiers based

on the speed of race accomplishment. Interestingly, physically well-trained skiers had a lower incidence of VaD compared to less well-trained skiers, which further strengthen our results. Many previous studies suggest a beneficial effect of physical activity on the incidence of AD specifically [10, 19, 20, 46], but this was not confirmed in our study together with others [15, 18, 47]. Reasons for discrepancies between these studies may be that physical activity reduces cerebrovascular comorbidity in individuals with AD and thereby delays the onset of cognitive symptoms, rather than affecting AD pathology per se. Joint pathologies (generally concurrent cerebrovascular disease) are common in individuals diagnosed with AD [48]. Hence, studies that do report significant associations between physical activity and AD may represent effects that lower the cerebrovascular burden and thus postpone the onset of cognitive symptoms due to AD rather than affecting the specifc AD pathology per se. Studies using AD biomarkers and MRI as an outcome, rather than clinical dementia diagnoses, may help elucidate the specific effects. In recently published clinical studies, physical activity did not affect amyloid-β levels in the cerebrospinal fluid [49], but still resulted in improved cognition [7]. Further, physical inactivity was not associated with amyloid-β deposition measured with PET [50]. Another possible explanation to the beneficial effects of physical activity on dementia incidence shown in previous studies is the study setup. Lack of exclusion of participants developing dementia soon after physical activity assessment may increase the risk that some of them are affected by reverse causation, where reduced physical activity may be caused by cognitive decline and preclinical dementia symptoms [15, 17]. Indeed, when studies with follow-up time ≥ 10 years were assessed separately in a meta-analysis, the impact of physical activity on dementia was more conservative [11]. Consistently, physical activity was associated with reduced risk for dementia with cerebrovascular disease, but not AD, in a recent study following 800 women over 44 years [51]. In the present study, we tried to limit reverse causation by excluding individuals who developed dementia within 5 years of the ski race or the physical activity assessment. Further, publication bias may have influenced the prevailing literature, since a large number of smaller studies showed larger-than-average effects [11].

Lately, intervention trials have been carried out to test if physical activity may reduce cognitive decline and dementia. The overall effects seem limited [3, 4], but one study with a multi-domain intervention found beneficial effects on cognitive performance [5]. In the study with the longest follow-up (mean 6.7 years), the risk of developing non-AD dementia was significantly reduced, with a trend towards protection against VaD specifically [3]. Moreover, when assessing the intervention effects of physical activity on cognitive performance in *APOE*-ε4 carriers and non-carriers separately, there was no effect difference depending on the genetic risk [52], which agrees with the present study.

In experimental settings, we have not been able to find any data on the effect of physical activity on pathological processes in animal models of VaD. However, the effects of exercise on AD pathology have been thoroughly studied in mice [25]. Many studies report the ability of exercise to improve cognition in aged wild-type mice as well as transgenic AD mice [25]. Nevertheless, some studies show no effect of exercise on cognition in transgenic AD models [27, 33] and some experimental studies can be biased by chronic stress, as reported by us [41]. In the present study, voluntary physical activity did not improve cognition in transgenic 5xFAD mice. Furthermore, physical activity did not reduce the levels of amyloid-β or formation of plaques, which is congruent with some previous studies [25, 27]. Important parameters to consider for the discrepancies between studies are the duration and timing of the exercise interventions and sample collection. As noted by Ryan et al., longer durations of exercise interventions are needed to investigate the long-term effects of an active lifestyle [25]. Many published studies have limitations in the timings and durations in order to study the effect of a long-term active lifestyle from middle age and onwards [25]. We initiate the exercise at an age of 2 months, just before the onset of Aβ pathology. Further, our intervention lasts for as long as 6 months, until the mice are 8 months old, an age with fully developed pathology. Given the genetically driven pathology in most transgenic AD models, the effects of exercise investigated might not be fully transferable to late-onset AD.

Limitations of the study include that physical activity was self-reported in the MDCS cohort, which introduces subjectivity into the estimation. We tried to compensate this with the use of a validated physical activity score [33] and by using data from two separate time points (5 years apart), thereby estimating the degree of physical activity over an extended time period in midlife. Further, we assume there is a healthy selection bias considering that individuals included in MDCS were generally healthier and more physically active at baseline than those excluded due to lack of data (see "Attrition" in the MDCS methods section). This may underestimate any true associations, but this was partly accounted for in sensitivity analyses where we included all individuals with baseline data on physical activity (only one assessment), thus minimizing attrition during follow-up. Still, no significant association was found for all-cause dementia nor AD. The association between physical activity and vascular

dementia was weaker in the analyses with *one* physical activity assessment (see results for MDCS). This may be due to the possibility that the potential effects of physical activity require an active lifestyle during a prolonged period, better reflected when physical activity was reported twice. In the Vasaloppet cohort, we lack data on physical activity among non-skiers and thereby include physically active individuals in the reference category as well, which may attenuate the true association. Skiers were considered physically active based on the assumption that it is necessary to undergo regular physical training in order to complete such a demanding long-distance race, and previous studies have indeed showed that this is the case [30]. This may induce bias dependent on other confounders, such as diet, BMI, and smoking habits. Since this information cannot be found in the Swedish registries, we could not adjust for these potential confounders. Still, the results of the association between physical activity and incidence of VaD and AD were in accordance with those from the fully adjusted model in the MDCS cohort. Nevertheless, we were able to adjust for age, sex, and education in the statistical models in the Vasaloppet. In addition, we clearly demonstrated that faster skiers had reduced incidence of VaD but not AD, implicating that the associations seen can be attributable to physical fitness level per se. In the MDCS, the study protocol provided data on several possible confounders that were included in the analyses. Lastly, the use of register-based diagnoses can be considered a limitation. All dementia diagnoses were derived from hospital registries, which most likely underestimates the true incidence. However, the Swedish National Patient Register covers 99% of all hospital-based diagnoses, and both primary and secondary diagnoses are represented. Another explanation to the relatively low incidence of dementia within the Vasaloppet cohort is that the study design excluded individuals that were already diagnosed with a severe disease that could prevent them from being active at baseline. This was necessary in order to reduce the potential bias due to inability to participate in the ski race. Hence, this design is likely to result in a lower incidence number due to elimination of comorbidity. In Sweden as a whole, the incident rate of dementia is 2 cases per 1000 person-years (2017, Statistics Sweden). The incident rate in MDCS is around this number, mainly due to participants being older (around 58 years). In the Vasaloppet cohort, the incident rates are below this, mainly due to the exclusion of comorbidities and a low age at baseline (around 36 years). Finally, since we aimed to study differences between dementia subtypes, possible diagnostic misclassification needs to be acknowledged. Clinically derived diagnoses may be insufficiently characterized, and concordance between clinical and neuropathological diagnoses does vary [53]. Nevertheless, in the MDCS, over 80% of individuals with dementia attended specialized Memory Clinics, and all medical records and brain imaging were retrospectively reviewed to determine the type of dementia diagnosis.

Taken together, we used two very different study designs, one in which physical activity was measured in a more objective way (participation in long-distance ski race), and the other where it was subjectively measured (by a self-reported questionnaire). Still, both these study setups revealed concurrent results where physical activity was associated with a lower incidence of VaD but not AD, despite differences in strengths and limitations within the separate cohorts. This consistency likely reduces the risk that the found associations are driven by confounding factors.

Conclusion

In conclusion, higher physical activity in midlife was associated with a lower incidence of VaD. No association between physical activity and AD was found, neither among individuals predisposed to develop AD by carrying the *APOE*-ε4 risk allele. Altogether, physical activity could be an important strategy to prevent the development of VaD, especially considering the lack of available treatments for this disease.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13195-019-0538-4.

Additional file 1: Figure S1. Representative pictures of the 6E10 staining of cortex and hippocampus in sedentary and running mice respectively. Scale bar represents 100 μm. No differences were found between groups with the Mann-Whitney U-test.

Abbreviations

5xFAD: 5x familial Alzheimer's disease; AD: Alzheimer's disease; HR: Hazard ratio; IQR: Interquartile range; MDCS: Malmo Diet and Cancer Study; NPR: National Patient Register; VaD: Vascular dementia

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Authors' contributions

OH was responsible for the study coordination as well as drafting and revising the manuscript. MS was responsible for the planning and conduction of the experimental study, including running intervention, behavioral tests, collection of samples, processing of tissue, image analysis, and statistical analyses of the experimental data. AMG was responsible for the statistical analyses of the MDCS data. MS and AMG did the literature search and wrote the main parts of the manuscript. EA was involved in the conduction of the experimental study and performed the immunohistochemistry. YY performed the western blots. KN was responsible for the MDCS collaboration and critically revised the manuscript. UH and SJ

were responsible for establishing and extracting data in the Vasaloppet cohort and critically revised the manuscript. TD was responsible for the planning of the experimental study and the Vasaloppet study as well as coordinating the collaborations and revising the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The data sets supporting the conclusions of this article can be made available upon request. MDCS data can be requested through an application to the MDCS steering committee. Vasaloppet database can be requested from Uppsala Clinical Research Center. Data used in the mouse model analyses can be requested through the corresponding author.

Ethics approval and consent to participate
The Ethical Review Board in Uppsala, Sweden, approved the Vasaloppet study (2010, Dnr 2010/305). The Regional Ethical Review Board of Lund University gave ethical approval for the Malmo Diet and Cancer Study in several stages (2002, Dnr 244-02, 2004, Dnr 154-2004, 2009, Dnr 633-2009, 2011, Dnr 83-2011, and 2013, Dnr 489-2013.) All MDCS participants provided informed consent at the study entry, when no cognitive disorder was present/diagnosed. Animal experiments were approved by Malmö/Lund animal ethics committee (2012, Dnr: M427-12) and performed in accordance to the Directive of the European Parliament.

Consent for publication
Not applicable

Competing interests
Oskar Hansson acquired research support (for the institution) from Roche, GE Healthcare, Biogen, AVID Radiopharmaceuticals, Fujirebio, and Euroimmun. In the past 2 years, he has received consultancy/speaker fees (paid to the institution) from Biogen, Roche, and Fujirebio. The remaining authors declare that they have no competing interests.

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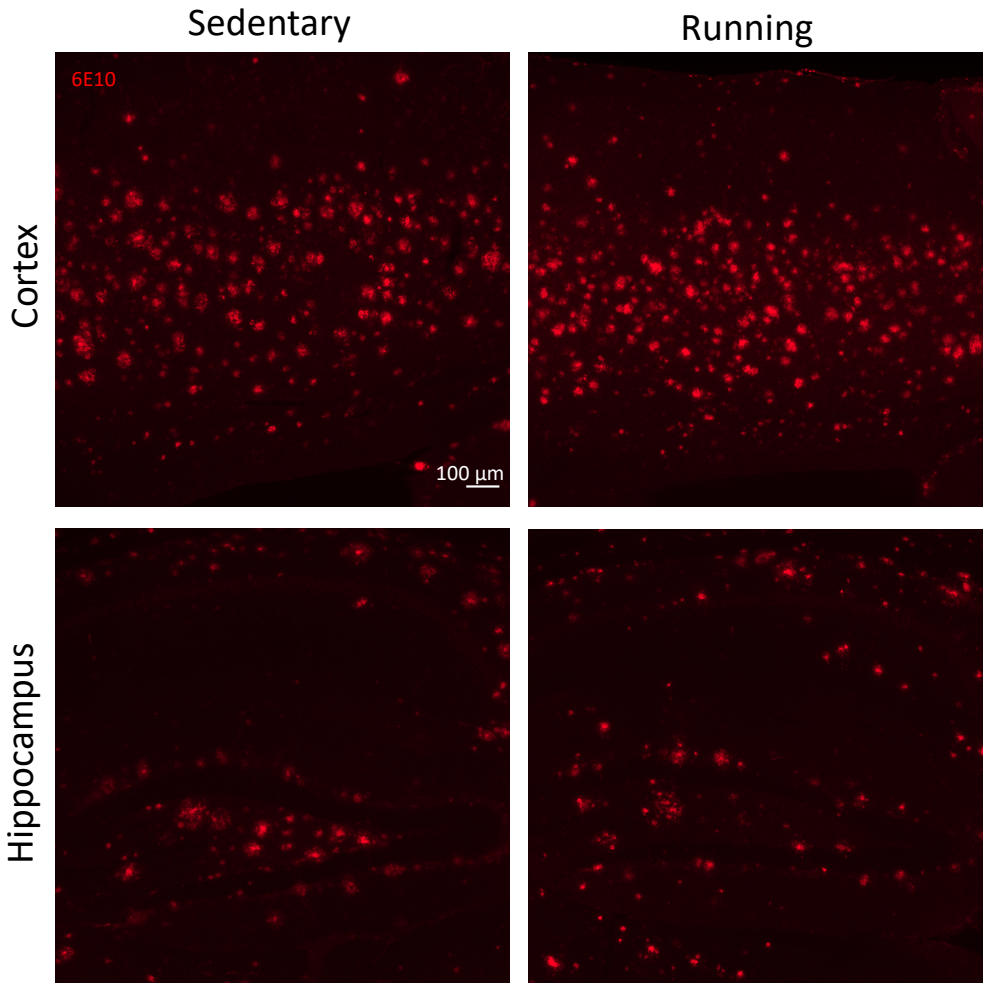
References

1. Rakesh G, Szabo ST, Alexopoulos GS, Zannas AS. Strategies for dementia prevention: latest evidence and implications. Ther Adv Chronic Dis. 2017; 8(8–9):121–36.
2. Norton S, Matthews FE, Barnes DE, Yaffe K, Brayne C. Potential for primary prevention of Alzheimer's disease: an analysis of population-based data. Lancet Neurol. 2014;13(8):788–94.
3. Moll van Charante EP, Richard E, Eurelings LS, van Dalen JW, Ligthart SA, van Bussel EF, et al. Effectiveness of a 6-year multidomain vascular care intervention to prevent dementia (preDIVA): a cluster-randomised controlled trial. Lancet. 2016;388(10046):797–805.
4. Andrieu S, Guyonnet S, Coley N, Cantet C, Bonnefoy M, Bordes S, et al. Effect of long-term omega 3 polyunsaturated fatty acid supplementation with or without multidomain intervention on cognitive function in elderly adults with memory complaints (MAPT): a randomised, placebo-controlled trial. Lancet Neurol. 2017;16(5):377–89.
5. Ngandu T, Lehtisalo J, Solomon A, Levalahti E, Ahtiluoto S, Antikainen R, et al. A 2 year multidomain intervention of diet, exercise, cognitive training, and vascular risk monitoring versus control to prevent cognitive decline in at-risk elderly people (FINGER): a randomised controlled trial. Lancet. 2015; 385(9984):2255–63.
6. Groot C, Hooghiemstra AM, Raijmakers PG, van Berckel BN, Scheltens P, Scherder EJ, et al. The effect of physical activity on cognitive function in patients with dementia: a meta-analysis of randomized control trials. Ageing Res Rev. 2016;25:13–23.
7. Hoffmann K, Sobol NA, Frederiksen KS, Beyer N, Vogel A, Vestergaard K, et al. Moderate-to-high intensity physical exercise in patients with Alzheimer's disease: a randomized controlled trial. J Alzheimers Dis. 2016; 50(2):443–53.
8. Lamb SE, Sheehan B, Atherton N, Nichols V, Collins H, Mistry D, et al. Dementia And Physical Activity (DAPA) trial of moderate to high intensity exercise training for people with dementia: randomised controlled trial. BMJ. 2018;361:k1675.
9. Iuliano E, di Cagno A, Cristofano A, Angiolillo A, D'Aversa R, Ciccotelli S, et al. Physical exercise for prevention of dementia (EPD) study: background, design and methods. BMC Public Health. 2019;19(1):659.
10. Hamer M, Chida Y. Physical activity and risk of neurodegenerative disease: a systematic review of prospective evidence. Psychol Med. 2009;39(1):3–11.
11. Blondell SJ, Hammersley-Mather R, Veerman JL. Does physical activity prevent cognitive decline and dementia?: a systematic review and meta-analysis of longitudinal studies. BMC Public Health. 2014;14:510.
12. Aarsland D, Sardahaee FS, Anderssen S, Ballard C, Alzheimer's Society Systematic Review g. Is physical activity a potential preventive factor for vascular dementia? A systematic review. Aging Ment Health 2010;14(4):386–395.
13. Stephen R, Hongisto K, Solomon A, Lonnroos E. Physical activity and Alzheimer's disease: a systematic review. J Gerontol A Biol Sci Med Sci. 2017; 72(6):733–9.
14. Xu W, Wang HF, Wan Y, Tan CC, Yu JT, Tan L. Leisure time physical activity and dementia risk: a dose-response meta-analysis of prospective studies. BMJ Open. 2017;7(10):e014706.
15. de Bruijn RF, Schrijvers EM, de Groot KA, Witteman JC, Hofman A, Franco OH, et al. The association between physical activity and dementia in an elderly population: the Rotterdam study. Eur J Epidemiol. 2013;28(3):277–83.
16. Tan ZS, Spartano NL, Beiser AS, DeCarli C, Auerbach SH, Vasan RS, et al. Physical activity, brain volume, and dementia risk: the Framingham study. J Gerontol A Biol Sci Med Sci. 2017;72(6):789–95.
17. Sabia S, Dugravot A, Dartigues JF, Abell J, Elbaz A, Kivimaki M, et al. Physical activity, cognitive decline, and risk of dementia: 28 year follow-up of Whitehall II cohort study. BMJ. 2017;357:j2709.
18. Yamada M, Kasagi F, Sasaki H, Masunari N, Mimori Y, Suzuki G. Association between dementia and midlife risk factors: the radiation effects research foundation adult health study. J Am Geriatr Soc. 2003;51(3):410–4.
19. Rovio S, Kareholt I, Helkala EL, Viitanen M, Winblad B, Tuomilehto J, et al. Leisure-time physical activity at midlife and the risk of dementia and Alzheimer's disease. Lancet Neurol. 2005;4(11):705–11.
20. Andel R, Crowe M, Pedersen NL, Fratiglioni L, Johansson B, Gatz M. Physical exercise at midlife and risk of dementia three decades later: a population-based study of Swedish twins. J Gerontol A Biol Sci Med Sci. 2008;63(1):62–6.
21. Carlson MC, Helms MJ, Steffens DC, Burke JR, Potter GG, Plassman BL. Midlife activity predicts risk of dementia in older male twin pairs. Alzheimers Dement. 2008;4(5):324–31.
22. Raber J, Huang Y, Ashford JW. ApoE genotype accounts for the vast majority of AD risk and AD pathology. Neurobiol Aging. 2004;25(5):641–50.
23. Head D, Bugg JM, Goate AM, Fagan AM, Mintun MA, Benzinger T, et al. Exercise engagement as a moderator of the effects of APOE genotype on amyloid deposition. Arch Neurol. 2012;69(5):636–43.
24. Podewils LJ, Guallar E, Kuller LH, Fried LP, Lopez OL, Carlson M, et al. Physical activity, APOE genotype, and dementia risk: findings from the Cardiovascular Health Cognition Study. Am J Epidemiol. 2005;161(7):639–51.
25. Ryan SM, Kelly AM. Exercise as a pro-cognitive, pro-neurogenic and anti-inflammatory intervention in transgenic mouse models of Alzheimer's disease. Ageing Res Rev. 2016;27:77–92.
26. Moore KM, Girens RE, Larson SK, Jones MR, Restivo JL, Holtzman DM, et al. A spectrum of exercise training reduces soluble Abeta in a

dose-dependent manner in a mouse model of Alzheimer's disease. Neurobiol Dis. 2016;85:218–24.
27. Xu ZQ, Zhang LQ, Wang Q, Marshall C, Xiao N, Gao JY, et al. Aerobic exercise combined with antioxidative treatment does not counteract moderate- or mid-stage Alzheimer-like pathophysiology of APP/PS1 mice. CNS Neurosci Ther. 2013;19(10):795–803.
28. Hallmarker U, Lindback J, Michaelsson K, Arnlov J, Asberg S, Wester P, et al. Survival and incidence of cardiovascular diseases in participants in a long-distance ski race (Vasaloppet, Sweden) compared with the background population. Eur Heart J Qual Care Clin Outcomes. 2018;4(2):91–7.
29. Carlsson S, Olsson L, Farahmand BY, Hallmarker U, Ahlbom A. Skiers in the long-distance ski race invest in their health. Lakartidningen. 2007; 104(9):670–1.
30. Farahmand BY, Ahlbom A, Ekblom O, Ekblom B, Hallmarker U, Aronson D, et al. Mortality amongst participants in Vasaloppet: a classical long-distance ski race in Sweden. J Intern Med. 2003;253(3):276–83.
31. Hallmarker U, Michaelsson K, Arnlov J, Hellberg D, Lagerqvist B, Lindback J, et al. Risk of recurrent ischaemic events after myocardial infarction in long-distance ski race participants. Eur J Prev Cardiol. 2016;23(3):282–90.
32. Berglund G, Elmstahl S, Janzon L, Larsson SA. The Malmo Diet and Cancer study. Design and feasibility. J Intern Med. 1993;233(1):45–51.
33. Taylor HL, Jacobs DR Jr, Schucker B, Knudsen J, Leon AS, Debacker G. A questionnaire for the assessment of leisure time physical activities. J Chronic Dis. 1978;31(12):741–55.
34. American Psychiatric Association. Diagnostic and statistical manual of mental disorders. 5th ed. Washington, DC: American Psychiatric Publishing; 2013.
35. Qiu C, Xu W, Fratiglioni L. Vascular and psychosocial factors in Alzheimer's disease: epidemiological evidence toward intervention. J Alzheimers Dis. 2010;20(3):689–97.
36. Manjer J, Carlsson S, Elmstahl S, Gullberg B, Janzon L, Lindstrom M, et al. The Malmo Diet and Cancer Study: representativity, cancer incidence and mortality in participants and non-participants. Eur J Cancer Prev. 2001;10(6):489–99.
37. Webster SJ, Bachstetter AD, Nelson PT, Schmitt FA, Van Eldik LJ. Using mice to model Alzheimer's dementia: an overview of the clinical disease and the preclinical behavioral changes in 10 mouse models. Front Genet. 2014;5:88.
38. Girard SD, Jacquet M, Baranger K, Migliorati M, Escoffier G, Bernard A, et al. Onset of hippocampus-dependent memory impairments in 5XFAD transgenic mouse model of Alzheimer's disease. Hippocampus. 2014;24(7): 762–72.
39. Oakley H, Cole SL, Logan S, Maus E, Shao P, Craft J, et al. Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. J Neurosci. 2006;26(40):10129–40.
40. George S, Petit GH, Gouras GK, Brundin P, Olsson R. Nonsteroidal selective androgen receptor modulators and selective estrogen receptor beta agonists moderate cognitive deficits and amyloid-beta levels in a mouse model of Alzheimer's disease. ACS Chem Neurosci. 2013;4(12):1537–48.
41. Svensson M, Rosvall P, Boza-Serrano A, Andersson E, Lexell J, Deierborg T. Forced treadmill exercise can induce stress and increase neuronal damage in a mouse model of global cerebral ischemia. Neurobiol Stress. 2016;5:8–18.
42. Vieira-Brock PL, McFadden LM, Nielsen SM, Smith MD, Hanson GR, Fleckenstein AE. Nicotine administration attenuates methamphetamine-induced novel object recognition deficits. Int J Neuropsychopharmacol. 2015;18(12):1-12.
43. Rijal Upadhaya A, Capetillo-Zarate E, Kosterin I, Abramowski D, Kumar S, Yamaguchi H, et al. Dispersible amyloid beta-protein oligomers, protofibrils, and fibrils represent diffusible but not soluble aggregates: their role in neurodegeneration in amyloid precursor protein (APP) transgenic mice. Neurobiol Aging. 2012;33(11):2641–60.
44. Boza-Serrano A, Yang Y, Paulus A, Deierborg T. Innate immune alterations are elicited in microglial cells before plaque deposition in the Alzheimer's disease mouse model 5xFAD. Sci Rep. 2018;8(1):1550.
45. Gorelick PB, Scuteri A, Black SE, Decarli C, Greenberg SM, Iadecola C, et al. Vascular contributions to cognitive impairment and dementia: a statement for healthcare professionals from the American Heart Association/American Stroke Association. Stroke. 2011;42(9):2672–713.
46. Guure CB, Ibrahim NA, Adam MB, Said SM. Impact of physical activity on cognitive decline, dementia, and its subtypes: meta-analysis of prospective studies. Biomed Res Int 2017;2017:9016924.
47. Ravaglia G, Forti P, Lucicesare A, Pisacane N, Rietti E, Bianchin M, et al. Physical activity and dementia risk in the elderly: findings from a prospective Italian study. Neurology. 2008;70(19 Pt 2):1786–94.
48. Rabinovici GD, Carrillo MC, Forman M, DeSanti S, Miller DS, Kozauer N, et al. Multiple comorbid neuropathologies in the setting of Alzheimer's disease neuropathology and implications for drug development. Alzheimers Dement (N Y). 2017;3(1):83–91.
49. Steen Jensen C, Portelius E, Siersma V, Hogh P, Wermuth L, Blennow K, et al. Cerebrospinal fluid amyloid beta and tau concentrations are not modulated by 16 weeks of moderate- to high-intensity physical exercise in patients with Alzheimer disease. Dement Geriatr Cogn Disord. 2016;42(3–4):146–58.
50. Vemuri P, Knopman DS, Lesnick TG, Przybelski SA, Mielke MM, Graff-Radford J, et al. Evaluation of amyloid protective factors and Alzheimer disease neurodegeneration protective factors in elderly individuals. JAMA Neurol. 2017;74(6):718–26.
51. Najar J, Ostling S, Gudmundsson P, Sundh V, Johansson L, Kern S, et al. Cognitive and physical activity and dementia: a 44-year longitudinal population study of women. Neurology. 2019;92:e1322–30.
52. Solomon A, Turunen H, Ngandu T, Peltonen M, Levalahti E, Helisalmi S, et al. Effect of the apolipoprotein E genotype on cognitive change during a multidomain lifestyle intervention: a subgroup analysis of a randomized clinical trial. JAMA Neurol. 2018;75(4):462–70.
53. Jellinger KA. Clinicopathological analysis of dementia disorders in the elderly--an update. J Alzheimers Dis. 2006;9(3 Suppl):61–70.

Publisher's Note

Figure S1.



Paper VI

SCIENTIFIC REPORTS
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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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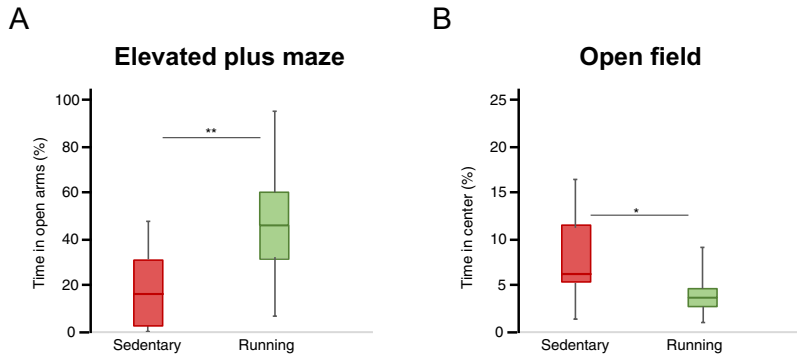

Figure 1. Exploratory behavior in Elevated Plus Maze and Open Field tests. Exploratory behavior is presented as the percentage of time spent in the open arms of the Elevated Plus Maze (**A**) or in the center zone in the Open Field (**B**) tests conducted during weeks 20–22. Box plots represent the median 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.

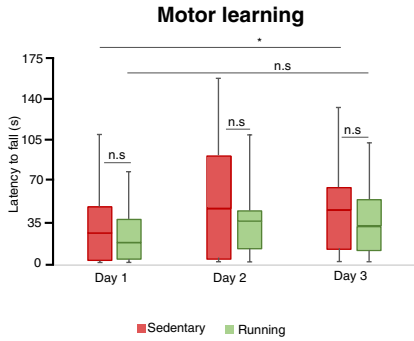

Figure 2. Motor learning in Rotarod test. The latency to fall off the rotarod at different days of training. Box plots represent the median values for each group with interquartile ranges and error bars indicating the minimum and maximum. *Rrepresents $p < 0.05$ (Wilcoxon test). For sedentary mice $n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

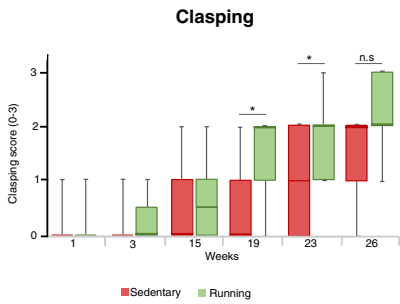

Figure 3. Hindlimb clasping at different time points. The hindlimb clasping scores at different time points. Box plots represent the median values for each group with interquartile ranges and error bars indicating the minimum and maximum. *Represents $p < 0.05$ (Mann Whitney U-test used for each given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

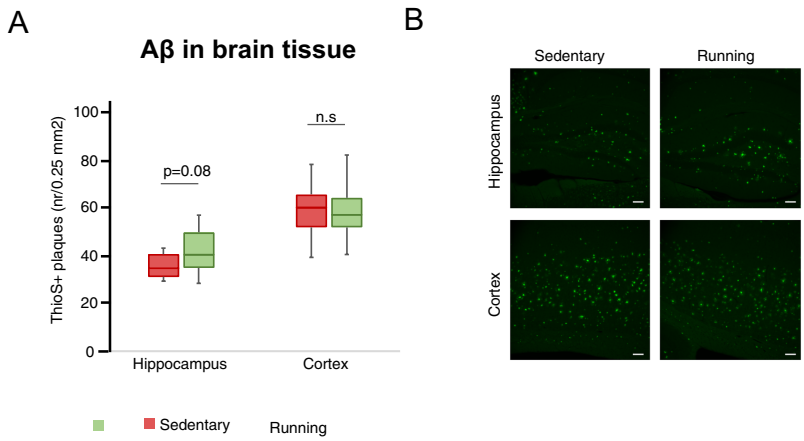

Figure 4. Aβ plaques in hippocampus and cortex. Thioflavin-S-positive Aβ plaques in hippocampus and cortex. Box plots represent the median values for each group with interquartile ranges and error bars indicating the minimum and maximum (**A**). Representative images at 10x, with scale bar representing 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

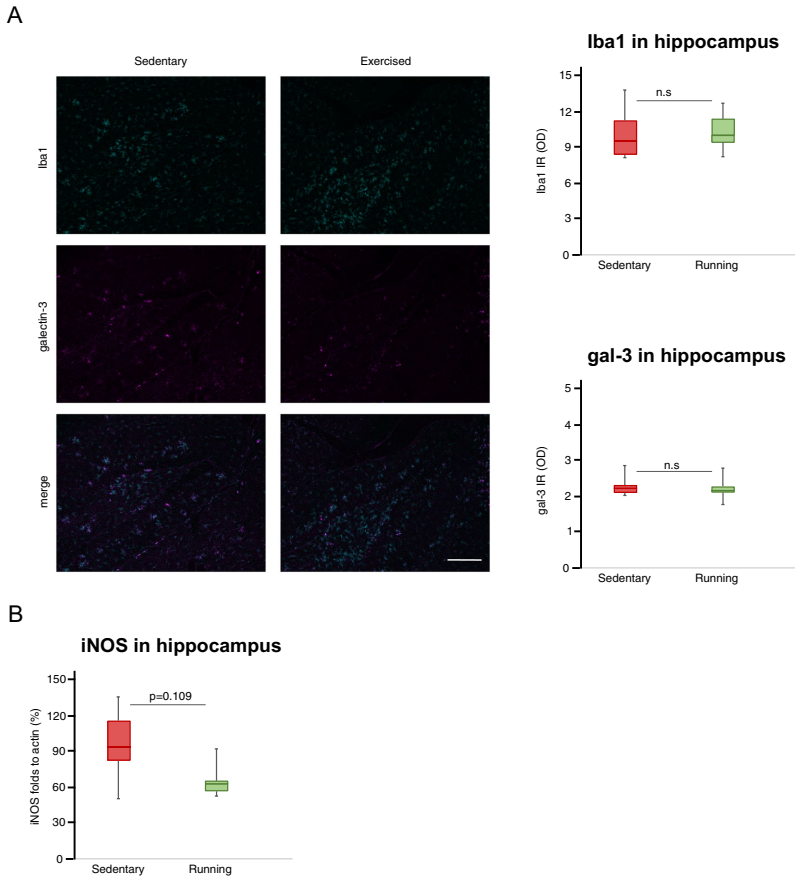

Figure 5. Neuroinflammation in hippocampus. Representative images of the Iba1 (labeling all microglia) and gal-3 (labeling activated microglia) staining in hippocampus at 10x with scale bar representing 200 μm (**A**) and box plots representing the median values of Iba1 and gal-3 intensities (n = 10 + 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.

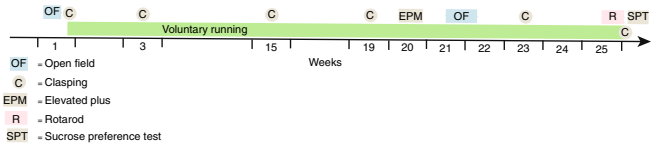

Figure 6. Experimental design. Mice had access to running wheels during experimental weeks 2–25. Before the introduction of exercise intervention, during week 1, Open Field test (OF) and Clasping test (C) were conducted. Clasping tests were then repeated in weeks 3, 15, 19, 23 and 26. During week 20 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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References

1. Tokuchi, R. *et al*. Differences between the behavioral and psychological symptoms of Alzheimer's disease and Parkinson's disease. *J Neurol Sci* **369**, 278–282, https://doi.org/10.1016/j.jns.2016.08.053 (2016).
2. Assal, F. & Cummings, J. L. Neuropsychiatric symptoms in the dementias. *Curr Opin Neurol* **15**, 445–450 (2002).
3. Calsolaro, V. & Edison, P. Neuroinflammation in Alzheimer's disease: Current evidence and future directions. *Alzheimers Dement* **12**, 719–732, https://doi.org/10.1016/j.jalz.2016.02.010 (2016).
4. Griffin, W. S. *et al*. Brain interleukin 1 and S-100 immunoreactivity are elevated in Down syndrome and Alzheimer disease. *Proc Natl Acad Sci USA* **86**, 7611–7615 (1989).
5. Hull, M., Berger, M., Volk, B. & Bauer, J. Occurrence of interleukin-6 in cortical plaques of Alzheimer's disease patients may precede transformation of diffuse into neuritic plaques. *Ann NY Acad Sci* **777**, 205–212 (1996).
6. Edison, P. *et al*. Microglia, amyloid, and cognition in Alzheimer's disease: An [11C](R)PK11195-PET and [11C]PIB-PET study. *Neurobiol Dis* **32**, 412–419, https://doi.org/10.1016/j.nbd.2008.08.001 (2008).
7. Fan, Z., Okello, A. A., Brooks, D. J. & Edison, P. Longitudinal influence of microglial activation and amyloid on neuronal function in Alzheimer's disease. *Brain* **138**, 3685–3698, https://doi.org/10.1093/brain/awv288 (2015).
8. Fan, Z. *et al*. Influence of microglial activation on neuronal function in Alzheimer's and Parkinson's disease dementia. *Alzheimers Dement* **11**, 608–621 e607, https://doi.org/10.1016/j.jalz.2014.06.016 (2015).

9. Novikova, G. *et al*. Integration of Alzheimer's disease genetics and myeloid genomics reveals novel disease risk mechanisms. *BioRxiv*, https://doi.org/10.1101/694281 (2019).
10. Terwel, D. *et al*. Critical role of astroglial apolipoprotein E and liver X receptor-alpha expression for microglial Abeta phagocytosis. *J Neurosci* **31**, 7049–7059, https://doi.org/10.1523/JNEUROSCI.6546-10.2011 (2011).
11. Chen, C. H. *et al*. Increased NF-kappaB signalling up-regulates BACE1 expression and its therapeutic potential in Alzheimer's disease. *Int J Neuropsychopharmacol* **15**, 77–90, https://doi.org/10.1017/S1461145711000149 (2012).
12. Rovio, S. *et al*. Leisure-time physical activity at midlife and the risk of dementia and Alzheimer's disease. *Lancet Neurol* **4**, 705–711, https://doi.org/10.1016/S1474-4422(05)70198-8 (2005).
13. Hamer, M. & Chida, Y. Physical activity and risk of neurodegenerative disease: a systematic review of prospective evidence. *Psychol Med* **39**, 3–11, https://doi.org/10.1017/S0033291708003681 (2009).
14. Ryan, S. M. & Kelly, A. M. Exercise as a pro-cognitive, pro-neurogenic and anti-inflammatory intervention in transgenic mouse models of Alzheimer's disease. *Ageing Res Rev* **27**, 77–92, https://doi.org/10.1016/j.arr.2016.03.007 (2016).
15. Yaguez, L., Shaw, K. N., Morris, R. & Matthews, D. The effects on cognitive functions of a movement-based intervention in patients with Alzheimer's type dementia: a pilot study. *Int J Geriatr Psychiatry* **26**, 173–181, https://doi.org/10.1002/gps.2510 (2011).
16. Palleschi, L. *et al*. Effect of aerobic training on the cognitive performance of elderly patients with senile dementia of Alzheimer type. *Arch Gerontol Geriatr* **22**(Suppl. 1), 47–50, https://doi.org/10.1016/0167-4943(96)86912-3 (1996).
17. Liang, K. Y. *et al*. Exercise and Alzheimer's disease biomarkers in cognitively normal older adults. *Ann Neurol* **68**, 311–318, https://doi.org/10.1002/ana.22096 (2010).
18. Kobe, T. *et al*. Combined omega-3 fatty acids, aerobic exercise and cognitive stimulation prevents decline in gray matter volume of the frontal, parietal and cingulate cortex in patients with mild cognitive impairment. *Neuroimage* **131**, 226–238, https://doi.org/10.1016/j.neuroimage.2015.09.050 S1053-8119(15)00872-1 [pii] (2016).
19. Ahlskog, J. E., Geda, Y. E., Graff-Radford, N. R. & Petersen, R. C. Physical exercise as a preventive or disease-modifying treatment of dementia and brain aging. *Mayo. Clin. Proc.* **86**, 876–884, https://doi.org/10.4065/mcp.2011.0252 (2011).
20. Yamada, M. *et al*. Association between dementia and midlife risk factors: the Radiation Effects Research Foundation Adult Health Study. *J Am Geriatr Soc* **51**, 410–414 (2003).
21. Sabia, S. *et al*. Physical activity, cognitive decline, and risk of dementia: 28 year follow-up of Whitehall II cohort study. *BMJ* **357**, j2709, https://doi.org/10.1136/bmj.j2709 (2017).
22. Najar, J. *et al*. Cognitive and physical activity and dementia: A 44-year longitudinal population study of women. *Neurology* **92**, e1322–e1330, https://doi.org/10.1212/WNL.0000000000007021 (2019).
23. Ravaglia, G. *et al*. Physical activity and dementia risk in the elderly: findings from a prospective Italian study. *Neurology* **70**, 1786–1794, https://doi.org/10.1212/01.wnl.0000296276.50595.86 (2008).
24. Hansson, O. *et al*. Midlife physical activity is associated with lower incidence of vascular dementia but not Alzheimer's disease. *Alzheimers Res Ther* **11**, 87, https://doi.org/10.1186/s13195-019-0538-4 (2019).
25. Webster, S. J., Bachstetter, A. D., Nelson, P. T., Schmitt, F. A. & Van Eldik, L. J. Using mice to model Alzheimer's dementia: an overview of the clinical disease and the preclinical behavioral changes in 10 mouse models. *Front Genet* **5**, 88, https://doi.org/10.3389/fgene.2014.00088 (2014).
26. Landel, V. *et al*. Temporal gene profiling of the 5XFAD transgenic mouse model highlights the importance of microglial activation in Alzheimer's disease. *Mol Neurodegener* **9**, 33, https://doi.org/10.1186/1750-1326-9-33 (2014).
27. Aytan, N. *et al*. Fingolimod modulates multiple neuroinflammatory markers in a mouse model of Alzheimer's disease. *Sci Rep* **6**, 24939, https://doi.org/10.1038/srep24939 (2016).
28. Boza-Serrano, A., Yang, Y., Paulus, A. & Deierborg, T. Innate immune alterations are elicited in microglial cells before plaque deposition in the Alzheimer's disease mouse model 5xFAD. *Sci Rep* **8**, 1550, https://doi.org/10.1038/s41598-018-19699-y (2018).
29. Moore, K. M. *et al*. A spectrum of exercise training reduces soluble Abeta in a dose-dependent manner in a mouse model of Alzheimer's disease. *Neurobiol Dis* **85**, 218–224, https://doi.org/10.1016/j.nbd.2015.11.004 (2016).
30. Xu, Z. Q. *et al*. Aerobic exercise combined with antioxidative treatment does not counteract moderate- or mid-stage Alzheimer-like pathophysiology of APP/PS1 mice. *CNS Neurosci Ther* **19**, 795–803, https://doi.org/10.1111/cns.12139 (2013).
31. Xiong, J. Y. *et al*. Long-term treadmill exercise improves spatial memory of male APPswe/PS1dE9 mice by regulation of BDNF expression and microglia activation. *Biol Sport* **32**, 295–300, https://doi.org/10.5604/20831862.1163692 (2015).
32. Nichol, K. E. *et al*. Exercise alters the immune profile in Tg2576 Alzheimer mice toward a response coincident with improved cognitive performance and decreased amyloid. *J Neuroinflammation* **5**, 13, https://doi.org/10.1186/1742-2094-5-13 (2008).
33. Boza-Serrano, A. *et al*. Galectin-3, a novel endogenous TREM2 ligand, detrimentally regulates inflammatory response in Alzheimer's disease. *Acta Neuropathol* **138**, 251–273, https://doi.org/10.1007/s00401-019-02013-z (2019).
34. Svensson, M. *et al*. Forced treadmill exercise can induce stress and increase neuronal damage in a mouse model of global cerebral ischemia. *Neurobiol Stress* **5**, 8–18, https://doi.org/10.1016/j.ynstr.2016.09.002 (2016).
35. Jawhar, S., Trawicka, A., Jenneckens, C., Bayer, T. A. & Wirths, O. Motor deficits, neuron loss, and reduced anxiety coinciding with axonal degeneration and intraneuronal Abeta aggregation in the 5XFAD mouse model of Alzheimer's disease. *Neurobiol Aging* **33**, 196 e129–e140, https://doi.org/10.1016/j.neurobiolaging.2010.05.027 (2012).
36. Schneider, F., Baldauf, K., Wetzel, W. & Reymann, K. G. Behavioral and EEG changes in male 5xFAD mice. *Physiol Behav* **135**, 25–33, https://doi.org/10.1016/j.physbeh.2014.05.041 (2014).
37. Peters, O. M. *et al*. Chronic administration of Dimebon does not ameliorate amyloid-beta pathology in 5xFAD transgenic mice. *J Alzheimers Dis* **36**, 589–596, https://doi.org/10.3233/JAD-130071 (2013).
38. O'Leary, T. P., Robertson, A., Chipman, P. H., Rafuse, V. F. & Brown, R. E. Motor function deficits in the 12 month-old female 5xFAD mouse model of Alzheimer's disease. *Behav Brain Res* **337**, 256–263, https://doi.org/10.1016/j.bbr.2017.09.009 (2018).
39. Intlekofer, K. A. & Cotman, C. W. Exercise counteracts declining hippocampal function in aging and Alzheimer's disease. *Neurobiol Dis* **57**, 47–55, https://doi.org/10.1016/j.nbd.2012.06.011 (2013).
40. Robison, L. S. *et al*. Long-term voluntary wheel running does not alter vascular amyloid burden but reduces neuroinflammation in the Tg-SwDI mouse model of cerebral amyloid angiopathy. *J Neuroinflammation* **16**, 144, https://doi.org/10.1186/s12974-019-1534-0 (2019).
41. Garcia-Mesa, Y. *et al*. Physical exercise protects against Alzheimer's disease in 3xTg-AD mice. *J Alzheimers Dis* **24**, 421–454, https://doi.org/10.3233/JAD-2011-101635 (2011).
42. Parachikova, A., Nichol, K. E. & Cotman, C. W. Short-term exercise in aged Tg2576 mice alters neuroinflammation and improves cognition. *Neurobiol Dis* **30**, 121–129, https://doi.org/10.1016/j.nbd.2007.12.008 (2008).
43. Steen Jensen, C. *et al*. Cerebrospinal Fluid Amyloid Beta and Tau Concentrations Are Not Modulated by 16 Weeks of Moderate- to High-Intensity Physical Exercise in Patients with Alzheimer Disease. *Dement Geriatr Cogn Disord* **42**, 146–158, https://doi.org/10.1159/000449408 (2016).
44. Paouri, E., Tzara, O., Zenelak, S. & Georgopoulos, S. Genetic Deletion of Tumor Necrosis Factor-alpha Attenuates Amyloid-beta Production and Decreases Amyloid Plaque Formation and Glial Response in the 5XFAD Model of Alzheimer's Disease. *J Alzheimers Dis* **60**, 165–181, https://doi.org/10.3233/JAD-170065 (2017).
45. Souza, L. C. *et al*. Neuroprotective Effect of Physical Exercise in a Mouse Model of Alzheimer's Disease Induced by beta-Amyloid(1-40) Peptide. *Neurotox Res*, https://doi.org/10.1007/s12640-012-9373-0 (2013).

46. Belarbi, K. *et al*. Beneficial effects of exercise in a transgenic mouse model of Alzheimer's disease-like Tau pathology. *Neurobiol Dis* **43**, 486–494, https://doi.org/10.1016/j.nbd.2011.04.022 (2011).
47. Hatchard, T., Ting, J. J. & Messier, C. Translating the impact of exercise on cognition: methodological issues in animal research. *Behav Brain Res* **273**, 177–188, https://doi.org/10.1016/j.bbr.2014.06.043 (2014).
48. Choi, S. H. *et al*. Combined adult neurogenesis and BDNF mimic exercise effects on cognition in an Alzheimer's mouse model. *Science* **361**, https://doi.org/10.1126/science.aan8821 (2018).
49. Berry, A. *et al*. Social deprivation stress is a triggering factor for the emergence of anxiety- and depression-like behaviours and leads to reduced brain BDNF levels in C57BL/6J mice. *Psychoneuroendocrinology* **37**, 762–772, https://doi.org/10.1016/j.psyneuen.2011.09.007 (2012).
50. Barber, R. C. The genetics of Alzheimer's disease. *Scientifica (Cairo)* **2012**, 246210, https://doi.org/10.6064/2012/246210 (2012).
51. 2012 Alzheimer's disease facts and figures. *Alzheimers Dement* **8**, 131–168, https://doi.org/10.1016/j.jalz.2012.02.001 (2012).
52. Guyenet, S. J. *et al*. A simple composite phenotype scoring system for evaluating mouse models of cerebellar ataxia. *J Vis Exp*, 10.3791/1787 (2010).
53. Lister, R. G. The use of a plus-maze to measure anxiety in the mouse. *Psychopharmacology (Berl)* **92**, 180–185, https://doi.org/10.1007/bf00177912 (1987).
54. Walf, A. A. & Frye, C. A. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat Protoc* **2**, 322–328, https://doi.org/10.1038/nprot.2007.44 (2007).

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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

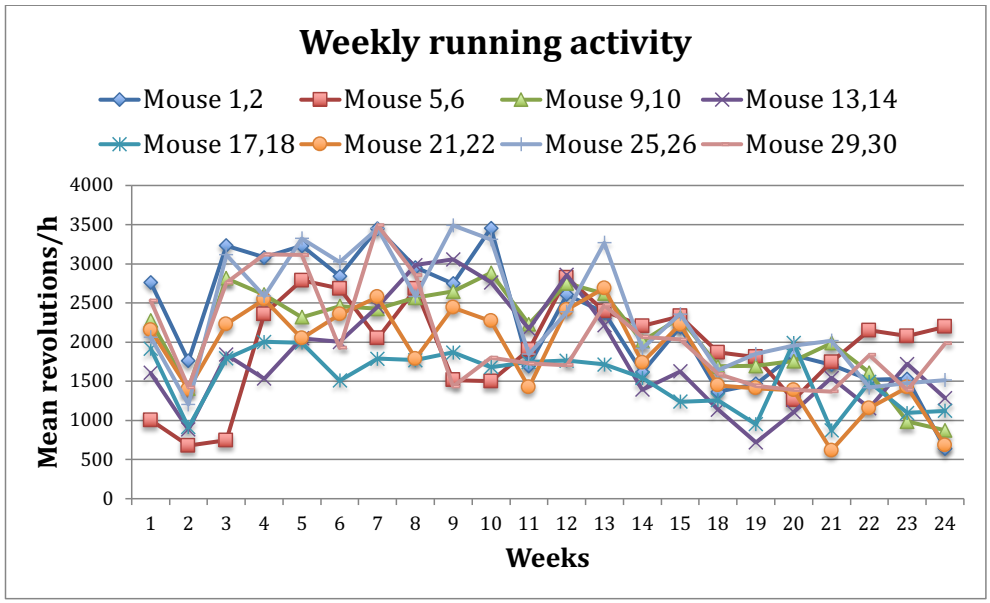

Figure S1. Running activity displayed as mean revolution per hour for each week and each couple sharing a running wheel in their home cage. Each revolution corresponds to a running distance of around 42 cm. ANOVA repeated measurements showed a change in the amount of running over time ($p<0.001$) and 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].

References

1 Bay-Richter, C. *et al.* Behavioural and neurobiological consequences of macrophage migration inhibitory factor gene deletion in mice. *J Neuroinflammation* **12**, 163, doi:10.1186/s12974-015-0387-4 (2015).

2 Touma, C., Sachser, N., Mostl, E. & Palme, R. Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. *Gen Comp Endocrinol* **130**, 267-278, doi:S0016648002006202 [pii] (2003).

3 Svensson, M. *et al.* Forced treadmill exercise can induce stress and increase neuronal damage in a mouse model of global cerebral ischemia. *Neurobiol Stress* **5**, 8-18, doi:10.1016/j.ynstr.2016.09.002 (2016).

Physical exercise as a preventive strategy for disorders affecting the brain

The findings in this thesis support the view that physical activity is a promising preventive strategy to reduce the burden of some of the most common disorders affecting the brain. By taking a broad perspective and conducting experimental animal research as well as epidemiological human studies, I investigate the effects of physical activity on disorders affecting the brain, such as brain ischemia, depression, dementia, and Parkinson's disease. The overall goal of this work is to encourage a more translational view of this research area.

Martina Svensson

Born and raised in the Scanian countryside (V. Sönnarslöv/Kvidinge), Martina Svensson has a background in biomedicine from Lund University. She is a neuroscience researcher focusing on the effects of exercise and has a passion for science communication.

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