PUBLICATIONS OF
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EASTERN FINLAND

Dissertations in Health Sciences

IRINA BELAYA

Exercise-induced cellular and molecular alterations in aging and Alzheimer´s disease

EXERCISE-INDUCED CELLULAR AND MOLECULAR ALTERATIONS IN AGING AND ALZHEIMER'S DISEASE

Irina Belaya

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To be presented by permission of the Faculty of Health Sciences,
University of Eastern Finland for public examination in SN201 Auditorium,
Kuopio
on November 12th, 2021, at 12 o'clock noon

Publications of the University of Eastern Finland
Dissertations in Health Sciences
No 644

A.I. Virtanen Institute for Molecular Sciences
University of Eastern Finland, Kuopio
2021

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Distributor:
University of Eastern Finland
Kuopio Campus Library
P.O.Box 1627
FI-70211 Kuopio, Finland
www.uef.fi/kirjasto

PunaMusta Oy, 2021

ISBN: 978-952-61-4311-8 (print/nid.)
ISBN: 978-952-61-4312-5 (PDF)
ISSNL: 1798-5706
ISSN: 1798-5706
ISSN: 1798-5714 (PDF)

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Belaya, Irina
Exercise-induced cellular and molecular alterations in aging and Alzheimer's disease
Kuopio: University of Eastern Finland
Publications of the University of Eastern Finland
Dissertations in Health Sciences 644. 2021, 121 p.
ISBN: 978-952-61-4311-8 (print)
ISSNL: 1798-5706
ISSN: 1798-5706
ISBN: 978-952-61-4312-5 (PDF)
ISSN: 1798-5714 (PDF)

ABSTRACT

The population is living longer than ever before, which in turn means that age-related diseases are now a major public health problem. Healthy aging is important not only for decreasing the risk of disease development, but also for the maintenance of the functional ability of people throughout their whole lifespan. Alzheimer's disease (AD) is the leading cause of dementia, which affects more than 50 million people worldwide. Even though aging is one major AD risk factor, there are also environmental and lifestyle factors which exert a huge impact on the development of AD. While research into AD has contributed to our understanding of the pathological features of this devastating disease, still no cure exists. This is most likely due to our still limited understanding of the disease mechanisms. One of the most important goals to prevent age-related disease is to clarify the molecular mechanisms associated with aging and AD, and deciphering how lifestyle factors such as physical exercise affect these processes and promote healthy aging.

Therefore, this thesis explored the impact of long-term voluntary exercise in a mouse model of aging and AD. In Study I, we evaluated the effect long-term voluntary exercise on redox regulation and cellular stress upon aging in mice. We found that long-term voluntary exercise was protective against age-related oxidative and endoplasmic reticulum (ER) stress in mice. In Study II, we assessed the effect of long-term voluntary exercise on cognitive function and astrocyte state in WT and the 5xFAD mouse model of AD. We found that long-term voluntary exercise was beneficial against the cognitive impairment developing in the 5xFAD

mice and was associated with astrocyte remodeling as well as a restoration of astrocytic brain derived neurotrophic factor (BDNF). In Study III, we examined the effect of long-term voluntary exercise on iron metabolism in WT and 5xFAD mice and evaluated the potential role of iron in the interplay between brain and peripheral tissues. We demonstrated that long-term voluntary exercise modulated iron homeostasis in WT and 5xFAD mice by decreasing levels of the iron regulator protein hepcidin in the brain possibly via an attenuation of the IL-6/STAT3 pathway.

In summary, our studies collectively highlight the positive impact of long-term physical exercise on aging and AD. Our results reveal new aspects of the beneficial effect of regular exercise in both the brain and skeletal muscle; for example, the potential mechanism behind the interplay between brain and periphery upon aging and AD. Regular physical exercise could be a promising preventive strategy against AD and should be further investigated as a way of promoting healthy aging.

Keywords: National Library of Medicine Classification: QT 120, QT 162, QU 55, QY 50, WL101, WT 104, WT 155, WT 158
Medical Subject Headings: Alzheimer Disease; Astrocytes; Brain; Brain-Derived Neurotrophic Factor; Cognitive Dysfunction; Endoplasmic Reticulum; Healthy Aging; Hepcidin; Homeostasis; Interleukin-6; Iron-Regulatory Proteins; Mice; Skeletal Muscle; Oxidative Stress

Belaya, Irina
Liikunnan aiheuttamat solu- ja molekyylimuutokset ikääntymisessä ja Alzheimerin taudissa
Kuopio: Itä-Suomen yliopisto
Publications of the University of Eastern Finland
Dissertations in Health Sciences 644. 2021, 121 s.
ISBN: 978-952-61-4311-8 (nid.)
ISSNL: 1798-5706
ISSN: 1798-5706
ISBN: 978-952-61-4312-5 (PDF)
ISSN: 1798-5714 (PDF)

TIIVISTELMÄ

Väestö elää pidempään kuin koskaan ennen. Siten ikään liittyvät sairaudet ovat nyt merkittävä kansanterveysongelma. Terve ikääntyminen on tärkeää sairauksien kehittymisriskin vähentämiseksi ja ihmisten toimintakyvyn ylläpitämiseksi koko eliniän ajan. Alzheimerin tauti on johtava syy dementialle, joka vaikuttaa yli 50 miljoonaan ihmiseen maailmanlaajuisesti. Vaikka ikääntyminen on yksi merkittävistä taudin riskitekijöistä, on myös olemassa ympäristötekijöitä ja elintapoihin liittyviä tekijöitä, joilla on valtava vaikutus taudin kehittymiseen. Siitä huolimatta, että Alzheimerin taudin tutkimus on auttanut meitä ymmärtämään tämän tuhoisan sairauden patologisia piirteitä, parannuskeinoa ei ole löytynyt. Tämä johtuu todennäköisesti siitä, että tietomme taudin solu- ja molekyylitason mekanismeista on edelleen rajallinen. Yksi tärkeimmistä tavoitteista ikääntymiseen liittyvien sairauksien ehkäisyssä onkin selvittää näitä mekanismeja ja tutkia sitä, miten elintapoihin liittyvät tekijät, kuten liikunta, vaikuttavat näihin prosesseihin ja edistävät tervettä ikääntymistä.

Tässä väitöskirjassa tutkittiin pitkäkestoisen vapaaehtoisen liikunnan vaikutusta Alzheimerin tautia ja ikääntymistä mallintavissa hiirissä. Tutkimuksessa I arvioimme pitkäaikaisen liikunnan vaikutusta lihaskudoksen redoksisäätelyyn ja solustressiin ikääntymisen aikana. Havaitsimme, että pitkäaikainen vapaaehtoinen liikunta suojasi ikääntymiseen liittyvältä oksidatiiviselta ja endoplasmisen retikulumin (ER) stressiltä. Tutkimuksessa II määritimme pitkäaikaisen vapaaehtoisen liikunnan vaikutusta kognitiiviseen toimintaan ja aivojen tukisoluihin, astrosyytteihin, 5xFAD-siirtogeenisillä hiirillä. Havaitsimme, että

pitkäaikainen vapaaehtoinen liikunta vähensi 5xFAD-hiirille kehittyvää kognitiivista heikentymistä ja siihen liittyi muutokset astrosyyteissä. Tutkimuksessa III tutkimme pitkäaikaisen vapaaehtoisen liikunnan vaikutusta raudan aineenvaihduntaan 5xFAD-siirtogeenisissä hiirissä ja arvioimme raudan mahdollista osallistumista aivojen ja perifeeristen kudosten väliseen viestintään. Osoitimme, että pitkäaikainen vapaaehtoinen liikunta sääteli raudan homeostaasia mm. vaikuttamalla raudan säätelyproteiinin hepsidiinin pitoisuuksiin aivoissa IL-6/STAT3-reitin solusignalointireitin kautta.

Yhteenvetona voidaan todeta, että tutkimuksemme tulokset korostavat pitkäaikaisen liikunnan hyödyllistä vaikutusta ikääntymiseen ja Alzheimerin tautiin ja osoittavat vaikutusten kohdistuvan sekä aivoihin että lihaskudoksiin. Säännöllisellä liikunnalla voidaan aikaansaada hyötyä ennaltaehkäisevänä strategiana Alzheimerin tautia vastaan, ja sitä olisi tutkittava edelleen keinona edistää tervettä ikääntymistä.

Avainsanat: Luokitus: QT 120, QT 162, QU 55, QY 50, WL101, WT 104, WT 155, WT 158
Yleinen suomalainen ontologia: Alzheimerin tauti; aivot; astrosyytit; eläinkokeet; hiiret; homeostaasi; ikääntyneet; interleukiinit; kognitiiviset prosessit; lihakset; liikunta; muistisairaudet; oksidatiivinen stressi; vanheneminen

Mens sana in corpore sano
A healthy mind in a healthy body

Juvenal (Satire, 10.356)

ACKNOWLEDGEMENTS

The study was carried out in the Neurobiology of Disease Research group, A.I. Virtanen Institute for Molecular Sciences and in the Institute of Biomedicine, Faculty of Health Sciences at the University of Eastern Finland. The study was supported by CIMO (now EDUFI) fellowship, the Academy of Finland, the Sigrid Juselius Foundation, the North Savo regional Fund of the Finnish Cultural Foundation and the University of Eastern Finland Doctoral Programme in Molecular Medicine.

I would like to warmly thank my principal supervisor, Associate Professor Katja M. Kanninen, Ph.D., for support and guidance throughout the studies. I am very grateful to you for giving me the opportunity to work and conduct my PhD work in your group. Your kindness, enthusiasm and optimism inspired me during my research. Thank you very much for your input, trust and expertise which helped me finalize my study.

I wish to thank my co-supervisor, Docent Mustafa Atalay, Ph.D., M.D., for your invaluable contribution in my PhD career, for sharing your knowledge, for your constant help and support during all my studies. I am very grateful to you for giving me the opportunity to embark on my PhD journey in the University of Eastern Finland.

My sincere gratitude to my co-supervisors, Professor Tarja Malm, Ph.D., for your valuable support and help, and to Professor Rashid Giniatullin, Ph.D., M.D. for your support at the beginning of my PhD study and for your infinite knowledge and ideas which were inspiring.

I wish to express my admiration to Professor Heikki Tanila, Ph.D. Your deep knowledge, expertise and critical comments were extremely helpful and valuable for my studies.

I wish to thank the official reviewers of my thesis, Adjunct Professor Ina M. Tarkka, Ph.D., and Associate Professor Riikka Kivelä, Ph.D. for their valuable feedback. I wish to thank Professor Alexei Verkhratsky, Ph.D., M.D. for agreeing to be my opponent in the public defence. I would like to thank Dr. Ewen MacDonald for revising the language of the thesis.

I would like to thank Docent Anitta Mahonen, Ph.D., the Head of the Institute of Biomedicine, for the support you provided when it was really needed.

I wish to thank all my co-authors who contributed to the studies included in this thesis: Masataka Suwa, Tao Chen, Shuzo Kumagai, Mariia Ivanova, Annika Sorvari,

Marina Ilicic, Sanna Loppi, Henna Koivisto, Alessandra Varricchio, Heikki Tikkanen, Frederick R. Walker, Alexandra Grubman, Heikki Tanila, Nina Kucháriková, Veronika Górová, Kai Kysenius, Dominic J. Hare, Peter J. Crouch, Anthony R. White and Jeffrey R. Liddell.

Special thanks to Taina Vihavainen and Mirka Tikkanen, for great assistance and commitment and for your expertise which you have shared with me. I want to thank Henna Koivisto for introducing me in animal work life and sharing your expertise in behavioural science. Furthermore, I want to thank all of the former and present members of the Neurobiology of Disease Group, Neuroinflammation Research Group and Molecular Pain Research Group, as well as the other members of the A.I. Virtanen Institute and the Biocenter Kuopio. Thank you for your help and for creating a friendly atmosphere at work.

I want to thank my wonderful friends who have brought so much joy to my life in Kuopio. I would like to thank my friend Dilyara for the support, exciting talks and being the best listener. My warmest thanks to Masha, Lera, Olja, Lucy and Kostja from ”Russian corner”, Flavia from “Italian corner”, Ville and Eero from “Finnish corner”, for your friendship and a lot of fun outside of work. My warmest thanks to all my dear friends from Saint-Petersburg, who have always made my trips home unforgettable.

Finally, I wish to thank my parents and my family for their support and love. Especially, I want to thank my lovely Nikita for the endless patience, help and support during all these years. I cannot imagine how I would have gone through all of this without you. Some words in Russian: мамочка, папочка, Валерка и вся моя большая семья, спасибо за то, что вы есть, за вашу поддержку. Я вас очень люблю. Мой любимый Никиточка, твой вклад в эту работу неоценим! Спасибо тебе за всё, за то, что был всегда рядом и спешил на помощь. Я очень тебя люблю ♥

Thank you! Kiitos! Спасибо!

September 2021, Kuopio

Irina Belaya

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications:

I **Belaya I**, Suwa M, Chen T, Giniatullin R, Kanninen KM, Atalay M, Kumagai S. Long-term exercise protects against cellular stresses in aged mice. *Oxidative Medicine and Cellular Longevity*. 2018 Mar 25; 2018: 2894247. doi: 10.1155/2018/2894247.

II **Belaya I**, Ivanova M, Sorvari A, Ilicic M, Loppi S, Koivisto H, Varricchio A, Tikkanen H, Walker FR, Atalay M, Malm T, Grubman A, Tanila H, Kanninen KM. Astrocyte remodeling in the beneficial effects of long-term voluntary exercise in Alzheimer's disease. *Journal of Neuroinflammation.* 2020 Sep 15; 17: 271. doi: 10.1186/s12974-020-01935-w

III **Belaya I**, Kucháriková N, Górová V, Kysenius K, Hare DJ, Crouch PJ, Malm T, Atalay M, White AR, Liddell JR, Kanninen KM. Regular physical exercise modulates iron homeostasis in the 5xFAD mouse model of Alzheimer's disease. *International Journal of Molecular Sciences*. 2021 Aug 13; 22(16): 8715. doi: 10.3390/ijms22168715.

CONTENTS

ABBREVIATIONS

4-HNE	4-hydroxy-2-nonenal
AD	Alzheimer's disease
ALDH1L1	Aldehyde dehydrogenase 1 family, member L1
ANOVA	Analysis of variance
APOE	Apolipoprotein E
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
Aβ	Amyloid beta
BACE	Beta-secretase
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
CBA	Cytokine bead array
CHOP	CCAAT-enhancer-binding protein homologous protein
CNS	Central nervous system
CR	Complement receptor
CSF	Cerebrospinal fluid
DCX	Doublecortin
DG	Dentate gyrus
DMT1	Divalent metal transporter 1
EE	Environmental enrichment
ER	Endoplasmic reticulum
EXE	Exercised
ELISA	Enzyme-linked immunosorbent assay
FAD	Familial Alzheimer's disease
FNDC5	Fibronectin type III domains containing protein 5
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAS	Gastrocnemius
GFAP	Glial fibrillar acidic protein
GRP	Glucose-regulated protein
GS	Glutamine synthetase
GSH	Glutathione

GSSG	Glutathione disulfide
hAPP	Human amyloid precursor protein mutation
hAPP (Flo)	Human amyloid precursor protein Florida mutation
hAPP (Ind)	Human amyloid precursor protein Indiana mutation
hAPP (Lon)	Human amyloid precursor protein London mutation
hAPP (Swe)	Human amyloid precursor protein Swedish mutation
HO-1	Heme oxygenase 1
HSC	Heat shock cognate
HSP	Heat shock protein
Iba-1	Ionized calcium binding adaptor molecule 1
ICP-MS	Inductively coupled plasma mass spectrometry
IFN-γ	Interferon-gamma
IL	Interleukin
IL-6R	Interleukin 6 receptor
iPSC	Induced pluripotent stem cells
JAK1	Januse kinase 1
LTD	Long-term depression
LTP	Long-term potentiation
MCP-1	Monocyte chemoattractant protein-1
MOC	Mander's colocalization coefficient
MWM	Morris water maze
NeuN	Neuronal nuclear antigen
NFTs	Neurofibrillary tangles
NGS	Normal goat serum
NMDARs	N-methyl-D-aspartic acid receptors
NSCs	Neural stem cells
OE	Old exercised
OS	Old sedentary
PBS	Phosphate buffered saline

PBST	Phosphate buffered saline with Tween-20
PDI	Protein disulphide isomerase
PFA	Paraformaldehyde
r	Pearson's correlation coefficient
PSD-95	Postsynaptic density 95
PSEN	Presenilin
PTPe	Receptor-type tyrosine-protein phosphatase epsilon
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species
RT-PCR	Real-time polymerase chain reaction
S100β	S100 calcium binding protein beta
SAD	Sporadic Alzheimer's disease
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SED	Sedentary
SOD	Superoxide dismutase
STAT3	Signal transducer activator of transcription 3
TA	Tibialis anterior
TBST	Tris buffered saline with Tween-20
TfR	Transferrin receptor
TG	Genetically modified
TNF-α	Tumor necrosis factor alpha
TREM2	Triggering receptor expressed on myeloid cells 2
TrkB	Tyrosine receptor kinase B
TRX	Thioredoxin
TxNiP	Thioredoxin-interacting protein
UPR	Unfolded protein response
WB	Western blot
WT	Wild type
Y	Young

1 INTRODUCTION

Healthy aging is nowadays an important mission considering the ever-increasing lifespan of the populations. Aging is associated with both structural and anatomical changes in the body, leading to a loss of complexity and a reduction in the ability of the organism to adapt to physiological stress (Lipsitz and Goldberger, 1992). The loss of muscle mass and strength, an impaired vasculature, cognitive impairment – all of these signs are associated with aging and increase the risk of age-related diseases including Alzheimer's disease (AD). AD is a neurodegenerative disorder characterized by cognitive impairment and memory loss (Nelson, Braak and Markesbery, 2009). If one considers all European countries, Finland has the highest death rate caused by AD (Official Statistics of Finland (OSF), 2018). Different lifestyle factors including physical inactivity are known to be linked with the risk of developing several age-related diseases including AD (Ballard *et al.*, 2011), while regular physical exercise has beneficial effects on healthy aging (Silverman and Deuster, 2014). Physical exercise has huge impacts on skeletal muscle, the major organ responding to exercise, by enhancing the antioxidant system and reducing inflammation (Ubaida-Mohien *et al.*, 2019). In the brain, physical exercise exerts positive effects on neuronal plasticity, behaviour and memory, together with an attenuation of the age-related decline in synaptic plasticity and neurogenesis in hippocampus, the main brain area implicated in learning and memory (Van Praag *et al.*, 2005; Choi *et al.*, 2018). Because of the lack of efficacious medical treatments against age-related diseases including AD, more studies should be focused on disease prevention, detailed investigation of the molecular mechanisms involved, as well as on deciphering the impact of lifestyle changes in slowing down or preventing this devastating disease.

AD is a complex disorder manifesting with an accumulation of amyloid beta (Aβ) plaques accompanied by neuronal and synaptic loss, oxidative stress, inflammation and glial cell activation (Hou *et al.*, 2019). The exact role of glial cells in the pathogenesis of the disease remains unclear. Currently, reactive astrocytes are considered to be involved in a defensive mechanism by isolating a damaged brain area, participating in Aβ clearance and providing support to neurons (Verkhratsky *et al.*, 2019). It has been shown in different AD models and different exercise regimes, that physical activity is able to reduce the numbers of Aβ plaques and the degree of inflammation, increase the levels of neurotrophic factors such as brain-derived neurotrophic factor (BDNF), and modulate astrocyte

activation in contradictory ways (Rodríguez *et al.*, 2013; Tapia-Rojas *et al.*, 2016; Hüttenrauch *et al.*, 2017). There remains little doubt that the astrocyte state is affected by exercise, but astrocyte-specific responses in the beneficial effects of long-term exercise in the context of AD have remained understudied.

Aging and age-related diseases including AD are associated with a dysregulation of cellular processes including redox control of cellular signalling, increased oxidative and endoplasmic reticulum (ER) stress, and altered protein homeostasis (Deldicque, 2013). Moreover, the dysregulated metabolism of redox-active metals such as iron plays an important role in the dysfunction of cellular processes in aging and AD (Zecca *et al.*, 2004). Impaired iron homeostasis has been associated with increased reactive oxygen species (ROS) production and oxidative stress (Andrews, 2000; Ang *et al.*, 2010), which can lead to protein misfolding and plaque aggregation (Brown and Naidoo, 2012). In AD, excess iron in the brain 1) induces the expression of pro-inflammatory cytokines, 2) is involved in Aβ production and 3) plays a role in cognitive impairment (Schröder, Figueiredo and Martins De Lima, 2013; Ward *et al.*, 2014). It is known that regular exercise training results in beneficial adaptations in antioxidant defence and improves redox signalling to protect cells against stress-related diseases (Radak, Chung and Goto, 2005), but limited information is available on the effects of life-long physical exercise on oxidative and ER stress upon aging. Moreover, regular exercise may positively affect iron homeostasis, but the underlying mechanisms are still unclear, especially in the context of aging and AD (Ziolkowski *et al.*, 2014).

With respect to the exercise-induced effects on aging and AD, little is known about the crosstalk between brain and periphery, especially the involvement of peripheral mechanisms. Contracting skeletal muscles have been shown to secrete bioactive factors, myokines into the bloodstream; these compounds can cross the blood brain barrier and regulate brain function (Pedersen, 2019). Interleukin-6 (IL-6) has an important role in immune modulation; it is also considered as a myokine secreted from the skeletal muscle during exercise (Pedersen, 2019). Regular physical exercise reduces IL-6 levels in plasma and induces a systemic anti-inflammatory effect (Marsland *et al.*, 2015). Given that under inflammation conditions, IL-6 is involved in the activation of hepcidin, a key regulator of iron metabolism, it has been postulated that iron and IL-6 play important roles in mediating the brain-muscle interplay upon exercise (Crielaard, Lammers and Rivella, 2017).

This thesis aims to investigate the effects of long-term physical exercise in aging and AD in the 5xFAD mouse model. First, it addresses the role of long-term

voluntary exercise in redox regulation and cellular stresses upon aging (Study I). Next, the effect of long-term voluntary exercise is evaluated in the context of AD by examining cognitive functions and astrocyte modulation in the 5xFAD mouse model (Study II). Finally, the effect of long-term voluntary exercise on iron metabolism in the 5xFAD mouse model and the potential role of iron in the interplay between brain and periphery via IL-6 are evaluated (Study III).

2 REVIEW OF THE LITERATURE

2.1 AGING

2.1.1 Epidemiology

The length of the human lifespan is extending and the population aged > 65 years is constantly increasing and expected to triple in numbers by 2040 (Newman and Cauley, 2012). In Europe, the percentage of the population aged 65 years or over is around 19% and this number keeps rising (United Nations, Department of Economic and Social Affairs, 2019). The percentages of long-life individuals over the age of 80 years old are highest in Japan, Italy, Portugal and Greece, indicating that the environment and lifestyle factors in these countries favor healthy aging (Poulain, Herm and Pes, 2013). Taking into account the remarkable extension in life expectancy, the majority of the most common diseases are associated with aging. The demographic changes pose a huge challenge to the health care budgets; therefore, it is highly important to improve our knowledge on the effects of lifestyle and environment on healthy aging.

2.1.2 Age-related diseases

Aging is known to be associated with both structural and anatomical changes in the body, leading to a loss of complexity and a reduction in the ability of the organism to adapt to physiological stress (Lipsitz and Goldberger, 1992). Aging is accompanied with multiple changes occurring at different levels of biological systems: molecular, physiological, pathological and psychological (da Costa *et al.*, 2016). Mechanisms explaining aging are considered as a complex biological process including the accumulation of DNA damage, epigenetic alterations manifested as DNA methylation and histone modifications, telomere shortening, dysregulation of protein folding and degradation systems, increased production of reactive oxygen species (ROS), mitochondrial dysfunction and changes in the immune system (Partridge, Deelen and Slagboom, 2018).

It is important to differentiate the changes linked with normal aging from pathological changes leading to age-related diseases. Changes associated with normal aging influence the quality of life and these processes are inevitable. Sensory changes, including loss of hearing, vision disabilities and problems with vestibular function, normally appear with age and can often be alleviated with

suitable treatment (Azemin *et al.*, 2012; Jaul and Barron, 2017). Normal aging is also associated with immune system dysfunction, which reduces the defense capacity of the body to combat infections and viruses, with the highest mortality rate in patients over 60 years old (Bonanad *et al.*, 2020). In addition, the loss of skeletal muscle mass and strength leading to movement problems is rather prevalent in the elderly (Dodds *et al.*, 2017). Age-related cognitive and memory impairments including speech difficulties and short-term memory loss (Jaul and Barron, 2017) are also common in the elderly and do not necessarily lead to dementia (Toepper, 2017). Co-existing with commonly accepted age-related changes, there exist complex biological processes that may evoke pathological changes and age-related diseases.

Physiological and molecular changes associated with aging elevate the risk of developing cancer (De Magalhães, 2013). Aging is considered as a risk factor for cardiovascular disease and is also associated with age-related dysfunctions of the vascular system, leading to a high risk of stroke and myocardial infarction (Costantino, Paneni and Cosentino, 2016). Sarcopenia, loss of skeletal muscle mass and strength, occurs with aging and is tightly associated with impaired locomotion in the elderly (Walston, 2012). As skeletal muscle is responsible for the majority of the glucose utilization, sarcopenia itself increases the risk for metabolic diseases such as type 2 diabetes, which is characterized by disturbances in glucose tolerance and impairments in the function of the insulin system (Gong and Muzumdar, 2012). According to several studies, diabetes is not only a risk of premature mortality but it is also associated with other age-related diseases such as cardiovascular diseases, cancer and Alzheimer's disease (AD) (Halim and Halim, 2019). As mentioned above, age-related cognitive changes are a part of normal aging but AD is linked with a myriad of pathological changes in the brain, eventually leading to a complete loss of autonomy (Toepper, 2017). Aging is the highest risk factor for AD, the most common neurodegenerative disorder.

2.2 ALZHEIMER'S DISEASE (AD)

2.2.1 Epidemiology and etiology

AD is a leading cause of dementia with more than 50 million people worldwide suffering from the disease, and this number is expected to triple by the year 2050 (Alzheimer's Disease International, 2019). Based on this estimation, in just a few years, AD will be one of the most challenging issues for public health worldwide. The main symptom of AD is progressive memory and cognitive impairment, which

both have a huge impact on the daily life of the affected person (Lane, Hardy and Schott, 2018). Mostly affecting the aged population, AD is the leading cause of death in Finland (19% of deaths) which is the highest rate of all European countries (Official Statistics of Finland (OSF), 2018).

There are two forms of AD: familial (FAD) and sporadic (SAD). The familial form of AD affects less than 5% of individuals of any age, and is associated with mutations in three genes – amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*) (Bekris *et al.*, 2010). The most common form of AD is sporadic, and an age over 65 is the main risk factor for the disease. It is still not clear what is the reason of developing sporadic AD; at present, it is considered as an interplay between genetic and environmental factors (Figure 1) (Lane, Hardy and Schott, 2018).

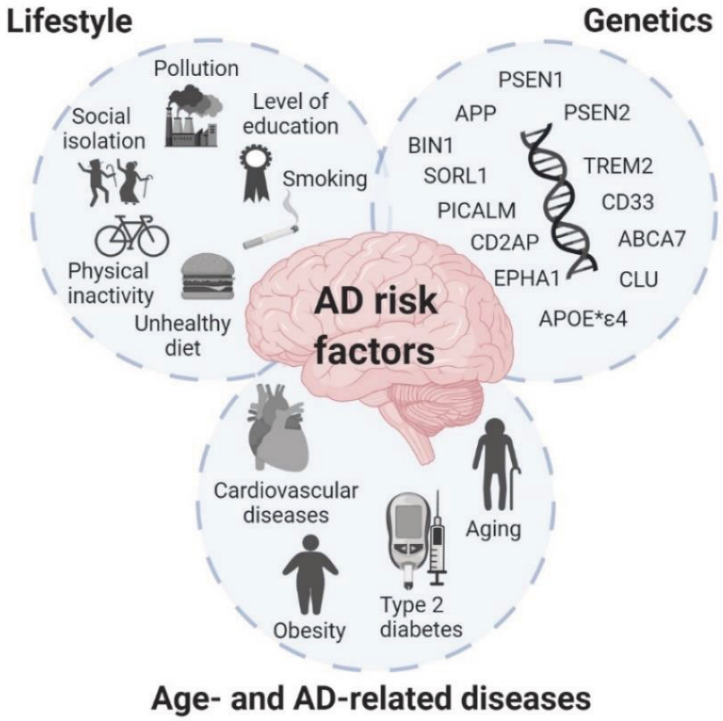

Figure 1. AD risk factors. Lifestyle (physical inactivity, unhealthy diet, smoking, low level of education, social isolation, living in pollutant areas), genetics (e.g. *PSEN1*, *PSEN2*, *APP*, *APOE*ε4*), age and certain diseases (type 2 diabetes, obesity, cardiovascular diseases) are increasing the chance that an individual will develop AD. Created with BioRender.com.

More than twenty AD risk genes were identified by genome-wide associated studies; these have been implicated in cholesterol metabolism (*APOE*, *CLU*, *ABCA7*), immune response (*TREM2*, *CD33*, *ABCA7*), APP processing (*BIN1*, *SORL1*) and synaptic function (*PICALM*, *CD2AP*, *EPHA1*) (Karch and Goate, 2015; Jansen *et al.*, 2019). Among all of the AD risk factors, a genetic predisposition is considered to be implicated in 70% of all cases, while the rest of them are associated with lifestyle factors and the presence of AD-related diseases (Ballard *et al.*, 2011). Epidemiological studies have revealed that environmental and lifestyle factors have a huge impact on the development on late-onset or sporadic AD. Several studies have been confirmed the high risk of AD development associated with obesity, cardiovascular diseases, type 2 diabetes (Van Norden *et al.*, 2012; Exalto *et al.*, 2013). In addition, women tend to suffer from AD more often than men, and this difference is explained not only by women's greater longevity but also by the higher rates of obesity and diabetes among females (Viña and Lloret, 2010). Lifestyle and environmental factors such as physical and social inactivity, unhealthy diet, smoking, a low level of education, living in a polluted area are related to AD risk factors (Xu *et al.*, 2015; Mir *et al.*, 2020), but all of them are modifiable, and should be investigated in more detail as a preventive approach for AD development.

2.2.2 Pathophysiology

Alois Alzheimer was the first clinician to identify AD and describe the main features of the disease – brain atrophy coupled with extracellular amyloid beta (Aβ) plaque deposition and intracellular neurofibrillary tangles (NFTs) (Alzheimer, 1907). In addition, neuronal and synaptic loss, inflammation, microglial activation, astrogliosis, mitochondrial dysfunction, oxidative stress and metal toxicity accompany AD pathology (Hou *et al.*, 2019; Kabir *et al.*, 2021).

Some of the major pathological features of AD pathology are neuronal loss and synaptic dysfunction, leading to an imbalance in neurotransmitters and cognitive impairment (Selkoe and Hardy, 2016). Studies on postmortem AD brain demonstrated a 25-35% decrease in the density of synapses in cortex accompanied with a reduction of hippocampal and cortical synaptic proteins, these being correlated with the degree of cognitive decline in AD patients (Terry et al., 1991).

According to the amyloid hypothesis proposed by the Selkoe research group (Selkoe, 1991), a disruption in the balance between Aβ production and its clearance is the initial step in AD, leading to an accumulation of Aβ plaques in the

brain (Figure 2) (Selkoe and Hardy, 2016). The plaques consist of the deposition of abnormally folded Aβ peptides, 39-43 amino acids in length, in the extracellular space. Abnormally folded Aβ peptides are cleaved from APP by γ- and β-secretase (BACE) enzymes and can form oligomers or become aggregated in plaques (Holtzman, Morris and Goate, 2011). Presenilin is the catalytic subunits of γ-secretase, and mutations in *PSEN1* and *PSEN2* genes lead to an increased production of the hydrophobic Aβ42 peptides, while mutations in *APP* affect the cleavage and aggregation of Aβ peptides (Selkoe and Hardy, 2016).

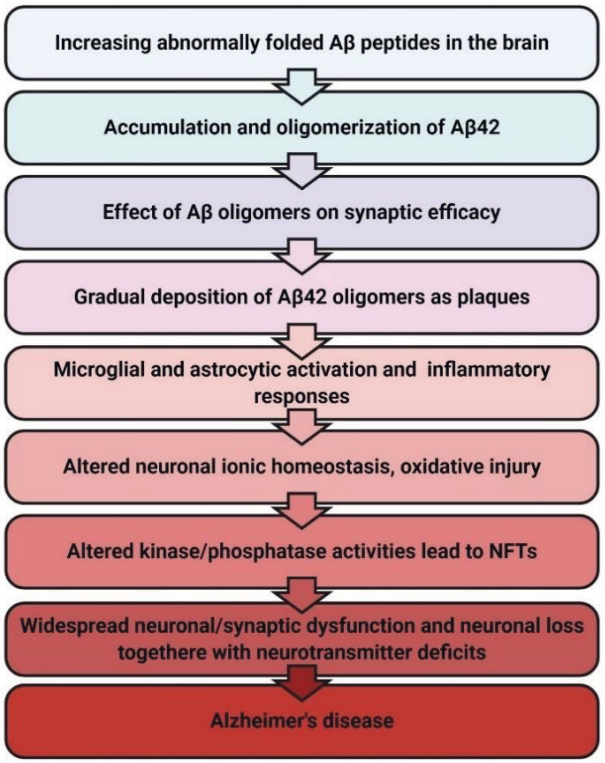

Figure 2. Pathological events leading to AD according to the amyloid hypothesis. Adapted from (Selkoe and Hardy, 2016). Created with BioRender.com.

According to the amyloid hypothesis, the imbalance in Aβ homeostasis is a trigger of the AD process, and the formation of NFTs is a consequence of Aβ deposition (Lane, Hardy and Schott, 2018). NFTs consist of hyperphosphorylated tau protein inside the neurons, causing neuronal dystrophy (Selkoe and Hardy, 2016). In physiological conditions, the tau protein stabilizes microtubules in neurons, while in AD, the tau protein becomes hyperphosphorylated, dissociates from

microtubules and creates intraneuronal NFTs (Holtzman, Morris and Goate, 2011). Based on human and animal studies, mutations in the genes related to Aβ homeostasis lead to Aβ plaque deposition, activation of glial cells, synaptic dysfunction, changes in neuronal tau in the brain and behavioral impairments; whereas tau mutations alone have been linked with neurodegenerative dementias but do not cause AD (Holtzman, Morris and Goate, 2011). Taken together, Aβ plaque deposition and tau pathology are both crucial in AD pathology, but the exact link between these two processes is still unclear.

There is evidence suggesting that one of the possible mechanisms explaining the link between Aβ plaque deposition and tau pathology could be neuroinflammation and dysregulation of the innate immune system (includes microglia and astrocytes), both major features of AD (Lane, Hardy and Schott, 2018). Aβ can induce an activation of glial cells, and this plays an important role in inflammatory processes occurring during AD pathogenesis (Guzman-Martinez *et al.*, 2019). In physiological conditions, abnormally folded Aβ peptides are cleared from the brain by different pathways: enzymatic (proteolytic degradation) or phagocytic by different cells (microglia, astrocytes, neurons, oligodendrocytes) (Jun Wang *et al.*, 2017). In sporadic AD, the dysregulation of Aβ clearance (Mawuenyega *et al.*, 2010) is linked with risk genes associated with the immune response (*TREM2*, *CD33*, *ABCA7*) and encoded proteins modulating phagocytosis (Karch and Goate, 2015). Furthermore, neuroinflammation is responsible for the dysregulated production of proinflammatory cytokines, which in turn is associated with cognitive impairment in the AD brain (Kelly, 2018).

AD is also associated with a dysregulation of redox-active metals such as iron, high concentrations of which are present in Aβ plaques (Ward *et al.*, 2014; Svobodová *et al.*, 2019). An excess of redox-active iron and dysregulation of iron metabolism can generate ROS and lead to oxidative stress (Andrews, 2000; Ang et al., 2010) and ferroptosis (Li *et al.*, 2020). Although the production of ROS is essential to maintain physiological homeostasis, as a consequence of increased ROS production, disrupted redox regulation of protein turnover can lead to increased protein misfolding and aggregation, which causes protein transformation to insoluble fibrils or aggregated plaques (Cheignon *et al.*, 2018). Moreover, excess iron induces glial activation and the increased expression of pro-inflammatory cytokines, and is involved in Aβ production and aggregation (Ward et al., 2014). Along with synaptic dysfunction, neuronal loss and neuroinflammation, excess of iron in the brain is also linked with a cognitive dysfunction (Schröder, Figueiredo and Martins De Lima, 2013).

2.2.3 Current treatment strategies

AD drug development is a difficult and complex process, and no new therapies for AD have been approved since 2003. Currently, four drugs have been approved for AD treatment such as donepezil, galantamine and rivastigmine, the cholinesterase inhibitors, and memantine, the glutamate receptor antagonist; but all of these drugs only delay the disease without curing it (Scheltens *et al.*, 2016). Recently, a new AD drug, abucanumab, was approved for use in AD patients. Although this drug reduces Aβ plaques in the brain, it remains questionable whether this drug can slow down the cognitive decline (Mullard, 2021). There are, however, several classes of agents that are currently in different phases of clinical trials: anti-amyloid, anti-tau, anti-inflammatory, neuroprotective, neurotransmitter based, metabolic, and regenerative (stem cell therapy) (Cummings *et al.*, 2019) (Table 1). All of them have different mechanisms of action and a common therapeutic purpose: 14% of agents aim at cognitive enhancement, 11% of agents aim to improve behavioral symptoms (agitation, apathy, sleep disorders), and the rest of the agents are intended to treat AD pathology – remove/reduce Aβ and tau spread, improve synaptic and mitochondrial function, reduce neuroinflammation and neuronal death (Cummings *et al.*, 2019).

Table 1. Agents in clinical trials for AD. Adapted from (Cummings *et al.*, 2019).

Class of agent	Mechanisms of action	Therapeutic purposes
Anti-amyloid	Antibody directed at Aβ oligomers	Remove Aβ
	Inhibitor of APP production	Reduce Aβ production
	BACE inhibitor	Prevent Aβ production
	Active immunotherapy	Remove Aβ and tau, prevent tau spread
Anti-tau	Tau protein aggregation inhibitor	Reduce NFTs formation and neuronal damage
	Monoclonal antibody	Remove tau and prevent tau spread
	Histone deacetylase inhibitor	Reduce tau-induced microtubule depolymerization
Neuroprotective	Herbs with antioxidant and anti-inflammatory properties	Reduce Aβ production

	Omega-3 fatty acid in high concentration in the brain	Reduce Aβ production, improve synaptic function
	Mitochondrial and ER stress inhibitors	Block neuronal cell death and neuroinflammation
	Antioxidants rich in anthocyanins	Improve mitochondrial function
	Iron chelating agent	Reduce ROS-induced neuronal damage; effect on Aβ and BACE pathology
	L-Serine amino acid	Stabilize protein misfolding
	Glutamate modulator	Reduce synaptic levels of glutamate
Neurotransmitter based	Ion channel modulator	Improve neuropsychiatric symptom (agitation, mania, psychosis)
	Cannabinoid (receptor agonist)	
	NMDA receptor antagonist	
	Nicotinic acetylcholine receptor agonist	Improve acetylcholine signaling (cognitive enhancer)
	Acetylcholinesterase inhibitor	
Anti-inflammatory	Bacterial protease inhibitor	Reduce neuroinflammation and hippocampal degeneration
	Selective tyrosine kinase inhibitor	Activity of mast cells, modulation of inflammatory processes
	Human plasma protein fraction infusion	Counteract inflammatory and age-related processes in the brain
Metabolic	Plant extracts with antioxidant properties	Improve brain blood flow and mitochondrial function
	Synthetic thiamine	Improve multiple cellular processes (cognitive enhancer)
	Affecting cAMP activity	Improve synaptic function (cognitive enhancer)
	Glucagon-like peptide 1 receptor agonist	Enhance cell signaling (cognitive enhancer)
	Increase insulin signaling in the brain	Enhance cell signaling and growth; promote neuronal metabolism
Regenerative	Stem cell therapy	Regenerate neurons; reduced Aβ deposition; decrease inflammation
	Hepatocyte growth factor	Regenerate neurons

Unfortunately, many recent promising agents, which were in phase 3 clinical trials, failed in AD patients. These failures in clinical trials are often attributed to a

too late starting point for treatment, and insufficient understanding of the pathophysiology of AD (Yiannopoulou and Papageorgiou, 2020). Moreover, targeting a multifactorial disorder such as AD, is extremely complicated. In addition, non-pharmacological interventions are currently in use for improving behavioral symptoms and quality of life in AD. These include cognitive training, exercise programs, and light and music therapies (Kishita, Backhouse and Mioshi, 2020).

2.2.4 The involvement of lifestyle factors in AD and age-related diseases

Lifestyle factors such as a healthy diet, social, physical and intellectual activity, limiting alcohol intake and cessation of smoking, and living in non-polluted areas, can impact enormously on human health. Lifestyle changes can also improve the quality of life in the elderly and reduce the risk of developing age-related diseases, including AD (Norton *et al.*, 2014; Kivipelto, Mangialasche and Ngandu, 2018; Mir *et al.*, 2020).

The disappointing lack of success in the clinic of new AD drugs leads to the conclusion that more efforts should be placed on disease prevention. A combination therapy approach that focuses on lifestyle changes should be seriously considered as a new preventive and therapeutic approach. The Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability (FINGER) trial is one of the first attempts to reduce the risk of cognitive decline in the elderly population using a complex lifestyle intervention. The results of this study indicated that a multidomain lifestyle intervention exerted beneficial effects on cognition regardless of participant characteristics, and the investigators proposed that this would be an effective strategy against AD (Ngandu *et al.*, 2015). A systematic meta-analysis reviewing 16 studies demonstrated that physical exercise alone was able to reduce risk of AD by 45% (Hamer and Chida, 2009). A cohort study with more than half a million participants showed that a healthy lifestyle (no smoking, regular physical exercise, healthy diet, low alcohol consumption) was associated with reduced AD development, even among participants that have a high genetic risk (Llewellyn *et al.*, 2019).

Age-related diseases and AD have common molecular mechanisms related to oxidative stress, inflammation, disruption of protein homeostasis and mitochondrial dysfunction, which could be targeted by preventive approaches such as a healthy lifestyle (Llewellyn *et al.*, 2019). However, the optimal mechanisms that could be used for brain protection during aging are still not

completely clear. It is thus increasingly important to assess how lifestyle factors may help to delay the development of AD.

2.2.5 AD research models

2.2.5.1 *In vitro* models

In vitro models are good tools with which to study the cellular and molecular mechanisms of disease, and nowadays many technologies are available. While in the past decades only rodent primary cells or immortalized cell lines were available, the most recent *in vitro* models have been based on human induced pluripotent stem cells (iPSCs) (Arber, Lovejoy and Wray, 2017). Different laboratories have established protocols for the differentiation of iPSCs from AD patients and healthy individuals into specific cell types affected by AD such as neurons (e.g. neuronal stem cells, NSCs), microglia and astrocytes (Penney, Ralvenius and Tsai, 2020). For example, iPSC-derived neurons of patients suffering from familial AD have high levels of Aβ40 and phosphorylated tau in comparison to controls, providing a good model mimicking AD-related neuronal pathology (Israel *et al.*, 2012). Moreover, 3D cultures of human iPSC-derived neurons together with microglia and astrocytes in specific microfluidic devices have been generated to induce neuroinflammation, a crucial pathological component of AD (Park *et al.*, 2018). Furthermore, based on the ability of NSCs under certain conditions to self-organize into a 3D structure, so-called “organoids” (Lancaster and Knoblich, 2014), has allowed researchers to generate a structure sharing some similarities with the AD brain and even developing amyloid plaque- and NFT-like structures (Gonzalez *et al.*, 2018). Although *in vitro* models can mimic some features of AD, these models have limitations related to the inability to display age-dependent cellular signatures, and the high variability between iPSCs from different individuals (Penney, Ralvenius and Tsai, 2020). Nevertheless, *in vitro* models are widely exploited in screening for compounds to combat against AD (Brownjohn *et al.*, 2017) and human stem cell technology is used for regeneration medicine (Sproul, 2015). However, it should be noted that *in vitro* approaches are not suitable for studying cognition and the impact of lifestyle factors on the brain.

2.2.5.2 *In vivo* models

When studying AD related pathology *in vivo*, different animal models including *Drosophila*, rats, even primates have been developed and used, but in practice nowadays, mouse models are mostly utilized. Because of morphological

similarities of plaque and tau pathology between familial and sporadic forms of the disease, genetically modified (transgenic, TG) mouse models carrying mutations in AD-related genes are also being commonly used in attempts to understand mechanisms behind sporadic AD (Sasaguri *et al.*, 2017).

AD mouse models usually overexpress single or multiple variants of human APP (hAPP), PSEN1, PSEN2 and tau mutations (Table 2). Widely used TG mice have hAPP mutations including London (Lon, V171I), Florida (Flo, I716V) and Indiana (Ind, V717F), leading to an increase in the Aβ42/Aβ40 ratio; the most commonly used Swedish (Swe, K670N/M671L) mutation causes increased production of Aβ (Hall and Roberson, 2012). TG mice expressing hAPP develop Aβ plaques at 6-9 months of age, synaptic loss, gliosis and cognitive deficits (Games *et al.*, 1995; Hsiao *et al.*, 1996). The limitations of APP models include late plaque deposition and the absence of neuronal loss. However, mice expressing both mutant APP and PSEN genes have accelerated Aβ deposition and neuronal loss making it possible to achieve AD-related pathological features at an earlier age (Sasaguri *et al.*, 2017). In order to study Aβ pathology together with NFTs, triple TG mice (3xTG-AD) were developed expressing mutant hAPP (Swe), PSEN1 (M146L) and tau (P301L) and showing Aβ deposition starting at 6 months, tau pathology at 10-12 months together with neuronal and synaptic loss (Oddo *et al.*, 2003). Although the 3xTG-AD mice demonstrate pathological features similar to those encountered in human AD such as NFTs, the development of plaques in this model is rather slow (Braidy *et al.*, 2012). Therefore, Oakley at al. generated the 5xFAD mouse model, which carries five FAD-related mutations in hAPP (Swe, Flo, Lon) and in PSEN1 (M126L and L286V) transgenes under the Thy-1 promoter; 5xFAD mice develop Aβ plaques accompanied with gliosis starting from 2 months of age, and synaptic degeneration, neuronal loss and cognitive deficits starting from 3-5 months of age (Oakley *et al.*, 2006). Although 5xFAD mice fail to generate NFTs, this is an aggressive and fast developing disease model, which seems to be a good tool with which to study AD pathology.

Table 2. The most commonly used mouse models of AD. Adapted from (Li, Bao and Wang, 2016).

	Strain	Genetic alterations	Pathological features	Cognitive deficits start
APP	PDAPP	hAPP (Ind), PDGFβ promoter	Aβ plaques at 6-9 months; synaptic loss; gliosis; inflammation	At 6 months
	TG2576	hAPP(Swe), HamPrP promoter		At 9 months
	APP23	hAPP(Swe), Thy-1 promoter		At 3 months
APPxPSEN	APP/PS1	hAPP(Swe), PSEN1(deltaE9),PrP promoter	Aβ plaques at 6 months; neuronal loss; gliosis	At 6 months
	5xFAD	hAPP(Swe, Lon, Flo) and PSEN1(M146L, L286V), Thy1 promoter	Aβ plaques at 2 months; synaptic and neuronal loss; gliosis	At 3-5 months
Triple TG	3xTG-AD	hAPP(Swe), hTau(P301L),Thy1 promoter; PSEN1(M146V), mPS1 promoter	Aβ plaques at 6 months; NFTs at 10-12 months; neuronal and synaptic loss; gliosis; inflammation	At 4 months

2.3 ALTERATIONS OF THE NEUROGLIAL NETWORK IN AGING AND AD

2.3.1 General features

In general, brain cells can be divided into two subtypes: neurons and glial cells. Neurons are responsible for transmission of electrical signals, whereas glial cells support proper neuronal functioning (Allen and Barres, 2009). In AD, Aβ and tau induce neuronal dysfunction and both morphological and functional alterations to glial cells with dramatic impacts on the neuroglial network and disease progression (De Strooper and Karran, 2016).

2.3.2 Cell-type specific features

2.3.2.1 Neurons

Brain plasticity is the ability of the neuronal network to change and adapt during the lifespan on both structural (neurogenesis) and functional (synaptic plasticity) levels (Toricelli *et al.*, 2020).

All manifestations of live organisms including sensations, reflexes, and processes in higher brain centers such as emotions and ideas, are mediated through the transmission of electrical and chemical signals within the neuronal network. One crucial part of this transmission is the synapse. Synapses are formed between neurons and act as a gap over which neuronal impulses are transmitted by releasing neurotransmitters, ions and other signalling molecules from the presynaptic neuronal membranes. These released substances activate ion channels or neurotransmitter receptors on the postsynaptic cell membrane. The NMDA receptors (NMDARs) are an example of these receptors (Waites, Craig and Garner, 2005). The ability of synapses to strengthen and weaken their synaptic contacts through changing their structure, number and function of synapses is a process called synaptic plasticity, which is the most important element in learning and memory. Long-term potentiation (LTP) or long-term depression (LTD) are the main cellular processes regulating synaptic plasticity. The excitatory neurotransmitter glutamate activates NMDARs, which can induce either LTP, promoting a dendritic spine growth, or LTD, leading to a synaptic loss (Palop and Mucke, 2010). Synaptic plasticity is the functional part of the neuroplasticity, whereas hippocampal neurogenesis corresponds to the structural component. Newborn neurons mature and become excitatory glutamatergic neurons in the dentate gyrus (DG) of the hippocampus – a brain area implicated in learning and memory (Toricelli *et al.*, 2020). Neurogenesis is regulated by neurotrophins and growth factors including brain-derived neurotrophic factor (BDNF), which also plays an important role in neuronal survival and synaptic plasticity (Oliveira *et al.*, 2013).

To a certain degree, the brain is resistant to age-dependant alterations, although changes associated with aging do occur. However, healthy brain aging is not associated with synaptic dysfunction and neuronal loss (Rodríguez-Arellano *et al.*, 2016). In AD, however, a shrinkage of the brain and a cognitive impairment associated with a loss and dysfunction of neuronal synapses are all observed (Terry *et al.*, 1991). The mechanisms of Aβ synaptic toxicity remain to be fully characterized, but there is some evidence that soluble Aβ oligomers surrounding

Aβ plaques play an important part in synaptotoxicity (Wang *et al.*, 2016). Aβ oligomers may lead to synaptic dysfunction through the glutamatergic system by inhibiting LTP or promoting LTD of excitatory synapses (Wang *et al.*, 2016). Aβ may affect glutamate uptake on the postsynaptic site, leading to a rise in glutamate levels in the synaptic cleft and consequent overactivation of NMDARs, causing an intracellular calcium influx (Li *et al.*, 2009); prolonged NMDAR activation leads to a disruption of intracellular calcium homeostais which eventually results in synaptic loss and neuronal death. One of the mechanisms of Aβ synaptotoxicity is direct binding of Aβ with postsynaptic receptors, thereby decreasing the strength of the synapses via a NMDA-dependent pathway (Forner *et al.*, 2017).

A decline in the amounts of synaptic proteins and neurotrophins including BDNF has been detected in the brains of AD patients (Ferrer *et al.*, 1999; Proctor, Coulson and Dodd, 2010). Interestingly, BDNF reductions have been observed in an early stage of AD and this reduction is correlated with the cognitive decline (Peng *et al.*, 2005). Moreover, it has been shown that reduced BNDF levels are more pronounced in AD mice with high levels of Aβ42 oligomers (Peng *et al.*, 2009). Taking into account the important role of BNDF and synaptic proteins in synaptic plasticity and neuronal survival, an attenuation of these proteins could be associated with synaptic loss and reduced neurogenesis in AD. The decline in neurogenesis affects neuroplasticity and contributes to the impairments in memory and learning associated with AD (Lazarov *et al.*, 2010). Interestingly, FAD-related proteins may directly promote impairments in hippocampal neurogenesis, which appears before Aβ plaque formation and neuronal loss, and may play crucial roles in the initiation of AD-related pathology (Lazarov and Marr, 2009).

2.3.2.2 Astrocytes

Astrocytes are the most functionally and morphologically diverse type of glial cells essential for numerous functions in the brain. Astrocytes are responsible for maintaining central nervous system (CNS) homeostasis at many levels e.g. systemic (regulation of ion concentrations, blood pH, energy balance), metabolic (glycogen synthesis) and molecular (ion transport, neurotransmitter release/removal) levels (Verkhratsky and Nedergaard, 2018). Furthermore, astrocytes regulate the blood brain barrier (BBB) and the CNS glymphatic system, thereby promoting brain clearance of neurotoxic fluids and proteins, including Aβ oligomers (Iliff *et al.*, 2012). Astrocytes also play crucial roles in producing, storing and supplying energy to neurons (Bélanger, Allaman and Magistretti, 2011). By interacting with synapses, astrocytes modulate neurotransmission and control

synapse formation through the release of astrocyte-specific molecules, trophic factors and gliotransmitters (Souza *et al.*, 2019). Therefore, astrocytes are critically involved in cognitive and behavioural regulation. Moreover, in response to CNS damage, astrocytes exert neuroprotective functions through a regulation of homeostasis or by changing to a reactive state, the so-called reactive astrogliosis (Burda and Sofroniew, 2014). For a long time, reactive gliosis was believed to mediate neuroinflammation and act as a trigger for neuronal damage (Akiyama *et al.*, 2000). Currently reactive astrogliosis is defined by many as a defense mechanism that is responsible for isolating a damaged area and neuronal support, although whether this mechanism is protective or harmful depends on various factors, including the type of neuropathology and the phase of the disease (Verkhratsky *et al.*, 2019).

Reactive astrocytes are characterised with morphological and functional alterations together with an upregulation of the glial fibrillar acidic protein (GFAP) (Escartin, Guillemaud and Carrillo-de Sauvage, 2019). In AD, reactive astrocytes are characterised with a hypertrophic soma and processes, and form a glial scar around Aβ plaques (Pekny *et al.*, 2016). Furthermore, these reactive astrocytes participate in Aβ clearance by increasing the production of Aβ-degrading enzymes (Yin *et al.*, 2006) and elevating phagocytic activity (Pomilio *et al.*, 2016).

In AD, reactive astrogliosis exists together with astrocyte atrophy and astrodegeneration, and it depends on the brain region (Verkhratsky *et al.*, 2016). Atrophic astrocytes are located distantly from the Aβ deposits in contrast to the reactive astrocytes surrounding plaques (Olabarria *et al.*, 2010). In AD, the atrophic astrocytes display a reduction of volume, a lower number of processes and complexity (Olabarria *et al.*, 2010), that in turn causes a reduction in the coverage of synapses leading to a decrease in astroglial glutamate uptake and synaptic dysfunction (Verkhratsky *et al.*, 2019). Moreover, Aβ deposits induce a calcium dysregulation in astrocytes contributing to the impairment in astrocyte function (Kuchibhotla *et al.*, 2009).

2.3.2.3 Other glial cells

In addition to the astrocytes, the CNS includes other glial ceJgfcyjcnm100292lls such as microglia and oligodendrocytes.

Microglia are the resident immune cells in the CNS, responsible for defensive immune responses. In the CNS damage, microglia move to the damaged region and are converted into an activated state (Song and Colonna, 2018). Activated microglia are characterised by a more spherical shape with shorter processes

compared to resting cells. Depending on brain pathology, activated microglia interact with neurons and release numerous molecules such as cytokines, chemokines and ROS (Kettenmann, Kirchhoff and Verkhratsky, 2013). AD is associated with microglial activation accompanied with morphological (Davies *et al.*, 2017), transcriptional (Mathys *et al.*, 2019) and functional alterations in these cells (Hemonnot *et al.*, 2019). Activated microglia surround Aβ plaques, forming a barrier between the lesion and healthy tissue, and they also secrete Aβ-degrading enzymes (Czirr *et al.*, 2017). In AD, Aβ oligomers and plaques are associated with neuroinflammation, which is considered to be a process primarily conducted by microglial cells (Nizami *et al.*, 2019). AD patients have increased levels of pro-inflammatory mediators such as ROS, chemokines, cytokines and complement components, all of which are indicative of the activation of inflammatory pathways (Heneka, Kummer and Latz, 2014). Variants in the *TREM2*, *APOE*, *CR1* and *CD33* genes, which are associated with Aβ clearance and immune responses, are expressed in microglial cells and have been associated with the risk of AD (Karch and Goate, 2015; Song and Colonna, 2018). For example, TREM2 is upregulated in activated microglia modulating the inflammatory response and microglial Aβ phagocytosis activity, but it is still not clear whether TREM2 overexpression has benefical or harmful effects in AD pathology (Hemonnot et al. 2019). In addition, it is still not clear whether the inflammatory processes mediated by microglia in AD are advantageous or detrimental.

The most abundant non-neuronal cells in the brain are oligodendrocytes (Pelvig *et al.*, 2008); their main function is to cover the neuronal axons with a lipid membrane (myelin) for accelerating the electrical impulse between neurons and target cells in CNS (Simons and Nave, 2016). In addition to myelination, oligodendrocytes also provide trophic and metabolic axonal support (De Strooper and Karran, 2016) and participate in lactate delivery to axons together with astrocytes (Lee *et al.*, 2012). Oligodendrocytes are considered as highly vulnerable cells, and the numbers of oligodendrocytes together with neurons decrease with advanced age, while the population of astrocytes remains unchanged (Pelvig *et al.*, 2008). Aβ deposits and AD-related oxidative stress cause oligodendrocytes dysfunction and demyelination with a further axon loss (Nasrabady *et al.*, 2018).

2.3.3 Neuron-Glia crosstalk and synaptic plasticity

Glial involvement in synaptic regulation is described by the tripartite synapse model according to which astrocytes communicate with presynaptic and postsynaptic neurons by releasing neurotransmitters (Araque *et al.*, 1999). Next,

the tripartite synapse model develops into the multipartite synapse, which consists of presynaptic and postsynaptic neuronal components, the extracellular matrix in the synaptic cleft, the astrocyte covering a synapse and microglia contacting with the synapse structure from time to time (Verkhratsky and Nedergaard, 2014). According to this model, each component plays an important role in synaptic plasticity.

Focusing on glial cells, astrocytes participate in the modulation of synaptic transmission by releasing and taking up neurotransmitters, regulating ion homeostasis and neurotransmitters dynamically in the synaptic cleft, thereby preventing neurotransmitter spillover (Nedergaard and Verkhratsky, 2012). Furthermore, astrocytes covering synapses secrete molecules and neurotrophic factors such as thrombospondins, hevin, cholesterol, *L*- and *D*-Serine and BDNF, which are crucial for synaptic plasticity, synaptogenesis, synaptic density and maturation (Augusto-Oliveira *et al.*, 2020). Together with astrocytes, oligodendrocytes also promote synaptogenesis via the production of cholesterol (Orth and Bellosta, 2012) and the secretion apolipoprotein E (APOE) which mediate cholesterol delivery to neurons (Chaves and Narayanaswami, 2008). In addition, astrocytes, with the support of oligodendrocytes, supply neurons with lactate as an energy source, which is involved in the regulation of neuronal activity, maintenance of LTP and long-term memory formation (Suzuki *et al.*, 2011). Microglia participate in early synaptogenesis by secreting growth factors (Kettenmann, Kirchhoff and Verkhratsky, 2013). During later development, microglia control the number of synapses by a process called synaptic pruning in a complement cascade dependent manner (Schafer *et al.*, 2012). Finally, microglia mediate synaptic connectivity by producing pro-inflammatory cytokine TNF-α (Stellwagen and Malenka, 2006; Pascual *et al.*, 2012). Thus, glial cells have a huge impact on synaptic plasticity and therefore on cognition and memory.

In AD, glial functions are impaired, and this affects their regulation of synaptic function and contribute to cognitive deficits. Atrophic astrocytes, which appear in the brain before there are any extracellular Aβ deposits, decrease synaptic coverage thereby affecting synaptic transmission (Pekny *et al.*, 2016). As was mentioned above, Aβ oligomers induce a synaptic loss and neuronal death by increasing the glutamate concentration in synapses (Li *et al.*, 2009), which in turn may be exacerbated through impaired astrocytic glutamate uptake caused by decreased atrophic astrocyte coverage of synapses (Verkhratsky *et al.*, 2019). Impaired glutamate uptake can also be caused by a reduction in the expression of glutamate transporters in reactive astrocytes surrounding the Aβ plaques

(Hefendehl *et al.*, 2016). Moreover, it has been shown in AD mice that astrocytes surrounding plaques decrease their expression of glutamine synthetase (GS), the enzyme responsible for converting glutamate to glutamine, leading to a disruption of glutamate homeostasis (Olabarria *et al.*, 2011). The common consequence of these astrocytic impairments is an increase in the extracellular glutamate concentration and a disruption of calcium homeostasis, leading to synaptic loss and neuronal death.

Under physiological conditions, microglia are responsible for pruning synapses in the development stages and phagocytosis of apoptotic neurons, but in AD, these microglial fucntions are inappropriate, leading to a synaptic loss (Song and Colonna, 2018). In the early AD stage in mice, Aβ oligomers induce an activation of the complement system which leads to increased microglial synaptic pruning (Hong *et al.*, 2016). An ablation of microglial complement receptor 3 (CR3) in AD mice was demonstrated to cause a reduction of the Aβ level (Czirr *et al.*, 2017). AD-associated neuroinflammation and increased production of pro-inflammatory mediators both are known to induce an attenuation of synaptic proteins and synaptic loss (Rajendran and Paolicelli, 2018). Based on genome-wide association studies (Karch and Goate, 2015) and single-cell transcriptomic analysis (Grubman *et al.*, 2019; Mathys *et al.*, 2019), many AD risk genes associated with either cholesterol metabolism (*APOE4*) or immune response (*TREM2*, *CR1*, *CD33*), are expressed in microglial cells, and have a crucial role in synaptic dysfunction (Henstridge, Hyman and Spires-Jones, 2019).

2.4 ALTERATIONS OF SKELETAL MUSCLE TISSUE IN AGING

2.4.1 Skeletal muscle structure

Skeletal muscle contraction is essential for body movement and posture, and regulates whole body protein and energy metabolism (Argiles *et al.*, 2016). In general, skeletal muscle consists of slow- and fast-twitch fiber types, which have different resistance to fatigue, oxidative capacity and substrates for energy production (Baskin, Winders and Olson, 2015). Slow-twitch fibers are specialized for continuous activity, have a high level of oxidative enzymes and produce adenosine triphosphate (ATP) through aerobic metabolism; whereas fast-twitch fibers have less fatigue-resistance, a low oxidative capacity and are specialized for rapid ATP production through anaerobic glycolysis (Schiaffino and Reggiani, 2011; Baskin, Winders and Olson, 2015). For instance, soleus skeletal muscle has more

slow-twitch fibers, whereas vastus lateralis and tibialis anterior (TA) skeletal muscles mostly consist of fast-twitch fibers (Armstrong and Phelps, 1984).

2.4.2 General features related to skeletal muscle atrophy in aging

Aging causes structural and functional changes in skeletal muscle including a decline in the regenerative capacity and a loss of muscle mass and strength. Although these changes occur in both types of skeletal muscle fibers, fast-twitch fibers are more susceptible to age-related atrophy (Doria *et al.*, 2012; Argiles *et al.*, 2016). The wasting of skeletal muscle exerts effects throughout the body i.e. there is a decline in the supply of muscle derived amino acids, cytokines and myokines, which are essential in combatting diseases (Curtis *et al.*, 2015). In addition to aging, a wide range of genetic (*APOE4*, *BIN1*) and lifestyle factors (physical inactivity, poor diet) as well as many diseases including cancer, type 2 diabetes, obesity and AD are associated with skeletal muscle atrophy (Curtis *et al.*, 2015; Chen *et al.*, 2019).

Several mechanisms and pathways have been identified as being involved in skeletal muscle aging processes and impaired muscle adaptation to stress (Brown, Guzman and Brooks, 2020) such as increased ROS production, mitochondrial dysfunction (Gouspillou *et al.*, 2014), altered protein and redox homeostasis, dysfunction of antioxidant defense systems (Jackson and Mcardle, 2011), increased apoptosis (Marzetti and Leeuwenburgh, 2006), inflammation (Jinyu Wang *et al.*, 2017) and denervation (Valdez *et al.*, 2010). Moreover, AD and a dysfunction in age-related calcium signalling as well as disturbances in the endoplasmic reticulum (ER) have been proposed to affect the functions of both muscular and neuronal systems (Puzianowska-Kuznicka and Kuznicki, 2009). Similar to neurons during aging, skeletal muscle also loses the ability to clear damaged proteins and it starts to accumulate protein aggregates (Chen *et al.*, 2019).

2.4.3 Redox-regulatory systems

Because of the high oxygen consumption essential for optimal muscle contraction, skeletal muscle has a high capacity to produce ROS (Fulle *et al.*, 2004). Antioxidant defence in skeletal muscle acts to reduce the oxidative damage of macromolecules that occurs in situations of increased ROS production (Powers and Jackson, 2008). The major antioxidant enzymes are superoxide dismutase (SOD), glutathione-peroxidase, peroxiredoxins and catalase (Powers and Jackson, 2008). SOD is responsible for converting superoxide radicals into oxygen (O_2) and hydroperoxides (H_2O_2), which is further dissociated by catalase into H_2O and O_2

(Ulrich and Jakob, 2019). In addition to SOD and catalase, H_2O_2 or organic hydroperoxides can be detoxified to water and alcohol, respectively, with thiol-based reactions catalysed by thiol peroxidases, the reduced state of which needs to be restored with the help of thiol-based redox systems (Brigelius-Flohé and Maiorino, 2013). Glutathione (GSH) and thioredoxin (TRX) systems are the key players in thiol-dependent antioxidant mechanisms and redox signalling, protecting cells against oxidative stress (Radak *et al.*, 2013) and ferroptosis (Kuang *et al.*, 2020). The main non-enzymatic antioxidant in muscle is GSH. This compound is the major cellular source of thiols groups; oxidized GSH forms glutathione disulfide (GSSG) and the GSH/GSSG ratio is an important indicator of oxidative stress (Morillas-Ruiz and Hernández-Sánchez, 2015). In addition to a central role in antioxidant defence, GSH together with TRX participates in oxidative protein folding in ER, forming protein disulfide bonds (Poet *et al.*, 2017). The TRX system, consisting of NADPH, thioredoxin reductase, and TRX itself, plays the main role in cellular redox signalling, preventing protein oxidation and apoptosis control (Arnér and Holmgren, 2000). TRX-interacting protein (TxNiP) inhibits protein reducing activity of TRX, thus the TRX-TxNiP interaction is important in redox regulation (Yoshihara *et al.*, 2014).

Although ROS are needed to maintain homeostasis, aging is associated with increased ROS production in muscle leading to oxidative stress and impaired redox signalling, which in turn is linked with the oxidation of DNA, lipids and proteins (Dröge, 2002; Jackson and Mcardle, 2011). Even though it is still not clear whether aging results in weakness or strengthening of the antioxidant enzyme activity (Brown, Guzman and Brooks, 2020), an impairment of the cell redox state and increased oxidative damage are evident in aged skeletal muscle (Fanò *et al.*, 2001; Thirupathi, Pinho and Chang, 2020). For example, aging has been reported to reduce the GSH/GSSG ratio in skeletal muscles (Mosoni *et al.*, 2004; Rebrin, Forster and Sohal, 2011). Although limited information is available about the effect of aging on TRX and TRX/TxNiP in skeletal muscle, Dimirio *et al,* have detected age-related TRX increases in TA muscle and explained this alteration as a compensatory mechanism against increased oxidative stress (Dimauro *et al.*, 2012). It is important to note that not all age-related changes in muscle are detrimental - some of them are compensatory responses and could contribute to an activated defensive response.

2.4.4 Endoplasmic reticulum (ER) stress and UPR

The endoplasmic reticulum (ER) is a cellular organelle responsible for calcium and protein homeostasis with the help of numerous chaperones and folding enzymes including glucose-regulated protein 78 (GRP78), GRP94, and protein disulfide isomerase (PDI) (Brown and Naidoo, 2012). Different stress conditions and the accumulation of unfolded proteins can alter ER functioning, thereby affecting normal protein homeostasis and leading to ER stress (Kaufman, 2002). In an attempt to limit the level of unfolded proteins and aggregates and return the ER towards normal functioning, cells activate adaptive mechanisms such as the ER stress or unfolded protein response (UPR) (Walter and Ron, 2011). The main role of the UPR system is to enhance the protein folding capacity, to reduce protein production and to facilitate the degradation of damaged proteins (Brown and Naidoo, 2012), whereas under chronic ER stress conditions associated with many pathological diseases, the URR is also responsible for the activation of apoptosis and cell death (Tabas and Ron, 2011).

Aging and neurodegenerative diseases including AD are associated with calcium dysregulation, impaired redox signaling and the accumulation of misfolded proteins leading to ER stress and activation of UPR (Puzianowska-Kuznicka and Kuznicki, 2009; Sprenkle *et al.*, 2017). Moreover, aging is associated with a reduction of ER chaperone efficacy due to oxidation, and a shift of the UPR to a pro-signalling state (Brown and Naidoo, 2012). Age-related alterations in ER chaperones have been detected in many tissues (Naidoo, 2009). As skeletal muscle contains 50-75% of the total proteins in the body and a large ER network, it is expected that aging can induce ER stress and UPR in skeletal muscle as well (Bohnert, McMillan and Kumar, 2018). It has been shown that the levels of ER chaperones are increased in skeletal muscle of old animals as compared to younger controls, together with the upregulation of C/EBP homologous protein (CHOP) (Ogata *et al.*, 2009; O'Leary *et al.*, 2013; Chalil *et al.*, 2015), which is responsible for mediating ER stress-induced apoptosis (Tabas and Ron, 2011). These alterations in the normal functioning of the ER may contribute to skeletal muscle atrophy (Gallot and Bohnert, 2021).

2.4.5 Heat shock proteins (HSPs)

Heat shock proteins (HSPs) are molecular chaperones, which together with the ER are responsible for protein homeostasis, and play essential roles during stress conditions such as in the presence of environmental or oxidative stresses.

Therefore, HSPs are also classified as stress proteins. As a result of cellular stress, the levels of HSPs are increased, promoting an adaptive response and providing protection against stress-induced damage such as refolding protein intermediates and degrading damaged proteins (Castrogiovanni and Imbesi, 2012). Studies on mice have shown that increased ROS production during contraction is associated with activated antioxidant enzyme activity together with increased levels of HSPs in skeletal muscle, and in this way HSPs participate in the adaptive response in skeletal muscle (McArdle *et al.*, 2001). Moreover, HSPs are essential in the regulation of apoptosis by interacting with apoptotic mediators (Marzetti and Leeuwenburgh, 2006). One of the important HSP groups is the HSP70 family, which contains the homologous proteins in ER (GRP78), mitochondria (GRP75) and cytoplasm (HSP70, HSC70); these are important for protein transport, folding, degradation and protection of cells against stresses (Daugaard, Rohde and Jäättelä, 2007).

Age-related accumulation of damaged proteins is exacerbated by the reduced HSPs levels, which are generally observed in neuronal and muscle tissues (Calderwood, Murshid and Prince, 2009). Moreover, aging attenuates the ability of skeletal muscle to adapt to different types of stress due to the decline in the HSPs content (Jackson and Mcardle, 2011). It was reported that in skeletal muscle of old rats, the level of HSP70 was decreased together with increased ER stress and apoptotic markers when compared to younger control animals (Ogata *et al.*, 2009). Although HSPs contribute to age-related skeletal muscle changes, it is also possible that some of these chaperones also participate in compensatory protective mechanisms in response to stress. For example, it was demonstrated that in skeletal muscle of old rats, the basal level of the HSPs was upregulated, and this activation of HSPs was suggested to be involved in compensatory mechanisms in response to oxidative damage and the presence of a pro-apoptotic environment (Chung and Ng, 2006).

2.5 PHYSICAL ACTIVITY AS A PREVENTIVE APPROACH IN AGING AND AD

2.5.1 Type of physical exercises

Physical activity can be described as the contraction of skeletal muscle produced by body movement that increases total energy consumption above the basal level (Caspersen, Powell and Christenson, 1985). In turn, exercise is described as

physical activity, which increases maximum oxygen consumption, and is distinguished by a planned and repetitive manner with a duration and intensity sufficient to improve physical health when compared to a sedentary individual (Yuede *et al.*, 2018). Although there are studies showing positive effects for human health even from one bout of exercise (Basso and Suzuki, 2017), most of the health benefits especially referring to aging are associated with regular, long-term physical exercise (Van Praag *et al.*, 2005; Garcia-Valles *et al.*, 2013; Gries *et al.*, 2018; Nilsson *et al.*, 2019). For example, an acute intensive exercise induces increased ROS production and apoptosis, whereas regular exercise training induced adaptations and promoted the antioxidant defence system to protect cells against stress-related diseases and delayed the aging processes (Radak, Chung and Goto, 2005; Morillas-Ruiz and Hernández-Sánchez, 2015). In general, physical exericse can be divided into aerobic exercise, which leads to an improvement of endurance performance, and resistance training, which elevates muscle mass and strength (Lambert, 2016). Both types of exercise may be undertaken with different intensities and duration, thus having diverse impacts on the individual's health. Based on epidemiological and clinical studies, vigorous intensity aerobic exercises (e.g. running) have greater health benefits than exercise with moderate intensity (Swain and Franklin, 2006). Although both aerobic and resistance exercise have beneficial effects on brain health (Pinho, Aguiar and Radák, 2019), a systematic review has shown that in general aerobic exercises have greater positive impacts on global cognitive and executive functions (Barha *et al.*, 2017).

2.5.1.1 Exercise protocols in animal studies

Currently many researchers are attempting to determine the optimal regular exercise programmes in order to obtain the best possible health benefits, but because of many factors related to genetic background, age, physiological conditions, and the level of the current physical state, it is hard to find a common solution to fit everyone (Silverman and Deuster, 2014). Animal models provide a unique opportunity to control multiple parameters (psychological, emotional, environmental) in order to investigate exercise efficacy in different diseases and in aging (Poole et al. 2020).

In animal studies, researchers broadly use three main exercise protocols: treadmill running, voluntary wheel running and swimming. The main advantages of using the treadmill running protocol include the control of all running parameters such as duration and speed, thereby reducing variablity between exercised animals (Poole *et al.*, 2020). However, in most cases, treadmill running is

performed under stressful conditions including a stimulus to ensure forced animal running and an incompatibility of running with the natural dark/light cycle of the animals (Goh and Ladiges 2015). Similar to treadmill running, swimming exercise is associated with physiological stress together with a continuous investigator attendance, both of which may affect the outcome (Poole *et al.*, 2020). On the other hand, the wheel running protocol allows animals to exercise with the desired intensity, speed and time under non-stressed conditions and without any direct investigator involvement. In order to monitor the exercise parameters for the wheel running protocol, the animals need to be housed individually, which can eventually affect their behaviour. Nevertheless, the main advantage of voluntary wheel running as a choice for an exercise training protocol is the opportunity to investigate long-term effects of exercise in both disease and aging studies (Manzanares, Brito-Da-Silva, and Gandra 2019). This voluntary wheel running also provides the opportunity to study a "healthy lifestyle" animal model starting from an early age.

2.5.2 Beneficial effects of physical activity

Everyday life became less physical active due to the development of many technologies and the availability of transport. An increasingly sedentary lifestyle has systemic impacts on health (Radak, Chung and Goto, 2008), and is one the risk factors for age-related diseases including neurodegenerative diseases and mortality worlwide (Biswas *et al.*, 2015). Indeed, regular physical exercise has a significant impact on human healthy aging through preventing or delaying many disorders and chronic diseases (Table 3). Specifically, regular physical exercise enhances various biological pathways including adaptation against stress and stress-related disease, promotion of an anti-inflammatory state, stabilization of the metabolic system, and the induction of neuroplasticity (Silverman and Deuster, 2014; Galli *et al.*, 2021).

Table 3. Beneficial effects of regular physical exercise (U.S. Department of Health and Human Services, 2018).

Improve the following characteristics	Reduce risks
Blood pressure and level of "bad" cholesterol	Cardiovascular diseases
Level of blood sugar and insulin sensitivity	Type 2 diabetes
Brain function and quality of sleep	AD and other dementias
Anxiety level	Depression
Energy balance and weight control	Obesity
Strengthen muscles and bones	Fall-related injuries and sarcopenia

2.5.2.1 Skeletal muscle tissue

Skeletal muscle is the major organ which responds to physical exercise. Regular physical exercise causes beneficial changes in contracting skeletal muscles including an increase of metabolic activity, elevated oxygen consumption, improved blood flow and the release of biologically active molecules (myokines), all of which influence many functions of the organism (Hawley *et al.*, 2014; Viña *et al.*, 2016). Currently, except for physical exercise, there is no treatment which can delay age-related skeletal muscle atrophy. It has been shown that regular and long-term physical exercise can delay age-associated skeletal muscle atrophy and weakness (Tarpenning *et al.*, 2004; Cartee *et al.*, 2016). Recently, proteomic analysis of skeletal muscle biopsies from regularly exercised young and old healthy individuals have demonstrated that physical activity is associated with improved mitochondrial functions, enhanced antioxidant protection and a lower level of inflammation (Ubaida-Mohien *et al.*, 2019).

Regular physical exercise induces cellular mechanisms in skeletal muscle, promoting adaptation to increased ROS production through redox signalling regulation and antioxidant defence mechanisms (Radak *et al.*, 2013, 2019). While acute intensive exercise induces ROS production that leads to oxidative stress and cellular damage, regular physical exericse results in adaptations inducing antioxidant defence and improvements in redox signalling to protect cells against stress-related diseases as well as delaying age-associated changes (Radak, Chung and Goto, 2005; Thirupathi, Pinho and Chang, 2020). Physical exercise might have similar protective effects against ER stress in skeletal muscle, promoting the adaptation response (Bohnert, McMillan and Kumar, 2018). One bout of intensive exercise can induce ER stress, activation of the UPR and ER stress-induced

apoptosis, whereas regular physical exercise causes only increased levels of ER chaperones and is considered to represent adaptation mechanisms in skeletal muscle (Wu *et al.*, 2011; Estébanez *et al.*, 2018). Moreover, regular physical exercise induces the production of HSPs in skeletal muscles of young and adult individuals, leading to quick adaptation and protection against cellular stresses, while aging causes an insufficient production of HSPs in skeletal muscle, leading to impairment in adaptation and a general failure to respond to stress (Castrogiovanni and Imbesi, 2012). Hence, the increased ROS production in skeletal muscle in response to physical exercise has an important role in promoting adaptation processes, which in turn can be crucial in the ability of the muscle to recover from age-related ROS induced redox dysregulation and cellular stresses (McArdle, Vasilaki and Jackson, 2002). However, there are rather few studies investigating the effect of long-term physical exercise on redox state, HSP system and ER stress in skeletal muscle.

2.5.2.2 Brain tissue

Although numerous epidemiological and experimental studies have demonstrated that regular physical exercise has beneficial effects on the brain at both the structural and functional levels, and to decrease the risk of AD and other age-related diseases (for reviews see (Hamer and Chida, 2009; Brown, Peiffer and Martins, 2013; Guiney and Machado, 2013)), it is important to investigate the mechanisms underlying exercise-induced positive effects. It has been shown in many studies that regular aerobic exercise protects against age-related brain atrophy and increases hippocampal volume in aged individuals (Colcombe *et al.*, 2003; Gordon *et al.*, 2008; Erickson *et al.*, 2011) and AD patients (Burns *et al.*, 2008). These structural alterations in the brain are associated with an exercise-induced improvement in cognitive function and memory (Erickson *et al.*, 2011; Jia *et al.*, 2019). Moreover, physical exercise exerts beneficial effects on executive function among the older population (Barha *et al.*, 2017) and AD patients (Guitar *et al.*, 2018). Similar to the research on humans, rodent studies shown that physical exercise induces positive structural brain changes, improves cognitive function and reduces the memory decline in aged rodents (Van Praag *et al.*, 2005; Kim *et al.*, 2010; Cahill *et al.*, 2015; Xu *et al.*, 2017) and AD transgenic mice (Yuede *et al.*, 2009; García-Mesa *et al.*, 2011). Because the formation of Aβ plaques is one of the major characteristics of AD, several studies have investigated the possible effect of physical exercise to evoke a reduction of plaque deposits. In some studies, it has been reported that regular physical exercise can induce a lowering of the Aβ

plaque load in the hippocampi of AD mice (Adlard *et al.*, 2005; Yuede *et al.*, 2009; Tapia-Rojas *et al.*, 2016; Zhang *et al.*, 2018).

Among the mechanisms that underlie the behavioural improvement linked with exercise, are increased neurogenesis, synaptic plasticity and elevated levels of neurotrophins and growth factors in hippocampus, the brain area associated with cognitive and memory function. Rodent studies have demonstrated that voluntary wheel running is able to reduce age-related declines in the numbers of newborn neurons in DG of the hippocampus of aged animals (Van Praag *et al.*, 2005; Kronenberg *et al.*, 2006) and AD mice (Tapia-Rojas *et al.*, 2016; Choi *et al.*, 2018). In addition to animal studies, it has been shown that the degree of exercise-induced neurogenesis correlates with cerebral blood volume, which significantly increases in the DG of humans after aerobic exercise (Pereira *et al.*, 2007). Voluntary wheel running affects synaptic plasticity by inducing increases in hippocampal LTP in rodents (Van Praag *et al.*, 1999; Farmer *et al.*, 2004). In fact, exercise-induced increases in LTP are more pronounced after a long-term period of voluntary running exercise as compared to a short-term duration (Patten *et al.*, 2013). Moreover, voluntary running stimulates increases of dendritic spine density, dendritic spine length and synaptic proteins in hippocampus and the cortex of healthy and AD rodents, which could lead to enhanced synaptic plasticity and memory (Revilla *et al.*, 2014; Brockett, LaMarca and Gould, 2015; Xu *et al.*, 2017). In animal studies, physical exercise was also demonstrated to enhance the expression of BDNF (Neeper *et al.*, 1995; Farmer *et al.*, 2004; Kim *et al.*, 2010) and insulin growth factor (Carro *et al.*, 2000) in the brain, whereas in human studies, regular exercise increased BNDF levels in blood serum and plasma (Erickson *et al.*, 2011; Szuhany, Bugatti and Otto, 2015). In AD studies, physical exercise rescued the disease-associated BDNF reduction in mice hippocampi when compared to sedentary control animals (Choi *et al.*, 2018), although based on a recent human study, 2 months of exercise had no effect on BDNF plasma levels in AD patients (Enette *et al.*, 2020). Thus, physical exercise could change the level of neurotrophic and growth factors in the brain, which are considered to be mediators of the exercise-induced beneficial effects on angiogenesis, neurogenesis, neuronal survival and synaptic plasticity (Cotman, Berchtold and Christie, 2007; Rothman *et al.*, 2012; Vecchio *et al.*, 2018).

In addition to the above mechanisms, physical exercises exert anti-inflammatory effects and modulate glial cell activation; they can act on the peripheral immune system and the inflammation status in the brain, decreasing the levels of pro- and increasing the levels of anti-inflammatory cytokines (Kelly,

2018), decreasing ROS levels and increasing levels of antioxidant enzymes (Marosi *et al.*, 2012). Rodent studies have shown that physical exercise was able to reduce the levels of pro-inflammatory cytokines in aged (Speisman *et al.*, 2013) and AD animals (Nichol *et al.*, 2008; Zhang *et al.*, 2019). Based on recent systematic reviews, it is believed that physical exercise decreases the levels of inflammatory cytokines in healthy individuals, with the most profound anti-inflammatory effect being encountered in older individuals after high-intensity aerobic exercise (Cronin *et al.*, 2017; Zheng *et al.*, 2019). Focusing on glial cells, it was found that physical exercise can induce GFAP-positive astrocyte density increases and morphological changes, including an enhancement of astrocyte complexity and an increase in glial cell area in healthy rodents (Viola *et al.*, 2009; Saur *et al.*, 2014; Brockett, LaMarca and Gould, 2015; Lundquist *et al.*, 2019) and AD mice (Rodríguez *et al.*, 2013; Rodríguez, Noristani and Verkhratsky, 2015). In different AD mouse studies, physical exercise was shown to induce different effects on astrocyte (Tapia-Rojas *et al.*, 2016; Hüttenrauch *et al.*, 2017; Zhang *et al.*, 2018) and microglia activation (Ziegler-Waldkirch *et al.*, 2018; Svensson *et al.*, 2020), and thus there is no doubt that these cells are affected by exercise but more studies are needed to reveal their role in mediating the beneficial effects of exercise.

2.5.2.3 Crosstalk between skeletal muscle and brain tissue

Physical exercise has effects on the whole body, and its effects on the brain may be regulated by several different pathways and by other organ systems (Stillman *et al.*, 2020). Exercise-induced factors with beneficial effects on the brain can be divided into those that are produced in the brain itself and those, which are secreted from the periphery, for example from skeletal muscle, circulate in the blood and subsequently cross the BBB (Young, Valaris and Wrann, 2019). Contracting skeletal muscle releases bioactive factors, known as myokines, which regulate the communication between skeletal muscle and other organs thereby inducing functional changes and mediating the effects of exercise (Huh, 2018; W. Chen *et al.*, 2021). Interacting with different tissues, myokine functions are diverse including adaptation to training, regulation of glucose homeostasis, lipid metabolism, angiogenesis, and the immune response (W. Chen *et al.*, 2021). Certain myokines can cross the BBB and regulate brain function, learning and memory (Delezie and Handschin, 2018; Pedersen, 2019).

As was mentioned above, physical exercise increases the levels of BDNF in the hippocampus and plasma of healthy and AD individuals (Rasmussen *et al.*, 2009; Kim *et al.*, 2010; Erickson *et al.*, 2011), but the mechanism is still unknown.

Although the BNDF level in skeletal muscle is also elevated after exercise, muscle-derived BDNF levels are not changed in the whole body circulation (Matthews *et al.*, 2009). Thus, the link between muscle-derived BDNF and the brain level of this neurotrophic factor due to exercise remains unclear. One of the important regulators of BDNF expression in the brain is irisin, a protein-hormone, a proteolytically cleaved form of fibronectin type III domains containing protein 5 (FNDC5) (Wrann *et al.*, 2013). Irisin is classified as a myokine that is induced by exercise in skeletal muscle through the transcriptional coactivator PGC-1α-dependent pathway (Boström *et al.*, 2012). It can cross the BBB and regulate cognitive function (Lourenco *et al.*, 2019). It has been demonstrated that in the response to exercise, the levels of FNDC5 and PGC-1 were elevated in skeletal muscle and hippocampus (Wrann *et al.*, 2013; Tiano, Springer and Rane, 2015; Belviranlı and Okudan, 2018; Choi *et al.*, 2018), although in some studies the levels of these proteins remained unchanged after exercise (Pekkala *et al.*, 2013; Kurdiova *et al.*, 2014). A recent study demonstrated that exercise-induced FNDC5/irisin increased rescue synaptic plasticity and prevented memory deficits (Lourenco *et al.*, 2019), and conteracted inflammation and oxidative stress (Joro *et al.*, 2020) suggesting that FNDC5/irisin was a potential mediator of the beneficial effects of exercise.

Cathepsin B is another potential exercise-induced mediator between contracting skeletal muscle and the brain; it is a lysosomal cysteine protease degrading intracellular peptide and protein (Turk *et al.*, 2012). In AD, cathepsin B has been associated with Aβ plaques, participating in Aβ clearance and thus demonstrating neuroprotective and anti-amyloidogenic properties (Mueller-Steiner *et al.*, 2006; Wang *et al.*, 2012). Physical exercise was reported to induce increases of cathepsin B in plasma, skeletal muscle and hippocampus together with enhancing adult neurogenesis in mice. In humans, an exercise-induced increase in cathepsin B level in plasma positively correlated with the hippocampal-dependent memory improvement (Moon *et al.*, 2016). Although a recent study of AD mice failed to demonstrate elevations of cathepsin B in muscle after exercise (Pena *et al.*, 2020), more studies are needed to clarify the involvement of cathepsin B in the brain-muscle crosstalk in response to exercise in AD.

Elevated levels of interleukin 6 (IL-6) in cerebospinal fluid (CSF) and serum have been associated with chronic inflammation and neurodegenerative diseases, including AD (Hampel *et al.*, 2005). Contracting skeletal muscle can regulate the immune response throughout the whole body via cytokine secretion, and may act in an anti-inflammatory manner (Z. Chen *et al.*, 2021). IL-6 is a myokine secreted

upon exercise, and muscle-derived IL-6 participates in glucose and lipid metabolism and it stimulates the production of anti-inflammatory cytokines IL-1ra and IL-10 (Steensberg *et al.*, 2001; Schnyder and Handschin, 2015). After exercise, the IL-6 level was highly elevated in skeletal muscle and plasma, but returned to basal levels during long-term training (Joro *et al.*, 2017; Pedersen, 2019). There was a negative correlation between the amount of regular exercise and resting plasma IL-6 levels: the more the physical activity, the lower was the basal plasma IL-6 level (T. H. Y. Lee *et al.*, 2019). IL-6 can also cross the BBB (Marsland *et al.*, 2015), implying that it may be involved in the potential crosstalk between the muscle and brain. Regular exercise was reported to reduce IL-6 basal levels in plasma and IL-6 expression in the brain of healthy rats (Chennaoui *et al.*, 2015), but IL-6 it has still been poorly investigated as a potential exercise-induced mediator in the context of AD.

Skeletal muscle stores 10-15% of the total body iron, which is contained in myoglobin and is important for skeletal muscle oxidative capacity and muscle growth during exercise (Buratti *et al.*, 2015). Physical exercise was able to reduce total body iron (Ziolkowski *et al.*, 2014) and exerted an impact on iron homeostasis in different tissues (Antosiewicz *et al.*, 2015; Ghio *et al.*, 2020). There is evidence that skeletal muscle plays an important role in the body iron metabolism in a hepcidin-dependent manner (Buratti *et al.*, 2015). Hepcidin is a hormone that regulates body iron homeostasis, and hepcidin expression is controlled by IL-6 during inflammation (Nemeth, Rivera, *et al.*, 2004). Considering that IL-6 is a myokine involved in the anti-inflammatory exercise effect and regulation of iron metabolism, iron seems to be involved in the crosstalk between brain and skeletal muscle upon physical exercise. With regards to AD, targeting iron overload and systemic iron availability through IL-6 and IL-6-related pathways could be considered as a potential therapeutic strategy (Crielaard, Lammers and Rivella, 2017). To date, only a few investigators have demonstrated that exercise was able to ameliorate total body (J.-Y. Lee *et al.*, 2019) and the brain (Choi *et al.*, 2021) iron level in exercised AD mice. Thus, exercise may impact on iron metabolism in the context of AD but the possible crosstalk between brain and periphery needs further investigation.

3 AIMS OF THE STUDY

We hypothesize that the beneficial effects of physical exercise involve a regulation of redox state and the stress responses occurring in aging skeletal muscle. Moreover, the beneficial exercise effects can be mediated by the interplay between the periphery and the brain. Furthermore, we hypothesize that astrocytes are an important cell type responding to physical exercise in the AD brain. The overarching goal of this study is to provide new understandings on the beneficial mechanisms induced by physical exercise both in the brain and periphery, in the context of aging and AD. The specific aims are:

1. To investigate oxidative and ER stress responses in aging and the potential protective effect of life-long physical exercise on these processes.

2. To study the role of astrocytes in the beneficial brain effects of exercise in a mouse model of AD.

3. To explore the exercise-induced interplay between brain and periphery via iron metabolism regulation and IL-6 in mice and to associate these with AD.

4 SUBJECTS AND METHODS

4.1 ANIMALS AND EXPERIMENTAL DESIGN (I-III)

In study I, male ICR mice (CLEA Japan Inc., Tokyo, Japan) were used, and at 3 months of age they were randomly divided into three groups: young (Y) n = 12, old sedentary (OS) n = 5, and old exercised (OE) n = 5. In study II and III, male wild-type (WT) JAXC57BL/6J mice and transgenic 5xFAD mice with mutations in hAPP (Swe, Flo, Lon) and PSEN1 (M146L, L256V) under the Thy-1 promoter (Oakley *et al.*, 2006) were used. Mice from study II and III at 6 weeks of age were divided into four groups: WT-sedentary (WT-SED) n = 21, WT-exercised (WT-EXE) n = 20, 5xFAD-sedentary (5xFAD-SED) n = 21, and 5xFAD-exercised (5xFAD-EXE) n = 20. Mice in all three studies were maintained under the following conditions: individual cages, 12 hours light/dark cycle, controlled temperature and humidity, and *ad libitum* access to food and water.

In all studies, the voluntary running protocol was set for the mice from exercised groups which had free access to a running wheel (study I – diameter 20 cm, Shinano Instruments, Tokyo, Japan; study II – diameter 24 cm, Techniplast, Italy) installed in each individual cage. In study I, OE mice had access to the running wheel for 21 months. In studies II and III, WT-EXE and 5xFAD-EXE mice had access to the running wheel for 6 months (Figure 3). The running distance and time for each mouse was recorded on a weekly basis with running counters (Sigma, Germany) installed in each cage. In addition, the weights of the mice were monitored weekly in studies II and III.

All mouse experiments in study I were carried out in accordance with Tohoku Institute of Technology guidelines for the Care and Use of Laboratory Animals and approved by the University Animal Experiment Committee. Studies II and II were conducted in accordance with the Council of Europe Legislation and Regulation for Animal Protection and approved by the National Animal Experiment Board of Finland.

4.2 BEHAVIORAL TESTING (II)

Mice used for all testings were 7 months old (Figure 3). Below is a brief description of the tests used. For a full description, please see Study II.

4.2.1 Nest building test

The nest building test allows to assess basic rodent activity related to hippocampal functioning (Deacon, Croucher and Rawlins, 2002). A paper towel was placed inside the home cage of each mouse for 24 hours. The built nests were scored with the following points: 0 point – untouched nest, 1 – flat nest with few bites, 2 – creased/moved and bitten nest, 3 – creased and bitten nest forming a crater inside the original nesting material.

4.2.2 Open field test

The open field test was used to assess exploration activity vs. anxiety of mice (Minkeviciene, Banerjee and Tanila, 2008). The mouse was placed in a circular box (diameter 120 cm, height 22 cm), facing a wall, and allowed to freely explore the area inside the box for 10 min. The behavioral parameters, including the duration in the center zone, frequency of entries into the center zone, and latency to first entry in the center zone, were analyzed using EthoVision XT 7.1 video tracking software (Noldus Information Technologies, Wageningen, The Netherlands).

4.2.3 Elevated plus-maze test

The elevated plus-maze test was used to assess the exploration vs. anxiety level of mice (Minkeviciene, Banerjee and Tanila, 2008). The mouse was placed in the center of the plus-maze (two open arms without walls and two closed arms) facing the closed arm, and allowed to freely explore through the maze for 5 min. Then behavioral parameters including duration in the open arms and frequency of entries into the open arms were analyzed using EthoVision XT 7.1 video tracking software (Noldus Information Technologies, Wageningen, The Netherlands).

4.2.4 Morris Water Maze (MWM) test

The Morris Water Maze (MWM) test evaluates hippocampal-dependent spatial learning and memory (Malm *et al.*, 2007). Briefly, during the test, the mouse seeks a submerged platform inside a circular pool. The test consisted of acquisition and probe phases. In the acquisition phases, the mouse was placed inside the pool facing the wall, and the trial continued until the mouse reached the hidden platform, but no longer than 60 s if the mouse was not able to find the platform. In the probe phase, the mouse was placed inside the swimming pool without the escape platform for 60 s to assess the search bias as an indication of memory.

Parameters including speed, escape latency, time in the wall zone for acquisition trials, as well as latency and distance to platform zone (previous platform location) for the probe phase were analyzed using EthoVision XT 7.1 video tracking software (Noldus Information Technologies, Wageningen, The Netherlands).

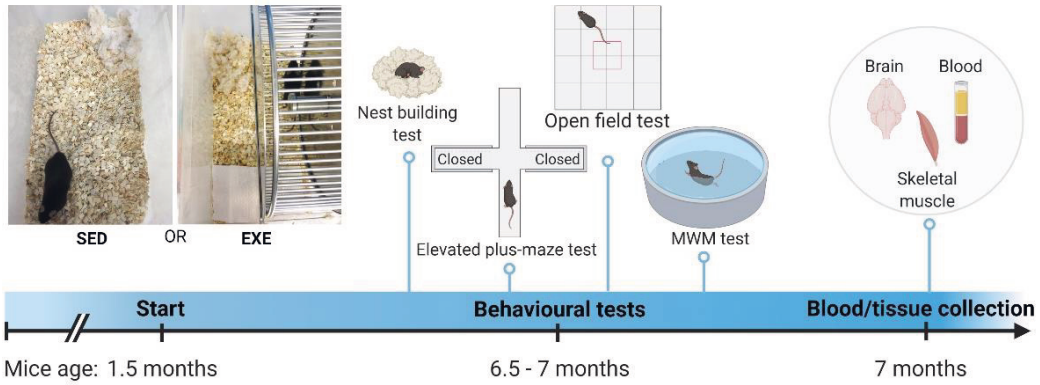

Figure 3. Housing conditions and behavioural tests timeline for mice in Study II-III. At 1.5 months WT and 5xFAD mice were transferred to either standard cage (SED) or cage with running wheel (EXE). Between 6.5 and 7 months of age behavioural tests were conducted. Two days after last running session, mice were sacrificed, and blood/tissues were collected. Created with BioRender.com.

4.3 TISSUE COLLECTION AND SAMPLE PREPARATION (I-III)

The mice from exercised groups were sacrificed two days after the running wheels were removed from their cages to avoid any effects of acute exercise.

In study I, mice were sacrificed with an overdose of pentobarbital sodium at the age of 3 months (Y mice) and 24 months (OS and OE mice), and TA and soleus muscles were collected, snap frozen in liquid nitrogen, and stored at -80 °C until analyses were performed.

In studies II and III, at the age of 7 months, mice were anesthetized with tribromoethanol (Sigma-Aldrich, St. Louis, MO, USA), blood was collected, and then mice were transcardially perfused with heparinized saline before the dissection of the tissues. The collected blood together with 3.8 % sodium citrate anticoagulant was centrifuged at 2000 x g for 6 min at +4 °C, and plasma supernatants were additionally centrifuged at 12000 x g for 3 min at +4 °C. Final plasma samples were snap frozen in liquid nitrogen and stored at -70 °C for further use. The left brain hemispheres were fixed with 4% PFA in 0.1 M phosphate buffer (pH 7.4) for 22 h,

then incubated in 30 % sucrose solution for 48 h, snap frozen in liquid nitrogen, and stored in -70 °C until cryosectioning. Cortices and hippocampi from the brain right hemisphere and the gastrocnemius (GAS) skeletal muscles were isolated, snap frozen in liquid nitrogen, and stored at -70 °C until further use.

4.4 QUANTITATIVE REAL-TIME PCR (RT-PCR, II, III)

Total RNA was isolated from brain (see Study II, III) and muscle homogenates (see Study III) with TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol (see Study II). The concentration and purity of RNA were determined by using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Genomic DNA was removed from all RNA samples by using DNase I, RNase-free kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Reverse transcription was performed for 1 µg of RNA with High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). Relative mRNA expression of target genes was measured by quantitative RT-PCR (StepOnePlus Real-Time PCR System, Thermo Fisher Scientific, Waltham, MA, USA) using TaqMan gene expression assays (Thermo Fisher Scientific, Waltham, MA, USA) listed in Study II and Study III. Results were normalized to the GAPDH endogenous control.

4.5 WESTERN BLOT (WB, I-III)

Cytosolic proteins were isolated from the brain (see Study II) and muscle homogenates (see Study I and III). Protein concentrations were determined by using the Pierce 660 nm Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) for brain samples and by using the BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) for muscle samples. Target protein levels from brain and muscle samples were determined by using WB; full procedures are described in Studies I and II. Briefly, proteins were separated in 12% SDS-PAGE, transferred onto PVDF (GE Healthcare, Chicago, IL, USA) or nitrocellulose (Millipore, Burlington, MA, USA) membranes, blocked in 5% fat-free milk and incubated overnight with the antibodies listed in Table 4 at +4 °C. Then, membranes were washed in PBST or TBST and incubated with the corresponding secondary antibodies at room temperature. In Study I, proteins were visualized and quantified with Odyssey Imaging System (LI-COR Biosciences Inc., Lincoln, NB, USA), whereas in Studies II and III, proteins were detected with BioRad ChemiDocTM Imaging System and quantified by using ImageLab software (BioRad, Hercules, CA, USA). GAPDH or

Ponceau S staining (for muscle protein samples) and β-actin (for brain protein samples) were used for results normalization.

4.6 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA, III)

HAMP mouse ELISA kit (Aviva Systems Biology, San Diego, CA, USA) was used to assess the hepcidin concentration in cortical protein samples according to the manufacturer's instructions. Absorbance of the samples was read at 450 nm with a Wallac Victor 1420 microplate reader (Perkin Elmer, Waltham, MA, USA). The results were normalized by the total protein concentration.

4.7 CYTOKINE BEAD ARRAY (CBA, II, III)

The cytokine bead array (CBA) mouse inflammation kit (BD Biosciences, Franklin Lakes, NJ, USA) was used to analyze IL-6, IL-10, MCP-1, IL12p70, IFNγ and TNFα cytokine levels in brain protein, muscle protein and plasma samples according to manufacturer's instructions. Samples were run using a CytoFlex S flow cytometer (Beckman Coulter, Brea, CA, USA), and acquired data were exported and analyzed by FCAP Array v3.0 software (Soft Flow Inc., Pcs, Hungary). The results were normalized according to the total protein concentration.

4.8 INDUCTIVELY COUPLED PLASMA-MASS SPECTROMETRY (ICP-MS, III)

In Study III, ICP-MS was used to assess the iron content, and samples for the analysis were prepared from mouse brain cortices and GAS skeletal muscles (see Study III). Measurements for cortical samples were made using an Agilent 7700x series ICP-MS instrument as reported previously (Choo *et al.*, 2018). Measurements for muscle samples were performed using 'microdroplet' laser ablation-ICP-MS (LA-ICP-MS) via a NWR-213 laser ablation unit (Electro Scientific Industries, Portland, OR, USA) linked to an Agilent 8800 ICP-QQQ-MS, and analyzed using Iolite software as described previously (Kysenius *et al.*, 2019). Iron content was normalized within samples using a multielement control (Mg, P, K for cortex; C, P for muscle).

4.9 IMMUNOHISTOCHEMISTRY (IHC, II, III)

Serial 20 µm sagittal brain sections, each 400 µm apart, were cut using a cryostat (Leica Microsystems, Wetzlar, Germany), and stored in anti-freeze solution at -20 °C before staining. Three sections per mouse were collected on Superfrost microscope slides (Thermo Fisher Scientific, Waltham, MA, USA), washed in PBS / PBST, incubated in 10mM sodium citrate buffer at +95°C for antigen retrieval, washed again and blocked in 10% NGS. After blocking, the sections were incubated in primary antibodies overnight at room temperature (see Table 4). Sections were washed, incubated in the corresponding fluorescent secondary antibodies for 2 hours at room temperature, washed, air dried and mounted with Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA). In the analysis conducted in Study II, hippocampal areas from the brain sections were imaged with 10x or 20x magnification on Zeiss Axio Imager M2 using a digital camera (Axiocam, Zeiss, Jena, Germany) and ZEN software. In Study III for the analysis, cortical and hippocampal areas from the brain sections were imaged with 10x magnification on Leica Thunder Imager 3D tissue Slide scanner (Leica Microsystems, Wetzlar, Germany). Immunoreactivity was quantified using the ImageJ software (National Institute of Health, Bathesda, MD, USA). The percentages of positively-stained area in the hippocampus, subiculum or cortex were measured for each section, and the average from three sections per mouse was reported.

Table 4. Antibodies used in Studies I-III

Antibody	Catalog number	Company	Application, dilution
HSP25, rabbit	SPA-801	Enzo Life Sciences Inc., USA	WB, 1:1000
HSP60, mouse	SPA-806	Enzo Life Sciences Inc., USA	WB, 1:500
HSP70, mouse	SPA-810	Enzo Life Sciences Inc., USA	WB, 1:1000
HSC70, rat	SPA-815	Enzo Life Sciences Inc., USA	WB, 1:1000
HSP90, rat	SPA-835	Enzo Life Sciences Inc., USA	WB, 1:1000
GRP78, rabbit	SPA-826	Enzo Life Sciences Inc., USA	WB, 1:500
GRP75, mouse	SPA-825	Enzo Life Sciences Inc., USA	WB, 1:1000
TRX-1, rabbit	ATRX-06	IMCO Corp, Sweden	WB, 1:500
TxNiP, mouse	K0205-3	MBL Co. Ltd, Japan	WB, 1:200
4-HNE, rabbit	HNE11-S	Alpha Diagnostic IntI Inc., USA	WB, 1:500
CHOP, mouse	L63F7	Cell Signaling Technology, USA	WB, 1:300

PDI, rabbit	C81H6	Cell Signaling Technology, USA	WB, 1:1000
GAPDH, rabbit	sc-25778	Santa Cruz Biotechnology, Inc., USA	WB, 1:500
Goat anti-rabbit IgG, DyLight 800	35571	Invitrogen, USA	WB, 1:25000
Goat anti-mouse IgG, DyLight 800	35521	Invitrogen, USA	WB, 1:10000
Goat anti-rat IgG, Alexa Fluor 680	A-21096	Invitrogen, USA	WB, 1:10000
Amyloid β,clone WO-2, mouse	MABN10	Millipore, USA	IHC, 1:1000
Iba-1, rabbit	019-19741	Wako, Japan	IHC, 1:250
GFAP, rabbit	Z033429-2	Dako, Denmark	IHC, 1:500 WB, 1:1000
GFAP, chicken	ab4674	Abcam, UK	IHC, 1:2500
S100β, rabbit	ab52642	Abcam, UK	IHC, 1:200 WB, 1:1000
GS, rabbit	ab73593	Abcam, UK	WB, 1:5000
ALDH1L1, rabbit	ab87117	Abcam, UK	WB, 1:1000
NeuN, rabbit	711054	Invitrogen, USA	IHC, 1:200
DCX, rabbit	4604	Cell Signaling Technology, USA	IHC, 1:200
BDNF, rabbit	SAB2108004	Sigma-Aldrich, USA	WB, 1:1000
BDNF, rabbit	ab108319	Abcam, UK	IHC, 1:150
Synaptophysin, rabbit	MA5-14532	Invitrogen, USA	WB, 1:200
PSD95, rabbit	3450	Cell Signaling Technology, USA	WB, 1:1000
Ferritin, rabbit	ab75973	Abcam, UK	IHC, 1:200 WB, 1:1000
TfR, rabbit	ab84036	Abcam, UK	IHC, 1:200 WB, 1:1000
DMT1, rabbit	ABS983	Sigma-Aldrich, USA	WB, 1:1000
Ferroportin	PA5-22993	Invitrogen, USA	WB, 1:1000
β-actin, mouse	A5441	Sigma-Aldrich, USA	WB, 1:5000
Goat anti-rabbit IgG, Alexa Fluor 488	A11008	Thermo Fisher Scientific, USA	IHC, 1:200, 1:250
Goat anti-mouse IgG, Alexa Fluor 568	A11004	Thermo Fisher Scientific, USA	IHC, 1:500
Goat anti-chicken IgG, Alexa Fluor 568	A11041	Thermo Fisher Scientific, USA	IHC, 1:250
Goat anti-rabbit IgG-HRP conjugate	170-65-15	BioRad, USA	WB, 1:3000
Donkey anti-mouse IgG-Cy5 conjugate	715-175-151	Jackson Immuno Res. Lab., USA	WB, 1:1000

4.10 COLOCALIZATION ANALYSIS FOR BDNF AND GFAP (II)

In Study II, colocalization analysis of double staining for BDNF and GFAP was performed for mouse brain sections. All procedures related to brain sections and IHC were the same as described in section 4.9.

In the correlation analysis, the hippocampal area was imaged with 40x magnification on a Zeiss Axio Observer inverted microscope with LSM800 confocal module (Zeiss, Jena, Germany) and ZEN software. Images from two channels ("red" GFAP and "green" BDNF) were converted to an 8-bit format to estimate the correlation of two proteins using JACoP plugin in ImageJ software (National Institute of Health, Bathesda, MD, USA); the JACoP plugin generates the following parameters: the Pearson's correlation coefficient (r; degree of correlation between two colors), overlap coefficient (R) and Mander's colocalization coefficient (MOC; the proportion of green pixels in the red channel).

In the quantitative colocalization analysis, hippocampal areas were imaged with 10x magnification in two channels (GFAP and BDNF) on Zeiss Axio Imager M2 using a digital camera (Axiocam, Zeiss, Jena, Germany) and ZEN software. Areas covered by "green" BDNF pixels were quantified in "red" GFAP-positive astrocytes pixels by Apoptosis correlator plugin in ImageJ software (National Institute of Health, Bathesda, MD, USA) for each section, and the average from three sections per mouse was reported. The threshold was chosen for each channel and the total numbers of overlapped pixels in both channels were calculated and normalized by the total number of pixels, as previously described (Sibarov *et al.*, 2012; Fahimi *et al.*, 2017).

4.11 MORPHOLOGICAL ANALYSIS OF ASTROCYTES (II)

In Study II, counting of astrocytes and morphological analysis of GFAP-positive astrocytes were performed on the same sections used for IHC in section 4.9. In addition, double staining for GFAP and Aβ was performed on 5xFAD mouse brain sections to measure astrocyte morphology depending on Aβ plaque proximity. All procedures related to brain sections and IHC were the same as described in section 4.9. Hippocampal areas were imaged with 20x magnification on Zeiss Axio Imager M2 using a digital camera (Axiocam, Zeiss, Jena, Germany) and ZEN software. Quantitative analysis of morphological parameters for GFAP-positive astrocytes and the number of astrocytes (GFAP+ and S100β+) in hippocampal areas were performed using digital modeling based on the MicroTrac analysis platform (Abdolhoseini, Walker and Johnson, 2016). Those cells that could not be

reconstructed correctly by the platform were manually removed from the analysis. Examples of images and representative GFAP-positive cells from each group of mice are presented in Study II, Figure 5A.

4.12 STATISTICAL ANALYSES

All the data were analyzed in a blinded manner with respect to the experimental groups. Statistical analyses were performed with GraphPad Prism 8.4.2 software (GraphPad Software Inc., San Diego, CA, USA) and SPSS 21.0 and 25.0 software (IBM, Armonk, NY, USA) using statistical tests indicated in the legends to the figure or in the descriptions of the results. All data are expressed as the mean ± SEM, and the level of significance was set at $p < 0.05$. The statistical outliers were determined using Grubbs' test and removed from the analysis.

5 RESULTS

5.1 LONG-TERM VOLUNTARY EXERCISE IS BENEFICIAL AGAINST CELLULAR STRESS IN AGED MICE (I)

In study I, we tested the hypothesis that long-term voluntary exercise would have protective effects against age-related disruption of redox state and chaperone function, increased ER stress and ER stress-related apoptosis in skeletal muscle. For this purpose, we measured key molecular markers of redox state, HSP response and ER stress in skeletal muscles of old sedentary (OS) and old long-term voluntary exercised mice (OE) and compared it to young (Y) mice. In addition, a correlation analysis was performed between all of the studied markers. In order to investigate possible exercise-induced fiber-specific changes, two different types of skeletal muscle were used in this study: soleus, mostly composed of slow-twitch aerobic muscle fibers, and tibialis anterior (TA), mostly composed of fast-twitch glycolytic muscle fibers. The results of Study I are summarized in Figure 4.

5.1.1 Long-term exercise restores age-related alterations in redox status in skeletal muscle

To investigate possible protective effects of long-term voluntary exercise on the age-related disruption of the redox system by increased oxidative stress, WB analysis was perfomed for TRX-1 and TxNiP, components of the TRX system, and 4-hydroxy-2-nonenal (4-HNE) adduct, a marker of lipid peroxidation, in soleus and TA skeletal muscles of mice. Aging induced an increase in the amounts of TRX-1 and TxNiP ($p < 0.01$; Study I, Figure 1) levels in both types of skeletal muscles in OS mice in comparison with Y mice. In particular, the TRX-1/TxNiP ratio was significantly lower in skeletal muscles of OS in comparison to Y mice ($p < 0.05$; Study I, Figure 1). No significant age-related changes in the 4-HNE adduct level were detected in skeletal muscle of OS mice when compared to Y mice. Long-term voluntary exercise remarkably reduced the age-related TxNiP rise in TA and soleus skeletal muscles ($p < 0.05$) of OE mice in comparison with OS mice, together with increasing the TRX-1/TxNip ratio (ns; Study I, Figure 1). Moreover, the level of the 4-HNE adduct was significantly lower in TA skeletal muscle of OE mice in comparison to OS mice ($p < 0.05$; Study I, Table 1).

5.1.2 Long-term exercise alters the expression of HSPs in skeletal muscle of old mice

Next, the effect of long-term voluntary physical exercise on the age-induced impairment in chaperone function was evaluated. WB was performed to measure the protein level of mitochondrial chaperones, such as GRP75 and HSP60, stress-inducible protein HSP70, and constitutive heat shock cognate 70 (HSC70) protein in skeletal muscles of mice. Aging induced a decline in the protein level of GRP75 ($p < 0.05$), HSC70 ($p < 0.05$; Study I, Figure 2) and HSP60 ($p = 0.07$; Study I, Table 1) by a third in soleus skeletal muscle of OS mice in comparison with young control mice, whereas in TA skeletal muscle, the levels of measured chaperones remained unchanged. Long-term voluntary physical exercise induced a significant upregulation of HSP70 ($p < 0.05$) and HSC70 ($p < 0.01$; Study I, Figure 2) in soleus but not in TA skeletal muscle of old mice. While physical exercise did not affect GRP75 and HSP60 levels in soleus skeletal muscle of old mice, in TA skeletal muscle, the level of GRP75 was remarkably increased after prolonged physical exercise ($p < 0.01$; Study I, Figure 2), together with a slight rise in HSP60 ($p = 0.10$; Study I, Table 1).

5.1.3 Long-term exercise affects ER Stress and UPR in skeletal muscle of old mice

To evaluate the effect of aging and long-term voluntary physical exercise on the cellular stress state, protein levels of several ER stress and UPR markers were measured in skeletal muscles of mice by WB. A significant increase in the level of GRP78 chaperone ($p < 0.05$; Study I, Figure 3) was detected in TA muscles of OS when compared to Y mice, with no GRP78 level changes in soleus muscle. Moreover, a dramatic upregulation of the ER stress-related apoptotic marker CHOP was observed in both TA ($p < 0.01$) and soleus ($p < 0.001$; Study I, Figure 3) skeletal muscles of OS mice in comparison to Y mice. While the GRP78 level was unaltered by exercise, the level of CHOP was significantly decreased after long-term physical exercise in soleus muscles of old mice ($p < 0.05$; Study I, Figure 3).

5.1.4 Correlation between ER stress and redox regulation markers in skeletal muscle

A correlation analysis between the measured ER stress and redox state markers was performed to evaluate the possible link between these two systems in skeletal muscle. A strong positive correlation was observed between the level of GRP78

and TRX-1 in TA ($r = 0.48$, $p < 0.05$) and soleus skeletal muscles ($r = 0.73$, $p < 0.001$; Study I, Table 2). In addition, the analysis revealed a significant positive correlation between the levels of CHOP and TxNiP in TA ($r = 0.79$, $p < 0.001$) and soleus skeletal muscles ($r = 0.63$, $p < 0.01$) together with a negative correlation between CHOP and TRX-1/TxNiP ratio in TA ($r = – 0.58$, $p < 0.01$) and soleus skeletal muscles ($r =– 0.69$, $p < 0.001$; Study I, Table 2).

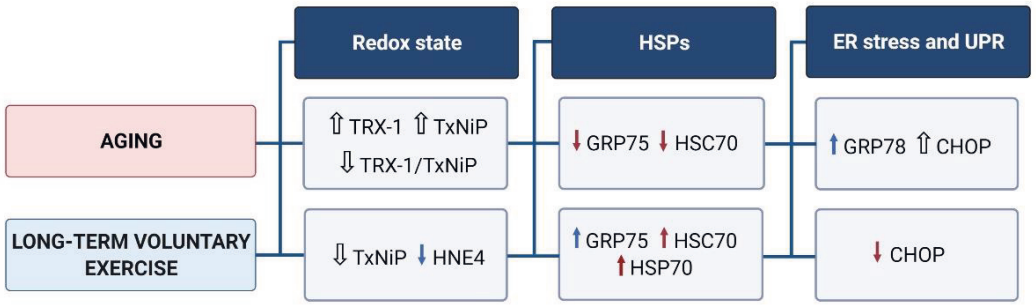

Figure 4. Effect of aging and long-term voluntary exercise on cellular stresses. White arrows represent changes in both types of skeletal muscles – soleus and T.A. Red arrows represent changes only in soleus, blue arrows – only in T.A. skeletal muscle. Created with BioRender.com.

5.2 LONG-TERM VOLUNTARY EXERCISE HAS BENEFICIAL EFFECTS IN AD MICE THROUGH ASTROCYTE REMODELING (II)

Study II was performed to investigate potential beneficial effects of long-term voluntary physical exercise in WT mice and a transgenic 5xFAD mouse model with respect to astrocytic modulation. A wide range of analyses were carried out, including behavioral testing to examine the effects of exercise on cognition and memory, and biochemical, histological and morphological assays to assess whether the exercise could influence the pathological features associated with AD, and the possible involvement of astrocytes in these processes. The main results of Study II are illustrated in Figure 5.

5.2.1 Long-term exercise reverses cognitive and memory impairments in 5xFAD mice

To determine whether long-term voluntary exercise affects body mass, locomotor activity, cognitive function and memory, the body weight and running distance of the WT and 5xFAD mice being evaluated weekly, with behavioral testing conducted

at 7 months of age. No exercise effect on body mass or running distance was seen throughout the study (ns; Study II, Supplementary Figure 1), although a genotype effect on body weights was detected, i.e. that 5xFAD mice have a lower body mass than WT mice ($p < 0.0001$; Study II, Supplementary Figure 1A). Based on a wide range of tests measuring typical mouse behavior (nest building test), exploration activity and anxiety (elevated plus-maze and open field tests), and spatial learning and memory (MWM test), 5xFAD mice were found to display significant impairments in cognitive function and memory (Study II, Figure 1). Long-term voluntary exercise resulted in significant improvements in nest building (Study II, Figure 1A), ameliorated anxiety and reversed exploratory activity in 5xFAD mice (Study II, Figure 1B, C). Moreover, a beneficial exercise effect was observed in spatial learning and memory of 5xFAD mice (Study II, Figure 1D).

5.2.2 Long-term exercise has no effect on neurogenesis, neuronal survival, Aβ burden and microglia activation in 5xFAD hippocampi

Histological staining of brain sections was performed to assess the effects of exercise on AD-associated impairment of adult hippocampal neurogenesis and neuronal loss, Aβ burden and microglia activation. Staining with doublecortin (DCX), a marker of immature neurons, and NeuN, a marker of mature neurons, revealed a significant reduction of DCX-positive neurons in DG ($p < 0.0001$) and NeuN level in subiculum ($p < 0.0001$) of 5xFAD mice when compared to WT mice (Study II, Figure 2). However, voluntary exercise did not alter these deficits (ns; Study II, Figure 2). Aβ plaque deposition in the hippocampal area was unchanged between sedentary and exercised mice (ns; Study II, Figure 3A, B). Iba1, a marker of activated microglia, was significantly increased in hippocampi of 5xFAD mice in comparison to WT ($p < 0.0001$), but no exercise-induced alterations were observed in either genotype (ns; Study II, Figure 3C, D). Of the hippocampal cytokines measured, it was only possible to evaluate MCP-1 and IL12p70 levels due to the low expression of the other cytokines (Study II, Supplementary Table 2). AD was associated with an upregulation of MCP-1 ($p < 0.01$) and IL12p70 level ($p < 0.05$) in hippocampi, whereas physical exercise induced a decline of IL12p70 in 5xFAD-mice ($p < 0.05$; Study II, Supplementary Table 2).

5.2.3 Long-term exercise ameliorates the reduction of postsynaptic protein PSD-95 in 5xFAD hippocampi

Next, WB was used to investigate the effect of voluntary exercise on synaptic proteins that are known to be altered in AD (Yuki *et al.*, 2014). While there was no genotype or exercise effect in the hippocampal level of a presynaptic marker, synaptophysin (ns), a significant reduction of the postsynaptic marker PSD-95 was detected in 5xFAD hippocampi ($p < 0.05$; Study II, Figure 2E, F). Moreover, voluntary exercise reversed the reduction of hippocampal PSD-95 in 5xFAD mice ($p < 0.01$; Study II, Figure 2E, F).

5.2.4 Increased GFAP expression and number of GFAP-positive astrocytes after long-term exercise in 5xFAD hippocampi

To test whether voluntary exercise has any effect on astrocytes, WB and RT-PCR were performed to measure hippocampal protein and mRNA levels of different astrocytic markers such as GFAP, S100β, glutamine synthetase (GS) and ALDH1L1. While no genotype effect was observed in hippocampal protein and mRNA levels of GS and ALDH1L1 (ns for protein and RNA level), GFAP ($p < 0.0001$ for protein and RNA level) and S100β levels ($p < 0.001$ protein level, $p < 0.05$ RNA level) were significantly increased in the hippocampi of 5xFAD mice when compared to WT mice (Study II, Figure 4A, B; Supplementary Table 3). Furthermore, among all the tested astrocytic markers, only the GFAP protein level was altered by exercise. There was a significant upregulation of GFAP in hippocampi of exercised 5xFAD mice in comparison to the sedentary 5xFAD mice ($p < 0.05$; Study II, Figure 4A,B).

Next, IHC for brain sections was used to make a closer examination of GFAP and S100β levels in hippocampi and possible exercise-induced alterations in those astrocytic markers. Similar to the WB results, a significant increase in GFAP was detected in hippocampi and subiculum areas ($p < 0.0001$; Study II, Figure 3E, F) together with an increase of S100β in the subiculum ($p < 0.0001$; Study II, Supplementary Figure 2A, B) in 5xFAD mice. Furthermore, voluntary physical exercise induced a further increase only in GFAP ($p < 0.01$; Study II, Figure 3E, F) with no effect on S100β (ns; Study II, Supplementary Figure 2A, B) in hippocampi and subiculum areas of 5xFAD mice.

Next, the number of hippocampal GFAP- and S100β-positive astrocytes was evaluated in brain sections by using the MicroTrac analysis platform (Abdolhoseini, Walker and Johnson, 2016). While the number of S100β-positive astrocytes remained unchanged regardless of genotype or exercise group (ns; Study II,

Supplementary Figure 2C), a significant 2.4-fold increase in the number of GFAP-positive astrocytes was observed in hippocampi of 5xFAD mice in comparison with WT mice ($p < 0.0001$; Study II, Figure 6B). Interestingly, a further increase in the number of hippocampal GFAP-positive astrocytes was detected in response to voluntary physical exercise in WT and 5xFAD mice ($p < 0.05$; Study II, Figure 6B). Taken together, these findings suggest that voluntary physical exercise affects specifically the GFAP-positive astrocyte population in the hippocampi.

5.2.5 Altered morphology of hippocampal GFAP-positive astrocytes in response to long-term exercise in 5xFAD mice

The exercise-induced increase in GFAP level was accompanied with an increase in the number of GFAP-positive astrocytes in 5xFAD hippocampi. To investigate whether this exercise-induced alteration was linked with structural changes in GFAP-positive astrocytes, morphological analysis using MicroTrac platform was performed on brain sections. AD caused dramatic changes in the morphology of hippocampal GFAP-positive astrocytes, including an enlarged soma area by 28% ($p < 0.0001$), increased cell solidity by 13% ($p < 0.0001$) and cell extent by 18 % ($p < 0.0001$; Study II, Figure 6C). Moreover, hippocampal GFAP-positive astrocytes in sedentary 5xFAD mice exhibited significantly less primary branches (10%, $p < 0.05$) and a shorter branch length (15%, $p < 0.05$; Study II, Figure 6C) in comparison to sedentary WT mice. Voluntary physical exercise significantly increased the cell area (12%, $p< 0.05$) of GFAP-positive astrocytes in the 5xFAD mice when compared to sedentary controls.

Next, double IHC staining for GFAP and Aβ was performed to assess whether the morphological changes in hippocampal GFAP-positive astrocytes were dependent on the proximity to Aβ plaques. Cells were considered as plaque-associated if they were located at a distance less than 50 µm from the Aβ plaque border (Study II, Figure 7A). Morphological analysis revealed that plaque-associated GFAP-positive astrocytes displayed significant structural changes in comparison to cells far from the plaques. These changes included a greater cell radius (15%, $p < 0.01$), enlarged cells area (74%, $p < 0.0001$) and soma size (100%, $p < 0.0001$), together with an increased number of primary branches (30%, $p < 0.0001$) and branch length (50%, $p < 0.001$; Study II, Supplementary Table 4). Moreover, voluntary exercise affected cell morphology selectively in the plaque-associated GFAP-positive astrocytes, inducing significant increases in the number of primary branches (14%, $p < 0.05$; Study II, Figure 7B) and soma size (52%, $p < 0.05$; Study II, Figure 7C). No exercise-induced morphological differences were

detected in GFAP-positive astrocytes distant from the Aβ plaques (Study II, Figure 7B, C; Supplementary Table 4).

These results indicate that an exercise-induced GFAP increase in 5xFAD hippocampi was accompanied with an increase in the number of GFAP-positive astrocytes coupled with morphological changes occurring in GFAP-positive astrocytes associated with Aβ plaques.

5.2.6 Long-term exercise ameliorates the reduction of BDNF in GFAP-positive astrocytes in hippocampi

Previous studies have demonstrated a decreased BDNF level in AD brain (Phillips *et al.*, 1991; Peng *et al.*, 2005, 2009). We investigate whether voluntary exercise could reverse this deficit. A significant reduction of BNDF was detected in hippocampi of 5xFAD mice when compared to WT ($p < 0.05$), whereas voluntary exercise trended towards reversing the BDNF decrease in 5xFAD mice ($p = 0.06$; Study II, Figure 5A).

Next, double IHC staining was performed for GFAP and BDNF to assess the possible link between exercise-induced changes in GFAP-positive astrocytes and the increased BDNF level in hippocampi of 5xFAD mice. First, we demonstrated that hippocampal GFAP-positive astrocytes expressed BDNF by conducting a correlation analysis (Study II, Figure 5B). Next, the level of BNDF in hippocampal GFAP-positive astrocytes was measured by colocalization analysis. A significant reduction of BNDF was detected in GFAP-positive astrocytes in hippocampi of 5xFAD mice when compared to WT mice ($p < 0.001$; Study II, Figure 5C, D). Voluntary exercise reversed this reduction in mice of both genotypes ($p < 0.05$; Study II, Figure 5C, D).

These findings demonstrate that voluntary exercise restores BDNF levels in hippocampi of 5xFAD mice, and specifically in GFAP-positive astrocytes.

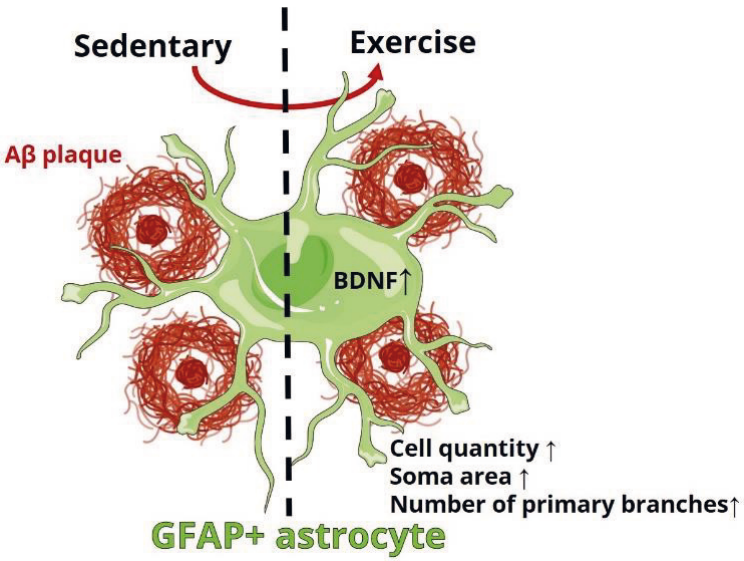

Figure 5. Effect of long-term voluntary exercise on GFAP-positive astrocytes in 5xFAD mice hippocampi. Exercise induced increase in number of GFAP-positive astrocytes, altered GFAP-positive astrocyte morphology around Aβ plaque and restored astrocytic BDNF in 5xFAD hippocampi. Created with Smart.servier.com and BioRender.com.

5.3 LONG-TERM VOLUNTARY EXERCISE MODULATES MUSCLE AND BRAIN IRON HOMEOSTASIS IN WT AND AD MICE (III)

Study III was conducted to evaluate the effect of long-term voluntary exercise on iron metabolism in WT and 5xFAD mice in the brain and skeletal muscle. We also investigated the impact of regular exercise on iron metabolism regulation via the IL-6 myokine. For these purposes, we measured the levels of total iron content, proteins essential for iron homeostasis, and IL-6 in WT and 5xFAD mice cortices, GAS skeletal muscles and plasma obtained from Study II. The results of Study III are summarized in Figure 6.

5.3.1 Long-term exercise impacts on iron load in muscle and brain

To assess the effect of long-term voluntary exercise on iron load, the level of the main iron storage protein was evaluated in cortices and GAS skeletal muscles of WT and 5xFAD mice. Histological staining revealed a significant increase in the amount of ferritin in the cortex of 5xFAD mice in comparison with WT mice ($p <$

0.001; Study III, Figure 2C,D) whereas the total iron content remained unchanged (Study III, Figure 2A). No genotype-induced alterations in the ferritin protein level were detected in GAS skeletal muscle (Study III, Figure 2G). However, long-term voluntary exercise induced a significant decrease in the cortical ferritin protein ($p < 0.05$; Study III, Figure 2C,D) and mRNA content ($p < 0.01$; Study III, Figure 2B) in 5xFAD mice. The muscular ferritin protein level was elevated in exercised mice in comparison with sedentary mice ($p<0.05$; Study III, Figure 2G) coupled with elevated total iron content in GAS skeletal muscle in response to long-term voluntary exercise ($p < 0.01$; Study III, Figure 1E). In addition, the mRNA expression of heme-oxygenase 1 (HO-1), the enzyme responsible for heme degradation into redox-active iron (Ward *et al.*, 2014), was assessed in cortex and GAS skeletal muscles of mice. AD was associated with an upregulation of HO-1 mRNA in the cortex of 5xFAD mice when compared to WT mice ($p < 0.001$), while long-term voluntary exercise induced a slight decline in HO-1 in 5xFAD exercised mice ($p < 0.05$; Study III, Figure 2B). No genotype or exercise effects on HO-1 mRNA level were detected in GAS skeletal muscles (Study III, Figure 2F).

5.3.2 Long-term exercise effects iron trafficking in muscle and brain

To evaluate the impact of long-term voluntary exercise on iron trafficking in the brain, the levels of key proteins involved in iron uptake including transferrin receptor (TfR) and divalent metal transporter 1 (DMT1), and iron efflux proteins including ferroportin and ferroxidase ceruloplasmin (Moos *et al.*, 2007), were measured in cortical and GAS muscle samples of mice. 5xFAD mice had increased cortical TfR ($p < 0.01$) and decreased cortical ceruloplasmin ($p < 0.05$; Study III, Figure 3A) mRNA expressions in comparison to WT mice. In addition, a genotype effect was detected in GAS skeletal muscle such as elevated TfR ($p < 0.01$) and ceruloplasmin ($p < 0.05$; Study III, Figure 3D) in 5xFAD-SED mice in comparison with WT-SED mice. Long-term voluntary exercise induced a slight reduction of TfR ($p < 0.05$), DMT1 ($p < 0.01$) and the ferroportin ($p < 0.01$) mRNA content in cortex of 5xFAD mice (Study III, Figure 3A). At the same time, histological staining of brain sections revealed a significant TfR protein increase in 5xFAD mice in response to exercise ($p < 0.05$; Study III, Figure 3B,C). Moreover, long-term physical exercise induced a dramatic decrease in TfR mRNA expression ($p < 0.001$; Study III, Figure 3D) and protein level ($p < 0.001$; Study III, Figure 3E) in GAS skeletal muscles of all exercised mice. In addition, an increase in DMT1 protein level ($p<0.01$; Study III, Figure 3E) was detected in GAS skeletal muscles in response to exercise.

5.3.3 Long-term exercise modulates iron homeostasis and decreases IL-6 in brain and periphery

Next, we tested whether long-term voluntary exercise modulated iron homeostasis in cortex and GAS skeletal muscle of WT and 5xFAD mice. The protein level of hepcidin, the key regulator of cellular iron levels (Vela, 2018; Kezele and Ćurko-Cofek, 2020), was evaluated in cortical samples by ELISA. Long-term voluntary exercise significantly decreased hepcidin in cortex of WT and 5xFAD mice ($p < 0.05$; Study III, Figure 4A). Moreover, the signal transducer activator of the transcription 3 (STAT3)/ Janus kinase 1 (JAK1) pathway, activation of which regulates hepcidin expression, was altered by exercise. The expressions of cortical STAT3 ($p < 0.001$) and JAK1 ($p = 0.06$) were increased in 5xFAD mice, while long-term voluntary exercise diminished the STAT3 ($p < 0.01$) and JAK1 ($p < 0.05$; Study III, Figure 4B) upregulation in the cortex of 5xFAD-EXE mice. In addition, the expression of receptor-type tyrosine-protein phosphatase epsilon (PTPe), which inhibits STAT/JAK signaling (Xu and Qu, 2008), was upregulated in cortex of WT and 5xFAD mice in response to exercise ($p < 0.05$; Study III, Figure 4B).

As the hepcidin level is connected to the inflammation status, in particular to IL-6 levels (Ward *et al.*, 2014), we evaluated the effect of long-term voluntary exercise on IL-6 receptor (IL-6R) and IL-6 levels in cortex, plasma and GAS skeletal muscle of mice. Although there were no genotype-related changes observed in IL-6 protein levels in cortex and plasma, in 5xFAD mice upregulations were observed in IL-6 protein in GAS skeletal muscle ($p < 0.05$; Study III, Figure 4E) and IL-6R mRNA expression in cortex ($p < 0.001$; Study III, Figure 4B). Long-term voluntary exercise induced a significant decrease in IL-6 concentrations in cortex and plasma in both genotypes of mice ($p < 0.05$; Study III, Figure 4E) coupled with a slight IL-6R reduction in the cortex of 5xFAD mice ($p = 0.1$; Study III, Figure 4B). No changes in IL-6 protein and IL-6R mRNA in GAS skeletal muscles were detected in response to exercise (Study III, Figure 4D,E). In addition, a correlation analysis of all studied mice revealed a significant negative correlation between cortical IL-6 and muscular total iron content ($r = – 0.56$, $p < 0.01$; Study III, Figure 4F) and cortical hepcidin and muscular total iron content ($r = – 0.49$, $p < 0.05$; Study III, Figure 4C), demonstrating that a low IL-6 and hepcidin cortical level accompanies with a high muscular total iron level.

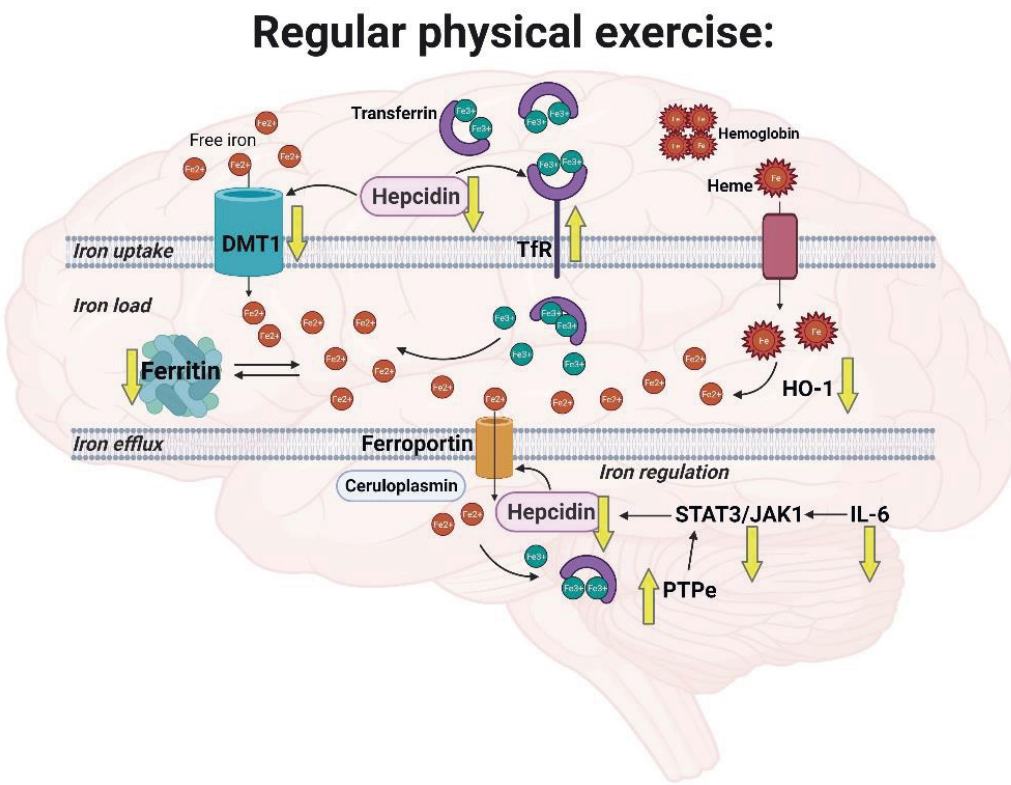

Figure 6. Effect of long-term voluntary exercise on iron metabolism in the 5xFAD brain. Yellow arrows indicate effect of regular exercise on iron trafficking, load, and homeostasis in cortex of 5xFAD mice demonstrated in Study III. Created with BioRender.com.

6 DISCUSSION

The main aim of this thesis was to study the role of physical exercise on brain and skeletal muscle health in the context of aging and AD.

In Study I, we explored the effect of life-long voluntary exercise on age-related cellular stress in mouse skeletal muscle. We found that life-long voluntary exercise exerted beneficial effects on redox regulation, ER stress adaptation and HSP defence in skeletal muscle upon aging.

In Study II, we investigated how long-term voluntary exercise impacts on the state of astrocytes in WT mice and the 5xFAD mouse model of AD. We found that cognitive improvements in 5xFAD mice were coupled with a modulation of GFAP-positive astrocytes in response to long-term voluntary exercise. In addition, long-term voluntary exercise restored astrocytic BDNF decrease in the 5xFAD brain.

In Study III, we examined the involvement of long-term voluntary exercise in the modulation of iron homeostasis in the brain and periphery in the context of AD. We demonstrated that long-term voluntary exercise was able to alter iron metabolism in the brain and skeletal muscle of mice, suggesting that hepcidin and IL-6 may act as central players in the modulation of iron homeostasis induced by exercise.

6.1 LONG-TERM VOLUNTARY EXERCISE AND CELLULAR STRESS RESPONSE IN AGING

Aging affects the ability of tissues to efficiently respond to cellular stress conditions. The present study described age-related alterations in skeletal muscle and the effect of long-term physical exercise on impaired redox system and disturbed expression of HSPs, increased ER stress and activated ER stress-related apoptosis.

Skeletal muscles from old animals are known to have disrupted ROS homeostasis, increased oxidative damage and dysregulated redox signalling (Jackson, 2009; Thirupathi, Pinho and Chang, 2020). The TRX system is important in cellular redox regulation, antioxidant defense and protection of cells from oxidative stress (Yoshihara *et al.*, 2014), although the effects of aging on the TRX system remain unclear. The novelty of the present study is that it is the first exploration of the effect of physical exercise on the TRX-TxNiP system in skeletal muscles of old mice. We found that aging was associated with an increased TRX

content, overexpression of TxNiP, an inhibitor of TRX reducing activity, and a reduction in the TRX/TxNiP ratio in skeletal muscles. Current knowledge about the TRX system in aging is scant, although it has been reported earlier that TRX increases in TA skeletal muscles in old mice in comparison to young controls (Dimauro *et al.*, 2012). This is suggested to play a compensatory role in the response to age-related increases in oxidative stress. Moreover, we found that long-term physical exercise reduced the elevation in TxNiP, and increased the TRX/TxNiP ratio in skeletal muscles of old trained mice, demonstrating beneficial effects of exercise on the cellular redox system. In line with our findings, a very recent study demonstrated that physical exercise diminished the TxNiP elevation in the hippocampi of AD mice (Rosa *et al.*, 2021). Therefore, age-related dysregulation of antioxidant defenses can be rescued by long-term physical exercise through the TRX-TxNiP system.

Age-related redox system dysregulation and oxidative stress disrupts the function of the ER, leading to an accumulation of unfolded proteins in the ER inducing ER stress, and activation of the UPR (Deldicque, 2013). Because of the high protein content, the presence of a large ER network and increased oxidative stress, skeletal muscle is highly susceptible to ER stress in aging (Bohnert, McMillan and Kumar, 2018). We investigated the effect of aging and long-term physical exercise on ER stress, ER stress-related apoptosis, and its possible association with the redox system in skeletal muscle of mice. We detected age-related increases in the level of the ER chaperone GRP78 in TA skeletal muscle, indicative of UPR activation. Increased levels of GRP78 are a sign of ER stress and initiation of the UPR. When the UPR fails to cope with the unfolded protein load, ER-stress induced cell death mechanisms become activated (Kim, Xu and Reed, 2008). We detected a dramatic upregulation of CHOP, a marker of ER-stress related apoptosis in skeletal muscles of old mice. While there was no exercise effect on GRP78 level, the CHOP level was drastically diminished after long-term physical exercise in soleus skeletal muscle of old mice. Previous studies have been shown GRP78 and CHOP upregulation in skeletal muscles of old rodents (Ogata *et al.*, 2009; Hwee *et al.*, 2014), although Chalil *et al.* demonstrated diverse age-related changes in the levels of GRP78 depending on the skeletal muscle type (Chalil *et al.*, 2015). Although the effect of physical exercise on UPR activation is controversial and dependent on the duration and intensity of training (Naito *et al.*, 2001; Tarpenning *et al.*, 2004; Demakakos *et al.*, 2010; Kim *et al.*, 2014; Estébanez *et al.*, 2019), little is known about the effects of exercise on ER stress in the context of aging. Moreover, only one study in skeletal muscle has shown that long-term treadmill exercise would be

able to decrease muscular mRNA CHOP levels in high intensity exercised rats, although the protein level remained unchanged (Kim *et al.*, 2014). Based on our finding, we conclude that age-induced ER-stress and ER-stress related apoptosis in skeletal muscle was partly ameliorated by long-term physical exercises.

Because the ER lumen has an oxidative environment that is important for proper ER enzyme function and protein folding (Kim, Xu and Reed, 2008; Lu and Holmgren, 2014), we performed a correlation analysis between ER stress and redox regulation markers. We found a strong positive correlation between TxNiP and CHOP levels, and TRX and GRP78 levels in skeletal muscles of all mice, indicating for the first time a possible association between a dysregulation of the redox system and ER stress. This finding is supported by the fact that TxNip is involved in apoptotic regulation (Zhou and Chng, 2013) and the regulation of ER stress enzyme activity (Lee *et al.*, 2014). Our results indicated that long-term physical exercise reduced the TxNiP level, that in turn was able to trigger an amelioration of the ER-stress related apoptosis in skeletal muscle of old mice.

Given that aging is associated with dysregulation of protein homeostasis and cellular stress, and acknowledging the important role of HSPs in these processes (Calderwood, Murshid and Prince, 2009), we assessed the effect of long-term physical exercise on the levels of HSPs in skeletal muscle. In soleus skeletal muscles of old mice, aging was associated with reduced levels of the mitochondrial chaperone GRP75 and cytosolic constitutively expressed HSC70, both of which belong to the HSP70 family. HSPs from the HSP70 family are important in cell survival and antioxidant protection against cellular stress, thus a reduction of these HSPs may be linked with increased oxidative stress (Daugaard, Rohde and Jäättelä, 2007). We were unable to detect any age-related alterations in the levels of other HSPs i.e. HSP25, HSP60, HSP70, in skeletal muscle, which is in line with previous studies (Naito *et al.*, 2001; Valls *et al.*, 2015). There are, however, conflicting studies showing unaltered HSC70 and decreased HSP70 levels (Ogata *et al.*, 2009), and increased HSP25, HSP60 and HSP70 levels (Chung and Ng, 2006; Cumming *et al.*, 2021) in skeletal muscle upon aging. The variance in the published results could be linked with different age and sex of animals and species together with different tissue collection procedures used in the studies. Moreover, we also demonstrated long-term physical exercise-induced cytoprotection via increased expression of members of the HSP70 family such as stress-inducible HSP70 and constitutively expressed HSC70 in soleus skeletal muscle coupled with a GRP75 rise in TA skeletal muscle in old mice. According to our results related to the TRX system, we suggest that the effects of aerobic long-term exercise on the redox

state and on the cytoplasmic HSP70 family are more prominent in soleus skeletal muscle. These muscles predominantly consist of slow-twitch fibers with a high oxidative capacity when compared to TA skeletal muscle (Armstrong and Phelps, 1984). In line with our findings, the aerobic exercise-induced upregulation of HSPs from the HSP70 family has previously been demonstrated in adult rodents (Huey and Meador, 2008; Abruzzo *et al.*, 2013) and old rats (Naito *et al.*, 2001). We can thus conclude that age-related impairments of the functions of the HSP chaperones are partially rescued by long-term physical exercise in skeletal muscles of old mice.

This study showed that long-term voluntary exercise is beneficial against cellular stresses in aging. Animal models are extremely useful for studying the long-term effects of lifestyle factors, and thus they can provide new, important insights into age-related disease prevention or for reducing the risk of disease progression. Further research should focus on the effects of life-long physical exercise on redox regulation and ER stress in the brain, to clarify how these processes contribute to neuronal dysfunction and neurodegenerative diseases (Sprenkle *et al.*, 2017).

6.2 ASTROCYTE REMODELING IN THE BENEFICIAL EFFECTS OF LONG-TERM VOLUNTARY EXERCISE IN AD

Physical exercise is known to be beneficial in AD. The present study evaluated the effect of long-term voluntary physical exercise on the modulation of the astrocyte state and the role of these cells in cognitive functioning in 5xFAD mice.

Human AD is associated with cognitive and memory impairments, and these have been confirmed in a wide range of AD animal models including the 5xFAD model (Oakley *et al.*, 2006; Jawhar *et al.*, 2012; Bhattacharya *et al.*, 2014; Devi and Ohno, 2015; Hüttenrauch *et al.*, 2017). Physical exercise has been demonstrated to have beneficial effects on cognitive and memory functions in different AD mouse models (Wolf *et al.*, 2006; Parachikova, Nichol and Cotman, 2008; García-Mesa *et al.*, 2011; Walker *et al.*, 2015; Tapia-Rojas *et al.*, 2016) and also in AD patients (Jia *et al.*, 2019). In line with previously published reports, we detected AD-related impairments in typical behaviour, exploration activity, anxiety level and spatial learning and memory in 5xFAD mice, which were rescued by long-term voluntary physical exercise.

Positive effects of physical exercise on cognition and memory are known to be associated with a stimulation of cell proliferation, hippocampal adult neurogenesis

and reduction of neuronal loss (Brown, Peiffer and Martins, 2013). Physical exercise (Tapia-Rojas *et al.*, 2016; Choi *et al.*, 2018) together with environmental enrichment (EE) (Herring *et al.*, 2009; Hu *et al.*, 2010) was able to reverse impaired adult neurogenesis and cell proliferation, although in some studies exercise and EE were not able to rescue the reduction of DCX-positive neurons and neuronal loss in AD mice (Wolf *et al.*, 2006; Cotel *et al.*, 2012). In contrast to some prior reports, in our study, physical exercise did not ameliorate the reduced number of DCX-positive newborn neurons in hippocampi or neuronal loss in the subiculum of 7-month-old 5xFAD mice. It is likely that the previously reported beneficial effects of physical exercise and EE on neurogenesis and neuronal survival are dependent on the age of the animals, the exercise protocol and the stage of AD at which exercise is undertaken. Therefore, further studies are needed to clarify the time and pathology-dependency of the positive exercise effects on neurogenesis and neuronal survival in AD. At the molecular level, the beneficial effects of physical exercise on cognition and behavior may be mediated by increased levels of growth factors, such as BDNF, and synaptic proteins, the expression of these factors has been associated with neuronal survival and synaptic plasticity (El-Husseini *et al.*, 2000; Peng *et al.*, 2009). We found that the reduction of both BDNF and PSD-95 in 5xFAD mice hippocampi was restored by long-term physical exercise, in agreement with a previously published study (Choi *et al.*, 2018). Thus, we can conclude that long-term physical exercise partially restores synaptic function with no effect on neurogenesis and neuronal survival in the hippocampi of 5xFAD mice.

The Aβ plaque burden is one of the major pathological characteristics of the AD brain. Reports on the effect of physical exercise on Aβ plaque load are controversial and have been shown to vary depending on the AD mice model used, age, sex, and duration and type of exercise (Wolf *et al.*, 2006; Parachikova, Nichol and Cotman, 2008; Cotel *et al.*, 2012; Xiong *et al.*, 2015; Tapia-Rojas *et al.*, 2016; Choi *et al.*, 2018; Zhang *et al.*, 2018). In our study, long-term physical exercise improved cognition and behavior of 5xFAD mice although there was no change in the Aβ plaque load in hippocampi. Considering that Aβ plaque deposition is associated with impaired adult neurogenesis and neuronal loss (Moon, Cha and Mook-Jung, 2014), a stable Aβ load is in line with unaltered adult neurogenesis, which we observed after long-term physical exercise in 5xFAD mice hippocampi. We believe that the effect of physical exercise might depend on the stage of AD pathology, and at 7 months of age, the 5xFAD mice already have a high Aβ burden, so high that it cannot be relieved by exercise alone. On the other hand, it is known that a reduction of Aβ burden is not necessary to achieve a therapeutic benefit.

For example, post-mortem staining of autopsy from AD patients who participated in a clinical trial of immunization have shown a reduction the numbers of Aβ plaques although the patients experienced a continuing cognitive decline (Holmes *et al.*, 2008). In line with this observation, an acute anti-Aβ monoclonal antibody treatment with m266 reversed the cognitive and memory impairment in PDAPP mice without affecting the Aβ burden in the brain (Dodart *et al.*, 2002). Therefore, it is also highly plausible that the beneficial effects of exercise observed in our study do not relate to the Aβ burden but are rather the result of glial modulation.

Neuroinflammation is an important contributor to AD pathology, which is characterized by glial cell activation and increased levels of pro-inflammatory cytokines (Guzman-Martinez *et al.*, 2019). Physical exercise is known to have an overall anti-inflammatory effect, although it remains unclear whether there is an exercise-induced modulation of inflammatory responses in the AD brain (Kelly, 2018). As has been reported by others, our study revealed increased levels in markers of activated microglia and reactive astrocytes coupled with increased expression of pro-inflammatory cytokines in hippocampi of 5xFAD mice. Long-term physical exercise did not impact on microglial markers or cytokine levels, but did increase the levels of GFAP, a marker of reactive astrocytes.

Reactive astrocytes play an important neuroprotective role in AD, surrounding Aβ plaques and isolating the damaged area (Pekny *et al.*, 2016). Ablation of astrocytes resulted in a reduction of Aβ degradation and synaptic connectivity in organotypic brain culture slices of 5xFAD mice (Davis *et al.*, 2021), and inhibition of astrocyte reactivity caused an acceleration of Aβ plaque pathology in mice (Kraft *et al.*, 2013). Both studies indicate that reactive astrocytes exert a protective role in AD. However, involvement of reactive astrocytes in AD depends on the disease stage. Our study revealed that long-term physical exercise enhanced the expression of GFAP in 5xFAD hippocampi together with increasing the numbers of GFAP-positive astrocytes when compared to sedentary mice. Moreover, among the known astrocytic markers, we found that only the levels of GFAP were altered in response to exercise, indicating reactive astrocytes as the most exercise-modulated astrocyte subtype in the AD brain. In line with our findings, exercise has been shown to increase GFAP levels in healthy (Saur *et al.*, 2014) and also in an AD animal model (Rodrigues *et al.*, 2010). However, some studies have demonstrated unaltered (Hüttenrauch *et al.*, 2017) or even decreased (Tapia-Rojas *et al.*, 2016; Zhang *et al.*, 2018) GFAP levels upon physical exercise or EE. We believe that the GFAP increase in 5xFAD mice hippocampi in response to physical exercise was related to an increased number of GFAP-positive astrocytes, and represented

a phenotypic shift of resting cells towards a reactive state. Moreover, the GFAP increase upon exercise could also be related to morphological alterations of reactive astrocytes. In agreement with previous studies (Olabarria *et al.*, 2010; Beauquis *et al.*, 2013; Pekny, Wilhelmsson and Pekna, 2014; Pomilio *et al.*, 2016), we found that the elevated GFAP level in AD was accompanied with dramatic changes in astrocytic morphology including atrophy of branches and cell hypertrophy. Moreover, we demonstrated that the proximity to Aβ plaques had a huge impact on the morphology of GFAP-positive astrocytes. In previous studies, it has been shown that exercise could alter astrocyte morphology (Viola *et al.*, 2009; Saur *et al.*, 2014; Brockett, LaMarca and Gould, 2015; Lundquist *et al.*, 2019), however limited information has been available in the context of AD. Interestingly, we found that long-term physical exercise impacted specifically on the GFAP-positive astrocytes surrounding Aβ plaques, increasing their soma area and branching number with no effect on cells distant from the plaques. Our results are in line with an earlier investigation that demonstrated increased GFAP-positive astrocyte soma in response to exercise and EE in AD mice hippocampi (Rodríguez *et al.*, 2013).

Astrocytes regulate synaptic plasticity, contributing to cognitive function and memory (Augusto-Oliveira *et al.*, 2020). Specifically, astrocyte-released BDNF modulates synaptic stability and spine density (De Pins *et al.*, 2019). Our study demonstrated that AD is associated with decreased BDNF levels in GFAP-positive astrocytes, whereas long-term physical exercise restored this deficit in the hippocampi of 5xFAD mice. Thus, exercise-induced alterations in the morphology of GFAP-positive astrocytes could be linked with the increased levels of astrocytic BDNF, which has been shown to regulate astrocyte morphology (Ohira *et al.*, 2007; Fahimi *et al.*, 2017). According to a recently published study, astrocytic BNDF release also mediated the EE-induced beneficial effect on synaptic plasticity in the aged brain (Lalo *et al.*, 2020).

Taken together, we conclude that the exercise-induced beneficial effects on cognitive function in AD are associated with an increased number and altered morphology of GFAP-positive astrocytes, which could be linked with the restoration of decreased release of BNDF from astrocytes. Modulators of the astrocyte state could thus have clinical implications for the promotion of brain health in AD. In summary, these results provide important evidence of the importance of non-neuronal cells in the beneficial effects of exercise. Future studies should attempt to decipher the impact of AD disease stage, age, and sex on exercise-induced modulation of the astrocyte state. In addition, the effect of

exercise duration, type (aerobic vs resistance), and intensity will need to be clarified to reveal potentially differential impacts of different types of exercise on brain health.

6.3 LONG-TERM VOLUNTARY EXERCISE MODULATES IRON HOMEOSTASIS IN THE 5XFAD MOUSE MODEL OF ALZHEIMER'S DISEASE

Dysregulated iron homeostasis is considered an important factor contributing to neurodegenerative diseases (Ke and Qian, 2003). In AD, iron overload is accompanied with disrupted iron metabolism, which is thought to be implicated in Aβ and ROS production, leading to oxidative stress, neuroinflammation and cognitive impairments (Schröder, Figueiredo and Martins De Lima, 2013; Ward *et al.*, 2014; Cheignon *et al.*, 2018). Regular physical exercise is considered to have beneficial effects on iron metabolism by reducing total body iron (Ziolkowski *et al.*, 2014; Antosiewicz *et al.*, 2015), although limited information is available with regards to exercise-induced iron modulation in the brain. As iron is important in skeletal muscle's oxidative capacity during exercise, there is a growing body of evidence indicating that modulation of iron metabolism in the whole body might be mediated by skeletal muscles undergoing physical exercise (Ziolkowski *et al.*, 2014; Buratti *et al.*, 2015). We investigated the major proteins mediating iron metabolism in cortex and skeletal muscle tissues in WT and 5xFAD mice to clarify the effects of long-term voluntary exercise on iron modulation in the context of AD.

Iron is the most abundant redox active metal in the body, thereby iron storage plays an important role in the control of iron availability (Dwyer *et al.*, 2009). Ferritin is responsible for storing iron in a non-toxic form, thereby attenuating and sequestering free iron (Ashraf, Clark and So, 2018) and thus it is an important indicator of a tissue's iron load (Liu *et al.*, 2018). AD is associated with elevated iron and ferritin levels in the brain (Du *et al.*, 2018; Choi *et al.*, 2021), and there is known to be a colocalization of iron deposits and ferritin with Aβ plaques both in cortex and hippocampus (Falangola *et al.*, 2005; Meadowcroft, Connor and Yang, 2015; Belaidi and Bush, 2016). Furthermore, elevated amounts of iron in brain as measured by CSF ferritin might accelerate Aβ plaque deposition and AD progression (Ayton, Diouf and Bush, 2018). In our study, a dramatic ferritin increase was observed in the cortex of 5xFAD mice, which is in line with previous reports (Svobodová *et al.*, 2019; Yu *et al.*, 2019; Choi *et al.*, 2021). Although we

detected no change in the cortical total iron content in 5xFAD mice, this ferritin increase might be an indicator of increases in labile iron in the cortex of 5xFAD mice. In support of this concept, the expression of HO-1, an enzyme which mediates the release of free iron from heme (Liu *et al.*, 2018), was also upregulated in the cortex of 5xFAD mice. Taken together, we demonstrated elevations of ferritin and HO-1 with no changes in the total iron content in cortex of 5xFAD mice.

We also found that long-term physical exercise reduced ferritin and HO-1 in the cortex of 5xFAD mice. In a recent study on the APP-C105 AD mouse model (Choi *et al.*, 2021), treadmill exercise promoted decreases in both ferritin and total iron, although we did not observe changes in the total iron content in 5xFAD cortices. The differences between the Choi *et al.* study and our results in the effects of exercise on the total iron level might be explained by one or a combination of the following differences: the AD mouse model used, the type of exercise protocol and the method used for the detection of total iron, all of which may affect the assessment and extent of iron release (Abbasi *et al.*, 2021). In addition to brain alterations, we showed that long-term voluntary exercise induced significant changes in the iron load in GAS skeletal muscles; the contents ferritin and total iron were increased in WT and 5xFAD exercised mice. A previous study by Ghio *et al.* revealed that there was a redistribution of body iron in response to voluntary exercise, which included an elevation of iron in tissues with high metabolic rates such as skeletal muscle and heart, and an attenuation of iron levels in plasma and liver (Ghio *et al.*, 2020). Therefore, it is plausible that the effect of long-term voluntary exercise observed in our study relates to a redistribution of body iron, which results in a lowering of ferritin levels in cortex and their elevation as well as the total iron content in skeletal muscle.

In AD, the dysregulation of iron homeostasis and iron overload in the brain can be linked with disturbances of cellular iron trafficking (Vela, 2018). Iron uptake is regulated by TfR and DMT1 receptors, while iron export is mediated by ferroportin and ferroxidase ceruloplasmin (Ward and Kaplan, 2012). In line with previous studies (Xian-Hui *et al.*, 2015; Yu *et al.*, 2019; Choi *et al.*, 2021), we found increased TfR and decreased ceruloplasmin expression in the cortex of 5xFAD mice, suggesting AD-induced alterations in the trafficking of iron in the brain. Moreover, ceruloplasmin and TfR levels were increased in skeletal muscles of 5xFAD mice, which could be a sign of AD-induced upregulation of iron trafficking in muscle. Until recently, only one study had investigated the role of physical exercise in iron load and trafficking in the brain of an AD mouse model (Choi *et al.*, 2021). It was shown that treadmill exercise induced a decrease in the iron load coupled with an

attenuation of TfR and DMT1 in the cortex of APP-C105 mice. In our study, we also found a reduction of cortical DMT1 in 5xFAD mice in response to long-term voluntary exercise, whereas staining of brain sections revealed a rise in TfR1 expression in the cortex of exercised 5xFAD mice cortex in comparison with sedentary mice. Moreover, we observed a dramatic reduction of TfR in GAS skeletal muscles upon regular exercise, which is in line with the elevated muscular ferritin increase in response to exercise. Here, we demonstrated that long-term voluntary exercise promoted a reduction of DMT1 expression and an increase in that of TfR in cortex of 5xFAD mice, while levels of TfR were markedly decreased in skeletal muscle of exercised mice. These findings clearly show that regular exercise is involved in the modulation of iron trafficking.

Next, we investigated the effect of regular exercise on the key proteins responsible for the modulation of iron homeostasis. Hepcidin regulates the body iron balance, and can directly interact with ferroportin, leading to a reduction of cellular iron export (Nemeth, Tuttle, *et al.*, 2004). Subsequent *in vitro* studies have shown that hepcidin can also control iron uptake by downregulating the expression of TfR and DMT1 (Du *et al.*, 2011). Given that hepcidin can negatively regulate cellular iron uptake and export, the possibility of cellular iron overload will increase if there is hepcidin overexpression. Brain iron homeostasis is also under the control of hepcidin, and AD-associated iron excess in the brain can be linked with hepcidin dysregulation (Vela, 2018). Although in our study we did not observe any increase in hepcidin in the cortex of 5xFAD mice, hepcidin upregulation in the rodent brain has been earlier reported to occur upon aging (Wang *et al.*, 2010; Lu *et al.*, 2016). Moreover, AD patients have increased serum hepcidin levels (Sternberg *et al.*, 2017; Fleszar *et al.*, 2019; Kweon *et al.*, 2019) suggesting it as a potential AD risk biomarker (Chatterjee *et al.*, 2020). The effect of physical activity on hepcidin levels has been only occasionally studied, although sedentary behavior (hypoactivity) has been linked with disrupted iron metabolism and increased hepcidin in liver and serum of healthy subjects (Cavey *et al.*, 2017; Nay *et al.*, 2020). Thereby, the effect of physical exercise is understudied in the brain and in the context of AD. In our study, we found a significant reduction of hepcidin in cortex of WT and 5xFAD mice in response to long-term voluntary exercise. This is the first study to demonstrate the ability of regular exercise to modulate hepcidin in the brain, which could potentially be beneficial in stabilizing the iron load and maintaining iron homeostasis in AD (Nay *et al.*, 2021).

We also investigated the possible mechanism behind the exercise-induced hepcidin reduction. Inflammation, one of the main pathological features of AD, is

known to induce hepcidin synthesis in the brain (Vela, 2018). In particular, IL-6 and the STAT3/JAK1 pathway are involved in hepcidin activation (Nemeth, Rivera, *et al.*, 2004; Qian *et al.*, 2014; You *et al.*, 2017). Moreover, it has been shown that hepcidin expression in the brain is lower in IL-6 knockout mice in response to inflammation stimuli in comparison with WT mice (Zhang *et al.*, 2017). In our study, we detected a significant elevation of STAT3 mRNA expression coupled with IL-6R mRNA overexpression in the cortex of 5xFAD mice. In Study II, we detected signs of increased inflammation in the brain of 5xFAD mice mediated via glial cell activation, while here the IL-6 level was unchanged between WT and 5xFAD mice, which is in line with previous studies (Choi *et al.*, 2018; Manji *et al.*, 2019). Recent proteomic analyses have described a significant STAT3 upregulation in 5xFAD mouse brains (Kim *et al.*, 2019). While IL-6 has a clear immune-modulatory role, it is also considered to be a myokine secreted from contracting skeletal muscle and potentially participating in brain-muscle crosstalk (Pedersen, 2019). Muscle-derived IL-6 might be a good candidate in exercise-induced iron modulation through hepcidin level regulation. In our study, we found a significant decrease in IL-6 in cortex and plasma in response to long-term voluntary exercise in both WT and 5xFAD mice, while no IL-6 changes were observed in skeletal muscle. In agreement with our results, it has been shown that resistance exercise could induce a decline in the concentration of IL-6 in APP/PS1 mice (Hashiguchi *et al.*, 2020) and while lifelong aerobic exercise stimulated a serum IL-6 decrease, it exerted no effect on muscular IL-6 levels in healthy individuals (Lavin *et al.*, 2020). Moreover, we demonstrated exercise-induced STAT3 downregulation together with an upregulation of STAT3/JAK1 inhibitor PTPe in the cortex of 5xFAD mice.

Taken together, we suggest that long-term voluntary exercise participates in the modulation of iron homeostasis in WT and 5xFAD mice both in the brain and skeletal muscle. Our study is the first to report that exercise-induced a decrease in the cortical hepcidin level possibly via an attenuation of the IL-6/STAT3 pathway, which could be important aspects of the brain-muscle crosstalk. Further research is needed to strengthen our findings related to exercise and IL-6 as a central element in iron and hepcidin modulation, possibly by using AD x IL-6 knockout mice. Furthermore, the association between brain iron levels with the cognitive decline in AD mice should be characterized. In addition, it would be important to evaluate the effect of regular exercise on the modulation of iron stores at the level of specific brain cell types in AD (Reinert *et al.*, 2019).

7 CONCLUSIONS

In the present study, the effects of long-term physical exercise were evaluated on aging and AD in mice, with a focus on the brain and skeletal muscle tissues. The results from this study are summarized as follows:

I. Long-term voluntary exercise is protective against oxidative and ER stress in mice upon aging. The protective effect of life-long exercise is associated with an enhancement of redox-regulation, ER stress adaptation and HSP defense.

II. Long-term voluntary exercise is beneficial against cognitive impairment in the 5xFAD mouse model of AD. The beneficial effect of long-term exercise is linked to a modulation of the reactive state of astrocytes, coupled to a restoration of astrocytic BDNF expression.

III. Long-term voluntary exercise modulates iron homeostasis in both WT and 5xFAD mice. The exercise-induced modulation of iron homeostasis is linked to decreases in the iron regulator protein hepcidin in the brain, possibly via an attenuation of the IL-6/STAT3 pathway.

In summary, we have demonstrated the positive impact of long-term physical exercise on aging and AD. Our study reveals new insights into the beneficial effects of regular exercise on both the brain and skeletal muscle and highlights a potential brain-periphery crosstalk in the context of aging and AD. This increased understanding is expected to provide new means for harnessing the beneficial effects of physical exercise in the prevention of age-related diseases like AD.

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ORIGINAL PUBLICATIONS (I – III)

I

Long-term exercise protects against cellular stresses in aged mice

Belaya I, Suwa M, Chen T, Giniatullin R, Kanninen K M, Atalay M and Kumagai S.

Oxidative Medicine and Cellular Longevity. Article ID 2894247, 2018.

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2018, Article ID 2894247, 10 pages
https://doi.org/10.1155/2018/2894247

Research Article

Long-Term Exercise Protects against Cellular Stresses in Aged Mice

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Received 26 September 2017; Revised 7 January 2018; Accepted 22 January 2018; Published 25 March 2018

Academic Editor: Paola Venditti

The current study examined the effect of aging and long-term wheel-running on the expression of heat shock protein (HSP), redox regulation, and endoplasmic reticulum (ER) stress markers in tibialis anterior (T.A.) and soleus muscle of mice. Male mice were divided into young (Y, 3-month-old), old-sedentary (OS, 24-month-old), and old-exercise (OE, 24-month-old) groups. The OE group started voluntary wheel-running at 3 months and continued until 24 months of age. Aging was associated with a higher thioredoxin-interacting protein (TxNiP) level, lower thioredoxin-1 (TRX-1) to TxNiP ratio—a determinant of redox regulation and increased CHOP, an indicator of ER stress-related apoptosis signaling in both muscles. Notably, GRP78, a key indicator of ER stress, was selectively elevated in T.A. Long-term exercise decreased TxNiP in T.A. and soleus muscles and increased the TRX-1/TxNiP ratio in soleus muscle of aged mice. Inducible HSP70 and constituent HSC70 were upregulated, whereas CHOP was reduced after exercise in soleus muscle. Thus, our data demonstrated that aging induced oxidative stress and activated ER stress-related apoptosis signaling in skeletal muscle, whereas long-term wheel-running improved redox regulation, ER stress adaptation and attenuated ER stress-related apoptosis signaling. These findings suggest that life-long exercise can protect against age-related cellular stress.

1. Introduction

Ageing is associated with the accumulation of anatomical and molecular changes that promote muscle atrophy, which is associated with a number of chronic diseases [1]. One of the most important steps in the prevention of age-related diseases and in the promotion of healthy aging is to increase knowledge of the molecular mechanisms associated with aging; it is also crucial to reveal how a healthy lifestyle, including regular physical exercise, may improve these processes. Cellular senescence is associated with impaired calcium homeostasis, mitochondrial dysfunction, aberrant redox control of cellular signaling, elevated oxidative and endoplasmic reticulum (ER) stress, a dysregulated unfolded protein response (UPR), and altered protein homeostasis [2]. Although the production of reactive oxygen species (ROS) is essential in physiological homeostasis and optimal muscle contraction, in times of oxidative stress, the increased ROS production can disrupt redox regulation of protein turnover, leading to increased protein misfolding and aggregation [3, 4]. While sustained deviation from redox homeostasis and activation of ER stress have been claimed to promote the development of aged-related diseases [3, 5], there is limited direct evidence to support this hypothesis.

The endogenous thiols including the glutathione (GSH) and thioredoxin (TRX) systems together with thioredoxin-

interacting protein (TxNiP), an endogenous inhibitor of TRX, are critical components of redox signaling and in the regulation of protection against oxidative stress [6]; they have also been associated with the regulation of UPR [7]. It has been shown that the GSH concentration is decreased during aging in skeletal muscle [8–10]. To date, there is limited information available regarding the effect of aging on the TRX system; in the only publication on this issue, the TRX protein content was higher in skeletal muscle of old mice compared with their younger counterparts [11]. To the best of our knowledge, age-dependent changes in the TxNiP system in skeletal muscle have not been investigated.

In the ER, lumen protein folding and homeostasis are carried out by chaperones and oxidatively folding enzymes including glucose-regulated protein 78 (GRP78, also known as BiP), and 94 (GRP94), calnexin, and thiol-disulfide oxidoreductase—protein disulphide isomerase (PDI), which belongs to the TRX superfamily [3]. During aging, the functional efficiency of ER chaperones declines, resulting in an accumulation of unfolded or misfolded proteins [12]. This process induces ER stress and leads to an activation of the UPR. The UPR system resolves ER stress by activating several signaling pathways aiming to restore protein homoeostasis by (1) increasing the synthesis of protein chaperones, (2) enhancing protein folding capacity, (3) stimulating protein degradation, and (4) decreasing protein production [3]. Additionally, cytoplasmic and mitochondrial chaperones, the heat shock proteins (HSPs), play a critical role in protein folding, intracellular trafficking of proteins, as well as dealing with proteins denatured by heat and metabolic stresses [13, 14]. It is known that aging suppresses HSP responses in skeletal muscle [15]. Chung and Ng [16] have detected increased HSP basal levels in the skeletal muscle of old rats, which can be linked to increased oxidative stress in sedentary muscle [17]. Several studies have reported that HSPs play an important role in apoptotic regulation. It has been demonstrated that a reduction in the level of HSP70 leads to ER and sarcoplasmic reticulum (SR) stress signaling and apoptosis induction in the skeletal muscle of aged mice [18]. The mitochondrial protein, HSP60, is also involved in antiapoptotic regulation [19]. Therefore, changes in HSP expression may influence apoptosis in skeletal muscle during aging.

Several age-related diseases are associated with chronic ER stress or impairment in UPR and HSP responses; these are reflected in overexpression of the transcription factor CCAAT-enhancer-binding protein homologous protein-(CHOP-) mediated, ER-originated proapoptotic signaling pathways [3]. Numerous studies have described induction of ER stress and ER stress signaling apoptosis pathways during aging. Levels of GRP78, PDI, and CHOP are increased in skeletal muscle tissues of old animals as compared to younger animals [16, 18]. On the other hand, O'Leary et al. [20] detected a 17-fold increase in CHOP protein expression, whereas a marginal decrease in GRP78 protein expression was observed in extensor digitorum longus (EDL) muscle of aged mice compared to younger animals. In addition, there are other studies reporting a reduction in the GRP78 level and an increase in the CHOP level during aging in various tissues including the brain [21], liver, kidney, heart, and spleen [22]. The rather sketchy information in the literature regarding ER stress and ER stress-related apoptosis in skeletal muscle during aging underlines the need for further studies in order to achieve a more thorough understanding of the topic.

Regular exercise improves the physical capacity and reduces the risk of developing chronic and age-related diseases [21, 22] by improving the metabolic state, antioxidant protection, and redox regulation [23]. Tarpenning et al. [24] examined the changes in the skeletal muscle of master athletes, who exercised regularly for 20 years. Lifelong training was reported to slow down aging-associated skeletal muscle fiber atrophy and prevent the reduction in muscular strength. Notably, acute intensive exercise induces the production of ROS that can evoke macromolecular damage, oxidative stress, ER stress, and activation of the UPR [6]. On the other hand, regular exercise training results in adaptations in antioxidant defense and improves redox signaling [6] to protect cells against stress-related diseases, thus delaying the aging processes [25]. In addition, the UPR, which is activated by exercise in skeletal muscles, may exert protective effects against ER stress and can promote metabolic adaptation to physical activity [26]. Long-term exercise was reported to upregulate HSP production in skeletal muscle [15, 25], which would be beneficial in coping with oxidative stress, ER stress, and ER stress-related apoptosis. Nevertheless, the ability to induce HSPs in aged skeletal muscle is compromised, which may impair the exercise-mediated adaptation processes [27].

There is only limited information available on the association of aging and exercise training concerning oxidative stress, ER (SR) stress, UPR, and/or ER stress-related apoptosis in skeletal muscle. The question of whether exercise training can reduce metabolic stress and apoptosis in skeletal muscle by increasing chaperone expressions and improving redox regulation has not been answered adequately. Our hypothesis is based on the fact that there is an age-induced disruption of redox regulation, increased redox ER stress, and ER stress-related apoptosis, and that long-term exercise can exert protective effects against these processes. The novelty of our study is that we investigated the key molecular markers associated with redox state, ER stress, and apoptosis in skeletal muscle of old animals in a life-long running model and compared them to young animals. Moreover, we determined age-related and exercise-induced changes in two types of skeletal muscle tissue: soleus, mostly composed of slow-twitch, aerobic muscle fibers, and tibialis anterior (T.A.), mostly consisting of fast-twitch glycolytic muscle fibers in order to reveal possible fiber specific changes.

2. Materials and Methods

2.1. Animals and Design. Three-month-old male ICR mice (CLEA Japan Inc., Tokyo, Japan) were maintained under standard conditions (24 ± 1°C; 12 h light–dark cycle) and had free access to food (CLEA Japan, CE-2, 3449 kcal/kg) and water. Male mice were used in order to avoid postmenopausal effects such as severe changes of sex hormones and osteoporosis in this life-long study. All mice were divided into three groups: young (Y, $n = 12$), old sedentary (OS,

$n = 5$), and old exercise (OE, $n = 5$). Mice from the Y group were sacrificed at 3 months age to obtain T.A. and soleus muscle samples. The mice in the OS and OE groups were maintained in individual wire mesh cages ($90 \times 220 \times 90$ mm). The mice from the OE group had free access to an activity wheel (628 mm circumference, 50 mm wide running surface of wire mesh; Shinano Instruments, Tokyo, Japan) from 3 to 24 months of age. Muscle samples from the mice in OS and OE group were collected when the mice were 24-month-old.

The mice were sacrificed with an overdose of pentobarbital sodium (60 mg/kg body weight intraperitoneally). The mice from the OE group were sacrificed 2 days after the last wheel-running session to rule out any effect of acute exercise. The T.A. and soleus muscles were removed, weighed, immediately frozen in liquid nitrogen, and stored at −80°C for later homogenization and biochemical assays. The experimental protocol was approved by the University Animal Experiment Committee and conducted in accordance with the Tohoku Institute of Technology guidelines for the Care and Use of Laboratory Animals.

2.2. Sample Preparation and Western Blot Analysis. Muscle samples were homogenized in eight volumes of lysis buffer (pH 7.4) containing 10 mM NaCl, 1.5 mM $MgCl_2$, 20 mM HEPES, 20% glycerol, 0.1% Triton X-100, 5% protease inhibitor cocktail, 0.5% PMSF, and 1.5% phosphatase inhibitor cocktail 2 (Sigma-Aldrich, St. Louis, MO, USA). Cytosolic extracts from samples were centrifuged at 2500 rpm for 10 min at +4°C. Supernatants containing cytosolic proteins were aliquoted prior to further measurements. Protein concentrations were measured by using the BCA protein assay kit (Thermo Fisher, Rockford, IL, USA). Western blot procedures were as previously reported [13], proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane (Millipore, Bedford, Mass., USA), and incubated for 1 hour at +37°C in blocking buffer (100 mM Tris, 154 mM NaCl, 5% nonfat dry milk). After treating with the antibodies listed below overnight at +4°C, the membranes were washed 4 times per 5 min with Tris-buffered saline containing 0.1% Tween 20 and incubated in secondary antibodies (antimouse IgG, 35521; anti-rabbit IgG, 35571; anti-rat IgG, A-21096, Thermo Fisher, Rockford, IL, USA) for 30 min in room temperature. GAPDH (sc-25778) was used as internal standard. Proteins were visualized with the Odyssey Imaging System (LI-COR Biosciences Inc., Lincoln, NB, USA) and quantified using Odyssey software.

2.3. Primary Antibodies. Antibodies against heat shock protein 25 (HSP25, SPA-801), heat shock protein 60 (HSP60, SPA-806), heat shock protein 70 (HSP70, SPA-810), heat shock protein 90 (HSP90, SPA-835), glucose-regulated protein 78 (GRP78, SPA-826), and glucose-regulated protein 75 (GRP75, SPA-825) were purchased from Enzo Life Sciences Inc., (Farmingdale, NY, USA). The antibody against cytosolic thioredoxin-1 (TRX-1, ATRX-06) was purchased from IMCO Corp (Stockholm, Sweden), thioredoxin-interacting protein (TxNiP, K0205-3) from MBL (Medical and Biological Laboratories Co. Ltd, Nagoya, Japan), and 4-hydroxy-2-nonenal (4-HNE, HNE11-S) from Alpha Diagnostic IntI Inc. (San Antonio, TX, USA). Antibodies against CCAAT/enhancer-binding protein homologous protein (CHOP, L63F7) and protein disulfide isomerase (PDI, C81H6) were purchased from Cell Signaling Technology (Danvers, MA, USA). The antibody against GAPDH (sc-25778) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

2.4. Protein Carbonyls. The level of protein carbonyls, a marker of protein oxidative damage, was measured from plasma samples after derivatization with dinitrophenylhydrazine using an ELISA method previously described with modifications [28]. The maximal intra-assay CV for protein carbonyls was 5.9% and the maximal inter-assay CV was 9.2%.

2.5. Lipid Hydroperoxide Assay. Lipid hydroperoxides (LPO) in muscle tissue were determined as described previously [29]. This method is based on oxidation of Fe II to Fe III by lipid hydroperoxides under acidic conditions, followed by complexation of Fe III by xylenol orange.

2.6. Statistical Analyses. All data are expressed as the mean ± SEM. The statistical significance of the data was determined using one-way analysis of variance (ANOVA) with a posthoc test (LSD). The equality of variances was analyzed with Levene's test. The nonparametric Mann–Whitney test was applied for nonhomogenously distributed data. Spearman's test was used for correlation analysis. Statistical analyses were performed with SPSS software version 21.0, and the level of significance was set at $p < 0.05$.

3. Results

No significant differences in body mass were observed among the groups [30]. The total food intake was 15% higher in OE group compared to the old-sedentary OS group [30]. The T.A. mass and T.A. mass/body mass were significantly lower in old mice, while wheel-running partly restored age-related decreases in T.A. mass (unpublished data). Notably, soleus mass and soleus mass/body mass were significantly lower in the OS group than in the Y group. Furthermore, those in the OE group were significantly higher than in the OS group, thereby indicating the efficiency of wheel-running [30].

3.1. Effects of Aging on Redox Regulation, HSP Expression, ER Stress, and UPR in Skeletal Muscle. Ageing remarkably increased TxNiP protein expression by 261.8% in the fast-twitch glycolytic T.A. muscle fibers and by 530.2% in the slow-twitch, aerobic soleus muscle fibers over the level in Y mice ($p < 0.01$, Figure 1). In OS mice, the level of TRX-1 expression was slightly increased by 57.7% in T.A. muscle ($p > 0.05$) and by 54.5% ($p > 0.05$) in soleus muscle compared to Y mice (Figure 1). Notably, we detected a decrease in the TRX-1/TxNiP ratio by 37.6% in T.A. muscle ($p < 0.05$) and by 77.6% in soleus muscle ($p < 0.05$) in OS mice compared to Y mice (Figure 1). We observed no significant changes in the level of protein carbonyls, 4-HNE adduct (lipid peroxidation marker), and lipid hydroperoxides (LPO)

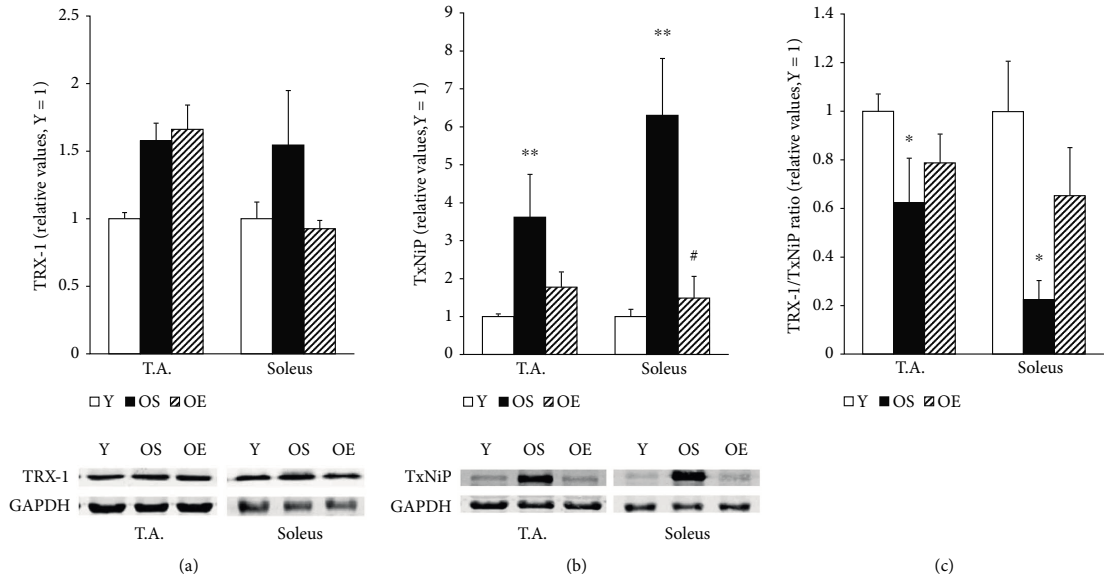

Figure 1: Effect of aging and voluntary wheel-running on the following redox regulation markers; thioredoxin-1 (TRX-1), thioredoxin-interacting protein (TxNiP), and TRX-1/TxNiP ratio in T.A. and soleus muscles of mice. White bars: young 3-month-old mice; black bars: OS sedentary 24-month-old mice; diagonal-striped bars: OE 24-month-old mice after 21 months' voluntary wheel-running. Data are mean ± SEM. $n = 12$ for Y, $n = 5$ for OS, and $n = 5$ for OE. Difference due to age: $^{*}p < 0.05$, $^{**}p < 0.01$. Difference due to exercise: $^{\#}p < 0.05$.

Table 1: Effect of aging and long-term voluntary wheel-running on the levels of 4-hydroxynonenal adducts (4-HNE), lipid hydroperoxides (LPO), protein carbonyls, and heat shock proteins in T.A. and soleus mice muscles.

Levels of 4-HNE adducts, LPO, protein carbonyls, and heat shock proteins		Y	OS	OE
T.A.	4-HNE	1.00 ± 0.04	0.88 ± 0.11	0.54 ± 0.013$^{\#}$
	LPO	1.00 ± 0.01	1.21 ± 0.01	1.26 ± 0.02
	Protein carbonyls	1.00 ± 0.01	1.02 ± 0.01	1.04 ± 0.01
	HSP25	1.00 ± 0.08	1.14 ± 0.23	1.14 ± 0.10
	HSP60	1.00 ± 0.05	1.05 ± 0.12	1.28 ± 0.11
	HSP90	1.00 ± 0.10	1.38 ± 0.24	1.14 ± 0.07
Soleus	4-HNE	1.00 ± 0.13	0.76 ± 0.18	1.07 ± 0.16
	LPO	1.00	0.94	0.95
	Protein carbonyls	1.00 ± 0.01	1.01 ± 0.01	1.01 ± 0.01
	HSP25	1.00 ± 0.06	1.08 ± 0.54	1.55 ± 0.19
	HSP60	1.00 ± 0.07	0.71 ± 0.07	0.72 ± 0.05
	HSP90	1.00 ± 0.07	0.89 ± 0.42	1.19 ± 0.13

Data are mean ± SEM. Groups are as follows: Y, young 3-month-old mice; OS, sedentary 24-month-old mice; OE, 24-month-old mice after 21 months of voluntary wheel-running. $n = 12$ for Y, $n = 5$ for OS, and $n = 5$ for OE. Difference due to exercise: $^{\#}P < 0.05$.

(Table 1) in skeletal muscle of old mice as compared to the young mice.

Moderate reductions were observed in the GRP75 (27.5%, $p < 0.05$) and HSC70 (32.5%, $p < 0.05$) expression levels in soleus muscle of OS mice as compared to the Y mice (Figure 2). However, aging did not affect the expression levels of HSP25, HSP60, and HSP70 proteins in T.A. or soleus skeletal muscles (Table 1, Figure 2).

To understand the cellular stress state of aged mice in view of ER stress and UPR, we tested the levels of GRP78 chaperone, PDI, and CHOP proteins (Figure 3). In T.A. muscle of OS mice, the level of GRP78 expression was increased by 53.9% ($p < 0.05$) compared to Y mice, whereas the GRP78 level was not changed in soleus muscle. PDI protein levels remained unaltered in OS mice in both T.A. and soleus muscles compared to Y mice. In OS mice, the level of the ER stress-related apoptosis marker CHOP was significantly elevated in T.A. muscle ($p < 0.01$) by 302.1% and in soleus muscle ($p \leq 0.001$) by 1199.8% compared to Y mice.

3.2. Effects of Long-Term Exercise on Redox Regulation and HSP Expression in Skeletal Muscle of Old Mice. Long-term exercise decreased the TxNiP level in both muscle types in the aged mice (Figure 1)—in T.A. by 50.9% ($p > 0.05$) and in soleus muscle by 76.4% ($p < 0.05$). The TRX-1/TxNiP ratio was increased by 26.3% in T.A. ($p > 0.05$) and by 190.4% in soleus muscle ($p > 0.05$) of OE mice compared to OS mice (Figure 1). Moreover, after long-term exercise, the level of the 4-HNE adduct (lipid peroxidation marker) declined by 39.3% ($p < 0.05$) compared to OS control mice in T.A. muscle (Table 1). Our findings did not reveal any significant effects of long-term exercise on the LPO and amounts of protein carbonyls, a marker of oxidative damage in proteins, in skeletal muscle. (Table 1). In addition, no significant

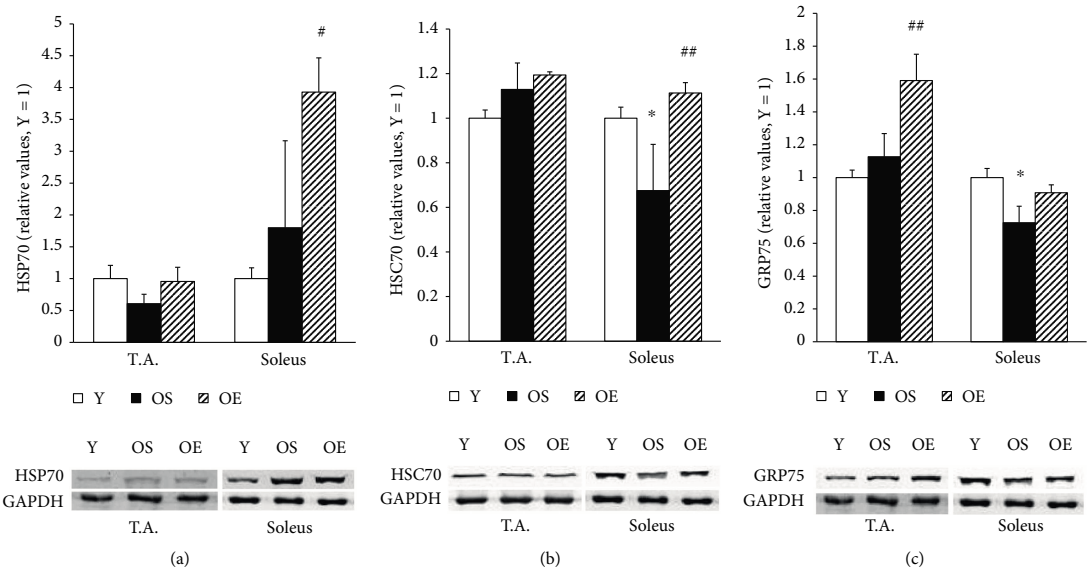

Figure 2: Effect of aging and voluntary wheel-running on the expressions of HSP70, HSC70, and GRP75 heat shock proteins in T.A. and soleus muscles of mice. Groups and bars are as in Figure 1. Data are mean ± SEM. $n = 12$ for Y, $n = 5$ for OS, and $n = 5$ for OE. Difference due to age: $^{*}p < 0.05$. Difference due to exercise: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.

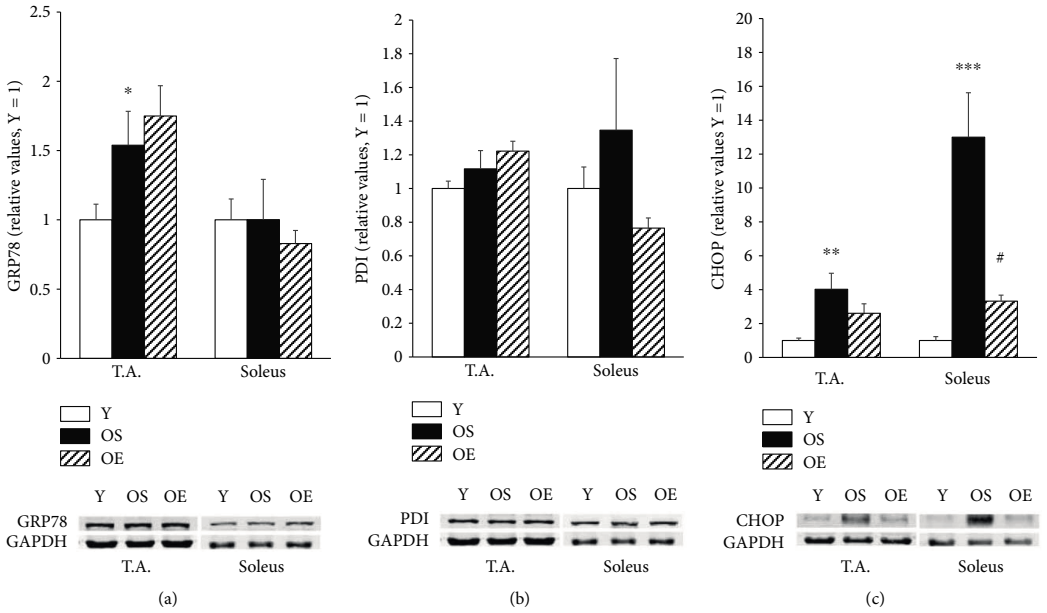

Figure 3: Effect of aging and voluntary wheel-running on ER stress and UPR markers GRP78, PDI, and CHOP in T.A. and soleus muscles of mice. Groups and bars are as in Figure 1. Data are mean ± SEM. $n = 12$ for Y, $n = 5$ for OS, and $n = 5$ for OE. Difference due to age: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p \leq 0.001$. Difference due to exercise: $^{\#}p < 0.05$.

changes in the TRX-1 protein level were observed in both T.A. and soleus muscles after long-term exercise in OE mice as compared to OS mice (Figure 1).

We next examined mitochondrial HSP protein expressions—HSP60 protein and GRP75 chaperone. When compared to OS muscles, the exercise-trained T.A. muscle

of old mice showed a marginal increase (21.6%, $p > 0.05$) in HSP60 protein expression and a more significant increase (41.1%, $p < 0.01$) in GRP75 protein expression (Table 1, Figure 2). The levels of these two mitochondrial HSP proteins strongly correlated with each other in T.A. muscle of old mice ($r = 0.867$; $p < 0.01$). At the same time, we did not observe any changes in these markers in soleus muscle of trained mice.

Several changes were observed in stress-inducible HSP70, HSP25, and housekeeping HSC70 proteins in soleus muscle of OE mice. After exercise, the level of HSP25 was slightly increased (43.1%, $p > 0.05$) in soleus muscle of old mice (Table 1). The expression of HSC70 was also significantly increased in soleus muscle of OE mice by 64.8% ($p < 0.01$) compared to OS mice (Figure 2). The expression of HSP70 in soleus muscle was also higher (118.5%, $p < 0.05$) in OE mice compared to OS mice (Figure 2). However, we did not observe any evidence that long-term exercise induced any significant changes in the expression levels of HSP25, HSC70, and HSP70 proteins in T.A.

3.3. Effects of Long-Term Exercise on ER Stress and UPR in Skeletal Muscle of Old Mice. To understand the effect of 21 months' wheel-running exercise on ER stress and UPR in skeletal muscle of old mice, we measured the levels of GRP78, PDI, and CHOP. Marginal upregulations of GRP78 (13.7%, $p > 0.05$) and PDI (9.4%, $p > 0.05$) proteins were observed to occur upon long-term exercise in T.A. muscle of OE mice (Figure 3). Furthermore, the levels of these two ER stress-related proteins remained unaltered in soleus muscle of OE mice compared to OS mice. Despite nonsignificant changes in GRP78 expression, the ER stress-related apoptotic marker CHOP was clearly attenuated by 74.5% ($p < 0.05$) in soleus muscle of trained old mice compared to their OS counterparts. The level of CHOP seemed to be lower (35.2%, $p > 0.05$) in T.A. muscle of the OE mice.

3.4. Correlation Analysis. We calculated the correlations among all studied cellular stress markers. Table 2 lists the most significant correlations between redox regulation markers and ER stress markers.

Table 2: Correlations between ER stress and redox regulation markers in the T.A. and soleus muscles of mice.

ER stress	Redox regulation	Correlation coefficient T.A./soleus muscle	p value T.A./soleus muscle
GRP78	TRX-1	0.479/0.728	0.018/0.001
PDI	TRX-1	0.505/0.789	0.012/0.001
CHOP	TxNiP	0.791/0.629	0.001/0.002
CHOP	TRX-1-TxNiP	−0.579/−0.687	0.004/0.001

Correlation analysis was performed by Spearman's test. Correlation data was obtained from all studied mice ($n = 22$). Significance level was set at $p < 0.05$.

4. Discussion

The present study describes a significant age-related suppression of antioxidant defense, impairment of redox regulation, an increase of ER stress and ER stress-related apoptosis, which are partly restored by long-term voluntary exercise.

The TRX system plays a crucial role in redox signaling and antioxidant defense. Under oxidative stress conditions, TRX exerts protective effects against apoptosis [6]; conversely, TxNiP inhibits the TRX system and promotes apoptosis. Thioredoxin-interacting protein directly binds to TRX and suppresses its reducing activity, and thus a lower TRX/TxNiP ratio may be a determinant of disrupted redox regulation and related disorders [31]. Furthermore, a dysfunction of thiol redox circuits, including TRX system, has been proposed to be the main cause of impaired redox homeostasis and dysregulation of cellular processes [6]. The present study is the first to determine changes in the TRX-TxNiP system in skeletal muscle of old mice. Notably, we observed a significant increase in the TxNiP protein content in both soleus and T.A. muscles of old animals. An important new finding was that aging significantly decreased the TRX-1/TxNiP ratio in both type of skeletal muscle tissues. The observed increases in the levels of TRX-1 (over 50%) in both skeletal muscle types in response to aging did not quite achieve statistical significance, possibly due to limited number of animals and the subsequent lack of statistical power. A previous study demonstrated a significant increase in the TRX-1 content in T.A. muscle of 28-month-old mice when compared with 6-month-old mice [11]. Despite the changes in TRX-1/TxNiP ratio and TxNIP and TRX-1 levels, no difference in protein carbonyl and 4-HNE protein adduct levels were observed between young and old sedentary animals. The lack of difference in oxidatively modified protein levels can be explained by their increased removal. The accumulation of oxidatively damaged proteins is also known to induce proteasome activity intended to achieve their removal [32]. Furthermore, we observed 21–26% increases of the lipid peroxidation marker LPO in old animals in T.A. muscle; however, these increases did not reach statistical significance. Nevertheless, our data collectively revealed increased TxNiP levels and a lower TRX-1/TxNiP ratio in aged animals that may explain the redox dysregulation and consequent cellular dysfunctions that occur during aging.

We evaluated the levels of ER stress and ER stress-specific apoptosis markers during aging and after voluntary exercise. The level of GRP78 significantly increased in T.A. but not in soleus muscle upon aging. GRP78 is a purely ER-located chaperone and its increased levels reflect an upstream stimulation of the unfolded protein response; unexpectedly, GRP78 levels increased only in T.A. muscle with aging. On the other hand, it has also been reported that during aging, the increased anabolic resistance and the downregulation of the protein folding capacity are more prominent in the soleus muscle compared to other types of skeletal muscle [33]. Nevertheless, a minor increase in the level of PDI—a key enzyme responsible for oxidative protein folding during ER stress and UPR—was observed in soleus and T.A. muscles upon aging. Furthermore, CHOP expression was remarkably increased in both soleus (13-fold) and T.A. (fourfold) muscles with aging. These findings of increased CHOP expression with aging are consistent with a previous study which demonstrated a significant upregulation of GRP78 and CHOP in the

soleus muscle of 32-month-old rats as compared to 6-month-old rats [18]. Moreover, Hwee et al. [34] reported a significant increase in the expressions of GRP78, PDI, and CHOP in gastrocnemius muscle of 24-month-old mice in comparison with 6-month-old mice. Interestingly, a recent study indicated that CHOP levels were higher in both T.A. and soleus muscles of old mice, while the GRP78 expression demonstrated skeletal muscle type-dependent diverse changes during aging [33]. Therefore, regardless of the diversity, the results presented by others and us imply that there is an age-induced activation of UPR and ER stress-related apoptosis signaling in skeletal muscle.

In order to clarify the association of impaired chaperone function with increased ER (SR) stress in aging, we examined HSP levels in skeletal muscle as a function of age. The levels of mitochondrial chaperone GRP75 protein and constitutively expressed cytosolic HSC70 declined in soleus muscle upon aging. We did not detect any significant changes in the expression level of HSP25, HSP60, and HSP70 proteins in both T.A. and soleus muscles during aging. Various studies have reported somewhat conflicting age-related HSP expression changes in skeletal muscle. Valls et al. [35] stated that aging did not induce changes in HSP27 and HSP70 levels in skeletal muscle. In addition, there were no differences in the levels of HSP70 in skeletal muscles including the soleus, plantaris, and gastrocnemius muscles in aged rats [36]. Other studies have demonstrated conflicting results; a significant decrease in HSP70 expression without any change in the expression level of HSC70 in soleus muscle of aged rats [18] or even increased levels of HSP25, HSP60, and HSP70 in skeletal muscle of old rats compared to adult rats [16]. The discrepancies of these results may be due to differences in the age of animals and species used for the studies. Based on our results, we can only postulate that age-induced disturbances in redox regulation may impair HSP functions and reduce the level of HSPs.

The present study focused on the protective effect of long-term exercise in mice; our animals were trained for 21 months, the majority of their lifespan. One major finding was that there was a remarkable decrease in TxNiP protein content in soleus muscle of trained mice compared to nontrained controls. Although a similar decrease (over 50%) in TxNiP levels was observed, the effect of long-term exercise training on TxNiP levels did not quite reach statistical significance in the T.A. muscle. This may be due to the small sample size and variations in the results due to the semiquantitative nature of Western blot techniques. In addition, consistent with the HSP responses, it is also expected that the influence of aerobic exercise on redox regulation would be more prominent in a muscular tissue consisting of mainly slow oxidative muscle fibers. The existing information on the effect of regular exercise on tissue TxNiP levels is, however, scanty. In the only publication in the literature, there was no exercise-mediated effect on the TxNiP level in the rat brain even though there was an increase in the level of TRX-1 protein [37]. Nevertheless, because brain tissue is not actively involved in the metabolic changes during exercise, caution is needed when making a direct comparison of the responses of muscle and brain tissue to exercise. Notably, we detected an increase in the TRX-1/TxNiP ratio in skeletal muscle in response to long-term exercise. Recent reports suggest that an increase in TRX together with a decrease in TxNiP expression could help to prevent various pathologies (reviewed recently by Yoshihara et al. [31]). In addition, we examined the levels of protein carbonyls and 4-HNE adducts, utilized as markers of protein oxidation and lipid peroxidation, respectively, in mice skeletal muscle. After long-term voluntary exercise, the amounts of 4-HNE adducts significantly decreased in T.A. muscle of OE mice, whereas there was no change in the protein carbonyls. These results are consistent with a previous study, which observed a decrease of lipid peroxidation levels after regular exercise training [23, 35]. Based on our findings, we suggest that life-long exercise improves the antioxidant TRX system and TRX-1/TxNiP ratio, which promote resistance to oxidative stress and could be protective against several pathologies related to aging.

A major focus of the current study was to explore ER stress and its association to redox regulation and oxidative stress in response to aging and long-term exercise. Markers of ER stress and UPR, GRP78 and PDI, were not significantly influenced in skeletal muscles by long-term exercise. In the existing literature, several studies have demonstrated that ER stress markers remain unaltered or are even suppressed after moderate regular exercise training, while a single bout of exercise with moderate intensity was reported to induce UPR activation [24, 36, 38]. We observed a minor upregulation of GRP78 and PDI proteins after long-term exercise in T.A. muscle of OE mice. In contrast, the level of these two ER stress-related proteins tended to decrease in soleus muscle of OE mice compared to OS. On the other hand, exercise induced a remarkable attenuation of the ER stress-related apoptosis marker CHOP in soleus muscle of aged mice. The level of CHOP also was observed to be lower in T.A. muscle in response to long-term exercise. These findings are consistent with previous studies, that is, significant reductions were demonstrated in the levels of CHOP mRNA in gastrocnemius muscle of rats after treadmill running exercise [23]. Moreover, in support of the relationship between ER stress and redox regulation disorders, the levels of CHOP and TxNiP protein were strongly correlated in T.A. and soleus muscles of all the studied mice. Although TxNiP has been demonstrated to induce apoptosis through activation of apoptosis-stimulating kinase 1 (ASK1) [39], here we report for the first time, a direct association between TxNiP and the ER stress-related apoptosis marker, CHOP. Our results are also in agreement with a recent study which provided direct evidence of the association of TxNiP with PDI activity and ER stress [40]. In addition, the TRX-1/TxNiP ratio correlated negatively and strongly with the level of CHOP in T.A. and soleus muscles. Therefore, our data demonstrates that lower TxNiP levels can be protective against ER stress-related apoptosis, and furthermore, that exercise training can be useful through decreasing TxNiP levels and improving the TRX-1/TxNiP ratio. We conclude that long-term exercise may lead to ER stress adaptation in skeletal muscle and exert protective effects against future stress and ER stress-related apoptosis.

Furthermore, we examined the effect of long-term exercise on HSP levels in skeletal muscle tissue of mice. As compared to OS mice, we observed an increase in GRP75 protein content (41.1%) in T.A. but not in soleus muscle of OE mice. Moreover, we detected a significant upregulation of stress-inducible HSP70 (118.5%), and constitutively expressed HSC70 (64.8%) proteins in soleus muscle after long-term exercise, while in T.A. muscle, these increases did not reach statistical significance. We postulate that similar to the changes we detected in the redox-regulation markers in response to long-term exercise, the difference in the upregulation of stress-inducible HSPs between soleus and T.A. muscles can be attributed to the difference in their metabolic properties; soleus muscle mostly contains slow oxidative fibers, whereas T.A. mostly contains fast glycolytic fibers [41]. There is a greater recruitment of soleus muscle to moderate aerobic exercise such as voluntary wheel-running. Furthermore, exercise-induced metabolic demands and ROS production are higher in soleus muscle, which ultimately result in the induction of HSPs [42]. Enhanced HSP levels in response to different types of exercise in various skeletal muscles have been well demonstrated. Increases in HSP70 expression after moderate treadmill endurance training in soleus and T.A. muscles of rats were recently reported [42]. The protein levels of HSP72 and HSP25 increased in T.A. muscle of old mice after resistance training [43] and in gastrocnemius muscles of adult mice after voluntary wheel-running [44]. Therefore, in agreement with previous studies, we observed an increase of HSP levels, particularly the constituent HSC70 levels, in response to long-term exercise in old animals. On the other hand, the voluntary exercise training used in our study resulted in a lower extent increase of inducible HSP levels when compared to the situation where more strenuous exercise protocols have been adopted. Moreover, the lower extent of HSP increase after long-term exercise can be associated with a compromised adaptive mechanism of HSP expression in old individuals, as compared to young animals and adults [27].

5. Conclusion

Collectively, the current study demonstrated that increased ER stress and ER stress-related apoptosis marker and impairment of the redox regulation system and HSP functions are associated with a sedentary lifestyle of old mice and that these changes occur in a muscle type-specific manner. At the same time, life-long exercise appeared to improve redox regulation and HSP defense, as well as to cause a reduction in the TxNiP level, leading to ER stress adaptation and attenuation of ER stress-related apoptosis in skeletal muscle. These findings provide further evidence that life-long exercise has a protective effect against age-related cellular stress processes.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Mustafa Atalay and Shuzo Kumagai equally contributed to this study and are shared last authors.

Acknowledgments

The study was supported by grants from Academy of Finland (TM-15-9664), Centre for International Mobility (TM-16-10086), Sigrid Juselius Foundation (TD1304), and COST Actions (CA16112). The authors thank Taina Vihavainen for the skillful technical assistance.

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II

Astrocyte remodeling in the beneficial effects of long-term voluntary exercise in Alzheimer's disease

Belaya I, Ivanova M, Sorvari A, Ilicic M, Loppi S, Koivisto H, Varricchio A, Tikkanen H, Walker F R, Atalay M, Malm T, Grubman A, Tanila H and Kanninen K M.

Journal of Neuroinflammation. 17:271, 2020.

Belaya et al. Journal of Neuroinflammation (2020) 17:271
https://doi.org/10.1186/s12974-020-01935-w

Journal of Neuroinflammation

RESEARCH Open Access

Astrocyte remodeling in the beneficial effects of long-term voluntary exercise in Alzheimer's disease

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Abstract

Background: Increased physical exercise improves cognitive function and reduces pathology associated with Alzheimer's disease (AD). However, the mechanisms underlying the beneficial effects of exercise in AD on the level of specific brain cell types remain poorly investigated. The involvement of astrocytes in AD pathology is widely described, but their exact role in exercise-mediated neuroprotection warrant further investigation. Here, we investigated the effect of long-term voluntary physical exercise on the modulation of the astrocyte state.

Methods: Male 5xFAD mice and their wild-type littermates had free access to a running wheel from 1.5 to 7 months of age. A battery of behavioral tests was used to assess the effects of voluntary exercise on cognition and learning. Neuronal loss, impairment in neurogenesis, beta-amyloid (Aβ) deposition, and inflammation were evaluated using a variety of histological and biochemical measurements. Sophisticated morphological analyses were performed to delineate the specific involvement of astrocytes in exercise-induced neuroprotection in the 5xFAD mice.

Results: Long-term voluntary physical exercise reversed cognitive impairment in 7-month-old 5xFAD mice without affecting neurogenesis, neuronal loss, Aβ plaque deposition, or microglia activation. Exercise increased glial fibrillary acid protein (GFAP) immunoreactivity and the number of GFAP-positive astrocytes in 5xFAD hippocampi. GFAP-positive astrocytes in hippocampi of the exercised 5xFAD mice displayed increases in the numbers of primary branches and in the soma area. In general, astrocytes distant from Aβ plaques were smaller in size and possessed simplified processes in comparison to plaque-associated GFAP-positive astrocytes. Morphological alterations of GFAP-positive astrocytes occurred concomitantly with increased astrocytic brain-derived neurotrophic factor (BDNF) and restoration of postsynaptic protein PSD-95.

Conclusions: Voluntary physical exercise modulates the reactive astrocyte state, which could be linked via astrocytic BDNF and PSD-95 to improved cognition in 5xFAD hippocampi. The molecular pathways involved in this modulation could potentially be targeted for benefit against AD.

Keywords: Alzheimer's disease, Voluntary exercise, 5xFAD mouse, Behavior, BDNF, Astrocyte, GFAP, Morphology

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Background

Alzheimer's disease (AD) is the most common neurodegenerative disorder pathologically characterized by accumulation of amyloid-beta (Aβ) plaques, formation of intracellular neurofibrillary tangles, neuronal loss, neuroinflammation, and oxidative stress [1]. Due to lack of broadly effective therapies, there is growing focus on modifiable lifestyle factors that may reduce disease risk or slow disease progression. Because it is known that a sedentary lifestyle is associated with impaired cognitive function and AD [2], one possible way to ameliorate AD pathology is regular physical exercise. Indeed, multifold benefits of regular physical exercise have been reported in both human and mouse models [3]. For instance, physical exercise has been shown to improve learning and memory by increasing long-term potentiation (LTP) and neurogenesis [4]. In addition, physical exercise is associated with structural and functional changes in the brain, promoting neuronal plasticity through increasing the levels of neurotropic and growth factors such as brain-derived neurotrophic factor (BDNF) [5]. Given that glial cells regulate many aspects of neuronal function and synaptic plasticity, the effects of physical exercise on glial cells may have broad implications for cognitive function, learning, and memory [6].

The exercise-induced cognitive improvements in AD mice is often associated with modulation of neuroinflammation [7]. Glial activation is a well-described feature of the AD brain [8], manifested as altered cell morphology [9, 10] and function [11]. Both astrocytes and microglia are implicated in AD progression [1] and recent cell type-specific gene expression data have undoubtedly provided evidence for the strong involvement of astrocytes in human AD [12, 13].

The most commonly reported exercise-induced effects in astrocytes include morphological changes, together with elevation or suppression in the levels of glial fibrillary acidic protein (GFAP) [14–19] . The reported difference in GFAP levels may be explained partly by astrocyte heterogeneity and partly by differences in the type of exercise intervention. Most studies have used voluntary wheel running, although daily access periods, duration of exercise, and age at the start of intervention have varied considerably among studies. Additionally, various murine models of AD have been used that display considerable variation in type and onset of pathology. The positive effect of physical exercise on the 5xFAD mouse model remains poorly investigated, although 5xFAD is a useful AD model with rapid Aβ plaque deposition starting from 2 months of age [20]. In particular, the impact of voluntary physical exercise on astrocyte-specific responses requires further clarification. Although astrocytes are important in AD progression and pathology, limited information exists on their role in exercise-mediated cognitive improvement.

We therefore tested the hypothesis that voluntary running exercise can improve cognitive deficits in the 5xFAD mouse model via astrocytic modulation. We assessed simultaneously a wide range of pathological features including neurogenesis, inflammation, AD pathology, synapses, and neurotrophic factors. The involvement of astrocytes in exercise-induced neuroprotection was assessed by a panel of extensive morphological, histological, and biochemical assays.

Methods

Experimental design

We used male transgenic 5xFAD (n = 41) mice carrying human amyloid precursor protein (APP) with the APP Swedish, Florida, and London mutations and human presenilin-1 (PSEN1) including the M146L and L286V mutations, driven by the mouse Thy1 promoter [21] and their wild-type (WT, n = 41) littermates on the JAXC57BL/6J background. Mice were housed in controlled temperature, humidity, and 12:12-h light-dark cycle, and had *ad libitum* access to food and water. This study was carried out in accordance with the Council of Europe Legislation and Regulation for Animal Protection and was approved by the National Animal Experiment Board of Finland.

At 6 weeks of age, mice were randomly divided into four groups: WT-Sedentary (WT-SED, n = 21), WT-Exercised (WT-EXE, n = 20), 5xFAD-Sedentary (5xFAD-SED, n = 21), and 5xFAD-Exercised (5xFAD-EXE, n = 20). The mice from WT-SED and 5xFAD-SED groups were housed in individual cages. The mice from WT-EXE and 5xFAD-EXE groups were housed in the same size individual cages, and had free access to running wheels (diameter 24 cm, Technoplast, Italy) for 6 months; the counters (Sigma, Germany) were attached to each cage to monitor the running distance and time for individual mice on weekly bases. In addition, body weight was measured for all mice weekly throughout the study. Two days before sacrificing, running wheels were removed from the cages of exercised mice to avoid any acute effects of running.

Behavioral testing

Nest building tests were performed for all mice at the age of 7 months to assess the ability of animals to build a nest—a basic rodent activity related to hippocampal function [22]. A paper towel (22 × 22 cm, Katrin PLUS, Mediq) was placed inside the home cage of each mouse, and after 24 h, the built nest was photographed and removed from the cage. Built nests were scored with the following points: 0 point—untouched nest, 1—flat nest with few bites, 2—creased/moved and bitten nest, and 3—creased and bitten nest forming a crater inside the

original nesting material. The person scoring the nest was blinded to the group of the mice.

To measure exploration activity vs. anxiety of mice, the open field test was performed as previously described [23]. At the age of 7 months, mice were individually placed in an empty white circular box (diameter 120 cm, height 22 cm), facing a wall, and allowed to freely explore the area inside the box for 10 min. Next, the tested mouse was placed back into the home cage; the box was cleaned with 70% EtOH and dried for the next mouse. Each mouse was recorded by video camera, and then the behavioral parameters, including duration in the center zone (80 cm diameter area, s), frequency of entries into the center zone, and latency to first entry in the center zone (s), were analyzed using EthoVision XT 7.1 video tracking software (Noldus Information Technologies, The Netherlands).

The elevated plus-maze test was performed at the age of 7 months as previously described [23] to assess exploration vs. anxiety level of mice. The mice were individually placed in the center of the plus-maze (two open arms without walls—30 × 5 cm, and two closed arms—30 × 5 × 20 cm, all painted black) facing closed arm, and allowed to freely explore the maze for 5 min. Subsequently, the tested mouse was placed back into the home cage; the plus-maze was cleaned with 70% EtOH and dried for the next mouse. Each mouse was recorded by video camera, and then behavioral parameters including duration in the open arms (s), and frequency of entries into the open arms, were analyzed using EthoVision XT 7.1 video tracking software (Noldus Information Technologies, The Netherlands). Only those mice that made more than five entries into different arms during the test were included in the analyses.

Spatial learning and memory were evaluated by the Morris water maze (MWM) test at the age of 7 months by using a testing protocol, described in detail previously [24]. Before the test phases, mice were pre-trained to find a submerged transparent platform 10 × 10 cm inside the white circular pool (diameter 120 cm, height 22 cm) filled with room temperature water, but within a limited swimming area (70 × 13 cm). Three days later, the MWM test began, composed of acquisition and probe phases. The acquisition phase consisted of five acquisition trials per day over 5 days from four different starting points, which were randomly chosen within the pool area. During the acquisition phase, the mouse was placed inside the pool, facing the wall, and each trial continued until the mouse reached the escape platform, or 60 s if the mouse was not able to find the platform. On the fifth day, a probe test was performed, during which the mouse was placed inside the swimming pool without the escape platform for 60 s to assess search bias as an indication of memory. During the test, mice rested for approximately 5 min in a warm cage with a cotton towel. The swimming path of each mouse was recorded by an overhead video camera, and parameters including speed (cm/s), escape latency (s), time in the wall zone (15 cm radius from wall area, s) for acquisition trials, as well as latency and distance to platform zone (30 cm radius area in previous platform location, s) for the probe trial were analyzed using EthoVision XT 7.1 video tracking software (Noldus Information Technologies, The Netherlands).

Tissue collection

Mice were deeply anesthetized with tribromoethanol (Sigma-Aldrich, USA), and then transcardially perfused with heparinized saline before collection of brains. The left brain hemisphere was immersion fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 22 h followed by incubation in 30% sucrose solution for 48 h, prior to snap freezing in liquid nitrogen and storage in − 70 °C until cryosectioning. The hippocampus was isolated from the right brain hemisphere, snap frozen in liquid nitrogen, and stored at − 70 °C until further use. The same animals were used for the biochemical assays such as, Real-Time PCR, Western blot and cytokine bead array, and immunohistochemistry.

Protein and RNA isolation

Frozen hippocampal samples (n = 5–7/group) were manually homogenized in eight volumes of lysis buffer pH 7.4 containing 20 mM Tris, 250 mM sucrose, 0.5 mM EDTA, 0.5 mM EGTA, 4% (v/v) protease, and 1% (v/v) phosphatase inhibitor cocktails (Sigma-Aldrich, USA). Cytosolic extracts from the tissues were centrifugated at 5000×g for 10 min at + 4 °C. The supernatants were divided into aliquots and stored at − 70 °C. Protein concentrations were determined by using Pierce 660 nm Protein Assay Kit (ThermoFisher Scientific, USA). For total RNA isolation, one of the hippocampal aliquots was mixed with TRI Reagent (Sigma-Aldrich, USA), and RNA was isolated according to the manufacturer's protocol. The concentration and purity of RNA was determined by using NanoDrop 2000 (ThermoFisher Scientific, USA); RNA samples with 260/280 ratios higher than 1.8 were selected.

Quantitative real-time PCR

Before reverse transcription, genomic DNA was removed from all RNA samples with DNase I, RNase-free kit (ThermoFisher Scientific, USA) following the manufacturer's instructions. Then, 1 μg of mRNA was reverse-transcribed using High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, USA). Quantitative real-time PCR (RT-PCR) was performed to measure relative mRNA expression of astrocyte and microglia

marker genes, as well as the brain-derived neurotrophic factor (*Bdnf*) gene, by using StepOnePlus Real-Time PCR System (ThermoFisher Scientific, USA). TaqMan gene expression assays (ThermoFisher Scientific, USA) used for RT-PCR are listed in Supplementary Table 1. Results were normalized to the glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) endogenous control.

Western blot

To measure BDNF, synaptic, and astrocytic markers protein level in hippocampal samples, 20 μg of proteins were used for Western blot (WB). Proteins were separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare Life science) by using Trans-Blot Turbo Transfer System (BioRad, USA). The membranes were blocked in 5% fat-free milk in PBST (phosphate-buffered saline with 0.2% of Tween-20) and incubated overnight at + 4 °C with following primary antibodies: rabbit anti-BDNF (1:1000), rabbit anti-synaptophysin (1:200), rabbit anti-postsynaptic density protein 95 (PSD-95, 1:1000), rabbit anti-glial fibrillary acidic protein (GFAP; 1:1000), rabbit anti-s100 calcium binding protein β (S100β; 1:5000), rabbit anti-glutamine synthetase (GS, 1:5000), and rabbit anti-aldehyde dehydrogenase 1 family, member L1 (ALDH1L1, 1:1000). The membranes were washed and incubated with secondary goat anti-rabbit antibodies conjugated with HRP (1:3000) for 2 h at room temperature. Proteins were visualized with SuperSignalTM West Pico PLUS Chemiluminescent substrate kit (Thermo Fisher Scientific, USA), and detected using BioRad Chemi-DocTM Imaging System and quantified by using ImageLab software (BioRad, USA). Mouse anti-β-actin antibody (1:5000) was used as an internal standard, following incubation with donkey anti-mouse Cy5-conjugated antibodies (1:1000). The list of all antibodies with company names and catalog numbers is presented in Supplementary Table 1.

Cytokine bead array

For determination of cytokine levels in hippocampal protein samples, the cytokine bead array (CBA) mouse inflammation kit (BD Biosciences, USA) was used according to the manufacturer's protocol. For CBA, 30 μg of cytosolic proteins from hippocampal samples were used to measure concentrations of the following cytokines: IL-6, IL-10, MCP-1, IL12p70, IFNγ, and TNFα. Samples were run using CytoFlex S flow cytometer (Beckman Coulter, USA), and acquired data were exported and analyzed by FCAP Array v3.0 software (Soft Flow Inc., USA).

Immunohistochemistry

Aβ plaque deposition, neuronal survival, neurogenesis, and activation/proliferation of microglia and astrocytes were assessed by immunostaining. Brains (n = 7–9/group) were cut in serial 20 μm sagittal sections, each 400 μm apart, using a cryostat (Leica Microsystems, Germany), and stored in anti-freeze solution at − 20 °C before staining. The sections were blocked in 10% normal goat serum (NGS) in PBST and incubated overnight at room temperature with the following antibodies: mouse anti-Aβ (1:1000), rabbit anti-neuronal nuclei (NeuN; 1:200 dilution), rabbit anti-doublecortin (DCX; 1:200), rabbit anti-ionized calcium binding adaptor molecule 1 (Iba1;1:250), rabbit anti-GFAP (1:500), and rabbit anti-S100β (1:200). Sections were washed and incubated for 2 h with suitable fluorescent secondary antibodies, goat anti-rabbit AlexaFluor 488 (1:200) or goat anti-mouse AlexaFluor 568 (1:500). Antibodies with company names and catalog numbers are listed in Supplementary Table 1. Hippocampal areas from three sections 400 μm apart were imaged for each mouse. Images were taken with × 10 magnification on Zeiss Axio Imager M2 using a digital camera (Axiocam, Zeiss, Germany) and ZEN software. Each image was stitched to avoid overlap of adjacent tiles and exported in TIFF format. Immunoreactivity was quantified using the ImageJ software (National Institute of Health, USA). The percentage of positively-stained area in the hippocampus and subiculum were measured for each brain section, and the average from three sections per mouse was reported.

Colocalization analysis for BDNF and GFAP

For colocalization analysis, double staining for BDNF and GFAP was performed for 5xFAD mouse brain sections. Then, 20 μm sagittal sections were incubated overnight in rabbit anti-BDNF (1:150) and chicken anti-GFAP (1:2500) antibodies. Sections were washed and incubated for 2 h with suitable fluorescent secondary antibodies, goat anti-rabbit AlexaFluor 488 (1:250), or goat anti-chicken AlexaFluor 568 (1:250). For correlation analysis, hippocampal areas were imaged with × 40 magnification on Zeiss Axio Observer inverted microscope with LSM800 confocal module (Zeiss, Germany) and ZEN software. Before the analysis, the red and green images were converted to 8-bit format and correlation of two proteins was measured by the JACoP plugin in ImageJ software (National Institute of Health, USA). For assessing the colocalization, the Pearson's correlation coefficient (Pr; degree of correlation between two colors), overlap coefficient, and Mander's colocalization coefficient (MOC; the proportion of green pixels in red channel) were used, which were generated by the JaCoP 2 plugin.

Furthermore, quantitative colocalization analysis was performed using GFAP and BDNF images taken from hippocampal areas with × 10 magnification on Zeiss Axio Imager M2 using a digital camera (Axiocam, Zeiss, Germany) and ZEN software. Each image was stitched to avoid overlap of adjacent tiles and exported in TIFF format. Areas covered by green (BDNF) pixels were quantified in red (GFAP-positive astrocytes) pixels by Apoptosis correlator plugin in ImageJ software (National Institute of Health, USA) for each brain section, and the average from three sections per mouse was reported. The threshold was chosen for each channel and total number of overlapped pixels in both channels were calculated and normalized by the total number of pixels. Similar analysis was performed before for colocalization [25, 26].

Morphological analysis of astrocytes

Morphological analysis of GFAP-positive astrocytes was performed on images taken from the same brain sections, which were used for single GFAP staining. In addition to measuring astrocyte morphology depending on plaque proximity, double staining for GFAP and Aβ was performed for 5xFAD mice brain sections. For the analysis, the hippocampal area from a single 20 μm sagittal section was imaged for each mouse. Images were taken with × 40 magnification on Zeiss Axio Imager M2 using a digital camera (Axiocam, Zeiss, Germany) and ZEN software. Each image was stitched to avoid the overlap of the adjacent tiles and exported in TIFF format. The number of GFAP-positive cells and quantitative analysis of cell morphological parameters were performed using digital modeling based on the MicroTrac analysis platform [27]. Cells that failed to be reconstructed correctly by the program were manually removed from the analysis. Example of images and representative GFAP-positive cells from each group of mice are presented in Fig. 6a.

Statistical analyses

All values are expressed as mean ± SEM. Two-way analysis of variance (ANOVA) was used to examine main genotype and exercise effect between WT and 5xFAD mice followed by unpaired t test comparison in case of a significant interaction between two factors (genotype*exercise) to examine exercise effect for each genotype separately. For nonparametric data, a Mann–Whitney test was used to compare the values between two groups. Changes in weekly running distance and body weight as well as MWM data were evaluated using mixed-model ANOVA for repeated measures with genotype and exercise as between-subject factors. Statistical analyses were performed with GraphPad Prism 8.4.2 software (GraphPad Software Inc, USA) and SPSS 25.0 software (IBM, USA); a difference was considered significant with $p < 0.05$. The statistical outliers were determined with Grubbs' test and removed from the analysis.

Results

Mice in the voluntary exercise groups had free access to a running wheel from the age of 1.5 months to 7 months. The weekly weighing of mice revealed that the body mass of 5xFAD mice was significantly lower ($F(1, 28) = 8.149$, $p = 0.006$) than the body mass of WT mice in accordance to previous studies [28, 29]. The difference further increased as the mice aged (genotype*age interaction: $F(24, 1872) = 6.068$, $p < 0.0001$; Supplementary Figure 1A). Undertaking voluntary exercise slightly reduced the body mass of both WT and 5xFAD mice ($F(1, 28) = 3.832$, $p = 0.054$; Supplementary Figure 1A). The efficacy of exercise was assessed by monitoring the running distance of each mouse by employing running counters that were documented on a weekly basis (Supplementary Figure 1B). The weekly running distance was similar in both genotypes of mice (28 ± 1 km/week for WT and 30 ± 1 km/week for 5xFAD mice, $F(1, 31) = 1.152$, $p = 0.29$)

Voluntary physical exercise recovered the impairment of nest building behavior and anxiety observed in 5xFAD mice

To evaluate the effect of voluntary physical exercise on typical behavioral features in mice, a nest building test was conducted at the age of 7 months. The sedentary 5xFAD (5xFAD-SED) mice demonstrated a significant impairment in the ability of build a nest, in comparison with sedentary WT (WT-SED) mice (Fig. 1a). The ANOVA revealed a significant main effect of genotype on nest score ($F(1, 32) = 20.82$, $p < 0.0001$). Voluntary physical exercise corrected this deficit (main effect of exercise: $F(1, 32) = 16.08$, $p = 0.0001$). The post-hoc test further revealed improvement in the exercised 5xFAD (5xFAD-EXE) mice (Mann–Whitney $U(31) = 84$, $p = 0.0004$) without having a significant effect on exercised WT (WT-EXE) mice (Mann–Whitney $U(33) = 156$, $p = 0.08$). These findings indicate that exercise is beneficial for nest building specifically for mice displaying AD-like pathology.

In the elevated plus-maze test, the 5xFAD-SED mice spent more time than WT-SED mice in the open arms. The ANOVA revealed a significant main effect of genotype on % time spent in open arms ($F(1, 34) = 7.079$, $p = 0.0097$) and on % number of visits to open arms ($F(1, 34) = 8.281$, $p = 0.005$; Fig. 1b). This is a characteristic anti-anxiety feature of this mouse line [21]. Voluntary physical exercise reversed this tendency in terms of % time spent in open arms (main effect of exercise: $F(1, 34) = 8.115$ $p = 0.006$) and % number of visits to open

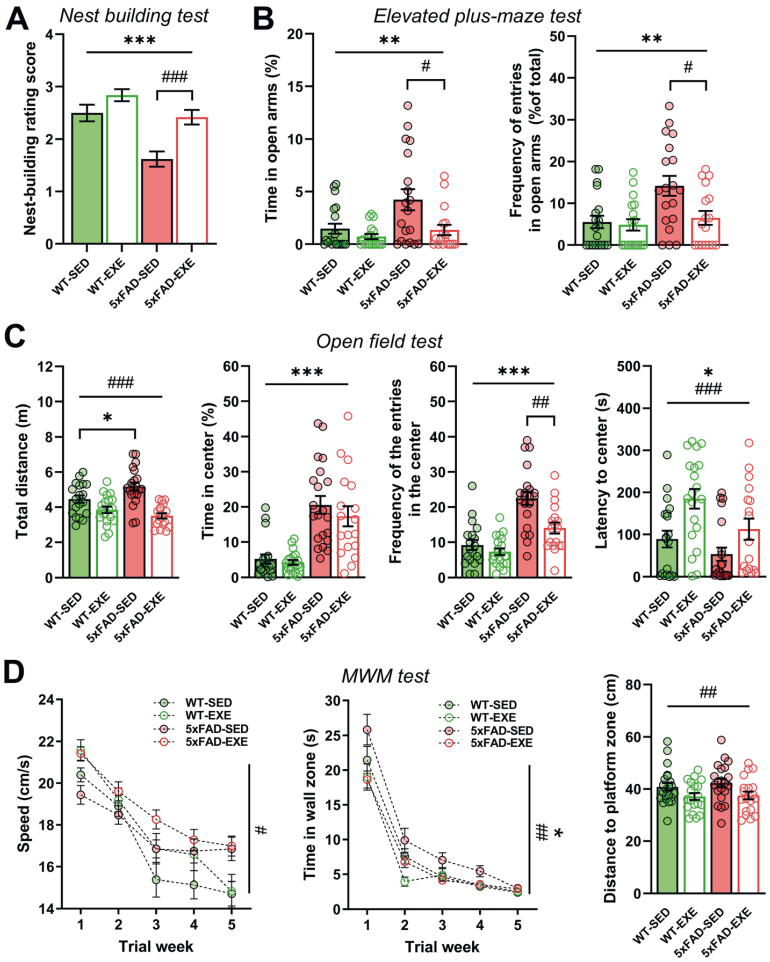

Fig. 1 Voluntary physical exercise reverses cognitive impairment in 5xFAD mice. Nest building, elevated plus-maze, open field, and MWM tests were performed on WT-SED, WT-EXE, 5xFAD-SED, 5xFAD-EXE male mice at 7 months of age to assess effect of voluntary exercise on behavior. **a** Nest-building rating score. **b** Percentage of spent time and frequency of entries in open arms of elevated plus-maze test. **c** Total moved distance, time in the center, frequency of entries in the center, and latency to center entries in open field test. **d** Evolution of swimming speed and time spent in wall zone during 5-day acquisition trials and distance in platform zone during probe trial in MWM test. All data are presented as mean (±SEM). Two-way ANOVA to measure genotype and exercise effect between WT and 5xFAD mice followed by Mann-Whitney test (**a**,**b**) or unpaired *t* test (**c**) comparison in case of significant interaction between two factors (genotype*exercise); two-way ANOVA for repeated measures (**d**), with the test day as the within-subject factor. n = 20-22 per group. «*» Genotype effect, «#» exercise effect: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$

arms (main effect of exercise: $F(1, 34) = 5.465$, $p = 0.02$; Fig. 1). The post-hoc tests further demonstrated the significant reduction in the time spent in open arms in the 5xFAD-EXE mice (Mann–Whitney U (35) = 95.5, p = 0.03) without changes in WT-EXE mice (Mann–Whitney U (36) = 147, p = 0.46). The data demonstrate that exercise is beneficial for ameliorating anxiety level in 5xFAD mice.

Voluntary physical exercise influenced locomotor activity and explorative behavior in both WT and 5xFAD mice

To investigate effect of voluntary physical exercise on locomotor activity and exploration in WT and 5xFAD mice, the open field test was performed at the age of 7 months. The ANOVA revealed no difference between the genotypes in the total distance traversed ($F(1, 37)$ = 0.921, p = 0.34), whereas the main effect of exercise was

significant ($F(1, 37) = 32.94$, $p < 0.0001$); there was significant genotype*exercise interaction ($F(1, 37) = 7.199$, $p = 0.009$). The post-hoc test further revealed that the 5xFAD-SED mice walked a longer distance than the WT-SED mice ($t(31) = 2.302$, $p = 0.03$), whereas exercised mice of both genotypes had significantly lower total distance values than sedentary mice (Fig. 1c). In accordance with the anti-anxiety phenotype [21], the 5xFAD mice spent more time in the center of the open field (main effect of genotype: $F(1, 37) = 49.18$, $p < 0.0001$), and entered the arena center significantly more often ($F(1, 37) = 43.39$, $p < 0.0001$) and with shorter latency ($F(1, 37) = 6.618$, $p = 0.012$) in comparison with WT mice (Fig. 1c). Interestingly, the number of entries to the arena center was significantly decreased (main effect of exercise: $F(1, 37) = 11.60$, $p = 0.0011$) and the latency to enter was significantly increased ($F(1, 37) = 13.00$, $p = 0.0006$) in both genotypes of mice after voluntary physical exercise (Fig. 1c). The post-hoc tests further demonstrated the significant reduction in the number of entries to the arena center in 5xFAD-EXE mice ($t(31) = 3.336$, $p = 0.002$) without changes in WT-EXE mice ($t(31) = 1.132$, $p = 0.27$). Together, these results indicate that exercise reduces exploratory activity in WT and 5xFAD mice.

Voluntary physical exercise improved spatial memory deficits in 5xFAD mice

To investigate the effect of voluntary physical exercise on spatial learning and memory, the Morris water maze (MWM) test was performed at the age of 7 months. Spatial learning over 5 days of task acquisition was assessed by measuring a range of parameters that characterize the ability of a mouse to find a hidden platform. Because exercised mice swam significantly faster than sedentary mice ($F(1, 28) = 5.101$, $p = 0.027$; Fig. 1d), the time spent in the wall zone was chosen as a reliable parameter for learning assessment that is independent of the swimming speed. The 5xFAD-SED mice spent significantly more time ($F(1, 38) = 4.253$, $p = 0.046$) than WT-SED mice near the pool wall. Voluntary physical exercise reduced this wall clinging tendency (thigmotaxis) in 5xFAD-EXE mice ($F = (1, 33) = 9.202$, $p = 0.004$; Fig. 1d). During a probe trial, voluntary physical exercise significantly reduced the mean distance to platform zone in both WT and 5xFAD mice (main exercise effect: $F(1, 28) = 8.106$, $p = 0.006$; Fig. 1d). Moreover, exercise recovered latency to the platform zone in 5xFAD-EXE mice (Mann–Whitney U (33) = 123.5, $p = 0.023$), which was increased in 5xFAD-SED mice in comparison to WT-SED mice (Mann–Whitney U (39) = 118, $p = 0.009$; data not shown). These findings show that exercise induces improvements in hippocampal-dependent learning and memory.

Voluntary physical exercise did not alter neurogenesis and neuronal survival in 5xFAD hippocampi

Adult hippocampal neurogenesis was previously shown to be impaired in AD [30]. Thus to investigate neurogenesis and neuronal survival as potential mechanisms underlying the behavioral changes upon voluntary physical exercise, we use histochemistry. Immunostaining of brain sections for NeuN (Fig. 2a), a marker of mature neurons, revealed that NeuN was significantly reduced in the subiculum of 5xFAD mice compared to WT mice (main effect of genotype: $F(1, 24) = 182.6$, $p < 0.0001$), with no changes observed in the hippocampi ($F(1, 24) = 0.09$, $p = 0.77$; Fig. 2b). Voluntary physical exercise did not alter the NeuN level in WT or 5xFAD mice (subiculum: $F(1, 24) = 1.932$, $p = 0.18$; hippocampi: $F(1, 24) = 0.08$, $p = 0.77$). Hippocampal neurogenesis was assessed by immunostaining for the immature neuronal marker DCX in the dentate gyrus (DG) (Fig. 2c). The number of DCX-positive cells in the DG was significantly reduced in 5xFAD mice when compared to WT mice (main effect of genotype: $F(1, 27) = 46.16$, $p < 0.0001$); Fig. 2d). However, no difference was observed in the number of DCX-positive cells between sedentary and exercised mice (main effect of exercise: $F(1, 27) = 0.02$, $p = 0.89$), indicating that the observed behavioral improvement occurring upon exercise is not linked to increased neurogenesis or amelioration of neuronal loss.

Voluntary physical exercise ameliorated the reduction of synaptic protein PSD-95 in 5xFAD hippocampi

To assess the effect of voluntary physical exercise on synaptic proteins known to be altered in AD [31], WB was used to measure the protein levels of the presynaptic marker synaptophysin and the postsynaptic marker PSD-95 in WT and 5xFAD hippocampi (Fig. 2e). While there was no genotype ($F(1, 18) = 1.967$, $p = 0.18$) or exercise ($F(1, 18) = 0.326$, $p = 0.57$) effect observed in the hippocampal level of synaptophysin, a significant reduction in the expression of PSD-95 was found in the hippocampi of 5xFAD mice in comparison with WT mice (main effect of genotype: $F(1, 19) = 5.786$, $p = 0.03$; Fig. 2f); there was genotype*exercise interaction ($F(1, 19) = 3.927$, $p = 0.06$). The post-hoc test further revealed that voluntary physical exercise ameliorated this reduction of PSD-95 in the 5xFAD mice hippocampi ($t(9) = 3.544$, $p = 0.006$) without changes in WT mice hippocampi ($t(10) = 0.732$, $p = 0.48$).

Voluntary physical exercise increased GFAP levels without affecting Aβ burden in 5xFAD brains

To investigate whether voluntary physical exercise affects Aβ plaque deposition and activation of glial cells, immunostaining was performed for Aβ, Iba1, a marker of activated microglia, GFAP, a marker of reactive

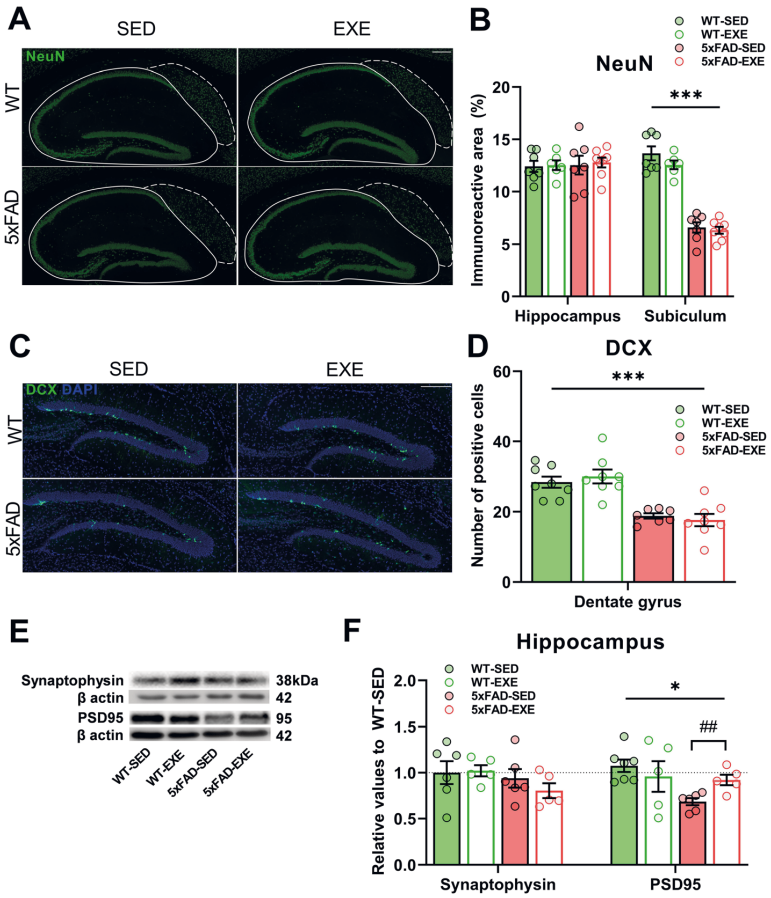

Fig. 2 Voluntary physical exercise ameliorates AD-induced reduction of synaptic proteins without affecting neurogenesis and neuronal survival. **a** Representative image of NeuN-positive nuclei (green) in hippocampal areas of WT-SED, WT-EXE, 5xFAD-SED, 5xFAD-EXE mouse brains. Solid and dotted lines represent the quantified hippocampus and subiculum areas, respectively. Scale bars 200 μm. **b** Comparison of NeuN level in hippocampi and subiculum of WT and 5xFAD mice brain quantified by measuring the percentage of positive immunoreactive area. **c** Representative images of DCX-positive cells (green) and DAPI (blue) in hippocampal dentate gyrus of WT-SED, WT-EXE, 5xFAD-SED, 5xFAD-EXE mice. Scale bar 100 μm. **d** Comparison of DCX-positive cells number in dentate gyrus of WT and 5XFAD mice. **e** Representative WB of synaptic proteins synaptophysin, PSD-95 and β actin in hippocampal protein samples. **f** Analysis of the WB bands normalized to β actin and presented as relative values to WT-SED. All data are presented as mean (± SEM). Two-way ANOVA used to measure genotype and exercise effect between WT and 5xFAD mice followed by unpaired *t* test comparison in case of significant interaction between two factors (genotype*exercise). **a**–**d** $n = 8$/group, **e**, **f** $n = 5$–7/group. «*» Genotype effect, «#» exercise effect: $^{*}p < 0.05$, $^{***}p < 0.001$, $^{\#\#}p < 0.01$

astrocytes, and S100β, astrocytic marker. The level of Aβ, as determined by WO2 immunostaining, was not affected by voluntary physical exercise in the 5xFAD hippocampi ($t(13) = 0.284$, $p = 0.78$) or subiculum ($t(13) = 1.016$, $p = 0.33$; Fig. 3a, b). The expression of Iba1 was significantly increased in the hippocampi (main effect of genotype: $F(1, 29) = 101.8$, $p < 0.0001$) and subiculum ($F(1, 29) = 336.7$, $p < 0.0001$) of 5xFAD mice in comparison with WT mice, but not altered by voluntary physical exercise (hippocampi: $F(1, 29) = 0.005$, $p = 0.95$; subiculum: $F(1, 29) = 0.110$, $p = 0.74$; Fig. 3c, d). GFAP levels were significantly increased in the hippocampi (main effect of genotype: $F(1, 25) = 52.23$, $p < 0.0001$) and subiculum ($F(1, 25) = 218.9$, $p < 0.0001$) of 5xFAD mice in comparison with WT mice (Fig. 3e, f, Supplementary Table 3). Interestingly, the GFAP level was even further increased in both hippocampus and subiculum ($t(13) = 3.679$, $p = 0.003$ and $t(13) = 3.847$, $p = 0.002$, respectively) of exercised 5xFAD mice in comparison with 5xFAD-SED mice, whereas in the subiculum of WT

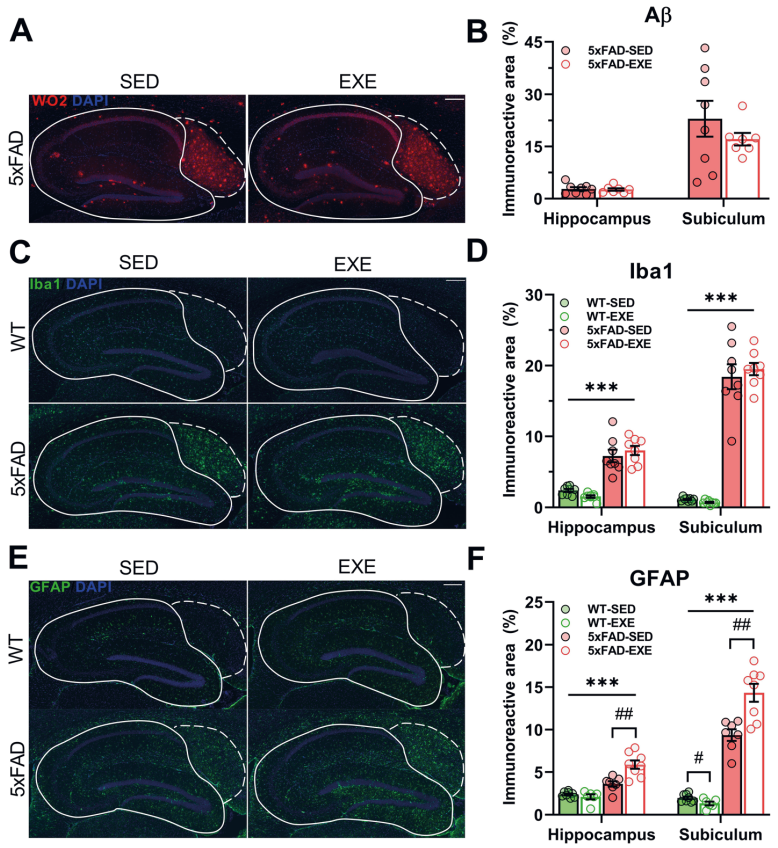

Fig. 3 Effect of voluntary physical exercise on Aβ load and glial activation in hippocampi. Representative images of Aβ (**a**, red), GFAP-positive astrocytes (**c**, green), Iba1-positive microglia (**e**, green), and DAPI (blue) in the hippocampal area of WT-SED, WT-EXE, 5xFAD-SED, 5xFAD-EXE mice. Solid and dotted lines represent quantified hippocampi and subiculum areas, respectively. Scale bars: 200 μm. Aβ (**b**), Iba1 (**d**), and GFAP (**f**) levels were quantified by measuring the percentage of positive immunoreactive area in hippocampi and subiculum. All data are presented as mean (± SEM). Two-way ANOVA used to measure genotype and exercise effect between WT and 5xFAD mice followed by unpaired *t* test comparison in case of significant interaction between two factors (genotype*exercise). *n* = 8/group, «*» Genotype effect, «#» exercise effect: $^{**}p < 0.01$, $^{***}p < 0.001$, $\#p < 0.05$, $\#\#p < 0.01$

mice GFAP was reduced after exercise ($t(12) = 2.352$, $p = 0.04$) in comparison with sedentary control. S100β levels were significantly increased in subiculum (main effect of genotype: $F(1, 29) = 85.35$ $p < 0.0001$) with no significant changes in hippocampi ($F(1, 29) = 0.78$, $p = 0.38$) of 5xFAD mice in comparison with WT mice (Supplementary Figure 2, Supplementary Table 3). Voluntary physical exercise did not alter the level of S100β in subiculum ($F(1, 29) = 1.7 \times 10^{-7}$, $p = 0.99$) and hippocampi ($F(1, 29) = 0.10$, $p = 0.75$) of mice from both genotypes. CBA measurements with hippocampal protein lysates (see Supplementary Table 2) demonstrated an increased level of the chemokine MCP-1 (19%, main effect of genotype: $F(1, 20) = 11.41$, $p = 0.003$) in 5xFAD mice in comparison with WT mice, and increased levels of the cytokine IL12p70 (54%, $t(11) = 2.676$, $p = 0.02$) in 5xFAD-SED mice in comparison with WT-SED mice. After voluntary physical exercise, the IL12p70 level was completely restored in hippocampi of 5xFAD-EXE mice ($t(9) = 2.543$, $p = 0.03$). These results indicate that voluntary physical exercise affects specifically the reactive astrocytes in 5xFAD mouse brains.

Voluntary physical exercise selectively affected the GFAP-positive population of astrocytes

Given the exercise-induced increase of GFAP immunoreactivity in 5xFAD brains, we next investigated whether other astrocytic markers such as S100β, glutamine

synthetase (GS), and ALDH1L1 were affected by voluntary physical exercise in the hippocampus of 5xFAD mice. WB analyses revealed that the protein levels of GFAP (main effect of genotype: $F(1, 21) = 72.42$, $p < 0.0001$) and S100β ($F(1, 20) = 21.03$, $p = 0.0002$) were significantly upregulated in the hippocampi of 5xFAD mice compared to WT mice, whereas GS ($F(1, 20) = 0.918$, $p = 0.35$) and ALDH1L1 ($F(1, 16) = 2.655$, $p = 0.12$) protein levels remained unchanged (Fig. 4a, b). Interestingly, WB results demonstrated a similar increase in GFAP levels as shown by immunohistochemistry (Fig. 3e, f) when 5xFAD-EXE mice were compared to 5xFAD-SED mice ($t(11) = 2.273$, $p = 0.04$). In contrast, voluntary physical exercise did not significantly affect S100β, GS, or ALDH1L1 protein levels in WT or 5xFAD mice (Fig. 4b), which suggests that it is specifically the GFAP-positive astrocytes that respond to physical exercise in the 5xFAD mouse brain.

Recently, Liddelow et al. found two different types of reactive astrocytes: A1 astrocytes, which are harmful and related to neurodegenerative diseases including AD, and neuroprotective A2 astrocytes [32]. We decided to examine hippocampal mRNA expression level of A1/A2 astrocyte markers in 5xFAD and WT mice (Supplementary Table 3). RT-PCR analysis revealed increased mRNA expression of A1 markers, such as Fkbp5 (main effect of genotype: $F(1, 19) = 6.088$, $p = 0.02$), Srgn ($F(1, 17) = 3.762$, $p = 0.07$), as well as A2 marker S100A10 ($F(1, 19) = 12.84$, $p = 0.002$) in hippocampi of 5xFAD mice compared to WT mice. Voluntary physical exercise slightly reduced hippocampal mRNA expression level of A1 markers Serping1 (main effect of exercise: $F(1, 18) = 3.306$, $p = 0.09$) and Srgn ($F(1, 17) = 1.826$, $p = 0.19$) in exercised mice compared to sedentary, as well as A2 marker S100A10 ($t(9) = 2.431$, $p = 0.04$) in 5xFAD-EXE mice compared to 5xFAD-SED mice.

Voluntary physical exercise ameliorated the reduction of BDNF in GFAP-positive astrocytes in hippocampi

To assess the effect of voluntary physical exercise on BDNF, WB was used to measure the protein levels of BDNF in WT and 5xFAD hippocampi (Fig. 5a). We observed a reduction in the protein level of BDNF in the hippocampi of 5xFAD mice in comparison with WT mice (main effect of genotype: $F(1, 21) = 4.88$, $p = 0.04$), which was increased by 18% after voluntary physical exercise in 5xFAD-EXE mice ($t(11) = 2.090$, $p = 0.06$; Fig. 5a). In addition, double staining was performed in brain sections to assess the level of colocalization between BDNF and GFAP in WT and 5xFAD hippocampi (Fig. 5b). We performed correlation analysis between BDNF and GFAP and obtained the following correlation parameters: Pr = 0.49, overlap coefficient = 0. 93, and MOC = 0.51, which clearly indicate that hippocampal GFAP-positive astrocytes are expressing BDNF. Based on this finding, we decided to assess the level of BDNF in GFAP-positive astrocytes among all experimental groups in hippocampi (Fig. 5c). Colocalization analysis revealed that BDNF level was significantly lower in GFAP-positive astrocytes in 5xFAD mice in comparison with WT mice (main genotype effect: $F(1, 26) = 17.35$, $p = 0.0003$), whereas voluntary physical exercise corrected this deficit in hippocampi of both WT and 5xFAD mice (main effect of exercise: $F(1, 26) = 6.335$, $p = 0.02$; Fig.

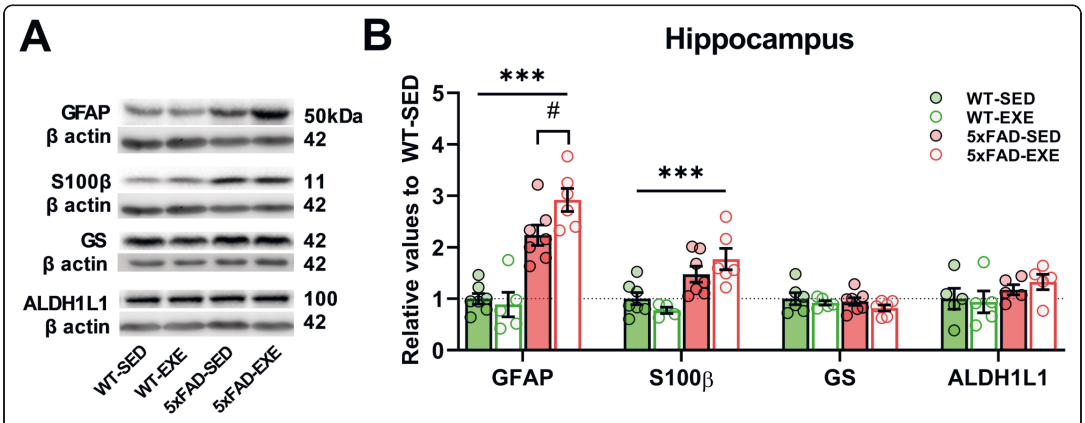

Fig. 4 Effect of AD and voluntary physical exercise on level of astrocytic markers in hippocampi. **a** Representative WB of astrocytic markers GFAP, S100β, glutamine synthetase (GS), ALDH1L1, and β actin in hippocampal protein samples. **b** Analysis of the WB bands normalized to β actin. Data were normalized to WT-SED values and presented as mean (± SEM). Two-way ANOVA used to measure genotype and exercise effect between WT and 5xFAD mice followed by unpaired *t* test comparison in case of significant interaction between two factors (genotype*exercise). *n* = 5–7/group. «*» Genotype effect, «#» exercise effect: ***$p < 0.001$, #$p < 0.05$

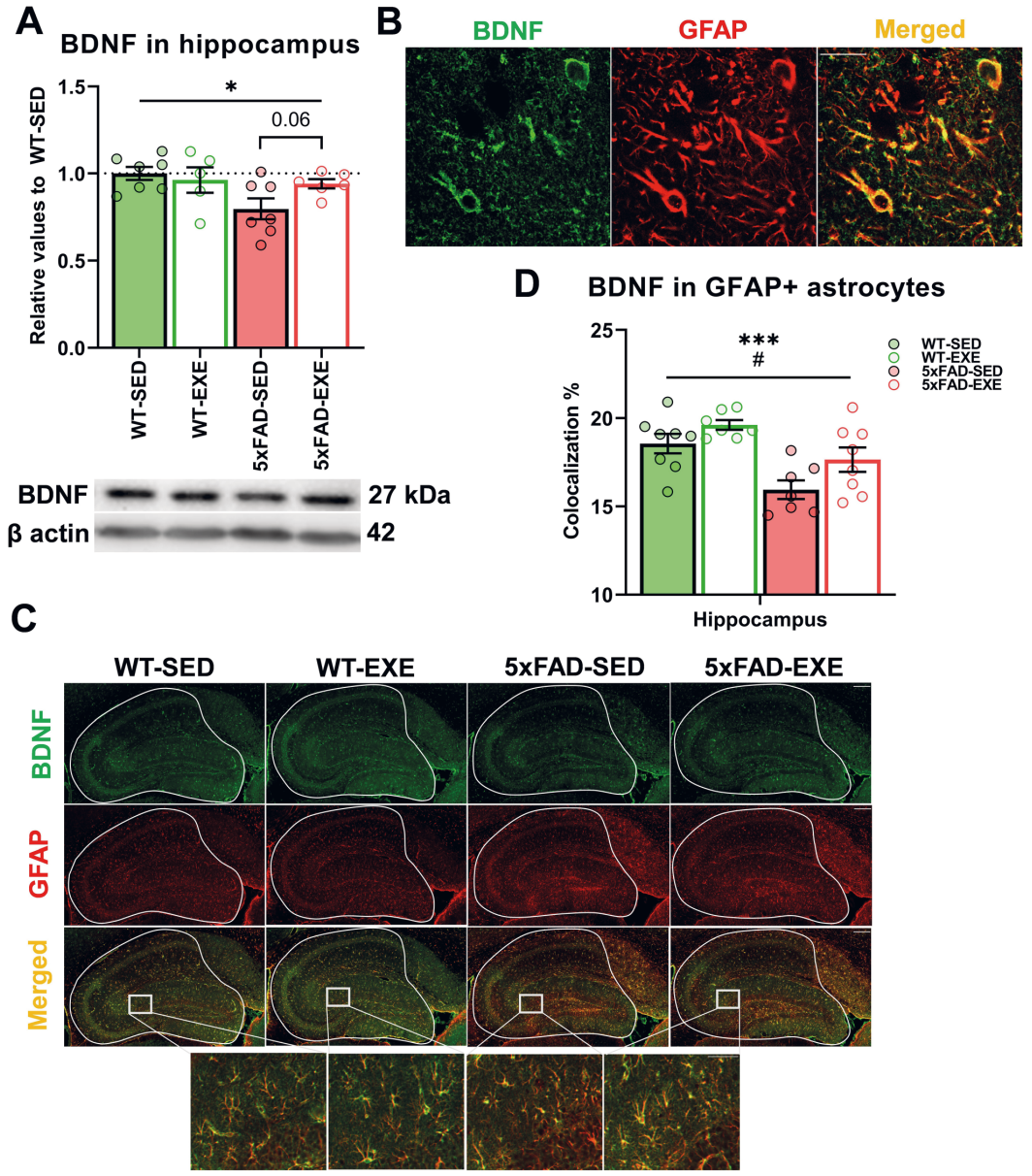

Fig. 5 Effect of AD and voluntary physical exercise on BDNF level in hippocampi and GFAP-positive astrocytes. **a** Representative WB of BDNF and β actin in hippocampal protein samples and the analysis of the WB bands normalized to β actin. **b** Colocalization of BDNF (green) and GFAP (red) in hippocampus of WT mice. Scale bars: 20 μm. **c** Representative images of BDNF (green), GFAP-positive astrocytes (red), and Merged (yellow) in the hippocampal area of WT-SED, WT-EXE, 5xFAD-SED, 5xFAD-EXE mice. Solid lines represent quantified hippocampi area. Scale bars: 200 μm. The framed areas are magnified from merged images, scale bar 50 μm. **d** Level of colocalization (yellow) between BDNF (green) and GFAP (red) was quantified by measuring total number of overlapped pixels (yellow) in both channels normalized by the total number of pixels. All data are presented as mean (± SEM). Two-way ANOVA used to measure genotype and exercise effect between WT and 5xFAD mice followed by unpaired *t* test comparison in case of significant interaction between two factors (genotype*exercise). **a** $n = 5–7$/group, **d** $n = 8$/group. «*» Genotype effect, «#» exercise effect: $^{*}p < 0.05$, $^{***}p < 0.001$, $\#p < 0.05$

5d). Altogether, these data demonstrate that voluntary physical exercise ameliorates the reduced BNDF level in hippocampi of 5xFAD mice, and specifically in GFAP-positive astrocytes.

Voluntary physical exercise altered the morphology of GFAP-positive astrocytes in 5xFAD hippocampi

To further examine the effects of voluntary physical exercise specifically on GFAP-positive astrocytes in the hippocampus, we employed a sophisticated morphological assessment using digital modeling based on the MicroTrac analysis platform as described [27]. Figure 6a depicts the typical morphology of GFAP-positive astrocytes in the brains of the different study groups. The analyses revealed a robust 2.4-fold increase in the number of GFAP-positive astrocytes in hippocampi of 5xFAD mice in comparison to WT mice (main effect of genotype: $F(1, 25) = 104.1$, $p < 0.0001$; Fig. 6b). Notably, voluntary physical exercise induced a further increase in the number of GFAP-positive astrocytes in 5xFAD and WT mice in comparison with sedentary mice (main effect of exercise: $F(1, 25) = 6.004$, $p = 0.02$). Interestingly, the number of S100β-positive astrocytes in hippocampi was constant for WT and 5xFAD mice (main genotype effect: $F(1, 40) = 0.06$, $p = 0.81$) and for sedentary and exercised mice (main exercise effect: $F(1, 40) = 0.03$, $p = 0.87$; Supplementary Figure 2).

Moreover, GFAP-positive astrocytes of 5xFAD mice displayed significant structural changes compared to WT mice (Fig. 6c), specifically smaller cell radius (4%, main effect of genotype: ($F(1, 25) = 5.429$, $p = 0.03$), enlarged soma area (28%, $F(1, 25) = 21.8$, $p < 0.0001$), increased cell solidity (13%, $F(1, 25) = 21.3$, $p = 0.0001$), and cell extent (18%, $F(1, 25) = 21.94$, $p < 0.0001$). The post-hoc tests further demonstrated that GFAP-positive astrocytes in 5xFAD-SED mice had significantly less primary branches (10%, $t(13) = 2.629$, $p = 0.02$) and shorter branch length (15%, $t(13) = 2.980$, $p = 0.011$) in comparison to WT-SED mice. Voluntary physical exercise partly restored these branching modifications in GFAP-positive astrocytes of 5xFAD mice, resulting in an increased number of primary branches (5%, $t(12) = 1.648$, $p = 0.13$) and increased branch length (14%, $t(12) = 1.727$, $p = 0.11$). Moreover, GFAP-positive astrocytes in 5xFAD-EXE mice had a significantly increased cell area (12%, $t(12) = 2.687$, $p = 0.02$) in comparison to 5xFAD-SED mice, whereas this parameter in 5xFAD-SED mice was unchanged ($t(13) = 0.832$, $p = 0.42$) when compared to WT-SED mice. Taken together, these results indicate that the presence of AD-like pathology induces robust alterations in GFAP-positive astrocytes, including an increase in the number of cells, concomitantly with soma hypertrophy and process atrophy. Voluntary physical exercise induced an increase in the cell area and increased the number of GFAP-positive astrocytes in the 5xFAD mouse brains.

To determine whether voluntary physical exercise affects all the GFAP-positive astrocytes, or specifically only the Aβ plaque-associated GFAP-positive astrocytes in 5xFAD mice, morphological analysis was performed on hippocampal sections double labeled for GFAP and Aβ. GFAP-positive astrocytes were considered as plaque-associated if they were located at a distance less than 50 μm from the edge of the plaque (Fig. 7a). The analyses demonstrated that the distance from the plaque had a dramatic effect on the morphology of GFAP-positive astrocytes: plaque-associated cells had significantly more primary branches (30%, $F(1, 25) = 23.71$, $p < 0.0001$), increased branch length (50%, $F(1, 25) = 17.69$, $p < 0.0003$), bigger cell radius (15%, $F(1, 25) = 12.91$, $p = 0.0014$), larger cell area (74%, $F(1, 25) = 26.62$, $p < 0.0001$), and enlarged soma (100%, $F(1, 25) = 27.60$, $p < 0.0001$; Supplementary Table 4). Voluntary physical exercise induced a significant increase in the number of primary branches (14%, $t(13) = 2.240$, $p = 0.04$) and soma size (52%, $t(13) = 2.440$, $p = 0.03$) of plaque-associated astrocytes in the hippocampi of 5xFAD mice, without affecting cells distant from the plaques (primary branches: $t(13) = 0.650$, $p = 0.53$; soma size: $t(13) = 0.197$, $p = 0.85$; Fig. 7b, c).

Discussion

The aim of the current study was to expand the knowledge on the possible positive role of astrocytes in exercise-mediated benefits in AD. Utilization of the 5xFAD mouse model in a voluntary physical exercise intervention paradigm that begins well before Aβ plaque formation provides an opportunity to mimic and test the benefits of a healthy lifestyle on the attenuation and possible prevention of AD pathology. Voluntary physical exercise was chosen instead of treadmill exercise as the voluntary paradigm has been shown to be more tolerable and induce lower stress levels, demonstrated by measurements of serum corticosterone [33]. In agreement with existing studies [34, 35], we monitored the amount of exercise undertaken by the mice and discovered that the weekly running distance and time was 30 ± 1 km and 20 ± 1 h, respectively. The refinement of an appropriate exercise regime to effectively regulate the neuroinflammatory response in AD model mice may be of high relevance to the human population and should be considered in all studies using experimental animals.

In accordance with previous studies in 5xFAD mice [16, 20, 21, 28, 36], we demonstrated significant behavioral impairments occurring at the age of 7 months. The nest building test, used to assess species-typical behavior in rodents requiring planning and execution of a series of actions, is associated with impairment in

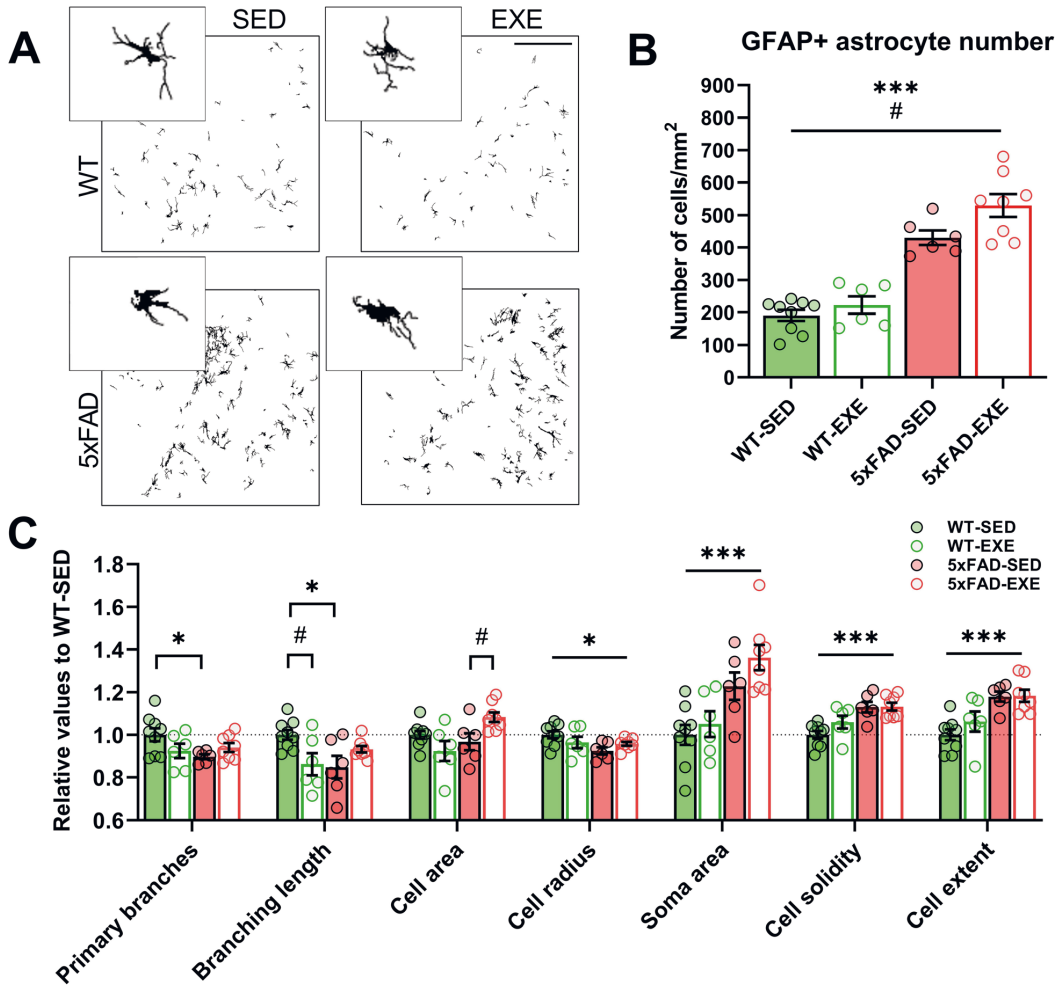

Fig. 6 Effect of AD and voluntary physical exercise on morphology of GFAP-positive astrocytes in hippocampi. **a** Representative reconstructed images of GFAP-positive astrocytes in hippocampi of WT-SED, WT-EXE, 5xFAD-SED, 5xFAD-EXE mice using MicroTrac analysis software. Scale bar 100 μm. Small images display a representative single cell from each experimental group. **b** Number of GFAP-positive astrocytes per squared mm in hippocampi. **c** Analysis of GFAP-positive astrocyte morphological parameters such as number of primary branches, total branch length, cell area, cell radius, soma area, cell solidity, and extent. Parameters for each group were normalized to WT-SED values. All data are presented as mean (± SEM). Two-way ANOVA used to measure genotype and exercise effect between WT and 5xFAD mice followed by unpaired *t* test comparison in case of significant interaction between two factors (genotype*exercise). *n* = 8/group. «*» Genotype effect, «#» exercise effect: $^{*}p < 0.05$, $^{***}p < 0.001$, $\#p < 0.05$

neurotransmitter systems and hippocampus-dependent function [37]. In the current study, the 5xFAD-SED mice displayed a significant impairment in nest construction, which was corrected almost to the WT levels by voluntary physical exercise. Our finding is in agreement with the study of Walker et al., in which 4 months of voluntary exercise improved nest building in 6-month-old TgCRND8 mice [38]. Typical rodent behavior is characterized by avoiding open areas and high levels of anxiety, whereas 5xFAD mice show a robust anti-anxiety phenotype [21]. Our results demonstrated that voluntary physical exercise stabilized the anxiety level and attenuated the exploration behavior of 5xFAD mice. Previous studies have also shown a positive exercise effect on exploration and anxiety levels in different mouse models, including the 3xTG-AD mice [35]. One recent study,

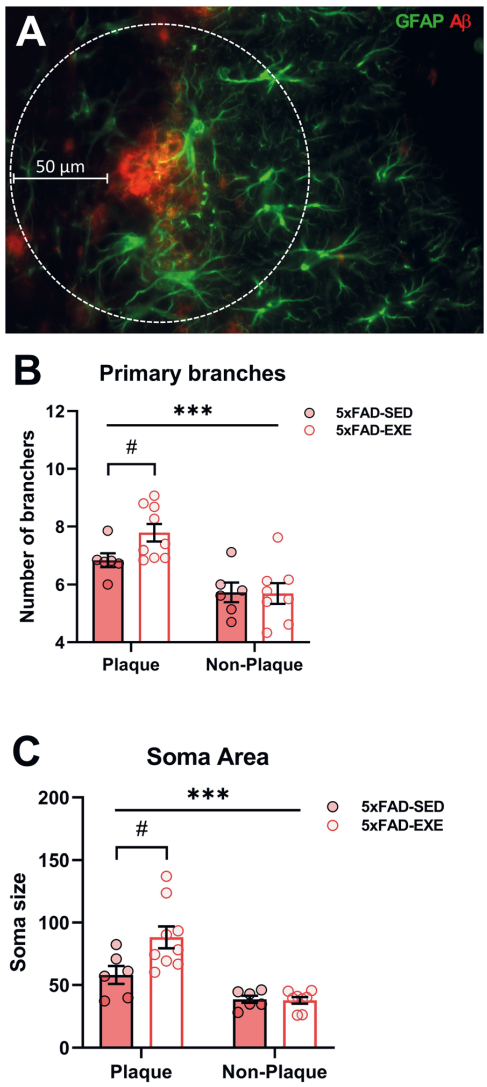

Fig. 7 Voluntary physical exercise selectively affects plaque-associated GFAP-positive astrocytes in hippocampi. **a** Representative image of Aβ plaque (red) surrounded by a GFAP-positive astrocyte (green) in the hippocampus of a 5xFAD mice. Dotted circle displays a 50 µm area, within which GFAP-positive astrocytes were considered as plaque-associated. Number of primary branches (**b**) and soma area (**c**) were quantified for plaque-associated and distant GFAP-positive astrocytes in hippocampi of sedentary and exercised 5xFAD mice by using MicroTrac analysis platform. All data are presented as mean (± SEM). Two-way ANOVA used to measure exercise and plaque effect between 5xFAD-SED and 5xFAD-EXE mice followed by unpaired *t* test comparison in case of significant interaction between two factors (exercise*plaque). $n = 8$/group. «*» Plaque effect, «#» exercise effect: $^{***}p < 0.001$, $\#p < 0.05$

however, demonstrated opposing effects of voluntary exercise on exploratory behavior and anxiety in the 5xFAD mouse model [39]. Comparing our results to the study of Svensson et al. is difficult due to the absence of WT mice as a baseline in their results. The hyperactivity of AD mice has been established in previous studies [29], [40], and is demonstrated in this study by the greater distance traveled by sedentary 5xFAD mice in comparison to WT controls in the open field test. In line with a previous study [38], our results demonstrated that 6 months of wheel running significantly reduced exploratory activity of 5xFAD and WT mice in terms of total distance traversed. In addition to non-cognitive testing, the results of this study showed exercise-induced improvements in hippocampal-dependent learning and memory, as has been previously shown in various mouse AD studies [17, 35, 41, 42] and in patients with AD [43]. Based on previous literature, it is possible that voluntary physical exercise may stabilize AD-related behavioral impairments of structural changes in the hippocampus, regulation of the neurotransmitter system, or the expression of growth factors that regulate neurogenesis, synaptic plasticity, and inflammation [4, 44].

Given that cognitive dysfunction in AD is associated with neuronal and synaptic loss accompanied with impaired neurogenesis, we first assessed whether the positive effects of voluntary exercise on cognition could be linked with parameters related to neuronal function. Here, we demonstrated that at 7 months of age, the 5xFAD mice had a significant reduction in the number of DCX-positive cells in the hippocampal dentate gyrus, as has been reported to occur in several AD mouse models [45, 46]. In previous studies, physical exercise and environmental enrichment (EE) have been shown to improve adult hippocampal neurogenesis in different AD mouse models [17, 47, 48]. Although a recent study in 5xFAD mice reported that voluntary exercise induces adult hippocampal neurogenesis [5], we did not observe this in our experiment. In the study of Choi et al., the number of mice in the running group was quite large (sedentary $n = 18$, exercise $n = 43$), and approximately 40% of the running mice had the same amount of DCX-positive cells as the sedentary mice. Nevertheless, the difference between our study and the work of Choi et al. could be explained by the difference in the age of mice used, as in that study, the authors detected increased neurogenesis after exercise in 5xFAD mice at 6 months of age, when mice have almost four times more DCX-positive cells in the dentate gyrus in comparison with 7-month-old 5xFAD mice. Firstly, the exercise-induced increase in the number of newborn neurons could depend on the stage of brain pathology and be more beneficial for neurogenesis at a younger age. Secondly, the positive effect of exercise could be diminished by single housing

of the mice, which in turn has been shown to negatively impact on the hippocampal neurogenesis in AD mice [49]. On the other hand, previous studies support our finding of unaltered neurogenesis through a demonstration that EE and voluntary exercise only increases the number of newborn neurons in wildtype, but not in 5xFAD [50], APP/PS1KI [51], APP-23 [41, 52], or presenilin-1 mutant mice [53]. In [54], 6 months of EE and physical exercise restored impaired neurogenesis in 3xTG-AD mice, although the proportion of immature proliferating cells (DCX-positive) were unchanged irrespective of genotype or housing conditions. Based on these results, we can hypothesize that exercise may have effects on later stages of neuronal development, because DCX stained only immature neurons. Further in-depth studies are required in order to confirm the effect of long-term voluntary physical exercise on neurogenesis in AD.

Considering that reduced neurogenesis is closely associated with increased Aβ deposition [46], it is also possible that the lack of neurogenesis we observed upon voluntary exercise is linked to Aβ burden, which was also unaffected by the exercise intervention. In previous studies, exercise has been shown to differentially affect Aβ plaque load, either inducing Aβ reduction [5, 15, 17], or having no effect [40–42, 51, 55]. Recent studies in 5xFAD mice are in line with our observations, showing no effect of long-term voluntary running [39] or EE [16] on hippocampal Aβ load at 8 months and 12 months of age, respectively. Because different studies have used different mouse models with dissimilar speeds and degrees of Aβ deposition and pathology progression, it is difficult to directly compare the results of these studies. For example, male 5xFAD mice display Aβ plaque deposition starting from the age of 2 months [20], with fast progression and reaching a plateau at 10 months of age [28, 46], whereas other AD mouse models have slower plaque load progression [56]. We thus hypothesize that exercise may slow down the speed of plaque formation at a young age, but at later ages, this exercise-mediated reduction is no longer able to cope with the increased amount of Aβ accumulation known to occur during disease progression.

Neuronal survival, as measured by NeuN immunostaining, was reduced in the subiculum, but not hippocampi of 5xFAD mice, in line with a previous study [20]. Voluntary exercise did not affect NeuN levels in the current study. Exercise-mediated effects in NeuN levels have not previously been reported in this mouse model, but previous studies in APP/PS1 mice reported that the hippocampal neuronal density is increased after long-term treadmill exercise [15] and short-term voluntary physical exercise [17], suggesting that either the type of exercise, the duration of the exercise, or the animal model used affects NeuN levels. On the other hand, other study is in agreement with our results showing that EE accompanied with voluntary exercise did not rescue neuronal loss in hippocampi of APP/PS1KI mice [51]. The synaptic loss and cognitive decline observed in AD is often associated with reductions of synaptic proteins and BDNF, one of the major neurotrophins regulating neuronal survival and synaptic plasticity [57]. Previous studies been shown that hippocampal PSD-95, which plays an important role in synapse stabilization and plasticity [58], is reduced in an age-dependent manner in 5xFAD mice [20], and in various other AD models [59, 60]. In the present study, we observed a significant reduction in both BDNF and synaptic protein PSD-95 in 5xFAD hippocampi. These reductions were both reversed after 6 months of voluntary exercise. This finding is in agreement with details in a previous report [5]. Taken together, the findings of our study suggest that voluntary exercise partially improves synaptic function by increasing PSD-95 and BDNF in the hippocampi of AD mice with no effect on adult neurogenesis or neuronal survival.

One of the pathological hallmarks of AD is neuroinflammation, which is accompanied by activation of glial cells [61]. Astrocytes can transit to a reactive state in response to CNS damage, and this astrocytic reaction is associated with increased GFAP expression, altered expression of many genes, and concomitant morphological and functional alterations [1, 62, 63]. Based on the "inflammation hypothesis," neuronal damage in AD is linked with brain inflammation mediated by glial activation with the increased expression of pro-inflammatory mediators and induced neurotoxic cascades [61, 64]. However, reactive astrocytes also play a neuroprotective role in AD by forming a glial scar around Aβ plaques and isolating damaged areas from the rest of the tissue [65]. In addition, Pomilio et al. have shown the presence of APP-related peptides inside astrocytes, together with significant increases of autophagic processes in GFAP-positive plaque-associated hippocampal astrocytes in PDAPP-J20 mice [66]. Moreover, attenuating astrocyte activation has been shown to accelerate plaque pathology and increase dystrophic neurites in APP/PS1 mice [67], indicating the protective effects of astrocyte activation. Currently, it remains elusive whether astrogliosis is beneficial or harmful and most likely depends on a variety of factors, including the phase of pathology.

GFAP is an intermediate filament, which is highly expressed in reactive astrocytes. As in previous studies [16, 68], our results revealed increased GFAP in 5xFAD hippocampi compared to WT mice. Furthermore, we report that exercised 5xFAD mice display an even higher level of GFAP compared to sedentary animals. This alteration is in line with studies showing that treadmill

exercise induced an increase in hippocampal GFAP levels in healthy [18] and in a sporadic rat AD model [69]. In contrast, some studies have demonstrated that exercise has differential effects on astrocyte reactivity. For example, exercise has been shown to suppress astrocyte reactivity and reduce the number of hippocampal GFAP+ astrocytes in APP/PS1 mice [15, 17]. Some studies, on the other hand, have not reported EE- or exercise-dependent alterations in GFAP protein in cortex, DG, or thalamus of 5xFAD mice, whereas the GFAP mRNA level was significantly upregulated [16]. In a recent review focusing on neuroprotection in AD, increased astrocytic GFAP was mentioned as one of the neuromodulatory effects of physical exercise [70].

In the current study, a range of astrocytic markers were investigated for potential exercise-induced alterations in 5xFAD mice, yet only GFAP expression was altered significantly, indicating that the so-called reactive astrocyte is the most affected cell subtype. As others have proposed [71], our data suggest that enhanced GFAP expression in the AD brain is linked with a phenotypic change of pre-existing resting cells, not proliferation of new cells. Human postmortem studies have demonstrated that in contrast to microglia [72], the total number of astrocytes remains unaltered in AD, but the number of GFAP-positive astrocytes is increased [73]. Our work corroborates this by showing that number of S100β-positive astrocytes was unchanged for all mice, whereas 5xFAD-SED mice have two times more GFAP-positive astrocytes in the hippocampi than the WT-SED mice, and this number is further increased by 23% after physical exercise. Based on our findings, we propose that the exercise-induced increase of GFAP in 5xFAD hippocampi may be partly explained by an increased number of GFAP+ astrocytes manifested as a phenotypic shift of the cells to a more reactive state. We thus suggest that exercise could enhance the protective reaction of astrocytes during AD pathology.

In AD, increase expression of GFAP is accompanied with changes in astrocyte morphology. Reactive astrocytes accumulate around Aβ plaques and are associated with hypertrophy of the cell body and thickening of processes [66, 74, 75]. At the same time, senescent-looking astrocytes localizing far from Aβ plaques (> 50 μm) display atrophied cell somas and simplified processes in hippocampus and entorhinal cortex [76, 77]. The results of the current paper demonstrate that in comparison to WT mice, the hippocampal GFAP-positive astrocytes of 5xFAD mice have an enlarged soma area, solid shape, and atrophic branches, and most of these cells are plaque-associated. Importantly, the difference in astrocyte morphology depends on cell localization: GFAP-positive astrocytes associated with plaques have distinct morphological alterations suggestive of reactive properties in comparison to distant cells.

Prior studies have also linked physical exercise and EE to altered astrocyte morphology [14, 18, 78, 79], yet little is known about exercise-induced morphological alterations of astrocytes in the AD brain. Here, we demonstrate that voluntary physical exercise significantly increases the soma area and number of primary branches in the 5xFAD hippocampi. Further analysis revealed that exercise targets specifically the astrocytes associated with Aβ plaques, whereas cells distant from the plaques remain unchanged. Our results are in line with previous study demonstrating that exercise and EE induces an increase in cell and soma surface of GFAP-positive astrocytes in the hippocampi of 12-month-old 3xTG mice [19]. Taken together, we propose that the exercise-induced increase of GFAP in 5xFAD hippocampi, in addition to the phenotypic change to a more reactive state, is a consequence of the morphological changes induced by exercise in plaque-associated astrocytes.

One mechanism that may explain our observed exercise-mediated alterations in GFAP-positive astrocytes and link these to improved behavioral outcomes is related to BDNF, which was significantly upregulated in GFAP-positive astrocytes in the hippocampi of 5xFAD-EXE mice. Astrocytes play a crucial role in synaptic plasticity by regulating synaptic transmission and synaptic structure [65] and BNDF and tyrosine receptor kinase B (TrkB) participate in synaptic function [80]. Alterations in the BDNF-TrkB system thereby affect key parameters such as synaptic plasticity, synaptic proteins including PSD-95, and memory function, which are all impaired in AD [81]. Evidence for the involvement of astrocytes in BDNF-mediated cognitive outcomes is numerous. For example, astrocytes expressing a truncated form of TrkB T1 respond to application of BNDF by releasing Ca^{2+} from intracellular space [82], astrocytes respond to increased BDNF by expression of TrkB receptors [83], astrocytes themselves produce the BDNF protein as it was shown in our study and in previous works [84, 85], and BDNF released from astrocytes is crucial for dendrite spine density and morphology [86]. Importantly, BDNF has been shown to regulate astrocytic morphology through TrkB-T1 receptors located specifically in GFAP+ astrocytes [26, 87]. Collectively, our data and the previously published reports suggest that the observed exercise-mediated increase in the number of GFAP-positive astrocytes together with their morphological changes in the 5xFAD hippocampi result from restoration of reduced BDNF level in GFAP-positive astrocytes and PSD-95 level in hippocampi in AD. These alterations, stimulated by voluntary physical exercise, are likely to stabilize LTP and thereby reduce the cognitive decline observed in the 5xFAD mice.

Conclusions

This paper highlights the importance of non-neuronal cells in the underlying benefits of voluntary exercise, providing additional insight into the significance of astrocytes as responders to physical exercise. Long-term voluntary physical exercise modulated the number of GFAP-positive astrocytes and the morphology of Aβ plaque-associated astrocytes in the hippocampi of 5xFAD mice. The molecular pathways involved in this modulation could potentially be targeted as a therapeutic strategy against AD.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12974-020-01935-w.

Additional file 1: Supplementary Figure 1. Body mass and running activity evolution of WT and 5xFAD mice as measured on a weekly basis. (A) 5xFAD mice have significantly lower body weight than WT mice, whereas exercise slightly reduces body weight in all mice (**p <0.01, $^{\#}p$ = 0.054). (B) Mice had free access to a running wheel from 1.5 to 7 months of age. The running distance was monitored for each mouse by using running counters. No significant differences were observed in the running distance between WT and 5xFAD mice (p = 0.166). Data is presented as mean ± SEM, one-way and two-way ANOVA for repeated measures, n = 20-22/group. « * » genotype effect, **p<0.01, « # » exercise effect. **Supplementary Figure 2**. Effect of voluntary physical exercise on S100β-positive astrocytes in hippocampi. Representative images of S100β-positive astrocytes (A, green) and DAPI (blue) in the hippocampal area of WT-SED, WT-EXE, 5xFAD-SED, 5xFAD-EXE mice. Solid and dotted lines represent quantified hippocampi and subiculum areas, respectively. Scale bars: 200 μm. S100β (B) levels were quantified by measuring the percentage of positive immunoreactive area in hippocampi and subiculum. Number of S100β -positive astrocytes per squared mm in hippocampi (C). All data are presented as mean (±SEM). 2-way ANOVA used to measure genotype and exercise effect between WT and 5xFAD mice. n= 8/group, « * » genotype effect: ***p<0.001. **Supplementary Table 1**. List of antibodies and TaqMan primers used in this study. **Supplementary Table 2**. Effect of AD and voluntary physical exercise on cytokine levels in the hippocampus. Data are presented as mean (±SEM). 2-way ANOVA was used to measure genotype and exercise effect between WT and 5xFAD mice followed by unpaired t test comparison in case of significant interaction between two factors (genotype*exercise). « * » genotype effect, « # » exercise effect, « § » interaction: *p<0.05, **p<0.01, $^{\#}p$<0.05, $^{\S}p$< 0.05. **Supplementary Table 3**. Effect of AD and voluntary physical exercise on mRNA expression of glial markers and BDNF in the hippocampus. Data are normalized by GAPDH C_T values and presented as mean (±SEM). 2-way ANOVA was used to measure genotype and exercise effect between WT and 5xFAD mice followed by unpaired t test comparison in case of significant interaction between two factors (genotype*exercise). « * » genotype effect, « # » exercise effect, « § » interaction: *p<0.05, **p< 0.01, ***p<0.001, $^{\#}p$<0.05, $^{\S}p$<0.05. **Supplementary Table 4**. Morphological analysis of plaque-associated and non-plaque-associated GFAP-positive astrocytes in hippocampus of 5xFAD mice. Data are presented as mean (±SEM). 2-way ANOVA was used to measure plaque-association and exercise effect between sedentary (SED) and exercised (EXE) mice followed by unpaired t test comparison in case of significant interaction between two factors (plaque*exercise). « * » plaque effect, « # » exercise effect, « § » interaction: **p<0.01, ***p<0.01, $^{\#}p$<0.05, $^{\S}p$<0.05.

Abbreviations

AD: Alzheimer's disease; Aβ: Amyloid beta; ALDH1L1: Aldehyde dehydrogenase 1 family, member L1; ANOVA: Analysis of variance; APP: Amyloid precursor protein; BDNF: Brain-derived neurotrophic factors; CBA: Cytokine bead array; DCX: Doublecortin; DG: Dentate gyrus; EE: Environmental enrichment; EXE: Exercised; GFAP: Glial fibrillary acidic protein; GS: Glutamine synthetase; Iba1: Ionized calcium binding adaptor milecule 1; LTP: Long-term potentiation; MOC: Mander's colocalization coefficient; MWM: Morris water maze; NeuN: Neuronal nuclear antigen; Pr: Pearson's correlation coefficient; PSD-95: Postsynaptic density 95; RT-PCR: Real-time PCR; S100β: s100 calcium binding protein β; SED: Sedentary; TrkB: Tyrosine receptor kinase B; WB: Western blot; WT: Wild type

Acknowledgements

We would like to thank Dzhessie Rait, Riikka Nivajärvi, Mika Venojärvi, and Taina Vihavainen for their help with tissue collection, Mirka Tikkanen for her help with mouse brain cryosectioning and immunostaining, and Valeriia Sitnikova for her help with colocalization analysis.

Authors' contributions

IB performed the experiments, analyzed the data, and prepared the manuscript; AS performed open field test and analyzed the data of behavioral testings; SL performed MWM test; HK and HTa were involved in the design and conception of behavioral experiments, analysis, and the interpretation of the results; MI took part in sample preparation, RT-PCR, and WB experiments and analysis of NeuN and GFAP-BDNF staining; AV took part in imaging and analysis of GFAP staining; MII and FRW design and performed the morphological experiments; AG revised grammar in the manuscript; HTi, MA, TM, AG, and HTa contributed to the study design and manuscript editing and revision, and KK conceived the study and the experiments, contributed to the interpretation of the results, and drafted and edited the manuscript. All authors read and approved the manuscript.

Funding

This study was supported by the Academy of Finland, the Sigrid Juselius foundation, Biocenter Finland, and the University of Eastern Finland.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The use of animals in this study was approved by the National Animal Experiment Board of Finland in accordance with the Council of Europe Legislation and Regulation for Animal Protection.

Consent for publication

No applicable.

Competing interests

Authors declare that they have no competing interests.

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Received: 2 June 2020 Accepted: 19 August 2020
Published online: 15 September 2020

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International Journal of Molecular Sciences. 22(16): 8715, 2021

International Journal of
Molecular Sciences

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Regular Physical Exercise Modulates Iron Homeostasis in the 5xFAD Mouse Model of Alzheimer's Disease

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Citation: Belaya, I.; Kucháriková, N.; Górová, V.; Kysenius, K.; Hare, D.J.; Crouch, P.J.; Malm, T.; Atalay, M.; White, A.R.; Liddell, J.R.; et al. Regular Physical Exercise Modulates Iron Homeostasis in the 5xFAD Mouse Model of Alzheimer's Disease. *Int. J. Mol. Sci.* **2021**, *22*, 8715. https://doi.org/10.3390/ijms22168715

Academic Editor: Athanasios Salifoglou

Received: 30 June 2021
Accepted: 9 August 2021
Published: 13 August 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Abstract: Dysregulation of brain iron metabolism is one of the pathological features of aging and Alzheimer's disease (AD), a neurodegenerative disease characterized by progressive memory loss and cognitive impairment. While physical inactivity is one of the risk factors for AD and regular exercise improves cognitive function and reduces pathology associated with AD, the underlying mechanisms remain unclear. The purpose of the study is to explore the effect of regular physical exercise on modulation of iron homeostasis in the brain and periphery of the 5xFAD mouse model of AD. By using inductively coupled plasma mass spectrometry and a variety of biochemical techniques, we measured total iron content and level of proteins essential in iron homeostasis in the brain and skeletal muscles of sedentary and exercised mice. Long-term voluntary running induced redistribution of iron resulted in altered iron metabolism and trafficking in the brain and increased iron content in skeletal muscle. Exercise reduced levels of cortical hepcidin, a key regulator of iron homeostasis, coupled with interleukin-6 (IL-6) decrease in cortex and plasma. We propose that regular exercise induces a reduction of hepcidin in the brain, possibly via the IL-6/STAT3/JAK1 pathway. These findings indicate that regular exercise modulates iron homeostasis in both wild-type and AD mice.

Keywords: Alzheimer's disease; 5xFAD mouse; regular voluntary exercise; iron; hepcidin; il-6; cortex; skeletal muscle

1. Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disorder mainly affecting the aged population and characterized by progressive memory loss and cognitive impairment [1]. Although age-related and genetic risk factors are implicated in the majority of all AD cases, a large proportion of cases are linked with lifestyle factors such as physical inactivity, unhealthy diet, social isolation, and living in polluted locations [2]. Due to the lack of effective medical treatments, more efforts should be focused on modulating lifestyle factors such as physical activity in efforts to slow or prevent disease progression. Regular physical exercise has huge impacts on health, reducing risk of cardiovascular diseases, chronic metabolic diseases, psychiatric disorders, and dementia enhancing adaptation against stress, anti-inflammatory action, and normalization of metabolic status [3,4]. In the brain, physical exercise positively affects neuronal plasticity and memory, also reducing

age-related declines in synaptic function and adult neurogenesis [5,6]. In skeletal muscle, the major organ responding to exercise, physical exercise increases metabolic activity, enhances antioxidant systems, and reduces inflammation [7].

Age-related diseases including AD are associated with dysregulation of biometal homeostasis, which is implicated in disruption of critical cellular processes [8–10]. For example, excess of redox-active iron (Fe^{2+}) or dysregulation of iron metabolism can generate intensively reactive oxygen species (ROS) and lead to oxidative stress, disruption of redox-homeostasis [11,12], and ferroptosis [13]. In AD, dysregulation of iron metabolism and excess iron is involved in amyloid beta (Aβ) production and aggregation, causing neuronal cell death [14]. While sustained deviation from redox homeostasis and metal imbalance may promote the development of aged-related neurodegenerative and metabolic diseases, direct evidence for this is limited. Published reports demonstrate that regular exercise can improve iron metabolism and protect against iron accumulation, yet the associated mechanisms remain unclear [15].

Regular exercise has beneficial effects on the whole body and is suggestive of a pathways participating in the interplay between different organs upon exercise training. It has been shown that contracting skeletal muscle produces and secretes different proteins and peptides, so-called myokines, into the bloodstream. The majority of the myokines can cross the blood brain barrier (BBB) and thereby affect brain functions [16]. Interleukin 6 (IL-6) is the first myokine discovered to be released from skeletal muscle in response to physical exercise [17] and is known to evoke systemic anti-inflammatory effects [18]. One session of exercise can increase muscle and plasma IL-6 levels by up to 100-fold, whereas long-term physical training reduces basal IL-6 levels in the plasma [19]. Moreover, given that IL-6 can cross the BBB [18], it is plausible that crosstalk between skeletal muscle and the brain can be mediated by IL-6. Aging and age-related diseases including AD are associated with alterations in myokine levels together with increased chronic inflammation [20]. IL-6, a multifunctional cytokine that is paramount in immune responses and nervous system function, is increased in the brain during aging and AD [21]. In contrast, brain IL-6 levels are reduced upon regular physical exercise [22,23]. Exercise-induced IL-6 modulation remains poorly investigated in the context of AD. Moreover, IL-6 is known to be involved in the regulation of brain iron metabolism [24].

In this study, we used the 5xFAD mouse model of AD, which is an early-onset model with rapid development of a variety of AD-related pathologies, including Aβ plaque deposits accompanied by glial activation starting from 2 months of age, neuronal loss, and cognitive impairments starting from 3–5 months of age [25,26]. The early AD pathology and relatively fast disease development make the 5xFAD mice a suitable model for studying the impact of lifestyle changes, such as physical exercise, in a relatively short period of time, and the model has been widely used in preclinical studies of AD. We have previously shown that, at the age of 7 months, the 5xFAD mice display significant cognitive impairments, which are reversed by long-term voluntary running [27]. While dysregulation of iron homeostasis is evident in AD, little is known about how physical exercise affects iron metabolism in the brain and periphery, and the mechanisms responsible for exercise-induced iron regulation in AD. Therefore, the aim of the present study was to evaluate the effect of long-term voluntary running exercise on iron metabolism in the brain and skeletal muscle in both wild-type (WT) and 5xFAD mice. We also assessed the impact of long-term voluntary running exercise on the regulation of iron metabolism by IL-6.

2. Results

5xFAD transgenic male mice and their WT littermates were divided into four groups: WT-sedentary (WT-SED), WT-exercised (WT-EXE), 5xFAD-sedentary (5xFAD-SED), and 5xFAD-exercised (5xFAD-EXE). The long-term voluntary exercise protocol lasted from the age of 1.5 months to 7 months for the exercised mice. No significant differences in running distance were observed among the exercised mice. 5xFAD mice had a lower body mass than the WT mice, and long-term exercise slightly decreased the weights of both WT

and 5xFAD mice [27]. To evaluate whether long-term physical exercise affects Aβ plaque load in the brain, we performed immunohistochemical staining using the WO2 antibody of brain sections (Figure 1A). Although physical exercise displayed only a tendency to decrease Aβ plaque load in the hippocampal area of 5xFAD mice [27], in the cortical layer V, the Aβ level was significantly lower in 5xFAD-EXE mice in comparison to 5xFAD-SED mice ($p < 0.05$; Figure 1B).

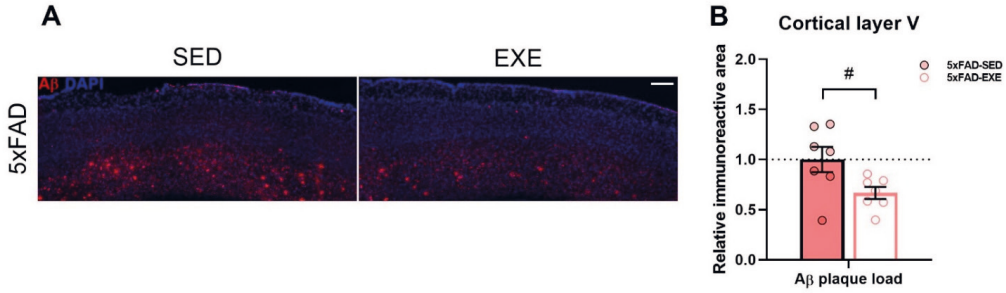

Figure 1. Effects of regular exercise on cortical Aβ load in the 5xFAD mouse model. (**A**) Representative images of Aβ staining in cortical layer V of 5xFAD-SED and 5xFAD-EXE mice. Scale bar 200 µm. (**B**) Percentage of immunoreactive area was quantified to measure Aβ content in cortical layer V. All data are relative to 5xFAD-SED and presented as mean ± SEM. $n = 8$/group. «$^{\#}$» exercise effect: $^{\#}$ $p < 0.05$.

2.1. Exercise Effects on Iron Load in Muscle and Cortex

Total iron was measured in cortical and gastrocnemius skeletal muscle (muscle) tissues by inductively coupled plasma mass spectrometry (ICP-MS). Although there was no genotype ($p = 0.6$) or exercise effect ($p = 0.9$, Figure 2A) detected in cortical total iron content, a significant exercise-induced increase in total iron level was found in muscles of both WT and 5xFAD mice (main effect of exercise: $p < 0.01$, Figure 2E).

To assess the effect(s) of exercise on iron load, we measured the mRNA expression and protein level of ferritin, the main iron storage protein, in cortex and muscle tissues. Quantitative PCR (qPCR) analysis revealed no changes in mRNA expression of ferritin between WT and 5xFAD mice, neither in cortex (genotype × exercise interaction: $p < 0.01$, post hoc test: $p = 0.5$, Figure 2B) or muscle tissues (main genotype effect: $p = 0.8$, Figure 2F). However, physical exercise induced a significant reduction in the mRNA expression level of ferritin in the cortex of 5xFAD-EXE mice in comparison with 5xFAD-SED mice (genotype × exercise interaction: $p < 0.01$, post hoc test: $p < 0.01$, Figure 2B).

Immunohistochemical staining of brain sections for ferritin (Figure 2C, Supplementary Figure S1A) revealed a significant ferritin increase in the cortex of 5xFAD mice when compared to WT mice (main genotype effect: $p < 0.001$, Figure 2D) while only a slight ferritin increase was detected in the hippocampi of 5xFAD mice when compared to WT mice (main genotype effect: $p = 0.053$, Supplementary Figure S1B). Moreover, physical exercise induced a significant reduction of ferritin in the cortex of exercised mice in comparison to sedentary mice (main exercise effect: $p < 0.05$, Figure 2D), whereas no difference in hippocampal ferritin level was detected between exercised and sedentary mice (main exercise effect: $p = 0.3$, Supplementary Figure S1B). Conversely, Western blot analysis revealed that physical exercise significantly increased the protein level of ferritin in the muscles of exercised mice when compared to sedentary mice (main exercise effect: $p < 0.05$, Figure 2G).

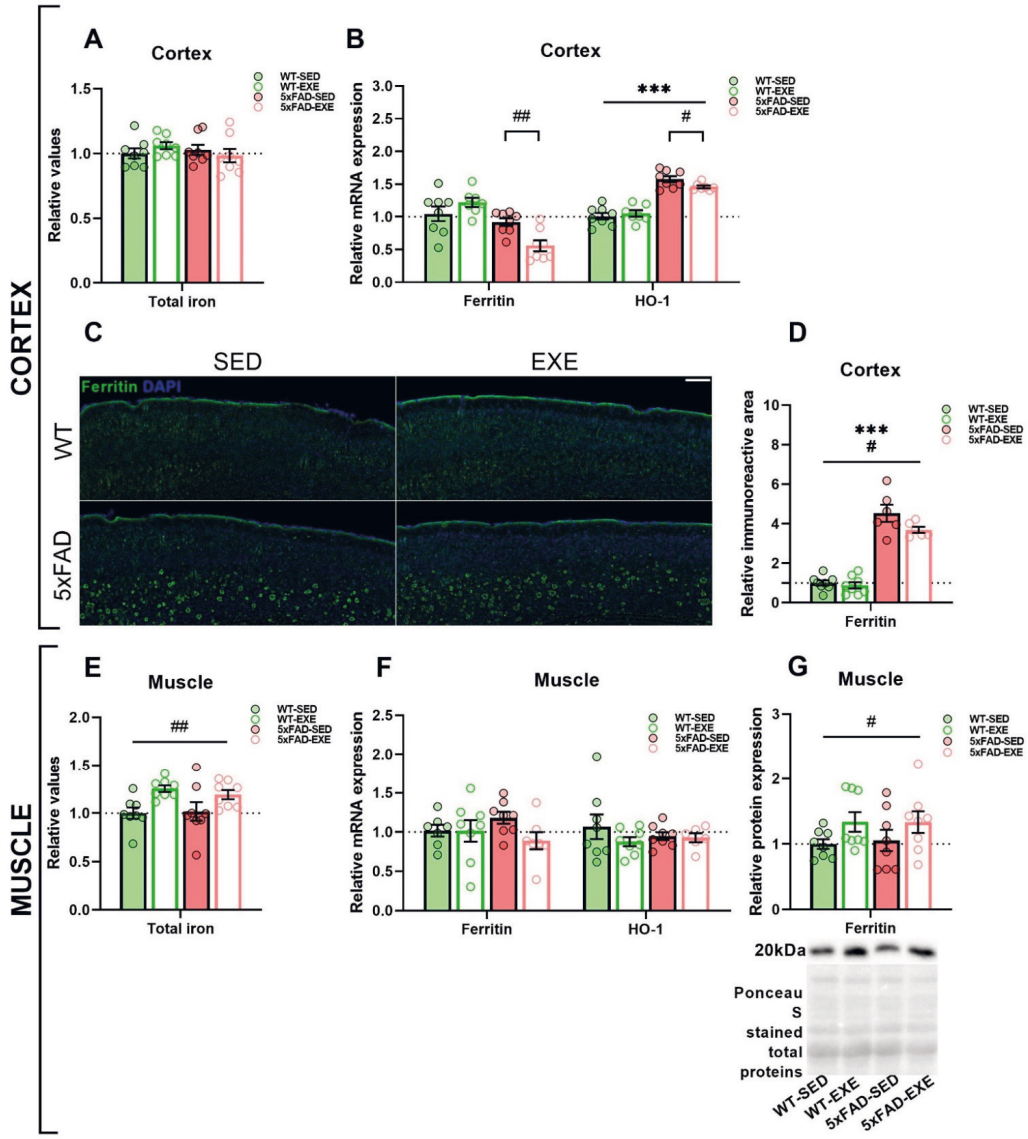

Figure 2. Effects of regular exercise on iron load in cortex and muscle tissues in 5xFAD mouse model. Total iron content, ferritin, and HO-1 level in cortex (**A**–**D**) and muscle samples (**E**–**G**) of WT and 5xFAD mice. Total iron content in cortex (**A**) and muscle (**E**) was measured by ICP-MS. mRNA expression of ferritin and HO-1 in cortex (**B**) and muscle (**F**) was measured by qPCR. (**C**) Representative images of ferritin levels in cortex of WT-SED, WT-EXE, 5xFAD-SED, and 5xFAD-EXE mice. Scale bar 200 μm. (**D**) Percentage of immunoreactive area was quantified to measure ferritin level in cortex. (**G**) Representative Ponceau S staining and Western blot of ferritin in muscle samples and the analysis of band intensities normalized to the total proteins. All data are presented as mean ± SEM. $n = 8$/group. «$^{\#}$» exercise effect: *** $p < 0.001$, $^{\#\#}$ $p < 0.01$, $^{\#}$ $p < 0.05$. General genotype/exercise effect among all groups is presented as a line with «$^{\#}$» sign on top, exercise effect in 5xFAD mice only is presented as a bracket with «$^{\#}$» sign on top.

We next assessed heme oxygenase 1 (HO-1), which degrades heme into redox-active Fe^{2+}, potentially leading to free iron overload, one of the features of AD [14]. This analysis revealed that HO-1 mRNA expression was significantly upregulated in the cortex of 5xFAD mice (main genotype effect: $p < 0.0001$, Figure 2B). Further post hoc analysis revealed a significant exercise-induced reduction of HO-1 mRNA expression in the cortex of 5xFAD-EXE when compared to 5xFAD-SED mice ($p < 0.05$, Figure 2B). No changes in HO-1 mRNA expression were observed in muscle samples (main genotype effect: $p = 0.9$, main exercise effect: $p = 0.4$, Figure 2F).

2.2. Exercise Effects on Iron Trafficking in Muscle and Cortex

Iron uptake in the brain is regulated by transferrin receptor (TfR) and divalent metal transporter 1 (DMT1) [28]. To investigate the effect of physical exercise on iron uptake, the mRNA and protein level of TfR, which is responsible for Fe^{3+} uptake, were evaluated in cortex and muscle tissues. qPCR analysis demonstrated that the mRNA expression of TfR was upregulated in the cortex (main genotype effect: $p < 0.01$, Figure 3A) of 5xFAD mice and in the muscles of 5xFAD-SED mice when compared to WT-SED mice (genotype × exercise interaction: $p = 0.07$, post hoc test: $p < 0.01$, Figure 3D). Physical exercise induced a significant reduction of cortical TfR mRNA expression in 5xFAD-EXE mice when compared to 5xFAD-SED mice (genotype × exercise interaction: $p = 0.06$, post hoc test: $p < 0.05$, Figure 3A) and in all exercised mouse muscles (main exercise effect: $p < 0.0001$, Figure 3D).

The immunohistochemical staining of brain sections for TfR (Figure 3B) revealed that the protein level of TfR was unchanged in the cortical region of 5xFAD-SED mice compared to WT-SED mice (genotype × exercise interaction: $p = 0.05$, post hoc test: $p = 0.6$, Figure 3C). Physical exercise induced a significant increase of TfR in the cortex of exercised 5xFAD mice in comparison with 5xFAD-SED mice (genotype × exercise interaction: $p = 0.05$, post hoc test: $p < 0.05$, Figure 3C). For muscle tissue, Western blot analysis showed a dramatic decrease of TfR protein in exercised mice when compared to sedentary mice (main exercise effect: $p < 0.0001$, Figure 3E).

In addition, the mRNA expression of DMT1, which transports Fe^{2+} into cells, was analyzed in cortex and muscle tissues by qPCR. Exercise induced a significant reduction in DMT1 mRNA expression in the cortex of 5xFAD-EXE mice in comparison with 5xFAD-SED mice (genotype × exercise interaction: $p = 0.0004$, post hoc test: $p < 0.01$, Figure 3A). Physical exercise caused a similar effect in muscle tissue, slightly reducing DMT1 mRNA expression in all exercised mice in comparison to sedentary mice (main exercise effect: $p < 0.05$, Figure 3D). Conversely, Western blot analysis revealed that regular exercise significantly increased the protein level of DMT1 in the muscles of exercised mice when compared to sedentary mice (main exercise effect: $p < 0.01$, Figure 3E).

To assess the effects of exercise on Fe^{2+} efflux, qPCR analysis was performed to measure mRNA levels of essential iron efflux proteins such as ceruloplasmin and ferroportin. Ceruloplasmin gene expression was reduced in the cortex of 5xFAD mice (main genotype effect: $p < 0.05$, Figure 3A), while increased expression was found in the muscle (genotype × exercise interaction: $p = 0.1$, post hoc test: $p < 0.05$, Figure 3D) in comparison to WT mice. While AD-related changes in ferroportin expression were not detected, physical exercise slightly reduced its expression in the cortex of exercised mice when compared to sedentary mice (main exercise effect: $p < 0.01$, Figure 3A). No significant changes in ferroportin mRNA (Figure 3D) or protein level (Figure 3E) were observed in muscle samples.

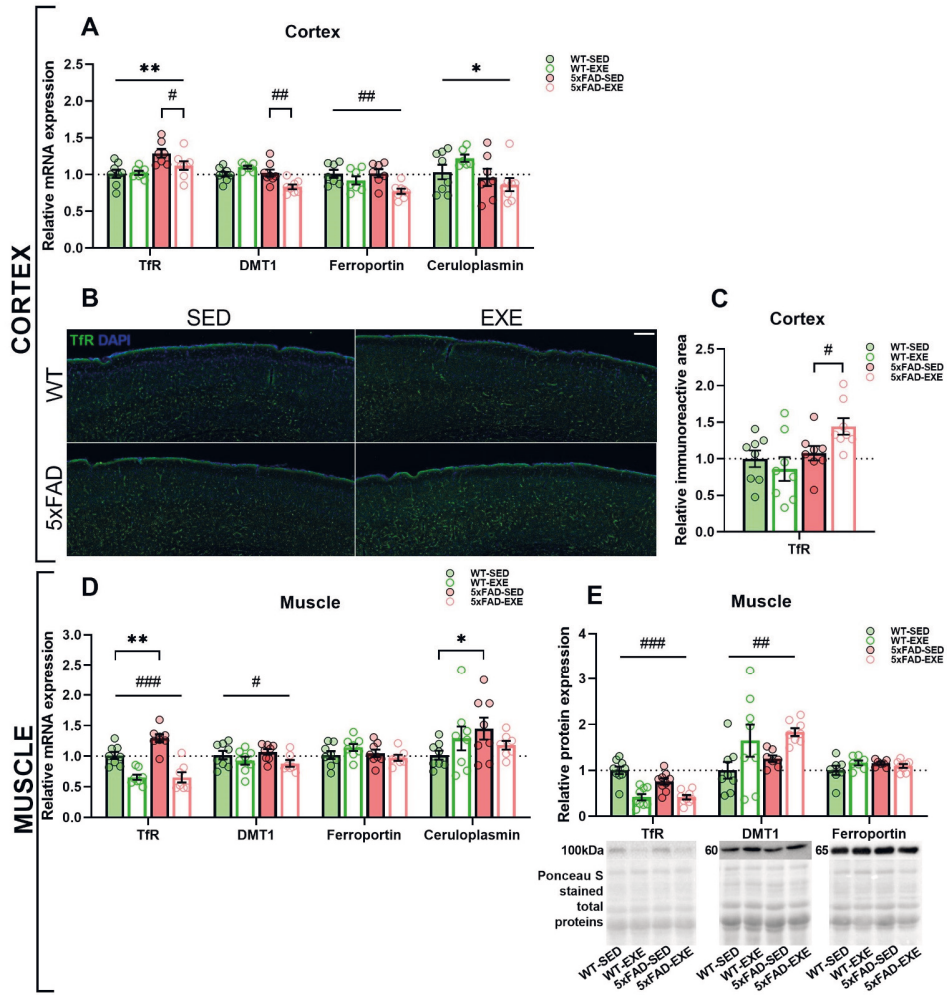

Figure 3. Effects of regular exercise on iron trafficking in cortex and muscle tissues in 5xFAD mouse model. Level of iron transporters and ferroxidase ceruloplasmin in cortex (**A**–**C**) and muscle samples (**D**,**E**) of WT and 5xFAD mice. mRNA expression of TfR, DMT1, ferroportin, and ceruloplasmin in the cortex (**A**) and muscle (**D**) was measured by qPCR. (**B**) Representative images of TfR levels in the cortex of WT-SED, WT-EXE, 5xFAD-SED, and 5xFAD-EXE mice. Scale bar 200 µm. (**C**) Percentage of immunoreactive area was quantified to measure TfR level in cortex. (**E**) Representative Ponceau S staining and Western blot of TfR, DMT1 and ferroportin in muscle samples and the analysis of band intensities normalized to the total proteins. All data are presented as mean ± SEM. $n = 8$/group. «*» genotype effect, «$^{\#}$» exercise effect: ** $p < 0.01$, * $p < 0.05$, $^{\#\#\#}$ $p < 0.001$, $^{\#\#}$ $p < 0.01$, $^{\#}$ $p < 0.05$. General genotype/exercise effect among all groups is presented as a line with «*»/«$^{\#}$» sign on top, exercise effect in 5xFAD mice only is presented as a bracket with «$^{\#}$» sign on top, genotype effect in SED mice only is presented as a bracket with «*» sign on top.

2.3. Exercise Effects on Regulation of Iron Homeostasis in Muscle and Cortex

Next, we evaluated the effects of exercise on iron homeostasis by measuring the levels of hepcidin, a hormone responsible for regulation of cellular iron levels [29,30]. ELISA analysis revealed a significant reduction of hepcidin in the cortex of exercised mice when compared to sedentary mice (main exercise effect: $p < 0.05$, Figure 4A). Moreover, the

signal transducer activator of transcription 3 (STAT3)/Janus kinase 1 (JAK1) pathway, activation of which is known to regulate hepcidin expression [31], was also altered by exercise. qPCR analysis demonstrated significant upregulation of STAT3 mRNA expression (main genotype effect: $p < 0.001$) and a slight increase in JAK1 ($p = 0.06$) in the cortex of 5xFAD mice in comparison with WT, whereas physical exercise ameliorated this increase in STAT3 (genotype × exercise interaction: $p = 0.02$, post hoc test: $p < 0.01$) and JAK1 (genotype × exercise interaction: $p = 0.03$, post hoc test: $p < 0.05$, Figure 4B) in 5xFAD cortices. In addition, an exercise-induced increase in the expression of receptor-type tyrosine-protein phosphatase epsilon (PTPe), which is involved in inhibition of STAT/JAK signaling [32,33], was detected in exercised mice in comparison to sedentary mice (main exercise effect: $p < 0.05$, Figure 4B).

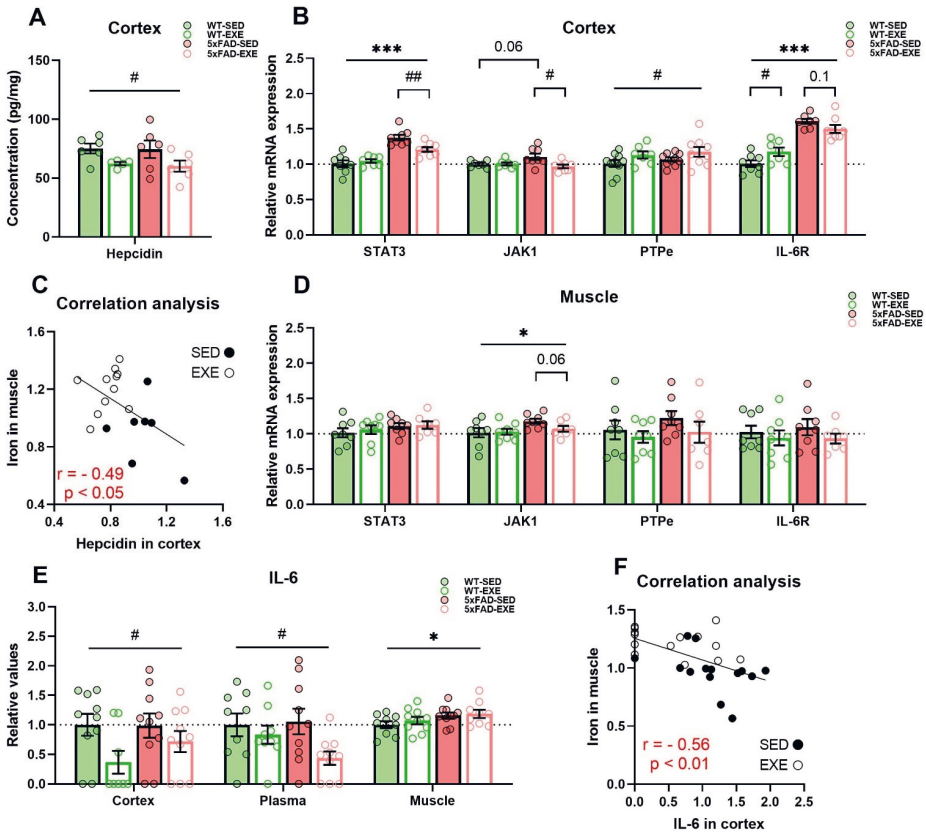

Figure 4. Effects of regular exercise on iron homeostasis regulation in cortex and muscle tissues in 5xFAD mouse model. (**A**) Hepcidin level in cortex was measured by ELISA and normalized to total protein concentration. (**C**) Correlation analysis for total iron content in muscle and hepcidin level in cortex was performed for all mice, r–Pearson correlation coefficient. mRNA expression of STAT3, JAK1, PTPe, and IL-6R in cortex (**B**) and muscle (**D**) was measured by qPCR. (**E**) IL-6 level in cortex, plasma, and muscle protein samples was measured by CBA. (**F**) Correlation analysis for total iron content in muscle and IL-6 level in cortex was performed for all mice. All data are presented as mean ± SEM. n = 6–10/group. «*» genotype effect, «$^{\#}$» exercise effect: *** $p < 0.001$, * $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#}$ $p < 0.05$. General genotype/exercise effect among all groups is presented as a line with «*»/«$^{\#}$» sign on top, exercise effect in 5xFAD or WT mice separately is presented as a bracket with «$^{\#}$» sign/p value on top, genotype effect in SED mice only is presented as a bracket with p value on top.

Hepcidin is known to be dependent on circulating iron and inflammation status, particularly on the level of IL-6 [14]. Therefore, we assessed IL-6 receptor (IL-6R) and IL-6 levels in the cortex, plasma, and muscle samples. qPCR analysis demonstrated that the mRNA expression of IL-6R was significantly increased in the 5xFAD cortex when compared to WT (main genotype effect: $p < 0.001$, Figure 4B). Exercise had slight effects in different genotypes: in WT IL-6R, it increased ($p < 0.05$), while in 5xFAD, it tended to decrease ($p = 0.12$, Figure 4B). No differences in IL-6R mRNA expression were detected in muscles (Figure 4D). Cytokine bead array (CBA) analysis revealed that physical exercise induced a significant reduction of IL-6 in the cortex (main exercise effect: $p < 0.05$) and plasma ($p < 0.05$) with no changes in muscle ($p = 0.5$) in both WT and 5xFAD-mice (Figure 4E). In addition, we found a significant negative correlation between cortical IL-6 and iron levels in muscle ($r = -0.56$, $p < 0.01$, Figure 4F) and cortical hepcidin and iron levels in muscle ($r = -0.49$, $p < 0.05$, Figure 4C) among all mice: the more iron in muscle, the less IL-6 and hepcidin in the cortex.

3. Discussion

Iron is an essential biometal, which is involved in many important biological processes in the body. Heme iron is bound to hemoglobin within red blood cells participating in oxygen transport, whereas non-heme iron is distributed through the body with 10% localized in the brain and participating in neurotransmitter signaling and myelin production [34]. Although AD is associated with iron dyshomeostasis and iron accumulation in the brain [35], the mechanisms underlying iron dysregulation remain unclear. Iron is essential for skeletal muscle oxidative capacity upon exercise, and growing evidence suggests an important role of skeletal muscle and physical exercise for regulation of iron metabolism in the whole body [15,36]. In the present study, we aimed to decipher the role of long-term voluntary running exercise in iron homeostasis in WT mice, and the 5xFAD mouse model of AD. In particular, the aim was to evaluate the effects of exercise on iron load, trafficking, and homeostasis in the brain and skeletal muscles.

Ferritin, the main iron storage protein, is elevated upon aging and in the AD brain. In particular, Aβ plaques found in the AD cortex and hippocampi are associated with iron deposits and ferritin [37–39]. In accordance with previous studies of AD model mice [40–42], we demonstrated significant increases of ferritin in the cortex of 7-month-old 5xFAD mice. Since ferritin is responsible for attenuation and sequestration of free iron [43], AD-associated increases of ferritin levels may indicate elevated labile iron level in the brain. HO-1 can be partly responsible for elevated labile iron level in the brain, because HO-1 degrades heme into Fe^{2+}, leading to labile iron level increase in AD [14]. This notion was supported by our finding of increased HO-1 overexpression in the 5xFAD cortex. Moreover, ferritin upregulation can occur in response to neuroinflammation [44], one of the major pathological features of AD.

Although recent studies demonstrated elevations in total iron levels in the brains of AD mice [41,45], we did not detect increased iron in the cortex of 5xFAD mice by ICP-MS analysis. In the study by Gurel et al., the iron level was measured in hippocampal lysates of 3-month-old 5xFAD mice, an age at which robust Aβ plaque accumulation does not yet occur. In contrast, in our study, the iron level was analyzed at 7 months of age, when Aβ plaque load is three times higher [46]. Iron levels have been shown to depend on age and stage of AD: the iron level has been shown to increase between the ages of 3 and 8 months, with a later iron level decrease occurring until 24 months of age in APP/PS1 mice [40]. Moreover, there is a possibility that AD is associated with changes in the distribution of iron between cell types or between its different molecular forms (free iron, ferritin, transferrin, heme) [14], which may partly explain the unchanged total iron with increased ferritin and HO-1 level in the cortex of 5xFAD mice. Taken together, the findings of our study indicate altered iron regulation via ferritin and HO-1 increases despite no changes in total iron levels in the cortex of 5xFAD mice at 7 months of age.

In recent reviews, it has been suggested that exercise can modulate iron metabolism and reduce iron stores in the body [15,47], but direct evidence is lacking. Furthermore, the effects of physical exercise on iron regulation in the brain remain poorly investigated. In the current study, long-term voluntary exercise reduced ferritin and HO-1 levels coupled with Aβ decrease in the 5xFAD cortex, with no effect on total iron content in the brain. In a recent study, exercise induced a similar reduction of ferritin level in AD mice; however, the total iron content in the brain also decreased [41]. The Choi et al. paper used a different AD mouse model (APP-C105) than that used in the current study and utilized a colorimetric iron measurement technique, which may explain the different results for total iron measurements. While ICP-MS analysis is considered as the golden standard for total iron measurements, colorimetric iron detection may depend on assay conditions, affecting the extent of iron release [48]. While our results demonstrated exercise-induced ferritin and HO-1 reductions in the mouse cortex, ferritin levels and total iron content in muscle were elevated upon exercise with no changes in HO-1 muscular level, which may indicate unchanged labile iron in response to regular exercise in muscle. Mitochondria play an important role in iron metabolism, participating in synthesis of iron sulfur clusters and heme, with the latter molecule being essential for oxygen transport and energy production [49,50]. It is known that exercise training induces increases in skeletal muscle mitochondrial mass, and enhances oxidative muscle capacity and oxygen delivery to skeletal muscle [51]. Thus, increased iron content in skeletal muscle of exercised mice may indicate elevated energy demands in this tissue. Recent work by Ghio et al. demonstrated an increase in total iron content with voluntary exercise in various tissues of rats [52]. In that study, voluntary exercise induced iron redistribution in the body whereby iron level was attenuated in plasma and liver and elevated in tissues with high metabolic activity, such as skeletal muscles, heart and lung. It is therefore plausible that in the current study, regular exercise also induced a redistribution of iron in the body, resulting in ferritin reduction in the cortex and elevation in the muscles coupled with unchanged total iron content in the cortex and elevation in the muscles.

Cellular iron trafficking is regulated by iron transporters including TfR, DMT1, and ferroportin aided by the ferroxidase ceruloplasmin [53]. Given that iron accumulation in the AD brain can be associated with dysregulation of iron trafficking [24], we assessed the effect of exercise on iron transport in the cortex and skeletal muscle tissues. In recent studies, upregulation of iron uptake transporters (TfR and DMT1) and downregulation of iron efflux transporters and responsible proteins (ferroportin and ceruloplasmin) were observed in the brains of various AD mouse models [41,42,54]. In our study, 5xFAD mice had increased TfR and decreased CP in the brain, which is consistent with previous reports. We also found that long-term physical exercise induced a significant reduction of DMT1 together with increased TfR in the 5xFAD brain. To date, only one study evaluated the effect of treadmill exercise on iron transporters in brains of AD model mice, and demonstrated a reduction of TfR and DMT1 with exercise in the motor cortex of APP-C105 mice [41]. The difference in TfR response to exercise may be linked with the use of a different AD mouse model or brain area assessed, or more likely, the exercise regimen used. In our study, we also measured iron transporters in skeletal muscle in response to physical exercise and found dramatic reductions in TfR and increases in DMT1 in the muscles of exercised mice. Similar DMT1 upregulation was observed in skeletal muscle of rats in response to 5-week treadmill exercise [55]. It has been shown that mitochondria express DMT1 transporters [56], which are the major mitochondrial iron importers involved in mitochondrial iron acquisition [57]. We demonstrated increased DMT1 in skeletal muscle in response to exercise, which may indicate increased iron utilization by mitochondria and energy metabolism. Taken together, we found that long-term voluntary exercise induced DMT1 reduction and TfR1 elevation in the 5xFAD cortex, together with DMT1 increase and TfR reduction in skeletal muscles. This may indicate that regularly undertaken exercise is able to modulate iron trafficking both in health and AD. Next, we investigated potential exercise-induced mechanisms responsible for this modulation.

Body iron balance is under the control of hepcidin, a key hormone in iron homeostasis responsible for negative regulation of cellular iron uptake and efflux [24,29,58]. In the periphery, hepcidin is synthetized by the liver and when in circulation, can readily cross the BBB [24]. The synthesis of hepcidin is regulated by iron load and inflammatory status [24]. Previous studies have demonstrated that hepcidin is distributed around Aβ plaques [59], that hepcidin level is increased in the serum of AD patients [60–62], and is suggested as a potential blood biomarker for identifying risk of AD [63]. Although we did not observe increased hepcidin in the 5xFAD mice cortex, Wang et al. previously reported elevated hepcidin expression in the mouse brain upon aging [64]. It has been suggested that iron overload occurring in AD is associated with a reduction of iron export due to dysregulation of hepcidin [24]. Thereby a decrease of hepcidin in the brain can potentially have positive effects on stabilization of iron homeostasis in AD [65]. It has been shown that hypoactivity, a model of sedentary behavior, is associated with dysregulation of iron metabolism accompanied by increased hepcidin levels in liver and bone of rats [66,67] and in the spleen and serum of healthy individuals [68]. To date, there are no publications reporting the effect of physical activity on brain hepcidin levels. Here, we report for the first time that long-term voluntary exercise induces a significant reduction of hepcidin in the cortex of both WT and 5xFAD mice. Our study is the first to link the iron status of the brain with reduction of hepcidin in mice undergoing voluntary exercise.

IL-6 is involved in regulation of iron metabolism through hepcidin [24], and IL-6 is necessary for hepcidin induction during inflammation in mice and humans [69]. Moreover, a recent study demonstrated that inflammation-induced iron accumulation and hepcidin upregulation in the brain is regulated by IL-6/STAT3 [31]. In accordance with this report, we observed STAT3 upregulation in the 5xFAD cortex. High levels of IL-6 are associated with cognitive decline and memory impairments [70], and in AD patients, serum and CSF levels of IL-6 are up-regulated and considered as a marker of inflammation [71]. Inflammation mediated by glial cell activation was demonstrated in the 5xFAD brain [27], yet in the current study, we did not observe AD-related increases of IL-6 in the cortex, in line with previously published reports [5,72]. However, we detected a significant increase in IL-6 receptor expression.

While IL-6 is often considered an immune-modulatory cytokine, it is also defined as a myokine secreted from contracting skeletal muscles to the blood stream [16]. Long-term regular aerobic exercise has been shown to reduce basal IL-6 levels in plasma: the more exercise, the lower the basal IL-6 level [73–75]. Moreover, IL-6 can cross the BBB, suggesting a potential crosstalk between muscle and the brain [18]. It has been proposed that lowering the peripheral levels of IL-6 may reduce the risk of developing neurocognitive defects [76]. Although regular aerobic exercise causes reductions in serum IL-6 levels in older healthy adults and individuals with mild cognitive impairment [75,77], the effects of exercise on IL-6 level in AD remain poorly studied. Our study demonstrated that long-term voluntary physical exercise significantly reduces IL-6 levels in plasma and the cortex of WT and 5xFAD exercised mice, while IL-6 levels in muscle remain unchanged. Consistent with our findings, previous studies have shown that IL-6 is attenuated in the brain after treadmill exercise of healthy rats [22] and after resistance exercise in APP/PS1 mice [23]. Moreover, a recent human study demonstrated that aging induces increases in serum IL-6, while lifelong aerobic exercise during 50 years decreases serum IL-6 with no differences observed in skeletal muscle IL-6 levels [78]. Together with a reduction of IL-6 in plasma and the cortex, we observed a significant decline of STAT3/JAK1 and upregulation of STAT3/JAK1 inhibitor PTPe in the cortex of exercised 5xFAD mice. We thus propose that long-term voluntary exercise induces a decrease of hepcidin in the brain, possibly via the IL-6/STAT3/JAK1 pathway. The exercise-induced decrease of hepcidin may be central in the regulation of brain iron metabolism.

4. Materials and Methods

4.1. Experimental Design

This study utilized male 5xFAD transgenic mice carrying five familial AD (FAD)-related mutations in human amyloid precursor protein (APP; Swe, Flo, and Lon) and human presenilin-1 (PSEN1; M146L and L286V) transgenes driven by the mouse Thy1 promoter [42] and their WT littermates on the JAXC57BL/6J background. Starting from six weeks of age, half of the mice were housed in individual regular cages (sedentary, SED), and half were housed in individual cages with a running wheel (Techniplast, Italy) and let to voluntarily exercise (exercise, EXE) freely for 6 months. The running distance and duration were recorded weekly for each exercised mouse using running counters (Sigma, Germany) installed in each cage. Exercised mice were sacrificed two days after removal of the running wheels to avoid acute effects of running. All mice had *ad libitum* access to food and water and were housed under a 12:12-h light-dark cycle with humidity and temperature control. The weights of the mice were monitored on a weekly basis. This study was conducted in accordance with the Council of Europe Legislation and Regulation for Animal Protection and was approved by the National Animal Experiment Board of Finland.

4.2. Tissue Collection

At seven months of age, mice were anesthetized with tribromoethanol (Sigma-Aldrich, St. Louis, MO, USA), blood was collected with 3.8% sodium citrate anticoagulant, and mice were transcardially perfused with heparinized saline. The left brain hemispheres were removed, the cortices were dissected on ice and snap frozen in liquid nitrogen and then stored at −70 °C. The right brain hemispheres were fixed for 22 h in 4% paraformaldehyde at +4 °C, followed by 24 h incubation in 30% sucrose at +4 °C, then snap frozen in liquid nitrogen and finally stored at −70 °C for cryosectioning. The right hemispheres (n = 8/group) were cut into serial 20 µm sagittal sections, each 400 µm apart, using a cryostat (Leica Microsystems, Wetzlar, Germany) and stored in anti-freeze solution at −20 °C until immunostaining analysis. Gastrocnemius skeletal muscles were collected from perfused animals, snap frozen in liquid nitrogen, and stored at −70 °C until analyzed. Collected blood was centrifuged at 2000× g for 6 min at +4 °C, plasma supernatants were additionally centrifuged at 12,000× g for 3 min at +4 °C, final plasma samples were snap frozen in liquid nitrogen and stored at −70 °C for further use.

4.3. Iron Quantitation Via ICP-MS

Iron content was assessed in samples via ICP-MS as reported previously [79]. Briefly, mouse brain cortices (n = 8/group) were digested overnight in concentrated nitric acid and then heated for 20 min at 90 °C. The volume of each sample was reduced to approximately 40 µL and then diluted to a final volume of 600 µL with 1% (v/v) nitric acid diluent. Measurements were performed using an Agilent 7700× series ICP-MS instrument.

Muscle samples were assessed for iron content using "microdroplet" laser ablation-ICP-MS (LA-ICP-MS) as described previously [80]. Briefly, muscle samples (n = 8/group) were homogenized in tris(hydroxymethyl)-aminomethane buffered saline (TBS)-based homogenization buffer as described previously [81]. Samples were assessed for protein content using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) and then diluted to a consistent protein concentration. One microliter of each sample was pipetted onto a glass slide and left to air dry overnight. Droplet residues were ablated off the slide surface using laser ablation and analyzed using Iolite software [80]. Measurements were performed using an NWR-213 laser ablation unit (Electro Scientific Industries, Portland, OR, USA) hyphenated to an Agilent 8800 ICP-QQQ-MS.

Iron content was normalized within samples using a multielement control (Mg, P, and K for brain; C and P for muscle) and expressed relative to the WT-SED group.

4.4. Protein and RNA Extraction

Cytosolic proteins were isolated from frozen cortical and muscle samples (n = 8–10/group) for Western blot, enzyme-linked immunosorbent assay (ELISA), and CBA analysis. Cortical samples were homogenized manually in eight volume of lysis buffer (20 mM Tris, 250 mM sucrose, 0.5 mM EDTA 0.5 mM EGTA, 4% (*v/v*) protease inhibitor cocktail, 1% (*v/v*) phosphatase inhibitor cocktail, pH 7.4) on ice and centrifuged at 5000× *g* for 10 min at +4 °C. Muscle samples were ground into fine powder using a porcelain cup with hammer under liquid nitrogen, then manually homogenized in five volume of lysis buffer (50 mM Tris, 150 mM NaCl, 0.3% Triton X-100, 4% (*v/v*) protease inhibitor cocktail, 1% (*v/v*) phosphatase inhibitor cocktail, pH 7.4) on ice and centrifuged at 1200× *g* for 10 min at +4 °C. The supernatants containing cytosolic proteins were collected and stored at −70 °C for further use. Protein concentrations were determined using the Pierce 660 nm Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) for cortical samples and using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) for muscle samples.

Before centrifugation, parts of the cortical and muscle homogenates were taken for RNA isolation, which was performed using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer's instructions. Genomic DNA was removed using DNase I, RNase-free kit (Thermo Fisher Scientific, Waltham, MA, USA) and cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA).

4.5. Quantitative Real-Time PCR (qPCR)

qPCR analysis was performed to measure mRNA level of essential proteins in iron metabolism in cortical and muscle samples using StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). TaqMan gene expression assays (Thermo Fisher Scientific, Waltham, MA, USA) used in this study included Fth1 (ferritin, Mm00850707_g1), Hmox1 (HO-1, Mm00516005_m1), Trfc (TfR, Mm00441941_m1), Slc11a2 (DMT1, Mm00435363_m1), Slc40a1 (ferroportin, Mm01254822_m1), Cp (ceruloplasmin, Mm00432654_m1), Stat3 (STAT3, Mm01219775_m1), Jak1 (JAK1, Mm00600614_m1), Ptpre (PTPe, Mm00448493_m1), Il6ra (IL-6R, Mm00439653_m1), and Gapdh (GAPDH, Mm99999915_g1). Relative expression levels to the WT-SED group were determined using the $2^{-\Delta\Delta Ct}$ method normalized to Gapdh as the endogenous control.

4.6. Western Blot

Ferritin and iron transporter protein content in muscles was measured by Western blot. Procedures were performed as previously described [27]. Briefly, 40 µg of muscle proteins were separated by SDS-PAGE, transferred to poly-vinylidene difluoride (PVDF) membranes (GE Healthcare, Chicago, IL, USA), blocked in 5% nonfat dry milk in phosphate-buffered saline with Tween-20 (PBST), washed in PBST, and incubated overnight at +4 °C with primary antibodies against ferritin (1:1000, ab75973, Abcam, Cambridge, UK), TfR (1:1000, ab84036, Abcam, Cambridge, UK), DMT1 (1:1000, ABS983, Sigma-Aldrich, St. Louis, MO, USA), and ferroportin (1:1000, PA5-22993, Invitrogen, Waltham, MA, USA). Then membranes were washed three times in PBST and incubated in goat anti-rabbit secondary antibody (1:3000, conjugated with HRP, 130-65-15, BioRad, Hercules, CA, USA) for 2 h at room temperature. Proteins were visualized with SuperSignalTM West Pico PLUS Chemiluminescent substrate kit (Thermo Fisher Scientific, Waltham, MA, USA), detected with BioRad ChemiDocTM Imaging System, and quantified using ImageLab software (BioRad, Hercules, CA, USA). The results were normalized to the total proteins of Ponceau S staining.

4.7. Enzyme-Linked Immunosorbent Assay (ELISA)

The hepcidin concentration was measured in the cortical homogenates using HAMP mouse ELISA kit (Aviva Systems Biology, San Diego, CA, USA) following the manufacturer's instructions. Absorbance of the samples was read at 450 nm with Wallac Victor 1420 microplate reader (Perkin Elmer, Waltham, MA, USA). All results were normalized by total protein concentration.

4.8. Cytokine Bead Array (CBA)

IL-6 concentration was measured in plasma, and cortical and muscle homogenates using the CBA mouse inflammation kit (BD Biosciences, Franklin Lakes, NJ, USA). Samples were run using CytoFlex S flow cytometer (Beckman Coulter, Brea, CA, USA), and acquired data were analyzed with FCAP Array v3.0 software (Soft Flow Ltd., Pcs, Hungary). All results were normalized by total protein concentration and expressed relative to the WT-SED group.

4.9. Immunohistochemistry (IHC)

Aβ, ferritin, and TfR levels were evaluated by immunostaining brain cryosections. Three sections (400 µm apart) were washed in 0.1M PB and mounted to superfrost slides (Thermo Fisher Scientific, Waltham, MA, USA). For Aβ and ferritin staining, sections were boiled at +95 °C in 10mM sodium citrate buffer and washed three times in PBST. After blocking in 10% normal goat serum in PBST for 1 h, sections were incubated overnight at room temperature with primary antibodies against Aβ (clone WO-2, 1:1000, MABN10, Sigma-Aldrich, St. Louis, NO, USA), ferritin (1:200, ab75973, Abcam, Cambridge, UK), and TfR (1:200, ab84036, Abcam, Cambridge, UK). Sections were washed three times in PBST and incubated in fluorescent goat secondary antibody (anti-mouse 1:500, AlexaFluor 568, a11004, anti-rabbit 1:250, AlexaFluor 488, a11008; Thermo Fisher Scientific, Waltham, MA, USA) for 2 h. Then, sections were washed three times in PBST and mounted in Vectashield mounting medium with DAPI (H-1200, Vector Laboratories, Burlingame, CA, USA). Images from sections were captured at 10 × magnification by Leica Thunder Imager 3D tissue Slide scanner (Leica Microsystems, Wetzlar, Germany) and analyzed using ImageJ software (National Institute of Health, Bathesda, MD, USA). The percentage of immunoreactive area (positive staining) in the cortex and hippocampus was measured for each section, and the average from three sections per mouse was calculated. All results were expressed relative to the 5xFAD-SED group for Aβ staining, and for WT-SED group for ferritin and TfR staining.

4.10. Statistical Analysis

To estimate genotype and exercise difference between WT and 5xFAD mice, two-way analysis of variance (ANOVA) was used. In case of significant interaction between two factors (genotype × exercise), unpaired t test was performed as a post hoc test to examine exercise effect in WT and 5xFAD mice separately or genotype effect in SED and EXE groups separately. Grubbs' test was performed to determine and remove possible statistical outliers from the analysis. Pearson's test was used for correlation analysis. All values are expressed as mean ± SEM. Differences were considered significant at $p < 0.05$. Statistical calculations were performed using GraphPad Prism 8.4.2 software (GraphPad Software Inc., San Diego, CA, USA).

5. Conclusions

This study highlights the importance of iron dysregulation in AD and demonstrates that long-term voluntary running exercise modulates iron homeostasis in the brain and skeletal muscles of both WT and 5xFAD mice. Our study is the first to link brain alterations of iron homeostasis with decreases in hepcidin and IL-6 in response to regular physical exercise.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms22168715/s1.

Author Contributions: Conceptualization, I.B., J.R.L., M.A., A.R.W. and K.M.K.; methodology I.B., J.R.L., M.A., A.R.W. and K.M.K.; validation I.B., J.R.L., A.R.W. and K.M.K.; formal analysis, I.B.; investigation, I.B., J.R.L., N.K., V.G. and K.K.; resources, P.J.C., D.J.H., M.A. and K.M.K.; writing—original draft preparation, I.B.; writing—review & editing, J.R.L., T.M., M.A., A.R.W. and K.M.K.; visualization, I.B.; supervision, K.M.K.; project administration, I.B.; funding acquisition, K.M.K. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the Academy of Finland, the Finnish Cultural Foundation, the Sigrid Juselius foundation, and the University of Eastern Finland.

Institutional Review Board Statement: The study was conducted in accordance with the Council of Europe Legislation and Regulation for Animal Protection. The Animal Experiment Board in Finland (Regional State Administrative Agency of Southern Finland) approved all the experiments, which were conducted in accordance with EU Directive 2010/63/EU for animal experiments (license ESAVI-2015-000744 from 10 March 2015).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The authors would like to thank Taina Vihavainen and Dzhessie Rait for help with tissue collection, Mirka Tikkanen for help with mouse brains cryosectioning, Galieva Tatjana and Navjot Thind for protein isolation from skeletal muscles and Liisi Soppela for RNA isolation from cortical samples. Part of the work was conducted with the support of UEF Cell and Tissue Imaging Unit, University of Eastern Finland, Biocenter Kuopio and Biocenter Finland. Graphical abstract was created using BioRender.com (accessed on 1 July 2021).

Conflicts of Interest: The authors declare no conflict of interest.

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Healthy aging is an important mission considering the ever-increasing lifespan of the populations. This study highlighted the positive impact of regular physical exercise in a mouse model of aging and Alzheimer's disease (AD). Our results revealed new aspects of the beneficial effect of regular exercise in both the brain and skeletal muscle. Regular physical exercise could be a promising preventive strategy against AD and should be further investigated as a way of promoting healthy aging.

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**PUBLICATIONS OF
THE UNIVERSITY OF EASTERN FINLAND**
Dissertations in Health Sciences

ISBN 978-952-61-4311-8
ISSN 1798-5706